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Synthesis and pharmacological evaluation of conformationally restricted κ-opioid receptor agonists†

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Abstract

In order to obtain novel polar κ agonists the κ-pharmacophoric ethylenediamine structural element was embedded in a rigid bicyclic scaffold. The pyridooxazine system was selected, since it contains polar O- and N-atoms in 1- and 7-position, respectively. An axially oriented pyrrolidine ring was attached at 5-position and the dichlorophenylacetyl moiety was introduced at N-4. The key steps of the 11-step synthesis are a double *Henry* reaction of iminodiacetaldehyde **7** with nitromethane

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and the introduction of the azido moiety of **13** by Mitsunobu reaction of the alcohol **11** with $Zn(N_3)_2 \cdot (pyridine)_2$. The X-ray crystal structure analysis of **17b** shows a dihedral angle N(acyl)-C-C-N(pyrrolidine) of -60.8(2)°, which is close to the postulated optimal angle. Moderate κ affinity was found for the secondary amine **17a** (K_i = 132 nM) and the methyl derivative **17b** (K_i = 266 nM). In the [^{35}S]GTPγS assay the secondary amine **17a** showed 28 % agonistic activity compared to U-69,593. Although **17a** and **17b** contain all crucial κ-pharmacophoric elements, their κ affinity is rather low, which might be attributed to the unfavorable *cis*-orientation of the pyrrolidine ring and the dichlorophenylacetamido moiety and/or the additional O- and N-atom in 1- and 7-position.

Key words: κ-opioid receptor agonists; iminodiacetaldehyde; *Henry* reaction; nitropiperidine-3,5-diols, pyridooxazines; conformational restriction; dihedral angle; receptor selectivity

1. Introduction

Strong analgesia is achieved by activation of μ-, κ- and δ-opioid receptors. Whereas dangerous side effects, such as respiratory depression as well as physical and psychical dependence, are mediated by typical μ agonists, κ agonists do not produce these side effects. However, activation of κ-opioid receptors is associated with centrally mediated sedation, dysphoria, and strong diuresis inhibiting the clinical use of κ agonists so far.[1-4]

κ-Opioid receptors are localized not only in the central nervous system, but also in some tissues in the periphery, including the skin, bowels and joints. Activation of

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these peripherally localized receptors can be used for the treatment of inflammatory and itching skin diseases (e.g. atopic dermatitis) and visceral pain.[5-7] Therefore, we are interested in κ agonists, which are able to activate κ-opioid receptors with high affinity and intrinsic activity, but cannot pass the blood-brain-barrier and enter the central nervous system. Due to this property they should stay in the periphery and thus cannot induce centrally mediated side effects, as outlined above.

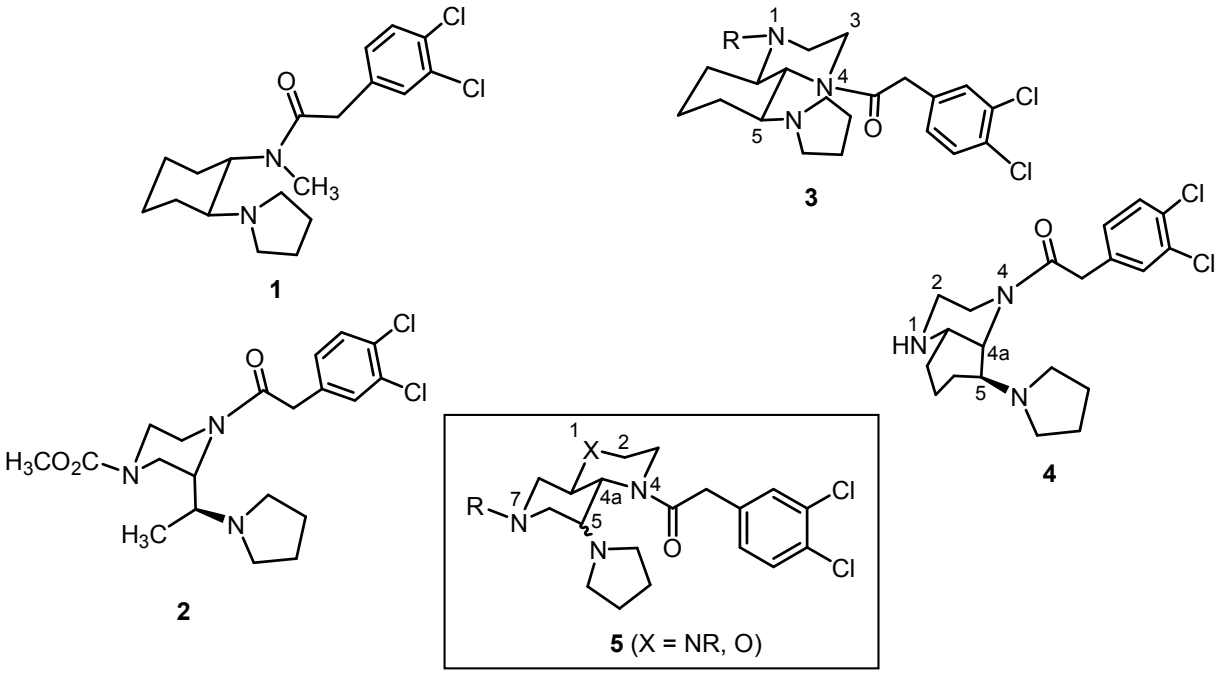

Figure 1: Development of the novel κ agonists **5** from prominent lead compounds **1**-**4**. **1** (U-50,488): K_i = 0.34 nM; **2**: K_i = 0.31 nM; **3a** (R = CO_2CH_3): K_i = 9.7 nM; **4a** (R = CO_2CH_3): K_i = 0.35 nM.

In addition to morphinoids,[8,9] peptides[10,11] and natural products (salvinorins),[12,13] ethylenediamines represent a privileged class of κ agonists.[14] In order to obtain high κ agonistic activity one N-atom of the ethylenediamine substructure has to be embedded in a pyrrolidine ring and the other N-atom has to be acylated with the 2-(3,4-dichlorophenyl)acetyl moiety. U-50,488 (**1**) represents the first potent κ agonist

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(K_i = 0.34 nM) of this class. (Figure 1) In U-50,488 the κ-pharmacophoric ethylenediamine structure is part of a *trans*-configured cyclohexane ring.[15] However, the ethylenediamine structure can also be part of a piperidine[16] or piperazine ring.[17,18] The piperazine derivative **2** shown in Figure 1 interacts in the subnanomolar range (K_i = 0.31 nM) with κ-opioid receptors.[18]

Combination of the cyclohexane ring of U-50,488 (**1**) with the piperazine ring of **2** resulted in the perhydroquinoxalines **3** and **4**. The κ affinity of the *trans,trans*-configured compounds **3** can be modulated by the substituent at the N-atom (N-1) outside the κ-pharmacophore. The methoxycarbonyl derivative **3a** (R = CO_2CH_3) reveals a κ affinity of 9.7 nM. However, the 3-pyridylmethyl derivative **3b** (R = 3-pyridylmethyl) interacts in the subnanomolar range with the κ-opioid receptor (K_i = 0.13 nM). Both compounds **3a** and **3b** represent full κ agonists in the [^{35}S]GTPγS assay.[19-21]

In contrast to the *trans*,*trans*-configured perhydroquinoxalines **3**, the cyclohexane and piperazine rings of **4** are *cis*-annulated. Changing of the configuration of the perhydroquinoxaline ring system increased the κ affinity considerably. The κ affinity of the racemic methoxycarbonyl derivative **4a** (R = CO_2CH_3) is 0.35 nM, which resides almost exclusively in the dextrorotatory (4a*R*,5*S*,8a*S*)-configured enantiomer (K_i = 0.25 nM).[22]

In this manuscript, we report on the synthesis and pharmacological evaluation of κ-opioid receptor agonists of type **5**. The scaffold of **5** consists of an annulated piperazine (X = NR) or morpholine ring (X = O) and a piperidine ring. The κ-

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pharmacophoric ethylenediamine system is found in the substructure "N(acyl)-CH-CH-N(pyrrolidine)". In contrast to the κ agonists **2**-**4**, ligands **5** have two positions in the core system, which allow the modulation of the pharmacodynamic and pharmacokinetic properties of the ligands: X in 1-position should be either a polar O- or N-atom increasing the polarity of the compounds. The additional N-atom in 7-position of the ring system contributes to the overall polarity of the ligands and moreover, allows the introduction of various substituents. A high polarity of the κ agonists is desired in order to minimize the penetration of the blood-brain-barrier and reduce centrally mediated κ agonist side effects.

2. Synthesis

For the synthesis of bicyclic κ agonists **5** a similar strategy was followed as for the synthesis of perhydroquinoxalines **3**.[19,20] Thus, iminodiacetaldehydes **7** were selected as starting materials, which were prepared by *Malaprade* cleavage of pyrrolidine-3,4-diols **6** with $NaIO_4$.[23] (Scheme 1) At first dialdehyde **7a** was reacted with nitromethane and NaOH in methanol under the same reaction conditions as described for the transformation of glutaraldehyde.[24,25] Under these reaction conditions the double *Henry* reaction did not lead to a clean product, probably due to hydrate, hemiacetal and/or acetal formation of dialdehyde **7a** with water and/or methanol. Therefore, the solvent methanol was replaced by *tert*-butanol, THF and ethyl acetate. The highest yield of **8a** was obtained with NaOH in ethyl acetate. After flash chromatographic purification, the achiral (3*r*)-configured 4-nitropiperidine-3,5-diol **8a** with one center of pseudochirality was isolated in 59 % yield. The benzoyl derivative **7b** was reacted in a THF/*tert*-butanol mixture affording 50 % yield of nitrodiol **8b**.

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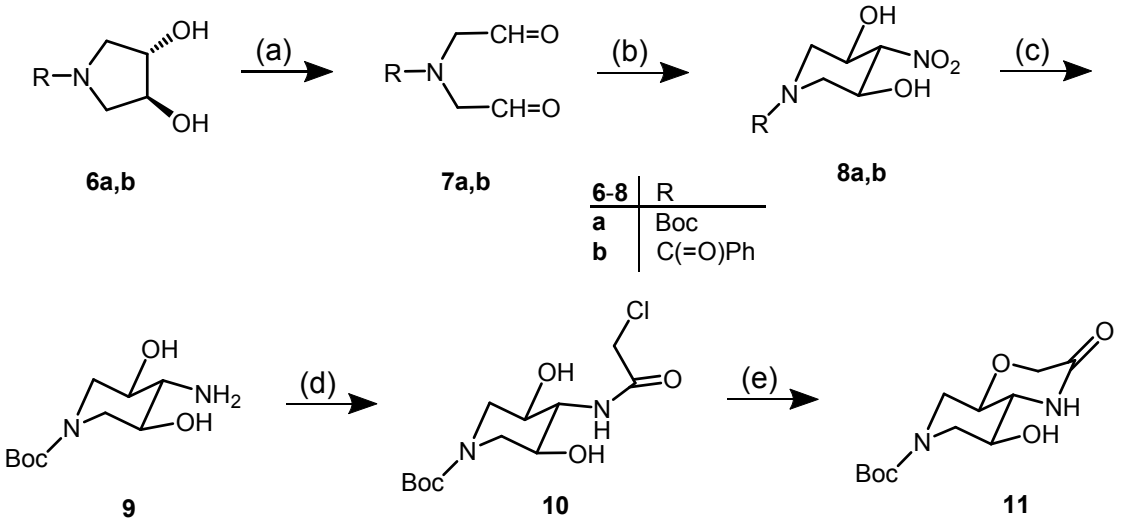

Scheme 1: Synthesis of bicyclic lactams **11**.
Reagents and reaction conditions: (a) $NaIO_4$, THF, rt, 24 h, 99 % (**7a**), 50 % (**7b**).[23] (b) CH_3NO_2, NaOH, EtOAc or THF/*tert*-butanol, rt, 12-20 h, 59 % (**8a**), 50 % (**8b**). (c) H_2, 1 bar (balloon), Raney-Ni, EtOH, rt, 5 h, 86 %. (d) $ClCH_2C(=O)Cl$, THF, NEt_3, rt, 1 h, 53 %. (e) Bu_4NI, NaH, THF, rt, 48 h, 61 %. Only one enantiomer of racemate **11** is shown in the Scheme.

In case of 2-nitrocyclohexane-1,3-diols the OH moieties could be replaced by various amino groups by simple stirring the nitrodiol with the respective primary or secondary amine in water.[19,20] However, in case of the piperidines **8a** and **8b** all reactions with amines failed to give the corresponding nitrodiamines. Since it was hypothesized that the sterically demanding and electron withdrawing Boc and benzoyl protective groups of **8a** and **8b** were the origin of this problem, the Boc group was replaced by other protective groups. Treatment of **8a** with trifluoroacetic acid afforded the secondary amine **8c**, which was reacted with allyl bromide, benzaldehyde/$NaBH(OAc)_3$, and 4-methoxybenzaldeyhyde/$NaBH(OAc)_3$ to provide the allyl, benzyl, and 4-methoxybenzyl derivatives **8d**-**f**, respectively. (Scheme 2) However, as reported for the Boc and benzoyl derivatives **8a**,**b**, neither the allyl (**8d**), benzyl (**8e**) nor the 4-

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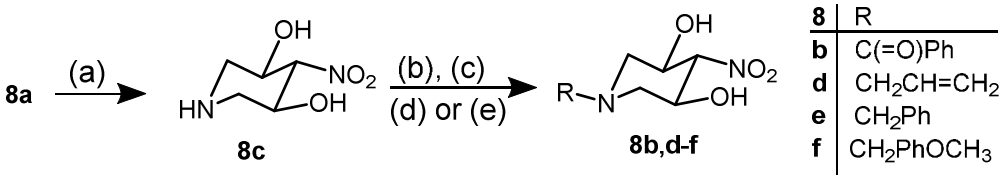
methoxybenzyl (**8f**) derivatives reacted with various primary and secondary amines to provide nitropiperidinediamines.



Scheme 2: Exchange of the N-Boc group of nitrodiol **8a** by other protective groups. Reagents and reaction conditions: (a) CF_3CO_2H, CH_2Cl_2, rt, 3 h, 62 %. (b) Ph(C=O)Cl, NEt_3, CH_2Cl_2, rt, 48 h, 43 % (**8b**). (c) allyl bromide, K_2CO_3, THF, rt, 24 h, 15 % (**8d**). (d) PhCH=O, $NaBH(OAc)_3$, NEt_3, CH_3CN, rt, 20 h, 50 % (**8e**). (e) 4-Methoxybenzaldehyde, $NaBH(OAc)_3$, NEt_3, THF, rt, 1 h, 38 % (**8f**).

Thus the nitrodiol **8a** was reduced with H_2 in the presence of the catalyst Raney Ni to give the aminodiol **9**. (Scheme 1) Reaction of **9** with chloroacetyl chloride provided the amide **10**, which was cyclized upon treatment with NaH and Bu_4NI to afford the morpholine annulated piperidine **11** in 61 % yield. This cyclization led to desymmetrization of the system: whereas the achiral (4*r*)-configured diols **8-10** contain a center of pseudochirality, the bicyclic compound **11** is chiral (racemic mixture) with three centers of chirality.

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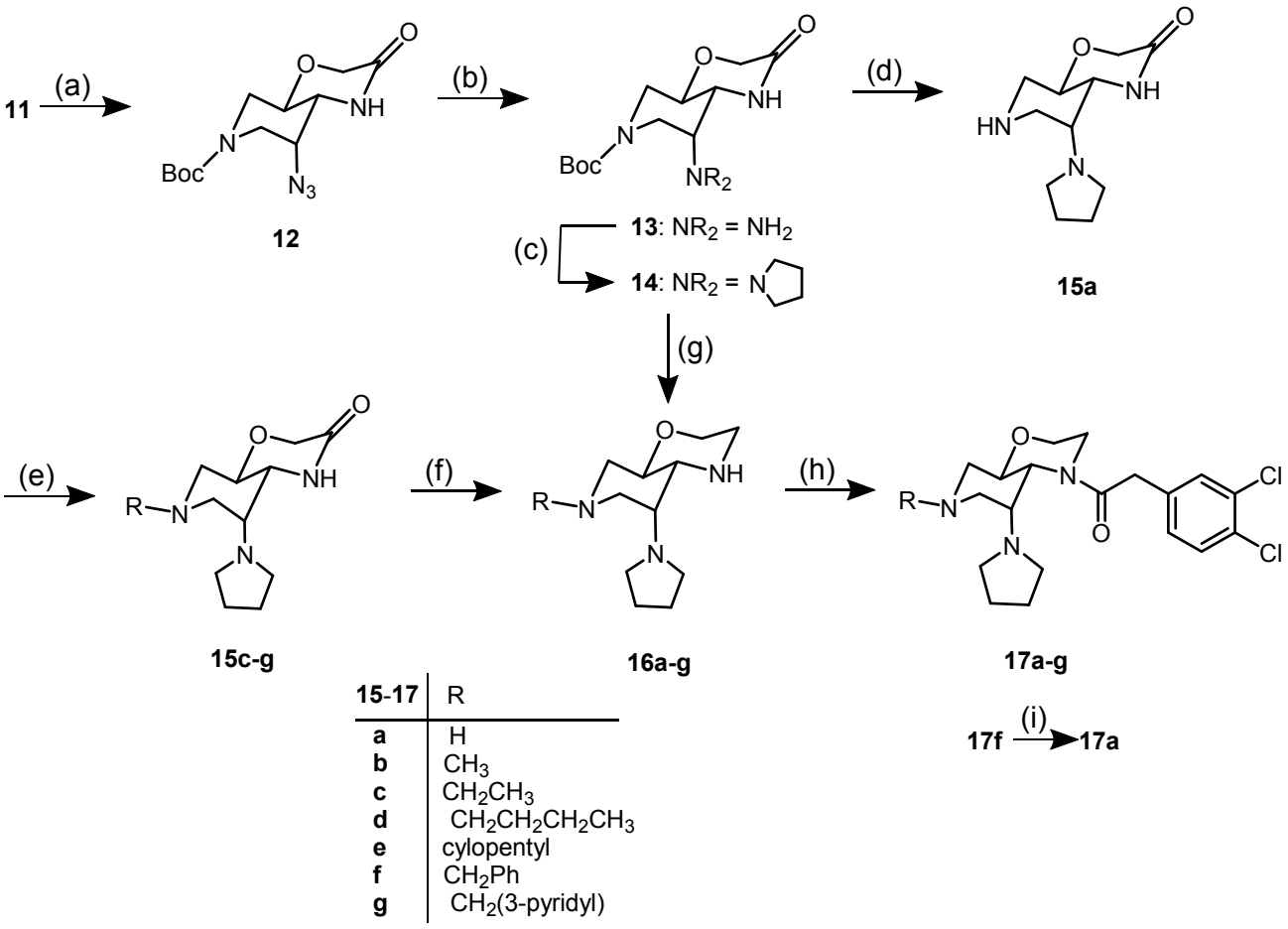

Scheme 3: Synthesis of arylacetamides **17**.

Reagents and reaction conditions: (a) $Zn(N_3)_2 \cdot (pyridine)_2$, PPh_3, DIAD, THF, rt, 4.5 h, 70 %. (b) H_2, 1 bar (balloon), Pd/C, CH_3OH, rt, 18 h, 90 %. (c) 1,4-diiodobutane, $NaHCO_3$, CH_3CN, reflux, 20 h, 55 %. (d) CF_3CO_2H, CH_2Cl_2, rt, 20 h, 18 %. (e) RCH=O or cyclopentanone, $NaBH(OAc)_3$, THF, rt, 50 min - 20 h, 74 - 95 % (from **14**). (f) $AlCl_3$, $LiAlH_4$ (1:3), THF, rt, 20 min, then addition of lactam **15**, THF, 0 – 20 °C, 15 - 60 min. (g) $AlCl_3$, $LiAlH_4$ (1:3), THF, rt, 20 min, then addition of lactam **14**, THF, 0 °C for 45 min, then rt for 20 min, 99 % (**16b**). (h) 2-(3,4-dichlorophenyl)acetyl chloride, CH_2Cl_2, NaOH, 0 – 20 °C, 71 - 92 % (from **14** or **15**); yield of **17g** only 38 % due to side reactions. (i) H_2, 1 bar (balloon), Pd/C, THF/H_2O, HCl, rt, 30 min, 90 %. Only one enantiomer of racemic mixtures **12**-**17** is shown in the Scheme.

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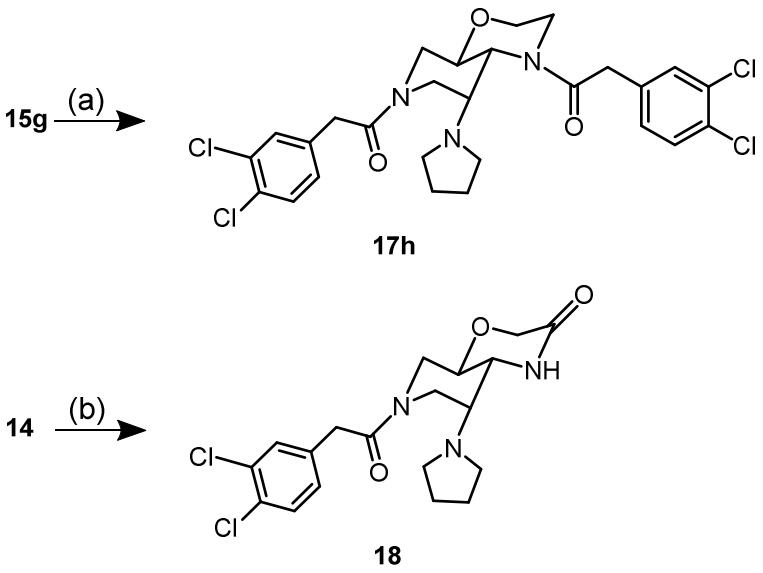
In the next step the remaining OH moiety of **11** should be transformed into a pyrrolidine ring. Mesylation of the secondary alcohol **11** and subsequent nucleophilic substitution with pyrrolidine as well as oxidation followed by reductive amination of the formed ketone did not lead to pyrrolidine derivatives. Therefore, the alcohol **11** was reacted in a *Mitsunobu* reaction with $Zn(N_3)_2 \cdot (pyridine)_2$, PPh_3 and DIAD[26] to give the azide **12**. (Scheme 3) Reduction of the azido group with H_2 and Pd/C provided the primary amine **13**, which reacted with 1,4-diiodobutane to afford the pyrrolidine **14** in 55 % yield.



Scheme 4: Synthesis of different (dichlorophenyl)acetamides.
Reagents and reaction conditions: (a) 1. $AlCl_3$, $LiAlH_4$ (1:3), THF, rt, 20 min, then addition of lactam **15g**, THF, 0 °C for 45 min, then rt for 20 min; 2. 2-(3,4-dichlorophenyl)acetyl chloride, 1.4 equiv., CH_2Cl_2, NaOH, rt, 12 h, 20 % (from **15g**). (b) 1. CF_3CO_2H, CH_2Cl_2, rt, 4 h; 2. 2-(3,4-dichlorophenyl)acetyl chloride, CH_2Cl_2, NaOH, rt, 50 min, 68 % (from **14**). Only one enantiomer of racemic mixtures **17h** and **18** is shown in the Scheme.

In order to introduce diverse substituents at the N-atom in 7-position of the ring system outside the κ pharmacophore, the Boc group of **14** was removed with

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trifluoroacetic acid and the resulting secondary amine **15a** was alkylated reductively with various aldehydes and a ketone in the presence of $NaBH(OAc)_3$[27] to provide the tertiary amines **15c**-**g**. Reduction of the lactams **15c**-**g** with AlH_3, which was prepared *in situ* by mixing $AlCl'_3$ and $LiAlH_4$ in the ratio 1:3,[28] led to the amines **16c**-**g**. The secondary amine **16a** and the methylated amine **16b** were obtained by reduction of the lactams **15a** and the Boc-derivative **14**, respectively. In the last step the secondary amines **16b**-**g** were acylated with (dichlorophenyl)acetyl chloride to produce the desired (dichlorophenyl)acetamides **17b**-**g**. The (dichlorophenyl)acetamide **17a** with a proton at the N-atom in 7-position was obtained by hydrogenolytic removal of the benzyl group of **17f**.

The yield of the pyridylmethyl derivative **17g** was only 38 %, which is due to side reactions during the AlH_3 reduction of the pyridylmethyl derivative **15g**. On the one side the pyridine ring was reduced to give a piperidine ring, which was acylated by (dichlorophenyl)acetyl chloride. On the other side the pyridylmethyl group was cleaved off leading to **16a**, which was acylated twice. Performing this reaction in a larger scale allowed the isolation of the diacylated compound **17h** in 20 % yield. (Scheme 4) The (dichlorophenyl)acetamide **18** bearing the κ-pharmacophoric elements in a different arrangement at the ring system, was obtained by acylation of the secondary amine **15a**, which was prepared *in situ* by TFA cleavage of the Boc-group of **14**. (Scheme 4)

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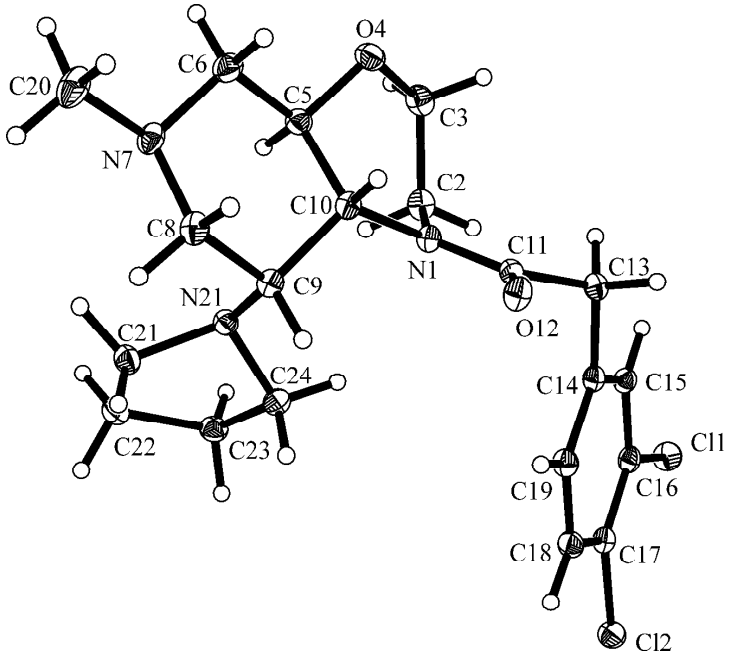

Figure 2: X-ray crystal structure of (dichlorophenyl)acetamide **17b** (thermal ellipsoids are shown at 15 % probability).
Elemental unit ($a_0 \neq b_0 \neq c_0$, $\alpha = \gamma = 90°$, $\beta = 105.364(8)°$) with the lattice constants a = 14.1810(20) Å, b = 12.8440(9) Å und c = 11.3534(8) Å; the crystal has the space group $P2_1/c$.

Recrystallization of the methylated derivative **17b** led to crystals, which were suitable for X-ray crystal structure analysis. (Figure 2) The crystal structure nicely confirms the *trans*-annulation of the piperidine and morpholine rings. The pyrrolidinyl group adopts an axial orientation in a relative *cis*-configuration to the N-acyl structural element. The piperidine ring exists in a chair conformation, whereas the morpholine ring adopts a twist conformation. The dihedral angle of the ethylenediamine κ-pharmacophore (N1-C10-C9-N21) was determined to be -60.8(2)°. This dihedral angle is very close to the dihedral angle of the ethylenediamine substructure of U-50,488 (60°), which has been postulated to be optimal for high κ affinity.[29]

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For the bicyclic system **18** bearing the required κ-pharmacophoric elements at different positions a similar dihedral angle N(acyl)-C-C-N(pyrrolidine) of 63.7° was calculated by MMX/AM1 method.

3. Pharmacological evaluation

The affinity of the pyridooxazines **17** and **18** towards the κ-opioid receptor was determined in competitive receptor binding studies. The tritium labeled potent and selective κ agonist [^{3}H]U-69,593 was used as radioligand and homogenates prepared from guinea pig brain served as receptor material. A high concentration (10 μM) of non-labeled U-69,593 was used to determine the non-specific binding.[30,31] The K_i values of the reference compounds U-69,593, naloxone and morphine are listed in Table 1 to demonstrate the validity of the affinity data.

In addition to the κ affinity the affinity towards μ- and δ-opioid receptors was recorded, to determine the selectivity of the ligands over related opioid receptors. Since small structural changes of potent κ ligands often leads to high σ_1 and/or σ_2 affinity, the affinity towards the σ receptors was also included into this study.[32-34]

The highest κ affinity was found for the secondary amine **17a** showing a K_i value of 132 nM. Introduction of a methyl moiety decreased the κ affinity to a K_i value of 286 nM. A further increase of the size of the substituent at N-7 led to a further decrease of κ affinity. Obviously, substituents larger than a methyl moiety are not tolerated at this position, which is in contrast to the perhydroquinoxalines **3**.

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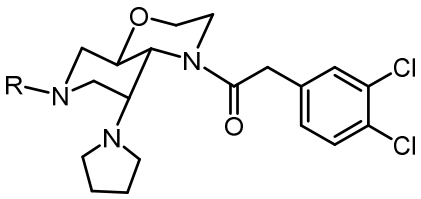
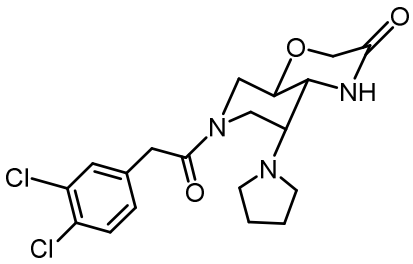
Table 1: Receptor affinity of pyridooxazines **17** and **18** towards opioid and σ receptors.



17a-h



18

compd.	R	K_i ± SEM [nM]				
		κ	μ	δ	σ_1	σ_2
		[^{3}H]U-69,593	[^{3}H]DAMGO	[^{3}H]DPDPE	[^{3}H]-(+)-pentazocine	[^{3}H]DTG
17a	H	132 ± 44	>1000	>1000	>1000	>1000
17b	CH_3	286 ± 63	>1000	>1000	>1000	>1000
17c	CH_2CH_3	1300	>1000	>1000	391	102
17d	$(CH_2)_3CH_3$	>1000	502	>1000	192	316
17e	cyclopentyl	>1000	447	717	181	45
17f	CH_2-phenyl	912	261 ± 79	260	983	>1000
17g	CH_2-3-pyridyl	>1000	633	666	291	>1000
17h	DCPA[a]	>1000	>1000	>1000	991	>1000
18	-	>1000	>1000	>1000	>1000	>1000
U-69,593		0.97 ± 0.40	-	-	-	-
Naloxone		6.9 ± 0.5	2.1 ± 0.5	2.4 ± 0.5	-	-
Morphine		35 ± 6.0	3.9 ± 2.1	2.0 ± 0.3	-	-
(+)-pentazocine		-	-	-	5.4 ± 0.5	-
Haloperidol		-	-	-	6.6 ± 0.9	78 ± 2.3

[a] DCPA = dichlorophenylacetyl;

K_i values are given as mean values ± SEM of three experiments (n = 3); due to low affinity, the K_i values of some compounds were recorded only once; if the reduction of radioligand binding at a test compound concentration of 1 μM is lower than 40 % a value >1000 nM is given; the exact reduction of radioligand binding in % is given in Table SI1 (Supporting Information);

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In the class of perhydroquinoxalines **3** a proton (**3c**: R = H) or a methyl moiety (**3d**: R = CH_3) at N-1 resulted in very high κ affinity with K_i values of 2.1 nM (**3c**) and 2.7 nM (**3d**).[20] Moreover, the pyridylmethyl group at N-1 led to extraordinarily high κ affinity (**3b**: K_i = 0.13 nM).[21] In the class of pyridooxazines **17** the ligands with a proton (**17a**) and a methyl moiety (**17b**) show the highest κ affinity, but the affinity is 60- and 100-fold lower than the κ affinity of the corresponding perhydroquinoxalines **3c** and **3d**, respectively. The pyridylmethyl substituted pyridooxazine **17g** was not tolerated by the κ-opioid receptor, it did not compete with the radioligand even at the very high concentration of 1 μM.

The reduced κ affinity of pyridooxazines **17** can be explained by an unfavorable orientation of the κ-pharmacophoric elements pyrrolidine and (dichlorophenyl)acetylamido moiety. In the potent lead compounds **1**, **3**, and **4** these pharmacophoric elements are *trans*-oriented with respect to the cyclohexane ring, which is in contrast to the *cis*-orientation in **17**. However, the dihedral angle N(acyl)-C-C-N(pyrrolidine) is -60.8(2)° according to the X-ray crystal structure analysis. This value is close to the postulated value of 60° for U-50,488,[29] which indicates that the dihedral angle alone cannot be the origin of the low affinity.

Alternatively, the low κ affinity of pyridooxazines **17** could be explained by the O-atom in 1-position replacing the (substituted) N-atom in the lead compounds **2**, **3**, and **4**. Although the O-atom has quite different properties than the (substituted) N-atom, the observed effect should not result from this substitution.

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Finally, the N-atom in 7-position of pyridooxazines **17** was postulated to be responsible for low κ affinity. It could be possible that the high polarity of the N-containing functional group together with large substituents at N-7 are not tolerated by the κ-opioid receptor. The N-substituents will clash with lipophilic amino acid residues in the κ-opioid receptor binding site.

The negligible κ affinity of **18** was unexpected, since it contains all required κ-pharmacophoric elements. Only the position of the (dichlorophenyl)acetyl moiety at the bicyclic system is different from those in ligands **17**.

In general the μ- and δ-opioid receptor affinity of the pyridooxazines **17** and **18** is rather low. In particular the most potent κ ligands **17a** and **17b** did not compete with the μ- and δ-selective radioligands [^{3}H]DAMGO and [^{3}H]DPDPE even at a concentration of 1 μM. Obviously, **17a** and **17b** show high selectivity over μ- and δ-opioid receptors.

Introduction of larger substituents at the N-atom, such as a butyl (**17d**), cyclopentyl (**17e**), benzyl (**17f**) or pyridylmethyl (**17g**) moiety led to moderate μ affinity in the range of 260-630 nM. With exception of the butyl derivative **17d**, the δ affinity of these ligands is in the same order (260-720 nM). However, **17d**-**g** cannot be regarded as potent μ and/or δ ligands. The moderate interaction of the ligands **17d**-**g** with μ- and δ-opioid receptors was very surprising, since they contain the structural features (dichlorophenylacetamide, pyrrolidine), which are responsible for high κ affinity. This observation may be explained by the structural similarity of κ-, μ- and δ-opioid receptors belonging to the same class of receptors.

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Since small structural changes of κ agonists could result in potent σ ligands,[32-34] the affinity towards σ_1 and σ_1 receptors was included into this study. The most interesting κ ligands **17a** and **17b** did not show any σ_1 or σ_2 affinity up to a concentration of 1 μM, indicating high selectivity over both σ receptor subtypes. However, the compounds **17d** and **17e** without κ affinity displayed moderate σ_1 affinity of 192 nM and 181 nM, respectively. For the cyclopentyl derivative **17e** and the allyl derivative **17c** σ_2 affinity of 45 nM and 102 nM was determined, respectively. It is assumed that large lipophilic substituents at the N-atom (e.g. butyl, cyclopentyl) increase the affinity of **17d** and **17e** towards σ_1 and σ_2 receptors. A similar observation was made in the class of benzomorphans.[33,34]

In order to determine the κ agonistic activity, the most potent κ ligand **17a** (K_i = 132 nM) of this series of compounds was investigated exemplarily in the [^{35}S]GTPγS assay.[35,36] At a concentration of 1 μM the secondary amine **17a** showed 27 % agonistic activity compared to the prototypical full κ agonist U-69,593. Obviously **17a** behaved in this assay as weak partial agonist.

The logP value of the most potent κ agonist **17a** was calculated with different software packages and the values were compared with the logP values calculated for the lead compounds **1**-**4**. The results are summarized in Table 2. It can be seen that the calculated logP values of **17a** are 1-2 orders of magnitude lower than the logP values of the lead compounds **1**-**4**, indicating an increased polarity for **17a**. These data indicate that the selected modifications introduced into **17a** are useful to reduce the lipophilicity, which may translate into reduced penetration into the central nervous

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system. In a model of the blood-brain-barrier, it was shown that the perhydroquinoxaline **3c** could not pass the endothelial cell layer. Compared to **3c**, the new κ agonist **17a** has a different stereochemistry, the NH-group is at a different position, but most importantly, it has an additional O-atom within the ring system. The calculations of the logP values in Table 2 reveal an even higher polarity for **17a** compared to **3c** and therefore a reduced penetration into the central nervous system is expected.

Table 2: logP values of **1**-**4** and **17a** calculated with different software packages.

compd.	clogP[a)]	clogP(H^+)[b)]	milogP[c)]	milogP(H^+)[d)]
1 (U-50,488)	4.91	5.36	4.57	1.41
2	3.73	4.09	2.80	-0.33
3c (R = H)	4.14	4.50	3.70	0.54
4	4.14	4.50	3.70	0.54
17a	2.67	3.16	2.29	-0.87

[a)] clogP = calculated logP value (ChemBioDraw 14.0.0.117).

[b)] clogP(H^+) = calculated logP value of the monoprotonated species (ChemBioDraw 14.0.0.117).

[c)] milogP = calculated logP value (www.molinspiration.com v2014.11).

[d)] milogP(H^+) = calculated logP value of the monoprotonated species (www.molinspiration.com v2014.11).

4. Conclusion

In this project the κ-pharmacophoric elements of the ethylenediamine class of κ agonists were newly arranged at a novel heterocyclic system, i.e. the hydrogenated pyridooxazine scaffold. In an eleven-step synthesis the dichlorophenylacetyl moiety

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and the pyrrolidine ring were attached at the *trans*-configured bicyclic ring system. Although the dihedral angle of the pharmacophoric elements (N(acyl)-C-C-N(pyrrolidine) was -60.8(2)° and thus close to the postulated optimum, the most promising compound **17a** reached only an κ affinity of 132 nM. Substituents larger than a CH_3 moiety were not tolerated at the additional N-atom within the bicyclic ring system. An unfavorable orientation of the pharmacophoric elements, the additional O-atom in 1-position and/or the substituted N-atom in 7-position of the ring system are discussed to be responsible for the moderate κ affinity. The secondary amine **17a** showing the highest κ affinity (K_i = 132 nM) in this series of ligands behaved as weak partial agonist in the [^{35}S]GTPγS assay.

5. Experimental, Chemistry

5.1. 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, eluent, fraction size, R_f value. Melting point: Melting point apparatus SMP 3 (Stuart Scientific), uncorrected. MS: MAT GCQ (Thermo-Finnigan); IR: IR spectrophotometer 480Plus FT-ATR-IR (Jasco). ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz): Unity Mercury Plus 400 spectrometer (Varian); δ in ppm related to tetramethylsilane; coupling constants are given with 0.5 Hz resolution.

5.2. HPLC method for the determination of the purity

Merck Hitachi Equipment; UV detector: L-7400; autosampler:L-7200; pump: L-7100; degasser: L-7614; column: LiChrospher® 60 RP-select B (5 µm), 250-4 mm; flow

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rate: 1.00 mL/min; injection volume: 5.0 µL; detection at λ = 210 nm; solvents: A: water with 0.05 % (v/v) CF_3CO_2H; B: CH_3CN with 0.05 % (v/v) CF_3CO_2H: 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 %.

5.3. X-ray diffraction

Data sets were collected with a Nonius Kappa CCD diffractometer. Programs used: data collection, COLLECT (R. W. W. Hooft, Bruker AXS, **2008**, Delft, The Netherlands); data reduction Denzo-SMN;[37] absorption correction, Denzo;[38] structure solution SHELXS-97;[39] structure refinement SHELXL-97[40] and graphics, XP (BrukerAXS, **2000**). *R*-values are given for observed reflections, and *w*R^2 values are given for all reflections.

5.4. General Procedures

General Procedure A for cleavage of the Boc-protective group of **14** and subsequent reductive alkylation of secondary amine **15a**

Trifluoroacetic acid (30 eq) was added to a solution of carbamate **14** in CH_2Cl_2. The mixture was stirred for 20 h and the solvent was removed in vacuum. The residue was dissolved in CH_2Cl_2 and the pH value was adjusted to pH 7 using Merck® ion exchanger III. The mixture was filtered and the solvent was removed under reduced pressure. The residue was dissolved in THF, and the aldehyde (1.0 eq – 1.5 eq) or ketone (1 eq) was added and the mixture was stirred for 15 min. In case of the ketone trifluoroacetic acid was used to accelerate the conversion. $NaBH(OAc)_3$ was added and the mixture was stirred for 50 min - 18 h until the amine was converted completely. A small amount of water was added and the mixture was stirred for

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additional 20 min. The solvent was removed under reduced pressure and the product was purified.

General Procedure B for the preparation of alane

Under N_2 atmosphere dry $AlCl_3$ (45 mg, 0.33 mmol) was suspended in THF (2.5 mL) and the mixture was stirred for 5 min at 0 °C. Then a solution of $LiAlH_4$ in THF (1.0 M, 1.0 mL, 1.0 mmol) was added dropwise. The mixture was stirred for 20 min at rt. The amount of AlH_3 equivalents in the resulting suspension is 1.33 mmol.

5.5. Synthetic procedures

5.5.1. *tert*-Butyl (4*r*)-3,5-dihydroxy-4-nitropiperidine-1-carboxylate (8a)

At 0 °C nitromethane (0.50 g, 8.2 mmol) and 15 % (m/m) NaOH (1 mL) were added dropwise to a solution of iminodiacetaldehyde **7a** (1.50 g, 7.5 mmol) in EtOAc (5 mL). The mixture was stirred for 12 h at rt, then neutralized using Amberlite® ion exchange resin (IR-120) and stirred for 1 h. The mixture was filtered, concentrated under reduced pressure and the residue was purified by fc (∅ = 5 cm, h = 16 cm, CH_2Cl_2/ethyl acetate = 4/1, V = 65 mL, R_f = 0.22 (cyclohexane/ethyl acetate = 4/1)). Colorless crystals, mp 145 – 150 °C, yield 1.17 g (59 %). $C_{10}H_{18}N_2O_6$ (M = 262.3 g/mol). ^{1}H NMR (d_6-DMSO): δ (ppm) = 1.41 (s, 9 H, C(C*H*$_3$)$_3$), 2.50 – 2.79 (m, 2 H, 2-C*H*$_2$, 6-C*H*$_2$), 3.69 – 3.76 (m, 2 H, 2 x C*H*OH), 3.90 – 4.10 (m, 2 H, 2-C*H*$_2$, 6-C*H*$_2$), 4.35 (t, ^3J = 9.8 Hz, 1 H, C*H*NO$_2$) 5.93 (d, ^3J = 5.6 Hz, 2 H, 2 x O*H*). ^{13}C NMR (d_6-DMSO): δ (ppm) = 27.9 (3 C, C(*C*H$_3$)$_3$), 47.1 (N*C*H$_2$), 48.0 (N*C*H$_2$), 67.5 (2 x *C*HOH), 79.7 (*C*(CH$_3$)$_3$), 96.5 (*C*HNO$_2$), 153.6 (*C*=O). MS (EM, ESI): m/z = calcd. for $C_{10}H_{17}N_2O_6$ $[M - H]^-$ 261.1087, found 261.1053; calcd. for $C_{10}H_{15}N_2O_5$ $[M - H_2O - H]^-$ 243.0981, found 243.0943. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 3437/3378 (*w*, O-H),

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2996/2920/2866 (*w*, C-H), 1691 (*s*, C=$O_{carbamate}$); 1554/1370 (*s*, C-NO_2). Purity (HPLC): 89 %, t_R = 11.31 min.

5.5.2. (4*r*)-4-Nitropiperidine-3,5-diol (8c)

Trifluoroacetic acid (0.12 mL, 1.52 mmol) was added to a solution of nitropiperidinediol **8a** (80.0 mg, 0.31 mmol) in CH_2Cl_2 (5 mL). The mixture was stirred for 3 h at rt. The solvent was removed under reduced pressure and the product was purified by fc (∅ = 2 cm, h = 16 cm, $CH_2Cl_2/CH_3OH/NH_3$ = 9.5/4.7/0.3, V = 10 mL, R_f = 0.54 (CH_3OH)). Yellow resin, yield 30.7 mg (62 %). $C_5H_{10}N_2O_4$ (M = 162.1 g/mol). ^{1}H NMR (d_6-DMSO): *δ* (ppm) = 2.24 (dd, ^2J = 12.6 Hz, ^3J = 10.6 Hz, 2 H, 2-CH_{2ax}, 6-CH_{2ax}), 2.93 (dd, ^2J = 12.7 Hz, ^3J = 5.1 Hz, 2 H, 2-CH_{2eq}, 6-CH_{2eq}), 3.69 – 3.80 (m, 2 H, 2 x C*H*OH), 4.18 (t, ^3J = 9.8 Hz, 1 H, C*H*NO_2), 5.54 (s broad, 3 H, O*H*, N*H*). MS (EM, APCI): m/z = calcd. for $C_5H_{11}N_2O_4$ $[M + H]^+$ 163.0719, found 163.0707; calcd. for $C_5H_9N_2O_3$ $[M + H - H_2O]^+$ 145.0613, found 145.0605. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 3283 (*w*, O-H), 2928/2827 (*w*, C-H), 1548/1372 (*s*, C-NO_2).

5.5.3. *tert*-Butyl (4*r*) 4-amino-3,5-dihydroxypiperidine-1-carboxylate (9)

A mixture of nitropiperidinediol **8a** (5.65 g, 21.5 mmol) and Raney-Ni (12.0 g, 1 mL precipitate ≙ 0.6 g) in ethanol (110 mL) was shaken for 5 h under H_2 atmosphere (1 bar, balloon). The mixture was filtered through Celite® and the solvent was removed under reduced pressure. R_f = 0.35 (CH_3OH). Yellow oil, yield 4.30 g (86 %). $C_{10}H_{20}N_2O_4$ (M = 232.3 g/mol). ^{1}H NMR (d_6-DMSO): *δ* (ppm) = 1.38 (s, 9 H, C(CH_3)$_3$), 2.33 (t, ^3J = 9.1 Hz, 1 H, C*H*NH_2), 2.35 – 2.55 (m, 2 H, 2-CH_2, 6-CH_2), 2.96 – 3.04 (m, 2 H, 2-CH_2, 6-CH_2), 3.88 (s broad, 2 H, 2 x C*H*OH), 5.06 (s broad, 2 H, 2 x O*H*). A signal for NH_2 protons is not observed in the ^{1}H NMR spectrum. MS (ESI):

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m/z = 233 $[M + H]^+$, 255 $[M + Na]^+$, 465 $[2 \times M + H]^+$. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 3350 (*w*, O-H), 2928 (*w*, C-H), 1668 (*s*, $C{=}O_{carbamate}$).

5.5.4. *tert*-Butyl (4*r*) 4-(2-chloroacetamido)-3,5-dihydroxypiperidine-1-carboxylate (10)

Chloroacetyl chloride (1.47 mL, 18.5 mmol) dissolved in THF (75 mL) was added to an ice-cooled solution of amine **9** (4.3 g, 18.5 mmol) and triethylamine (2.56 mL, 18.5 mmol) in THF (75 mL) dropwise within 2 h. The mixture warmed up to rt and stirred for 1 h. The solvent was removed under reduced pressure and the product was purified by fc (∅ = 8 cm, h = 18 cm, CH_2Cl_2/CH_3OH = 9.7/0.3 → CH_2Cl_2/CH_3OH = 9.5/0.5, V = 65 mL, R_f = 0.15 (CH_2Cl_2/CH_3OH = 9.5/0.5)). Yellow resin, yield 3.0 g (53 %). $C_{12}H_{21}ClN_2O_5$ (M = 308.8 g/mol). ^{1}H NMR (CD_3OD): δ (ppm) = 1.46 (s, 9 H, $C(CH_3)_3$), 2.53 – 2.70 (m, 2 H, 2-CH_2, 6-CH_2), 3.43 – 3.51 (m, 2 H, 2 x CHOH), 3.63 (d, ^3J = 9.7 Hz, 1 H, CHNHCO), 4.11 (s, 2 H, CH_2Cl), 4.13 – 4.23 (m, 2 H, 2-CH_2, 6-CH_2). A signal for NH proton is not observed in the ^{1}H NMR spectrum. ^{13}C NMR (CD_3OD): δ (ppm) = 28.6 (3 C, $C(CH_3)_3$), 43.5 (CH_2Cl), 49.6 (NCH_2), 50.5 (NCH_2), 62.3 (CHNHCO), 69.1 (2 C, 2 x CHOH), 81.7 ($C(CH_3)_3$), 156.1 ($C{=}O_{carbamate}$), 170.2 ($C{=}O_{amide}$). MS (EM, APCI): m/z = calcd. for $C_{12}H_{22}{}^{35}ClN_2O_5$ $[M + H]^+$ 309.1217, found 309.1174; calcd. for $C_8H_{14}{}^{35}ClN_2O_5$ $[M – C(CH_3)_3 + 2 H]^+$ 253.0591, found 253.0564; calcd. for $C_7H_{14}{}^{35}ClN_2O_3$ $[M – OCOC(CH_3)_3 + 2 H]^+$ 209.0693, found 209.0660. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 3319 (*m*, O-H), 2983/2928/2857 (*w*, C-H), 1701 (*s*, C=O), 1647 (*s*, C=O).

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5.5.5. *tert*-Butyl (4a*RS*,8*RS*,8a*SR*)-8-hydroxy-2-oxo-2,3,4a,5,6,7,8,8a-octahydro-1*H*-pyrido-[3,4-b][1,4]oxazine-6-carboxylate (11)

At 0 °C tetrabutylammonium iodide (1.25 g, 3.39 mmol) and NaH (119.1 mg, 2.83 mmol) were added to a solution of alcohol **10** (873 mg, 2.83 mmol) in THF (20 mL). The reaction mixture was allowed to warm up and stirred for 2 d at rt. After cooling to 0 °C the transformation was stopped by addition of a small amount of water. The solution was neutralized with 1 M HCl and stirred for 30 min. The mixture was extracted with CH_2Cl_2 (30 mL). The organic layer was washed with H_2O (10 mL). The aqueous layer was extracted with CH_2Cl_2 (3 x 10 mL). The combined organic layer was washed with brine, dried (Na_2SO_4) and filtered. The filtrate was concentrated in vacuum and the product was purified by fc (∅ = 8 cm, h = 17 cm, CH_2Cl_2/CH_3OH = 9.7/0.3, V = 65 mL, R_f = 0.35 (CH_2Cl_2/CH_3OH = 9.5/0.5)). Colorless solid, mp 181 – 182 °C, yield 466 mg (61 %). $C_{12}H_{20}N_2O_5$ (M = 272.3 g/mol). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.45 (s, 9 H, C(C*H*$_3$)$_3$), 2.47 – 2.81 (m, 2 H, 5-C*H*$_2$, 7-C*H*$_2$), 3.21 (t, ^3J = 9.4 Hz, 1 H, 8a-C*H*), 3.25 – 3.34 (m, 1 H, 4a-C*H*), 3.46 – 3.56 (m, 1 H, 8-C*H*), 4.16 – 4.50 (m, 3 H, 5-C*H*$_2$, 7-C*H*$_2$, O*H*), 4.19 (d, ^2J = 17.1 Hz, 1 H, 3-C*H*$_2$), 4.30 (d, ^2J = 17.1 Hz, 1 H, 3-C*H*$_2$), 7.51 (s broad, 0.5 H, N*H*), 7.87 (s broad, 0.5 H, N*H*). ^{13}C NMR ($CDCl_3$): δ (ppm) = 28.4 (3 C, C(*C*H$_3$)$_3$), 46.1 (*C*-5 or *C*-7), 47.9 (*C*-7 or *C*-5), 61.2 (*C*-8a), 67.7 (*C*-3), 68.1 (*C*-8), 72.1 (*C*-4a), 81.2 (*C*(CH$_3$)$_3$), 154.6 (*C*=O$_{carbamate}$), 169.3 (*C*=O$_{amide}$). MS (EM, APCI): m/z = calcd. for $C_{12}H_{21}N_2O_5$ $[M + H]^+$ 273.1451, found 273.1476; calcd. for $C_8H_{13}N_2O_5$ $[M – C(CH)_3 + 2 H]^+$ 217.0825, found 217.0854. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 3204 (*w*, O-H), 2962/2944/2832 (*m*, C-H), 1690 (*s*, C=O), 1630 (*s*, C=O). Elemental analysis: calcd. C 52.93, H 7.40, N 10.29, found 52.47, H 7.57, N 9.61.

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5.5.6. *tert*-Butyl (4a*RS*,8*RS*,8a*RS*)-8-azido-2-oxo-2,3,4a,5,6,7,8,8a-octahydro-1*H*-pyrido[3,4-b][1,4]oxazine-6-carboxylate (12)

$Zn(N_3)_2 \cdot (pyridine)_2$ (185 mg, 0.6 mmol) and PPh_3 (393 mg, 1.5 mmol) were added to a solution of alcohol **11** (136 mg, 0.50 mmol) in THF (6 mL). DIAD (0.29 mL, 1.5 mmol) was added dropwise and the mixture was stirred for 4.5 h at rt. The solvent was removed under reduced pressure and the product was purified by fc (∅ = 4 cm, h = 16 cm, CH_2Cl_2/CH_3OH = 9.85/0.15 → CH_2Cl_2/CH_3OH = 9.8/0.2, V = 20 mL, R_f = 0.53 (CH_2Cl_2/CH_3OH = 9.5/0.5)). Colorless solid, mp 162 – 163 °C, yield 105 mg (70 %). $C_{12}H_{19}N_5O_4$ (M = 297.3 g/mol). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.48 (s, 9 H, C(C*H*$_3$)$_3$), 2.53 – 2.75 (m, 1 H, 5-C*H*$_2$ or 7-C*H*$_2$), 2.81 – 3.08 (m, 1 H, 7-C*H*$_2$ or 5-C*H*$_2$), 3.46 (dd, ^3J = 9.2 Hz, ^3J = 3.1 Hz, 1 H, 8a-C*H*), 3.61 – 3.70 (m, 1 H, 4a-C*H*), 3.88 – 3.91 (m, 1 H, 8-C*H*), 4.15 – 4.30 (m, 2 H, 3-C*H*$_2$), 4.31 – 4.41 (m, 1 H, 5-C*H*$_2$ or 7-C*H*$_2$), 4.42 – 4.58 (m, 1 H, 7-C*H*$_2$ or 5-C*H*$_2$), 7.86 – 8.09 (m, 1 H, N*H*). ^{13}C NMR ($CDCl_3$): δ (ppm) = 28.4 (3 C, C(*C*H$_3$)$_3$), 45.5 (*C*-5 or *C*-7), 45.9 (*C*-7 or *C*-5), 57.4 (*C*-8a), 58.7 (*C*-8), 67.9 (*C*-3), 69.3 (*C*-4a), 81.3 (*C*(CH$_3$)$_3$), 154.7 (*C*=O$_{carbamate}$), 170.1 (*C*=O$_{amid}$). MS (EM, APCI): m/z = calcd. for $C_{12}H_{20}N_5O_4$ $[M + H]^+$ 298.1515, found 298.1503; calcd. for $C_8H_{12}N_5O_4$ $[M – C(CH)_3 + 2 H]^+$ 242.0889, found 242.0899. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 2970/2897 (*w*, C-H), 2118 (*s*, N_3), 1705 (*s*, C=O), 1670 (*s*, C=O). Elemental analysis: calcd. C 48.48, H 6.44, N 23, found 48.14, H 6.42, N 23.33.

5.5.7. *tert*-Butyl (4a*RS*,8*RS*,8a*RS*)-8-amino-2-oxo-2,3,4a,5,6,7,8,8a-octahydro-1*H*-pyrido[3,4-b][1,4]oxazine-6-carboxylate (13)

Azide **12** (45.6 mg, 0.15 mmol) and Pd/C (20 % (m/m), 9.12 mg) were stirred for 18 h under H_2 atmosphere (1 bar, balloon) in CH_3OH (3 mL). The mixture was filtered through Celite® and the solvent was removed under reduced pressure. The product was purified by fc (∅ = 2 cm, h = 20 cm, CH_2Cl_2/CH_3OH = 9/1, V = 10 mL, R_f = 0.31).

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Colorless solid, mp 155 – 156 °C, yield 36.7 mg (90 %). $C_{12}H_{21}N_3O_4$ (M = 271.3 g/mol). ^{1}H NMR (CD_3OD): δ (ppm) = 1.47 (s, 9 H, C(C*H*$_3$)$_3$), 2.57 – 2.77 (m, 1 H, 5-C*H*$_2$ or 7-C*H*$_2$), 2.89 – 3.09 (m, 1 H, 7-C*H*$_2$ or 5-C*H*$_2$), 3.13 – 3.24 (m, 1 H, 8-C*H*), 3.40 (dd, ^3J = 9.6 Hz, ^3J = 3.5 Hz, 1 H, 8a-C*H*), 3.69 – 3.78 (m, 1 H, 4a-C*H*), 4.10 – 4.18 (m, 1 H, 5-C*H*$_2$ or 7-C*H*$_2$), 4.20 (s, 2 H, 3-C*H*$_2$), 4.25 – 4.35 (m, 1 H, 7-C*H*$_2$ or 5-C*H*$_2$). Signals for N*H*$_2$ and N*H* protons are not observed in the ^{1}H NMR spectrum. ^{13}C NMR (CD_3OD): δ (ppm) = 28.6 (3 C, C(*C*H$_3$)$_3$), 46.7 (*C*-5 or *C*-7), 48.6 (*C*-7 or *C*-5), 49.6 (*C*-8), 59.6 (*C*-8a), 68.4 (*C*-3), 69.9 (*C*-4a), 81.8 (*C*(CH$_3$)$_3$), 156.9 (*C*=O$_{carbamate}$), 171.9 (*C*=O$_{amid}$). MS (EM, APCI): m/z = calcd. for $C_{12}H_{22}N_3O_4$ $[M + H]^+$ 272.1610, found 272.1572; calcd. for $C_8H_{14}N_3O_4$ $[M – C(CH)_3 + 2 H]^+$ 216.0984, found 216.0957; calcd. for $C_7H_{14}N_3O_2$ $[M – CO_2C(CH)_3 + 2 H]^+$ 172.1086, found 172.1068. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 3379/3194 (*w*, N-H), 2882 (*w*, C-H), 1686 (*s*, C=O), 1643 (*s*, C=O).

5.5.8. *tert*-Butyl (4a*RS*,8*RS*,8a*RS*)-2-oxo-8-(pyrrolidin-1-yl)-2,3,4a,5,6,7,8,8a-octahydro-1*H*-pyrido[3,4-b][1,4]oxazine-6-carboxylate (14)

A mixture of primary amine **13** (951 mg, 3.5 mmol), 1,4-diiodobutane (1.8 mL, 14.0 mmol), $NaHCO_3$ (2.0 g, 23.8 mmol) and CH_3CN (100 mL) was stirred under reflux for 20 h. After evaporation of the solvent in vacuum the residue was purified by fc (∅ = 8 cm, h = 14 cm, CH_2Cl_2/CH_3OH = 9.75/0.35 → CH_2Cl_2/CH_3OH = 9.5/0.5, V = 65 mL, R_f = 0.21 (CH_2Cl_2/CH_3OH = 9.5/0.5)). Colorless solid, mp 135 °C (decomposition), yield 626 mg (55 %). $C_{16}H_{27}N_3O_4$ (M = 325.4 g/mol). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.46 (s, 9 H, C(C*H*$_3$)$_3$), 1.66 – 1.73 (m, 4 H, N(CH$_2$C*H*$_2$)$_2$), 2.55 – 2.84 (m, 7 H, N(C*H*$_2$CH$_2$)$_2$, 5-C*H*$_2$, 7-C*H*$_2$, 8-C*H*), 3.50 (dd, ^3J = 10.3 Hz, ^3J = 2.8 Hz, 1 H, 8a-C*H*), 3.86 – 3.99 (m, 1 H, 4a-C*H*), 4.13 – 4.31 (m, 2 H, 3-C*H*$_2$), 4.31 – 4.43 (m, 1 H, 5-C*H*$_2$ or 7-C*H*$_2$), 4.46 – 4.62 (m, 1 H, 7-C*H*$_2$ or 5-C*H*$_2$), 6.69 – 7.40 (m, 1 H,

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N*H*). ^{13}C NMR ($CDCl_3$): δ (ppm) = 23.4 (2 C, $N(CH_2\mathit{C}H_2)_2$), 28.5 (3 C, $C(\mathit{C}H_3)_3$), 46.0 (*C*-5 or *C*-7), 47.0 (*C*-7 or *C*-5), 52.9 (2 C, $N(\mathit{C}H_2CH_2)_2$), 59.9 (*C*-8a), 60.8 (*C*-8), 67.8 (*C*-3), 69.4 (*C*-4a), 80.5 ($\mathit{C}(CH_3)_3$), 154.5 (C=$O_{carbamate}$), 168.9 (C=O_{amide}). MS (EM, ESI): m/z = calcd. for $C_{16}H_{28}N_3O_4$ $[M + H]^+$ 326.2080, found 326.2106; calcd. for $C_{12}H_{20}N_3O_4$ $[M - C(CH)_3 + 2\ H]^+$ 270.1454, found 270.1460. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 2967/2940/2897 (*w*, C-H), 1674 (*s*, C=O). Elemental analysis: calcd. C 59.06, H 8.36, N 12.91, found 57.73, H 8.18, N 12.58.

5.5.9. (4a*RS*,8*RS*,8a*RS*)-8-(Pyrrolidin-1-yl)-4a,5,6,7,8,8a-hexahydro-1*H*-pyrido[3,4-b][1,4]oxazin-2(3*H*)-one (15a)

Trifluoroacetic acid (0.23 mL, 3.0 mmol) was added dropwise to a solution of Boc-protected pyridooxazine **14** (43.1 mg, 0.13 mmol) in CH_2Cl_2 (3 mL). After stirring for 2 h, the solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (5 mL), washed with a saturated solution of $NaHCO_3$ (5 mL) and the aqueous layer was extracted with CH_2Cl_2 (3 x 5 mL). The organic layers were combined and the solvent was removed in vacuum. R_f = 0.13 (CH_2Cl_2/CH_3OH = 8/2). Colorless crystals, yield 5.5 mg (18 %). $C_{11}H_{19}N_3O_2$ (M = 225.3 g/mol). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.71 – 1.74 (m, 4 H, $N(CH_2C\mathit{H}_2)_2$), 1.79 – 1.99 (m, 2 H, 5-CH_2, 7-CH_2), 2.44 – 2.49 (m, 1 H, NH_{amine}), 2.65 – 2.72 (m, 5 H, $N(C\mathit{H}_2CH_2)_2$, 8-C*H*), 3.19 – 3.27 (m, 1 H, 5-CH_2 or 7-CH_2), 3.30 (dd, ^3J = 13.0 Hz, ^3J = 4.9 Hz, 1 H, 7-CH_2 or 5-CH_2), 3.57 (dd, ^3J = 9.7 Hz, ^3J = 2.4 Hz, 1 H, 8a-C*H*), 3.83 – 3.91 (m, 1 H, 4a-C*H*), 4.18 (d, ^3J = 16.7 Hz 1 H, 3-CH_2), 4.27 (d, ^2J = 16.8 Hz, 1 H, 3-CH_2), 6.90 (s, 1 H, NH_{amide}). MS (APCI): m/z = 226 $[M + H]^+$.

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5.5.10. (4a*RS*,8*RS*,8a*RS*)-6-Ethyl-8-(pyrrolidin-1-yl)-2,3,4a,5,6,7,8,8a-octahydro-1*H*-pyrido[3,4-b][1,4]oxazin-2-one (15c)

According to **General Procedure A** Boc-protected pyridooxazine **14** (70.9 mg, 0.22 mmol) was reacted with trifluoroacetic acid (0.50 mL, 6.5 mmol) in CH_2Cl_2 (5 mL). The subsequent alkylation was performed with acetaldehyde (25 µL, 0.44 mmol), $NaBH(OAc)_3$ (65.3 mg, 0.31 mmol) and THF (4 mL). After 40 min the transformation was complete and the product was purified by fc (∅ = 2 cm, h = 18 cm, $CH_2Cl_2/CH_3OH/NH_3$ = 9.5/0.47/0.03, V = 10 mL, R_f = 0.20 (CH_2Cl_2/CH_3OH = 9/1)). Colorless solid, mp 164 – 166 °C, yield 46.6 mg (84 %). $C_{13}H_{23}N_3O_2$ (M = 253.3 g/mol). ^{1}H NMR ($CDCl_3$): *δ* (ppm) = 1.08 (t, ^3J = 7.2 Hz, 3 H, C*H*$_3$), 1.75 – 1.84 (m, 4 H, N(CH_2CH_2)$_2$), 2.00 – 2.10 (m, 2 H, 5-C*H*$_{2\text{-ax}}$, 7-C*H*$_{2\text{-ax}}$), 2.37 – 2.47 (m, 1 H, C*H*$_2$CH$_3$), 2.50 – 2.60 (m, 1 H, C*H*$_2$CH$_3$), 2.75 – 2.84 (m, 2 H, N(C*H*$_2$CH$_2$)$_2$), 2.85 – 2.98 (m, 3 H, 8-C*H*, N(C*H*$_2$CH$_2$)$_2$), 3.14 – 3.20 (m, 1 H, 7-C*H*$_{2\text{-eq}}$), 3.23 (dd, ^2J = 10.3 Hz, ^3J = 4.6 Hz, 1 H, 5-C*H*$_{2\text{-eq}}$), 3.42 (dd, ^3J = 9.8 Hz, ^3J = 3.5 Hz, 1 H, 8a-C*H*), 4.02 – 4.10 (m, 1 H, 4a-C*H*), 4.20 (d, ^2J = 16.8 Hz, 1 H, 3-C*H*$_2$), 4.25 (d, ^2J = 16.7 Hz, 1 H, 3-C*H*$_2$), 7.76 (s broad, 1 H, N*H*). ^{13}C NMR ($CDCl_3$): *δ* (ppm) = 11.94 (*C*H$_3$), 23.4 (2 C, N(CH_2*C*H$_2$)$_2$), 51.9 (*C*H$_2$CH$_3$), 53.5 (2 C, N(*C*H$_2$CH$_2$)$_2$), 53.9 (*C*-7), 56.2 (*C*-5), 59.4 (*C*-8a), 61.2 (*C*-8), 67.7 (*C*-3), 70.5 (*C*-4a), 169.9 (*C*=O). MS (EM, APCI): m/z = calcd. for $C_{13}H_{24}N_3O_2$ $[M + H]^+$ 254.1869, found 254.1845. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 2962/2936/2870 (*m*, C-H), 1670 (*s*, C=O).

5.5.11. (4a*RS*,8*RS*,8a*RS*)-6-Methyl-8-(pyrrolidin-1-yl)-2,3,4a,5,6,7,8,8a-octahydro-1*H*-pyrido[3,4-b][1,4]oxazine (16b)

A solution of AlH_3 (1.33 mmol) was freshly prepared by **General Procedure B**. Then Boc-protected pyridooxazine **14** (60 g, 0.18 mmol) dissolved in THF (3 mL) was added dropwise to the alane solution at 0 °C. The mixture was stirred at 0 °C for

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45 min and at rt for 20 min. 2 M NaOH (5 mL) was carefully added at 0 °C. CH_2Cl_2 (5 mL) was added, the layers were separated and the aqueous layer was extracted with CH_2Cl_2 (4 x 5 mL). The combined organic layer was washed with brine, dried (Na_2SO_4) and filtered. The solvent was removed under reduced pressure. Colorless crystals, yield 45 mg (> 99 %). $C_{12}H_{23}N_3O$ (M = 225.3 g/mol). ^{1}H NMR (CD_3OD): δ (ppm) = 1.67 – 1.79 (m, 4 H, 2 x N(CH_2CH_2)$_2$), 1.80 – 1.87 (m, 1 H, 5-CH_2 or 7-CH_2), 2.03 – 2.10 (m, 1 H, 7-CH_2 or 5-CH_2), 2.22 (s, 3 H, CH_3), 2.39 – 2.47 (m, 1 H, 8-C*H* or 8a-C*H*), 2.67 – 2.78 (m, 3 H, N(CH_2CH$_2$)$_2$, 8a-C*H* or 8-C*H*), 2.80 – 2.95 (m, 5 H, N(CH_2CH$_2$)$_2$, 2-CH_2, 5-CH_2 or 7-CH_2), 3.66 (d, 1 H, 7-CH_2 or 5-CH_2), 3.57 (ddd, ^2J = 11.4 Hz, ^3J = 10.3 Hz, ^3J = 4.3 Hz, 1 H, 3-C$H_{2\text{-ax}}$), 3.66 (m, 1 H, 4a-C*H*), 3.76 (ddd, ^3J = 11.6 Hz, ^3J = 3.0 Hz, ^3J = 1.5 Hz, 1 H, 3-C$H_{2\text{-eq}}$). A signal for N*H* is not observed in the ^{1}H NMR spectrum.

5.5.12. 2-(3,4-Dichlorophenyl)-1-[(4a*RS*,8*RS*,8a*RS*)-8-(pyrrolidin-1-yl)-2,3,4a,5,6,7,8,8a-octahydropyrido[3,4-b][1,4]oxazin-1-yl]ethanone (17a) WMS-29-08

Pd/C (40 % (m/m), 12 mg) and conc. HCl (0.5 mL) were added to a solution of amide **17f** (29.9 mg, 0.06 mmol) in THF/water (1/1, 5 mL) and the suspension was stirred under H_2 atmosphere (1 bar, balloon) at rt for 30 min. The mixture was filtered and THF was removed under reduced pressure. The pH value of the aqueous solution was adjusted to pH 8 with 2 M NaOH. The solution was extracted with CH_2Cl_2 (5 x 3 mL), the organic layer was dried (Na_2SO_4), filtered and concentrated in vacuum. The product was purified by fc (∅ = 2 cm, h = 16 cm, CH_2Cl_2/CH_3OH/NH_3 = 8.9/1.09/0.01, V = 10 mL, R_f = 0.46). Pale yellow solid, mp 87 – 90 °C, yield 21.9 mg (90 %). $C_{19}H_{25}Cl_2N_3O_2$ (M = 398.3 g/mol). ^{1}H NMR (CD_3OD): δ (ppm) = 1.62 – 1.77 (m, 4 H, N(CH_2CH_2)$_2$), 2.40 – 2.59 (m, 5 H,

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N(CH_2CH_2)$_2$, 5-C$H_{2\text{-ax}}$), 2.71 (d, ^2J = 14.1 Hz, 1 H, 7-C$H_{2\text{-ax}}$), 3.07 – 3.23 (m, 3 H, 5-C$H_{2\text{-eq}}$, 7-C$H_{2\text{-eq}}$, 8-C*H*), 3.74 (d, ^2J = 15.4 Hz, 1 H, ArCH_2), 3.79 – 4.02 (m, 6 H, 2-CH_2, 3-CH_2, 8a-C*H*, ArCH_2), 4.28 – 4.39 (m, 1 H, 4a-C*H*), 7.21 (dd, ^3J = 8.2 Hz, ^4J = 2.0 Hz, 1 H, 6-CH_{ar}), 7.44 – 7.50 (m, 2 H, 2-CH_{ar}, 5-CH_{ar}). ^{13}C NMR (CD_3OD): δ (ppm) = 24.1 (2 C, N(CH_2CH_2)$_2$), 40.7 (Ar*C*H$_2$), 42.5 (*C*-8a), 49.8 (*C*-7), 51.4 (*C*-5), 54.1 (2 C, N(*C*H_2CH_2)$_2$), 62.6 (*C*-8), 65.7 (*C*-2), 66.8 (*C*-3), 67.4 (*C*-4a), 130.4 (C_{ar}-6), 131.6 (C_{ar}-2), 131.9 (C_q), 132.4 (C_{ar}-5), 133.3 (C_q), 136.9 (C_q), 173.1 (*C*=O). MS (EM, APCI): m/z = calcd. for $C_{19}H_{26}Cl_2N_3O_2$ $[M + H]^+$ 398.1402, found 398.1401. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 2955/2924/2770 (*m*, C-H), 1636 (*s*, C=O). Purity (HPLC): 99.3 %, t_R = 15.83 min.

5.5.13. 2-(3,4-Dichlorophenyl)-1-[(4a*RS*,8*RS*,8a*RS*)-6-methyl-8-(pyrrolidin-1-yl)-2,3,4a,5,6,7,8,8a-octahydropyrido[3,4-b][1,4]oxazin-1-yl]ethanone (17b) (WMS-29-01)

2-(3,4-Dichlorophenyl)acetyl chloride (33.5 µL, 0.22 mmol) was added to a solution of amine **16b** (40.6 mg, 0.18 mmol) in CH_2Cl_2 (5.5 mL) and the mixture was stirred for 30 min at rt. Then 2 M NaOH (5.5 mL) was added and the mixture was stirred for additional 12 h at rt. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (2 x 3 mL). The combined organic layer was dried (Na_2SO_4) and filtered. The filtrate was concentrated under reduced pressure and the product was purified by fc (∅ = 2 cm, h = 17 cm, CH_2Cl_2/CH_3OH = 9.8/0.2 → CH_2Cl_2/CH_3OH = 9.5/0.5, V = 10 mL, R_f = 0.57 (CH_2Cl_2/CH_3OH = 9/1)). Pale yellow solid, mp 111 – 112 °C, yield 62.2 mg (84 %). $C_{20}H_{27}Cl_2N_3O_2$ (M = 412.35 g/mol). ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.62 – 1.83 (m, 4 H, N(CH_2CH_2)$_2$), 1.90 (t, ^2J = 9.9 Hz, ^3J = 9.9 Hz, 1 H, 5-C$H_{2\text{-ax}}$), 2.18 (d, ^2J = 13.0 Hz, 1 H, 7-C$H_{2\text{-ax}}$), 2.26 (s, 3 H, CH_3), 2.45 – 2.77 (m, 4 H, N(CH_2CH_2)$_2$), 2.90 – 2.98 (d, ^2J = 13.0 Hz, 1 H, 7-C$H_{2\text{-eq}}$), 3.12 (dd, ^2J = 10.1 Hz,

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^3J = 4.3 Hz, 1 H, 5-C$H_{2\text{-eq}}$), 3.63 (d, ^2J = 15.3 Hz, 1 H, ArCH_2), 3.70 (d, ^2J = 15.3 Hz, 1 H, ArCH_2), 3.58 – 4.03 (m, 6 H, 2-CH_2, 3-CH_2, 8-C*H*, 8a-C*H*), 4.42 – 4.60 (m, 1 H, 4a-C*H*), 7.09 (dd, ^3J = 8.2 Hz, ^4J = 2.1 Hz, 1 H, 6-CH_{ar}), 7.35 (d, ^4J = 2.0 Hz, 1 H, 2-CH_{ar}), 7.39 (d, ^3J = 8.2 Hz, 1 H, 5-CH_{ar}). ^{13}C NMR ($CDCl_3$): δ (ppm) = 23.3 (2 C, N(CH_2CH_2)$_2$), 40.6 (ArCH_2), 41.5 (*C*-8a), 45.9 (CH_3), 53.3 (2 C, N(CH_2CH_2)$_2$), 58.1 (*C*-7), 60.2 (*C*-5), 64.0 (*C*-2), 65.8 (*C*-3), 66.2, (*C*-4a), 128.6 (C_{ar}-6), 130.7 (C_{ar}-2), 131.1 (C_{ar}-5), 131.4 (C_q), 132.8 (C_q), 134.7 (C_q), 170.6 (*C*=O). A signal for *C*-8 is not observed in the ^{13}C NMR spectrum. MS (EM, APCI): m/z = calcd. for $C_{20}H_{28}{}^{35}Cl_2N_3O_2$ $[M + H]^+$ 412.1559, found 412.1538. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 2947/2874/2789 (*w*, C-H), 1636 (*s*, C=O), 1134/1111 (*s*, C-O_{ether}), 729 (*s*, Ar). Purity (HPLC): 98.9 %, t_R = 17.63 min.

X-ray crystal structure of **17b**: formula $C_{20}H_{27}Cl_2N_3O_2$, *M* = 412.35, colorless crystal, 0.25 x 0.20 x 0.10 mm, *a* = 14.1810(20), *b* = 12.8440(9), *c* = 11.3534(8) Å, *β* = 105.364(8)°, *V* = 1994.0(3) Å^3, ρ_{calc} = 1.374 gcm^{-3}, μ = 3.094 mm^{-1}, empirical absorption correction (0.511 ≤ T ≤ 0.747), *Z* = 4, monoclinic, space group $P2_1/c$ (No. 14), λ = 1.54178 Å, *T* = 223(2) K, ω and φ scans, 16232 reflections collected (±*h*, ±*k*, ±*l*), 3445 independent (R_{int} = 0.038) and 3179 observed reflections [*I*>2σ(*I*)], 245 refined parameters, *R* = 0.036, wR^2 = 0.095, max. (min.) residual electron density 0.17 (-0.25) e.Å^{-3}, hydrogen atoms were calculated and refined as riding atoms.

CCDC-1485426 (compound **17b**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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5.5.14. 1-[(4a*RS*,8*RS*,8a*RS*)-6-Benzyl-8-(pyrrolidin-1-yl)-2,3,4a,5,6,7,8,8a-octahydro-pyrido[3,4-b][1,4]oxazin-1-yl]-2-(3,4-dichlorophenyl)-ethanone (17f)

2-(3,4-Dichlorophenyl)acetyl chloride (33.5 µL, 0.22 mmol) was added to a solution of amine **16f** (53.8 mg, 0.18 mmol) in CH_2Cl_2 (5 mL) and the mixture was stirred for 15 min at rt. 2 M NaOH (5 mL) was added and the mixture was stirred vigorously for 20 min. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (5 x 5 mL). The combined organic layers were washed with brine, dried (Na_2SO_4), filtered and the solvent was removed under reduced pressure. The product was purified by fc (∅ = 2 cm, h = 18 cm, CH_2Cl_2/CH_3OH = 9.7/0.3, V = 10 mL, R_f = 0.19 (CH_2Cl_2/CH_3OH = 9.5/0.5)). Colorless solid, mp°125 – 128 C, yield 62.3 mg (71 %). $C_{26}H_{31}Cl_2N_3O_2$ (M = 488.5 g/mol). ^{1}H NMR (CD_3OD): *δ* (ppm) = 1.55 – 1.74 (m, 4 H, $N(CH_2CH_2)_2$), 1.91 (t, ^2J = 9.9 Hz, ^3J = 9.9 Hz, 1 H, 5-C$H_{2\text{-ax}}$), 2.16 (d, ^2J = 13.5 Hz, 1 H, 7-C$H_{2\text{-ax}}$), 2.24 – 2.58 (m, 4 H, $N(CH_2CH_2)_2$), 3.00 (d, ^2J = 12.8 Hz, 1 H, 7-C$H_{2\text{-eq}}$), 3.04 – 3.07 (m, 2 H, 5-C$H_{2\text{-eq}}$, 8-C*H*), 3.42 (d, ^2J = 12.6 Hz, 1 H, PhCH_2), 3.58 (d, ^2J = 12.6 Hz, 1 H, PhCH_2), 3.70 – 4.02 (m, 7 H, 2-CH_2, 3-CH_2, 8a-C*H*, ArCH_2), 4.39 – 4.49 (m, 1 H, 4a-C*H*), 7.20 (dd, ^3J = 8.2 Hz, 1 H, ^4J = 2.1 Hz, 6-CH_{ar}), 7.23 – 7.33 (m, 5 H, 5 x CH_{ph}), 7.44 – 7.78 (m, 2 H, 2-CH_{ar}, 5-CH_{ar}). ^{13}C NMR ($CDCl_3$): *δ* (ppm) = 23.4 (2 C, $N(CH_2CH_2)_2$), 40.6 (ArCH_2), 41.4 (*C*-8a), 53.2 (2 C, $N(CH_2CH_2)_2$), 55.8 (*C*-7), 58.1 (*C*-5), 60.2 (*C*-8), 62.8 (PhCH_2), 64.7 (*C*-2), 65.8 (*C*-3), 66.7 (*C*-4a), 127.5 (C_{ph}), 128.4 (2 C, 2 x C_{ph}), 128.6 (C_{ar}-6), 129.6 (2 C, 2 x C_{ph}), 130.7 (C_{ar}-2), 131.1 (C_{ar}-5), 131.4 (C_q), 132.8 (C_q), 134.8 (C_q), 137.5 (C_q), 170.1 (*C*=O). MS (EM, APCI): m/z = calcd. for $C_{26}H_{32}{}^{35}Cl_2N_3O_2$ [M + H] 488.1872, found 488.1889; calcd. for $C_{18}H_{28}N_3O$ [M – (3,4-dichlorophenyl)acetyl + 2 H] 302.2232, found 302.2262. IR (neat): $\tilde{\nu}$ (cm^{-1}) = 2920/2855/2793 (*m*, C-H), 1643 (*s*, C=O), 729/698 (*m*, Ar). Purity (HPLC): 97.5 %, t_R = 20.39 min.

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6. Experimental, Pharmacological studies

6.1. Materials

The guinea pig brains for the σ_1, μ and κ receptor binding assay, rat liver for the σ_2 binding assay and rat brains for the δ-opioid receptor binding assay 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.

6.2. Preparation of membrane homogenates from guinea pig brain

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 x *g* for 10 min at 4 °C. The supernatant was separated and centrifuged at 23500 x *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 23500 x 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.

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6.3. Preparation of membrane homogenates from rat liver

Two rat livers were cut into small pieces and 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 1,200 x *g* for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,000 x *g* for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 8.0) and incubated at room temperature for 30 min. After the incubation, the suspension was centrifuged again at 31,000 x *g* for 20 min at 4 °C. The final pellet was resuspended in 5-6 volumes of buffer and stored at -80,°C in 1.5 mL portions containing about 2 mg protein/mL

6.4. Preparation of membrane homogenates from rat brain

5 rat brains (Sprague Dawley rats) 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 1,200 x *g* for 10 min at 4 °C. The supernatant was separated and centrifuged at 23,500 x *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 x *g* (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5-6 volumes of buffer and stored at -80,°C in 1.5 mL portions containing about about 1.5 mg protein/mL.

6.5. Protein determination

The protein concentration was determined by the method of Bradford,[41] modified by Stoscheck.[42] 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

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filled to a total volume of 50.0 mL with deionized H_2O. 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).

6.6. General procedures for the binding assays

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 the 96-well multiplates. The concentrations given are the final concentration in the assay. Generally, the assays were performed by addition of 50 µL of the respective assay buffer, 50 µL of 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 minutes. After

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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.[43] The K_i-values are given as mean value ± SEM from three independent experiments.

6.7. Determination of the κ-opioid receptor affinity (guinea pig brain)[20]

The assay was performed with the radioligand [^{3}H]-U-69593 (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-69593, 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-69593. The K_d-value of U-69593 is 0.69 nM.

6.8. Determination of the µ-opioid receptor affinity (guinea pig brain)

The assay was performed with the radioligand [^{3}H]-DAMGO (51 Ci/mmol, Perkin Elmer LAS). The thawed guinea pig brain membrane preparation (about 100 µg of the protein) was incubated with various concentrations of test compounds, 3 nM [^{3}H]-DAMGO, 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 Naloxon. The K_d-value of DAMGO is 0.57 nM.

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6.9. Determination of the δ-opioid receptor affinity (rat brain)

The assay was performed with the radioligand [^{3}H]-DPDPE (69 Ci/mmol, Amersham). The thawed rat membrane preparation (about 75 µg of the protein) was incubated with various concentrations of test compounds, 3 nM [^{3}H]-DPDPE, and TRIS-$MgCl_2$-PMSF-buffer (50 mM, 8 mM $MgCl_2$, 400 µM PMSF, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled Morphine. The K_d-value of DPDPE is 0.65 nM.

6.10. Determination of the σ_1 receptor affinity (guinea pig brain)[44-46]

The assay was performed with the radioligand [^{3}H]-(+)-pentazocine (22.0 Ci/mmol; Perkin Elmer). The thawed membrane preparation of guinea pig brain cortex (about 100 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H]-(+)-Pentazocine, and TRIS buffer (50 mM, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled (+)-pentazocine. The K_d-value of (+)-pentazocine is 2.9 nM.[44]

6.11. Determination of the σ_2 receptor affinity (rat liver)[44-46]

The assays were performed with the radioligand [^{3}H]-di-*o*-tolylguanidine ([^{3}H]DTG, specific activity 50 Ci/mmol; ARC, St. Louis, MO, USA). The thawed membrane preparation of rat liver containing 100 µg protein was incubated with various concentrations of the test compound, 3 nM [^{3}H]DTG and buffer containing (+)-pentazocine (500 nM (+)-pentazocine in 50 mM TRIS, pH 8.0) at room temperature. The non-specific binding was determined with 10 µM non-labeled DTG. The K_d values is 17.9 nM.[45]

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6.12. [^{35}S]GTPγS binding assay, agonistic activity at the κ-opioid receptor[35,36]

The [^{35}S]-guanosine-5´-3-O-(thio)triphosphate (GTPγS) assay was carried out as described in ref.[45]. The receptor material was obtained from human HEK 293 (human embryonic kidney) cells. Vehicle 1.00 % DMSO. Incubation time 30 min. Incubation temperature 30 °C. Incubation buffer 20 mM HEPES pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA. Quantification of bound [^{35}S]GTPγS. Significance criteria for an agonists: >50 % increase of bound [^{35}S]GTPγS relative to U-69,593 response. The EC_{50} values were determined by a non-linear, least squares regression analysis using MathIQTM (ID Business Solutions Ltd., UK). Reference standards were run as an integral part of each assay to ensure the validity of the results obtained.

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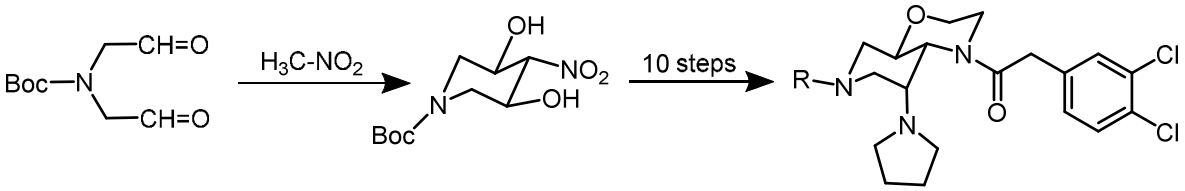

R = H: $K_i(\kappa)$ = 132 nM
partial agonist

Additional N- and O-atoms in the bicyclic scaffold increase polarity and allow fine tuning of pharmacodynamic and pharmacokinetic properties of novel κ agonist.