

A Novel Series of 2-Carboxytetrahydroquinolines Provides New Insights into the Eastern Region of Glycine Site NMDA Antagonists

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Summary

A series of potent 4-substituted tetrahydroquinolines has been synthesized and biologically tested in order to refine the eastern region of the pharmacophore model for glycine site NMDA antagonists concerning the assessment of lipophilicity, flexibility, and hydrogen bonding. Displacement studies on rat cortical membranes using [³H]-5,7-dichlorokynurenic acid as a radioligand indicated that binding affinities are markedly enhanced when additional hydrogen-accepting groups are introduced into the eastern region of the 2-carboxytetrahydroquinolines. Among the most potent ligands were some urea, sulfonyleurea, and crown ether compounds as interesting leads for new diagnostics, especially for the evaluation of PET tracers, which allow biodistribution studies and NMDA receptor studies in the living organism.

Introduction

Overactivation of the *N*-methyl-D-aspartate (NMDA) receptor has been implicated in a huge number of acute and chronic neurodegenerative disorders. The most prominent among these include stroke, Alzheimer's disease, and epilepsy^[1]. A visualization and quantification of NMDA receptor changes in these diseases by PET studies may lead to a deeper understanding of the pathogenesis. Due to their potency and high receptor selectivity, tetrahydroquinolines could become interesting targets for diagnostic interventions when combined with brain permeability enhancers. Efforts to refine the structure-activity relationships of glycine antagonists to the receptor protein in order to evaluate new diagnostic tools and to shift these findings to some *in vivo* potent lead structures led to the following series of new compounds.

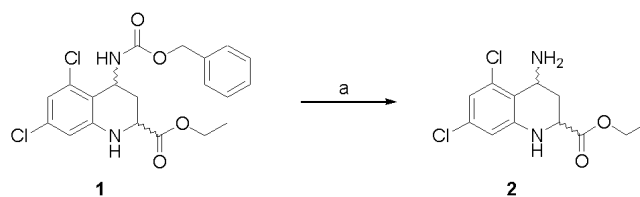
Chemistry

The general synthetic steps used for the preparation of this series are outlined in Scheme 1 and Scheme 2.

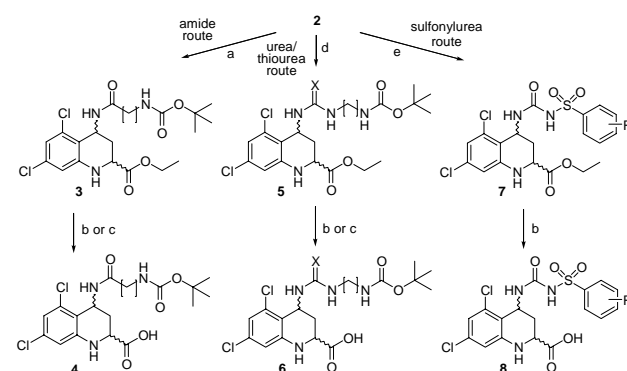
The synthesis of compounds **1** and **2** was performed according to the procedure of Stevenson et al.^[2] with modifications concerning the cleavage of the benzyloxycarbonyl protecting group, which was easily achieved in good yields by reaction with trimethylsilyl iodide.

For the preparation of the amide series, the primary amino group in compound **2** was condensed with the appropriate carboxylic acid linker using standard amide coupling proce-

dures. The ester group was then saponified with LiOH in THF or, when epimerization into the *trans*-isomers was required, reacted with sodium methoxide^[3]. Activation of the primary amino function with *N,N'*-carbonyldiimidazole (CDI) followed by the addition of the amine linker yielded the urea derivatives. For synthesis of the corresponding thioureas, 1,1'-thiocarbonyldiimidazole was used. Saponification and epimerization were performed as described in the amide series. Reaction of compound **2** with the appropriate arylsulfonyleurea afforded the sulfonyleurea derivatives, which had to be hydrolysed with aqueous LiOH in THF.



Scheme 1. (a) 1. flash chromatography on silica gel (eluent: CHCl₃) for the separation of *cis*- and *trans*-isomers; 2. trimethylsilyl iodide; MeOH.



Scheme 2. (a) water soluble carbodiimide; CH₂Cl₂; TEA; carboxylic acid linker (b) 1 equiv LiOH; H₂O; THF affords the *cis*-isomer; (c) 5% sodium methoxide; MeOH affords the *trans*-isomer; (d) formation of ureas: CDI; THF; 0 °C; amine linker/formation of thioureas: 1,1'-thiocarbonyldiimidazole; THF; 0 °C; amine linker; (e) arylsulfonyleurea; CH₂Cl₂. For X and R see (Tables 1–3).

Pharmacology

The compounds were tested for their affinity to the glycine site on the NMDA receptor using the [^3H]-5,7-dichlorokynurenic binding assay on rat cortical membranes [4].

Patch clamp studies were performed on cultured superior colliculus neurones [4]. These neurones were obtained from rat embryos, mechanically dissociated in 0.05% DNAase/0.3% ovomucoid following an 8 min preincubation with 0.66% trypsin/0.1% DNAase. The dissociated cells were then centrifuged at 18G for 10 min, resuspended in minimum essential medium and plated at a density of 150,000 cells cm^{-2} onto poly-L-lysine-precoated plastic petri dishes. The cells were nourished with NaHCO_3 /HEPES-buffered minimum essential medium supplemented with 5% foetal calf serum and 5% horse serum and incubated at 37 °C. The medium was exchanged completely following inhibition of further glial mitosis with cytosine- β -D-arabinofuranoside (20 μM) after about 7 days in vitro. Thereafter the medium was exchanged partially twice weekly. Patch clamp recordings were made from these neurones. Only results from stable cells were accepted for inclusion in the final analysis. For further experimental details, see experimental part.

Results and Discussion

The whole series of 2-carboxytetrahydroquinolines including the results of the biological testing is summarized in Tables 1–3. The physical data of compounds 9–26 are given in Table 4.

Compounds 9, 10, and 12 in the amide series confirm the general findings of Leeson et al. [3], describing the *trans*-isomers of 4-substituted 2-carboxytetrahydroquinolines as more active than the corresponding *cis*-isomers. This effect of stereoselectivity on affinity could be also observed in patch clamp recordings throughout the amide series (Table 1). The *trans*-isomer of 10 was the most potent compound with an alicyclic spacer, indicating, that a lipophilic carbon chain enhances the ability of the ligand to interact with the eastern region (bulk tolerance region) of the receptor protein. Compound 11, holding a lipophilic phenyl moiety within the side chain, is equipotent to compound 10. Compound 12 shows a similar affinity to compound 9 although it holds a much stronger tendency towards lipophilicity. The reason for that effect might be the stronger rigidity of compound 12 compared to 9.

The *cis*- and *trans*-isomers in the urea series are in line with the findings by Leeson et al. [3], indicating that long side chains in the eastern region of the ligand enhance stereoselectivity. The stereoselectivity of the *trans*-isomer 18 has been ameliorated over the corresponding *cis*-form by the factor 200. The piperazine moiety seems to be a useful structure to differentiate between the *cis*- and *trans*-isomers in the [^3H]-5,7-DCKA binding assay. Using patch clamp techniques, the IC_{50} values for *cis*- and *trans*-configured compounds are much less discriminative. Examining the *trans*-isomers of compounds 15, 16, and 17, a prolonged side-chain between the phenyl ring and the *tert*-butoxycarbonyl group does not necessarily lead to more active compounds. A maximum in affinity is already reached with compound 15 (C_1 -spacer) whereas compounds 16 and 17

(C_2 - and C_3 -spacer) show less affinity (factor 80 respectively factor 10000). The *trans*-isomers of compounds 15 and 13 [3] show equipotency, but in contrast to 13, compound 15 exhibits a stronger difference in affinity between *cis*- and *trans*-form due to the side chain in the eastern region, which leads to a pronounced stereochemical differentiation. Taking all binding data into account, the piperazine derivative (compound 18) was the most active structure in this series. A C_1 -spacer between the phenyl ring and the piperazine moiety (compound 19) caused a drastic loss in receptor affinity, which may be due to the basicity of the piperazine- N^1 -atom of 19 in contrast to the protolytically neutral one of compound 18. The crown ether derivatives 20 and 21 exhibit a considerable activity. As could be detected by mass spectrometry, the crown ether unit forms a stable complex with sodium or potassium cations thus enforcing the coulombic interactions with the receptor. A possible interaction of the crown ether unit with the receptor protein is depicted in Figure 1. On the other hand, the improved affinities of the crown ether derivatives may be at least partially effected by the added potential of the polyether structure to gain hydrogen-bond-accepting interactions.

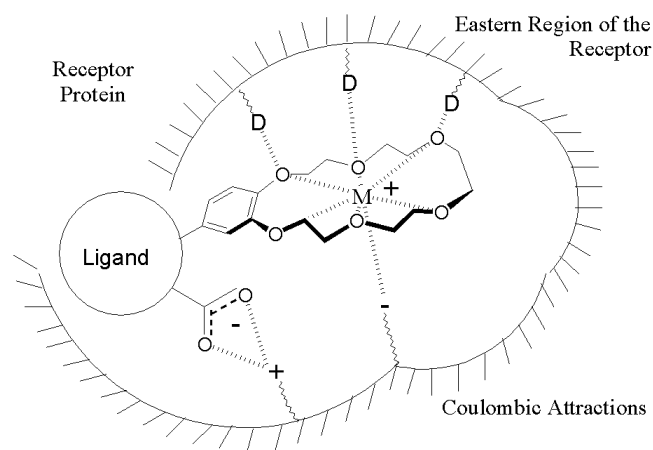


Figure 1. Hypothetical affinity enhancing elements of the crown ether unit. D is a hydrogen bond donor, + is a receptor cation and – a receptor anion.

The thiourea compounds 22 and 23 reflect the general trend in the urea series, showing a C_1 -spacer between the phenyl ring and the carbamate as favourable for binding. The all in all weaker affinity of thiourea derivatives compared to the urea compounds affirms the important requirement of hydrogen bonding adjacent to the 4-position in the heterocycle, which was first indicated by Leeson et al. [3]. We therefore decided to pursue the idea of extensive hydrogen bonding by creating a small series of sulfonylurea derivatives (compounds 24 and 25), which were directly compared with the corresponding urea derivatives, serving as standard compounds in the assay (Table 3). Compound 24 and compound 25 containing the hydrogen-bond-accepting sulfonyl group are even more potent than the highest affinity substance of this class of glycine antagonists, the urea derivative L-

689,560, which is in the form of 4'-[³H]-L-689,560 [5] used as highly specific radioligand for the glycine site.

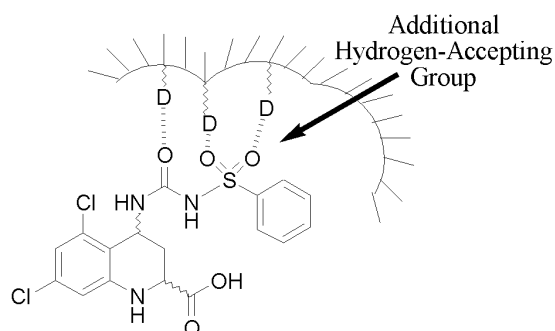
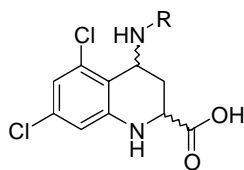


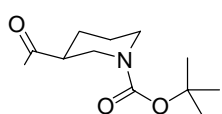
Figure 2. High affinity sulfonyleurea compound, which may be used as a lead for a tritium labeled radioligand or for the design of a PET tracer.

The supplementary structural features which serve to further enhance affinity are depicted in Figure 2.

Compound **24** and compound **25** may be useful structures for the design of novel radioligands and especially the development of PET-tracers, which allow the mapping of the glycine site as well as an imaging of the NMDA-receptor distribution and density under pathological conditions. The ¹⁸F-labeling is now under investigation and will certainly be the topic of further communications.

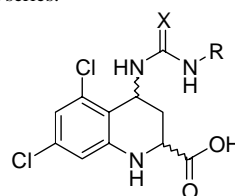
Table 1. Amide series:



Cpd.	R	IC ₅₀ (μM) ^a	
		<i>cis</i> -isomer	<i>trans</i> -isomer
9	CO(CH ₂) ₂ NHCOOC(CH ₃) ₃	6.60 (13.20) ^b	0.41 (2.00) ^b
10	CO(CH ₂) ₅ NHCOOC(CH ₃) ₃	1.50 (3.30) ^b	0.14 (1.00) ^b
11	COPh(CH ₂) ₂ NHCOOC(CH ₃) ₃ -4	–	0.12
12		5.30 (6.90) ^b	0.47 (2.14) ^b

Tables 1–3: a. IC₅₀ values are determined in the [³H]-5,7-DCKA binding assay, using rat cortical membranes. Each value is an average of triplicates. The values of compounds **13** [3] and **26** are included for purposes of comparison. b. The IC₅₀ values gained in patch clamp studies on cultured superior colliculus neurones are indicated in parantheses.

Table 2. Urea/thiourea series:



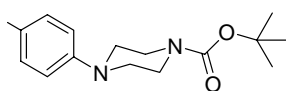
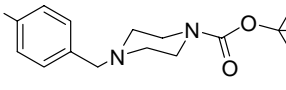
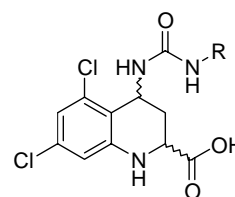
Cpd	X	R	IC ₅₀ (μM) ^a	
			<i>cis</i> -isomer	<i>trans</i> -isomer
13	O	Ph	0.01	0.007 [3]
14	O	Ph(NHCOOC(CH ₃) ₃)-4	0.32 (2.00) ^b	0.008 (0.03) ^b
15	O	Ph(CH ₂) ₂ NHCOOC(CH ₃) ₃ -4	0.440 (1.30) ^b	0.007 (0.05) ^b
16	O	Ph((CH ₂) ₂) ₂ NHCOOC(CH ₃) ₃ -4	–	0.561
17	O	Ph((CH ₂) ₃) ₂ NHCOOC(CH ₃) ₃ -4	–	71.70
18	O		1.22 (4.50) ^b	0.006 (0.10) ^b
19	O		–	0.17
20	O	4-benzo-15-crown-5	–	0.064
21	O	4-benzo-18-crown-6	–	0.18
22	S	Ph(NHCOOC(CH ₃) ₃)-4	–	2.75
23	S	Ph(CH ₂) ₂ NHCOOC(CH ₃) ₃ -4	–	1.19

Table 3. Sulfonyleurea series/standard compounds:



Cpd.	R	IC ₅₀ [μM] ^a
		<i>trans</i> -isomer
24	SO ₂ PhCH ₃ -4	0.0002
25	SO ₂ Ph	0.0001
26	PhCH ₃ -4	0.0018

Table 4. Physical data of the final compounds.

Cpd.	Mp. (°C)	IR (KBr)	¹ H-NMR, δ (ppm), J (Hz)
<i>cis</i> 9	258-259	3370 s (-NH), 2970 s (-CH ₂ -), 1700 s (-C=O), 1635 s (-NHCO)	1.39 (s, 9 H, -C(CH ₃) ₃), 1.92 (m, 1 H, CH _{abcd}), 2.08 (m, 1 H, -CH _{2a} -), 2.31 (dm, 1 H, J = 12.5, CH _{abcd}), 2.46 (m, 1 H, -CH _{2b} -), 3.39 (m, 1 H, -CH _{2c} -), 3.67 (m, 1 H, -CH _{2d} -), 3.74 (m, 1 H, CH _{abcd}), 4.99 (m, 1 H, CH _{abcd}), 6.04 (s, 1 H, NH, exchange), 6.52 (d, 1 H, J = 2.5, Ar-H), 6.58 (d, 1 H, J = 2.5, Ar-H), [DMSO-d ₆ , 200 MHz]
<i>trans</i> 9	260-262	3370 s (-NH), 2970 s (-CH ₂ -), 1700 s (-C=O), 1635 s (-NHCO)	1.32 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.38 (s, 9 H, -C(CH ₃) ₃), 2.24 (m, 2 H, -CH ₂ -), 2.28 (dm, 1 H, J = 12.0, CH _{abcd}), 3.08 (m, 2 H, -CH ₂ -), 3.52 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 4.92 (m, 1 H, CH _{abcd}), 6.51 (d, 1 H, J = 2.5, Ar-H), 6.54 (d, 1 H, J = 5.0, NH, exchange), 6.74 (d, 1 H, J = 2.5, Ar-H), [DMSO-d ₆ , 200 MHz]
<i>cis</i> 10	230-232	3330 m (-NH), 2470 m (-OH), 1660 s (-NHCO), 1640 s (-NHCO)	1.37 (s, 9 H, -C(CH ₃) ₃), 1.42 (m, 2 H, -CH ₂ -), 1.56 (m, 2 H, -CH ₂ -), 1.88-2.05 (m, 4 H, 2x-CH ₂ -), 1.96 (m, 1 H, CH _{abcd}), 2.41 (d, 1 H, J = 12.5, CH _{abcd}), 3.02 (m, 2 H, -CH ₂ -), 3.83 (m, 1 H, CH _{abcd}), 4.96 (m, 1 H, CH _{abcd}), 6.13 (d, 1 H, J = 5.0, NH, exchange), 6.54 (d, 1 H, J = 2.5, Ar-H), 6.58 (d, 1 H, J = 2.5, Ar-H), [DMSO-d ₆ , 200 MHz]
<i>trans</i> 10	233-235	3330 m (-NH), 2470 m (-OH), 1660 s (-NHCO), 1640 s (-NHCO)	1.36 (s, 9 H, -C(CH ₃) ₃), 1.41 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.38 (m, 2 H, -CH ₂ -), 1.52 (m, 2 H, -CH ₂ -), 1.85-2.06 (m, 4 H, 2x-CH ₂ -), 2.28 (dm, 1 H, J = 12.0, CH _{abcd}), 3.05 (m, 2 H, -CH ₂ -), 3.61 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 4.93 (m, 1 H, CH _{abcd}), 6.48 (d, 1 H, J = 5.0, NH, exchange), 6.55 (d, 1 H, J = 2.5, Ar-H), 6.72 (d, 1 H, J = 2.5, Ar-H), [DMSO-d ₆ , 200 MHz]
<i>trans</i> 11	238-240	3380 m (-NH), 2920 s (-CH ₂ -), 1710 s (-C=O), 1670 s (-NHCO)	1.38 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.40 (s, 9 H, -C(CH ₃) ₃), 2.28 (dm, 1 H, J = 12.5, CH _{abcd}), 3.58 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 4.28 (d, 2 H, -CH ₂ -NHCO), 4.89 (m, 1 H, CH _{abcd}), 5.98 (d, 1 H, J = 5.0, NH, exchange), 6.50 (d, 1 H, J = 2.5, Ar-H), 6.52 (d, 1 H, J = 5.0, NH, exchange), 6.58 (d, 1 H, J = 2.5, Ar-H), 7.28 (d, 2 H, J = 5.0, Arom.), 7.65 (d, 2 H, J = 5.0, Arom.), [DMSO-d ₆ , 200 MHz]
<i>cis</i> 12	250-253	3480 m (-NH), 2930 s (-CH ₂ -), 1730 s (-C=O), 1650 s (-NHCO)	0.86 (m, 2 H, -CH ₂ -), 1.36 (m, 2 H, -CH ₂ -), 1.37 (s, 9 H, -C(CH ₃) ₃), 1.64 (m, 2 H, -CH ₂ -), 1.85-2.06 (m, 2 H, -CH ₂ -), 1.96 (m, 1 H, CH _{abcd}), 2.38 (m, 1 H, J = 12.5, CH _{abcd}), 2.72 (m, 1 H, -CH-), 3.77 (m, 1 H, CH _{abcd}), 5.02 (m, 1 H, CH _{abcd}), 6.11 (s, 1 H, NH, exchange), 6.56 (d, 1 H, J = 2.5, Ar-H), 6.62 (d, 1 H, J = 2.5, Ar-H), [DMSO-d ₆ , 200 MHz]
<i>trans</i> 12	255-258	3480 m (-NH), 2930 s (-CH ₂ -), 1730 s (-C=O), 1650 s (-NHCO)	0.82 (m, 2 H, -CH ₂ -), 1.27 (m, 2 H, -CH ₂ -), 1.37 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.39 (s, 9 H, -C(CH ₃) ₃), 1.56 (m, 2 H, -CH ₂ -), 1.95-2.12 (m, 2 H, -CH ₂ -), 2.29 (dm, 1 H, J = 12.0, CH _{abcd}), 2.74 (m, 1 H, -CH-), 3.65 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 4.83 (m, 1 H, CH _{abcd}), 6.58 (d, 1 H, J = 2.5, Ar-H), 6.62 (s, 1 H, NH, exchange), 6.76 (d, 1 H, J = 2.5, Ar-H), [DMSO-d ₆ , 200 MHz]
<i>cis</i> 13	Lit. [3]	data were consistent with literature	
<i>trans</i> 13	Lit. [3]	see above	
<i>cis</i> 14	>260 dec.	3360 s (-NH), 2970 s (-CH ₂ -), 1705 s (-C=O), 1655 s (-NHCO)	1.42 (s, 9 H, -C(CH ₃) ₃), 1.88 (m, 1 H, CH _{abcd}), 2.74 (dm, 1 H, J = 12.5, CH _{abcd}), 3.76 (m, 1 H, CH _{abcd}), 4.96 (m, 1 H, CH _{abcd}), 6.01 (d, 1 H, J = 5.0, NH, exchange), 6.54 (d, 1 H, J = 2.5, Ar-H), 6.58 (d, 1 H, J = 2.5, Ar-H), 6.68 (d, 1 H, J = 5.0, NH, exchange), 7.22-7.35 (m, 4 H, Ar-H), 8.56 (s, 1 H, NH, exchange), [DMSO-d ₆ , 200 MHz]
<i>trans</i> 14	>260 dec.	3360 s (-NH), 2970 s (-CH ₂ -), 1705 s (-C=O), 1655 s (-NHCO)	1.29 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.42 (s, 9 H, -C(CH ₃) ₃), 2.28 (dm, 1 H, J = 12.5, CH _{abcd}), 3.52 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 4.92 (m, 1 H, CH _{abcd}), 6.32 (d, 1 H, J = 5.0, NH, exchange), 6.54 (d, 1 H, J = 2.5, Ar-H), 6.72 (d, 1 H, J = 5.0, NH, exchange), 6.78 (d, 1 H, J = 2.5, Ar-H), 7.26-7.36 (m, 4 H, Ar-H), 8.54 (s, 1 H, NH, exchange), [DMSO-d ₆ , 200 MHz]
<i>cis</i> 15	>260 dec.	3380 s (-NH), 2970s (-CH ₂ -), 1690 s (-C=O), 1655 s (-NHCO)	1.41 (s, 9 H, -C(CH ₃) ₃), 1.94 (m, 1 H, CH _{abcd}), 2.36 (dm, 1 H, J = 12.5, CH _{abcd}), 3.78 (m, 1 H, CH _{abcd}), 3.87 (d, 2 H, -CH ₂ -NHCO-), 4.91 (m, 1 H, CH _{abcd}), 6.04 (d, 1 H, J = 5.0, NH, exchange), 6.57 (d, 1 H, J = 2.5, Ar-H), 6.61 (d, 1 H, J = 2.5, Ar-H), 6.70 (d, 1 H, J = 5.0, NH, exchange), 6.86 (d, 2 H, J = 5.0, Ar-H), 7.30 (d, 2 H, J = 5.0, Ar-H), 8.61 (s, 1 H, NH, exchange), [DMSO-d ₆ , 200 MHz]
<i>trans</i> 15	>260 dec.	3380 s (-NH), 2970s (-CH ₂ -), 1690 s (-C=O), 1655 s (-NHCO)	1.26 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.37 (s, 9 H, -C(CH ₃) ₃), 2.28 (dm, 1 H, J = 12.5, CH _{abcd}), 3.48 (m, 1 H, CH _{abcd}), 3.96 (d, 2 H, -CH ₂ -NHCO-), 4.89 (m, 1 H, CH _{abcd}), 6.31 (s, 1 H, NH, exchange), 6.52 (d, 1 H, J = 2.5, Ar-H), 6.67 (d, 1 H, J = 5.0, NH, exchange), 6.84 (d, 1 H, J = 2.5, Ar-H), 7.16 (d, 2 H, J = 5.0, Ar-H), 7.38 (d, 2 H, J = 5.0, Ar-H), 8.49 (s, 1 H, NH, exchange), [DMSO-d ₆ , 200 MHz]

Table 4. Continued.

Cpd.	Mp. (°C)	IR (KBr)	¹ H-NMR, δ (ppm), J (Hz)
trans 16	>260 dec.	3350 s (-NH), 2970 s (-CH ₂ -), 1685 s (-C=O), 1650 s (-NHCO)	1.36 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.38 (s, 9 H, -C(CH ₃) ₃), 2.29 (dm, 1 H, J = 12.5, CH _{abcd}), 2.58 (t, J = 12.5, -CH ₂ -), 3.08 (t, J = 12.5, -CH ₂ -), 3.68 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 4.93 (m, 1 H, CH _{abcd}), 6.48 (d, 1 H, J = 5.0, NH, exchange), 6.56 (d, 1 H, J = 2.5, Ar-H), 6.62 (d, 1 H, J = 5.0, NH, exchange), 6.74 (d, 1 H, J = 2.5, Ar-H), 7.08 (d, 2 H, J = 8.0, Ar-H), 7.26 (d, 2 H, J = 8.0, Ar-H), 8.07 (s, 1 H, NH, exchange), [DMSO-d ₆ , 90 MHz]
trans 17	>260 dec.	3360s (-NH), 2970 s (-CH ₂ -), 1685 s (-C=O), 1655 s (-NHCO)	1.38 (s, 9 H, -C(CH ₃) ₃), 1.39 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.58 (quin, 2 H, J = 12.5, -CH ₂ -), 2.33 (dm, 1 H, J = 12.5, CH _{abcd}), 2.42 (t, 2 H, J = 12.5, -CH ₂ -), 2.85 (t, 2 H, J = 12.5, -CH ₂ -), 3.67 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 4.97 (m, 1 H, CH _{abcd}), 6.46 (d, 1 H, J = 5.0, NH, exchange), 6.54 (d, 1 H, J = 2.5, Ar-H), 6.63 (d, 1 H, J = 2.5, Ar-H), 7.00 (d, 2 H, J = 10.0, Ar-H), 7.16 (d, 2 H, J = 10.0, Ar-H), 8.16 (s, 1 H, NH, exchange), [DMSO-d ₆ , 90 MHz]
cis 18	>260 dec.	3390 s (-NH), 2970 s (-CH ₂ -), 1685 s (-C=O), 1655 s (-NHCO)	1.35 (s, 9 H, -C(CH ₃) ₃), 1.91 (m, 1 H, CH _{abcd}), 2.36 (dm, 1 H, J = 12.5, CH _{abcd}), 2.93 (t, 4 H, J = 10.0, 2x-CH ₂ -), 3.73 (m, 1 H, CH _{abcd}), 3.82 (t, 4 H, J = 10.0, 2x-CH ₂ -), 4.91 (m, 1 H, CH _{abcd}), 6.06 (d, 1 H, J = 5.0, NH, exchange), 6.52 (d, 1 H, J = 2.5, Ar-H), 6.55 (d, 2 H, J = 6.0, Ar-H), 6.56 (d, 1 H, J = 2.5, Ar-H), 7.13 (d, 2 H, J = 10.0, Ar-H), 7.92 (s, 1 H, NH, exchange), [DMSO-d ₆ , 200 MHz]
trans 18	>260 dec.	3390 s (-NH), 2970 s (-CH ₂ -), 1685 s (-C=O), 1655 s (-NHCO)	1.32 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.39 (s, 9 H, -C(CH ₃) ₃), 2.33 (d, 1 H, J = 12.5, CH _{abcd}), 2.92 (t, 4 H, J = 10.0, 2x-CH ₂ -), 3.76 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 3.89 (t, 4 H, J = 10.0, 2x-CH ₂ -), 4.88 (m, 1 H, CH _{abcd}), 6.32 (d, 1 H, J = 5.0, NH, exchange), 6.50 (d, 2 H, J = 6.0, Ar-H), 6.56 (d, 1 H, J = 2.5, Ar-H), 6.82 (d, 1 H, J = 2.5, Ar-H), 7.08 (d, 2 H, J = 6.0, Ar-H), 7.81 (s, 1 H, NH, exchange), [DMSO-d ₆ , 200 MHz]
trans 19	>260 dec.	3360 s (-NH), 2970 s (-CH ₂ -), 1690 s (-C=O), 1650 s (-NHCO)	1.37 (s, 9 H, -C(CH ₃) ₃), 1.38 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 2.35 (dm, 1 H, J = 12.0, CH _{abcd}), 2.58 (t, 4 H, J = 10.0, 2x-CH ₂ -), 3.09 (t, 4 H, J = 10.0, 2x-CH ₂ -), 3.41 (s, 2 H, -CH ₂ -NR ₂), 3.59 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 4.94 (m, 1 H, CH _{abcd}), 6.52 (d, 1 H, J = 5.0, NH, exchange), 6.55 (d, 1 H, J = 2.5, Ar-H), 6.75 (d, 1 H, J = 2.5, Ar-H), 7.09 (d, 2 H, J = 10.0, Ar-H), 7.37 (d, 2 H, J = 10.0, Ar-H), 8.08 (s, 1 H, NH, exchange), [DMSO-d ₆ , 200 MHz]
trans 20	>260 dec.	3380 s (-NH), 2930 s (-CH ₂ -), 1720 s (-C=O), 1690 s (-NHCO)	1.31 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 2.26 (dm, 1 H, J = 12.5, CH _{abcd}), 3.55 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 3.58 (m, 8 H, 4x-CH ₂ -), 3.70 (m, 4 H, 2x-CH ₂ -), 3.96 (m, 4 H, 2x-CH ₂ -), 4.96 (m, 1 H, CH _{abcd}), 6.41 (d, 1 H, J = 5.0, NH, exchange), 6.56 (d, 1 H, J = 2.5, Ar-H), 6.58-6.82 (m, 3 H, Arom.), 6.75 (d, 1 H, J = 2.5, Ar-H), 8.22 (s, 1 H, NH, exchange), [DMSO-d ₆ , 90 MHz]
trans 21	>260 dec.	3360 s (-NH), 2910 s (-CH ₂ -), 1725 s (-C=O), 1650 s (-NHCO)	1.28 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 2.24 (dm, 1 H, J = 12.5, CH _{abcd}), 3.48 (m, 4 H, 2x-CH ₂ -), 3.53 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 3.55 (m, 8 H, 4x-CH ₂ -), 3.75 (m, 4 H, 2x-CH ₂ -), 4.00 (m, 4 H, 2x-CH ₂ -), 4.95 (m, 1 H, CH _{abcd}), 6.35 (d, 1 H, J = 5.0, NH, exchange), 6.54 (d, 1 H, J = 2.5, Ar-H), 6.55-6.80 (m, 3 H, Arom.), 6.75 (d, 1 H, J = 2.5, Ar-H), 8.18 (s, 1 H, NH, exchange), [DMSO-d ₆ , 90 MHz]
trans 22	>260 dec.	3360 s (-NH), 2910 s (-CH ₂ -), 1725 s (-C=O), 1650 s (-NHCO)	1.29 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.42 (s, 9 H, -C(CH ₃) ₃), 2.28 (dm, 1 H, J = 12.5, CH _{abcd}), 3.52 (dd, 1 H, J = 12.5, 4.5, CH _{abcd}), 4.92 (m, 1 H, CH _{abcd}), 6.32 (d, 1 H, J = 5.0, NH, exchange), 6.54 (d, 1 H, J = 2.5, Ar-H), 6.72 (d, 1 H, J = 5.0, NH, exchange), 6.78 (d, 1 H, J = 2.5, Ar-H), 7.26-7.36 (m, 4 H, Ar-H), 8.54 (s, 1 H, NH, exchange), [DMSO-d ₆ , 90 MHz]
trans 23	>260 dec.	3360 s (-NH), 2970 s (-CH ₂ -), 1690 s (-C=O), 1670 s (-NHCO)	1.26 (ddd, 1 H, J = 12.5, 8.5, 4.5, CH _{abcd}), 1.37 (s, 9 H, -C(CH ₃) ₃), 2.28 (dm, 1 H, J = 12.5, CH _{abcd}), 3.48 (m, 1 H, CH _{abcd}), 3.96 (d, 2 H, -CH ₂ -NHCO-), 4.89 (m, 1 H, CH _{abcd}), 6.31 (s, 1 H, NH, exchange), 6.52 (d, 1 H, J = 2.5, Ar-H), 6.67 (d, 1 H, J = 5.0, NH, exchange), 6.84 (d, 1 H, J = 2.5, Ar-H), 7.16 (d, 2 H, J = 5.0, Ar-H), 7.38 (d, 2 H, J = 5.0, Ar-H), 8.49 (s, 1 H, NH, exchange), [DMSO-d ₆ , 90 MHz]
trans 24	>260 dec.	3400 s (-NH), 3030, m (-OH), 1660 s (-CONH), 1350 m (-SO ₂ -)	1.58 (ddd, 1 H, J = 13.3, 12.5, 3.7, CH _{abcd}), 2.08 (dm, 1 H, J = 13.3, CH _{abcd}), 2.38 (s, 1 H, Ar-CH ₃), 3.76 (d, 1 H, J = 10.6, CH _{abcd}), 4.70 (s, 1 H, NH), 4.72 (m, 1 H, CH _{abcd}), 6.64 (d, 1 H, J = 1.6, Ar-H), 6.80 (s, 2 H, NH), 6.69 (d, 1 H, J = 1.6, Ar-H), 7.58 (d, 2 H, J = 8.0, Ar-H), 7.77 (d, 2 H, J = 8.0, Ar-H), [DMSO-d ₆ , 400 MHz]
trans 25	>260 dec.	3400 s (-NH), 3000 s (-OH), 1720 s (-C=O), 1650 s (-NHCO)	1.57 (ddd, 1 H, J = 13.2, 12.6, 3.5, CH _{abcd}), 2.07 (dm, 1 H, J = 12.7, CH _{abcd}), 3.78 (dd, 1 H, J = 12.0, 2.8, CH _{abcd}), 3.80 (s, 1 H, NH), 4.72 (m, 1 H, CH _{abcd}), 6.63 (d, 1 H, J = 1.9, Ar-H), 6.77 (s, 1 H, NH), 6.89 (d, 1 H, J = 1.9, Ar-H), 7.59 (m, 3 H, Ar-H), 7.69 (s, 1 H, NH), 7.90 (m, 2 H, Ar-H), [DMSO-d ₆ , 400 MHz]
trans 26	Lit. [3]	data were consistent with the literature	

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Experimental Part

Chemistry

Melting points were measured on a Büchi apparatus (Dr. Tottoli) and are uncorrected. IR spectra (KBr) were recorded on a Beckmann IR Model 4220 or a Perkin-Elmer 1310 spectrophotometer. ¹H NMR spectra were obtained on a Bruker WH 90 (90 MHz), a Bruker AC-200 (200 MHz) or a Bruker AC-400/Bruker ARX-400 (400 MHz) and were consistent with proposed structures. Chemical shifts are described in parts per million. Tetramethylsilane was used as internal standard. Coupling constants (*J*) are reported in hertz. Although the MS (EI, FD, FAB) spectra are not included, they were obtained for all compounds and were consistent with the assigned structures. Thin-layer chromatography (TLC) was carried out with E. Merck silica gel 60 F₂₅₄ plates. Yields are reported in per cent from their theoretically calculated value.

Cis/trans-4-amino-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-ethyl-carboxylate *N-Vinylbenzylloxycarbamate*^[6]

Freshly distilled acryloyl chloride (30.0 mL, 369 mmol) diluted with 90 mL of dry toluene was added dropwise to an ice-cold solution of sodium azide (28.74 g, 441 mmol) in 150 mL deionized water. This mixture was vigorously stirred at 0 °C for 5 h. The organic layer was separated, washed with 30 mL of 10% aqueous sodium carbonate, deionized water and then dried over magnesium sulfate.

The anhydrous acryloyl azide solution was added dropwise (!) to a stirred solution composed of hydroquinone (1.83 g, 16.56 mmol), pyridine (180 mL, 22.26 mmol) and benzyl alcohol (45.9 mL, 444 mmol). After the addition, the mixture was stirred for 30 min at 110 °C. The toluene was evaporated under vacuum and the product, a colourless liquid, isolated via distillation (bp 135–137 °C/0.1 Torr). Yield: 50%.

Ethyl Glyoxylate^[7]

Ethyl diethoxyacetate (25.8 g, 147 mmol), glyoxylic acid monohydrate (13.0 g, 142 mmol) and *p*-toluenesulfonic acid monohydrate (200 mg) were heated at 90 °C for 27 h with an air condenser. Afterwards the mixture was cooled with ice-methanol and vigorously stirred while slowly adding phosphorus pentoxide (18.0 g, 126.8 mmol). Then the reaction mixture was heated for another 2 h to 90–100 °C. The product was isolated by distillation under vacuum (bp. 70 °C/0.1 Torr). Yield: 85–90%.

Cis/trans-4-benzylloxycarbonylamino-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-ethylcarboxylate^[2]

3,5-Dichloroaniline (20.0 g, 123 mmol) was dissolved in 800 mL dry dichloromethane and stirred for 15 min at room temperature over magnesium sulfate (20 g). After the addition of freshly prepared ethyl glyoxylate (14.0 g, 137 mmol) the mixture was stirred for another 30 min. The magnesium sulfate was filtered off and *N*-vinylbenzylloxycarbamate (25.0 g, 141 mmol) was added to the resulting solution. The mixture was cooled to 10 °C under an atmosphere of nitrogen and a catalytic quantity of boron trifluoride diethyl ether complex (2.12 g, 15 mmol) was slowly injected through a septum. The solution colour turned into a bright orange and the reaction was completed within 1 h at room temperature. The solvents were removed and the resulting dark red oil purified by column chromatography (silica gel 60 Merck 7734; 0.063–0.2 mm) using chloroform as eluent. The first fraction contained the *trans*-isomer, the second fraction the *cis*-isomer confirmed by NMR techniques. Yield: 30% (*cis:trans* 3:1).

Cis/trans-4-amino-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-ethyl-carboxylate Hydrochloride

Cis- or *trans*-benzylloxycarbonylamino-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-ethylcarboxylate (4.23 g, 999 μmol) was dissolved in dry CH₂Cl₂ and cooled under nitrogen to 0 °C. Trimethylsilyl iodide (2.20 g, 11.0 mmol) was slowly added through a septum and the reaction mixture turned into a suspension. After 4 h at room temperature, 10 mL methanol were added dropwise until the reaction mixture cleared up. The product was purified by precipitation of the corresponding hydrochloride using ethyl acetate presaturated with HCl. Yield: 50–70%.

Liberation of the Free Base

The corresponding hydrochloride (1 mmol) was dissolved in 10 mL water and alkalinised by addition of concentrated ammonia. The free base had to be extracted immediately with diethyl ether (3 x 50 mL). The organic layers were collected and dried over magnesium sulfate. After filtration and evaporation of the solvent, the free base resulted as a clear, pale yellow oil, which was unstable during storage.

A) *Amide Route*

Cis- or *trans*-4-amino-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-ethyl-carboxylate (289 mg, 1 mmol) and 1.2 mmol of the carboxylic acid linker were dissolved in 20 mL dry dichloromethane under an atmosphere of nitrogen. *N'*-(Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (1.2 mmol) dissolved in 10 mL dry dichloromethane was added followed by triethylamine (1.2 mmol). The reaction mixture was stirred for 12 h at room temperature under nitrogen. After the addition of 20 mL dichloromethane, the mixture was purified by acid-base treatment. The organic layer was dried over magnesium sulfate and evaporated to dryness. The product was crystallized from diethyl ether. Yield: 75%

Saponification without Epimerization

Cis- or *trans* product in **A** (1 mmol) was dissolved in 5 mL THF, diluted with 1 mL water and reacted with 0.5 mL aqueous 1 M LiOH. The mixture was stirred for 6 h at room temperature and further diluted with 5 mL water. The aqueous solution was washed with 5 mL ethyl acetate. The organic layer was discarded and the aqueous solution acidified with citric acid. After exhaustive extraction with ethyl acetate, the organic layers were dried over magnesium sulfate and concentrated. The residue was crystallized from diethyl ether. Yield: 70–80%.

Saponification with Epimerization

The *cis*-product in **A** (1 mmol) was suspended in 4 mL of sodium methoxide (5% in methanol). The mixture was stirred for 24 h at room temperature. After the reaction had completed, the solution was reduced to a volume of 2 mL, diluted with 5 mL water and acidified with citric acid. Exhaustive extraction with ethyl acetate, drying over magnesium sulfate and evaporation to dryness yielded the solid *trans*-product, which was triturated with diethyl ether. Yield: 70–80%.

B) *Urea/thiourea Route*

1,1'-Carbonyldiimidazole (325 mg, 2 mmol) was dissolved in 20 mL dry freshly distilled THF and cooled to 0 °C under N₂. *Trans*- or *cis*-4-amino-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-ethyl-carboxylate (580 mg, 2 mmol) dissolved in 5 mL dry freshly distilled THF were added dropwise (5 mL/30 min). After the reaction had completed, 2 mmol of the amine linker dissolved in 10 mL dry THF were added at room temperature and stirred overnight. The reaction mixture was evaporated in vacuo and the residue dissolved in 30 mL ethyl acetate. After a washing procedure (3% hydrochloric acid, saturated sodium carbonate solution, brine) the organic layer was dried over magnesium sulfate, filtered, concentrated and the resulting product crystallized from 10 mL diethyl ether. Recrystallization from methanol gave the pure product. Yield 65–70%.

For the formation of the thioureas 1,1'-thiocarbonyldiimidazole was used instead of CDI.

Saponification with or without epimerization was performed as described for **A**.

C) Sulfonylurea Route

Trans-4-amino-5,7-dichloro-1,2,3,4-tetrahydroquinoline-2-ethylcarboxylate (300 mg, 1.0 mmol) and the corresponding isocyanate (1.0 mmol) were dissolved in dry dichloromethane. The mixture was refluxed for 6 h and the solvent removed under reduced pressure. The residue was dissolved in ethyl acetate and washed successively with 5% hydrochloric acid (3 × 50 mL), saturated sodium carbonate (1 × 50 mL) and brine (1 × 50 mL). The organic layer was dried, filtered and evaporated. The resulting residue was washed with diethyl ether. Yield: 80–85%.

Saponification was performed as described in A (Saponification without epimerization).

The amine linkers, which were not commercially available were prepared as follows:

N-tert-Butyloxycarbonyl-(4-aminophenyl)methylamine

p-Nitrobenzylamine hydrochloride (1.88 g, 10 mmol) was suspended in 20 mL dry CH₂Cl₂. 10 mL Triethylamine were added to the suspension and stirring continued until the suspension turned into a clear solution. Di-*tert*-butyl-dicarbonate (2.4 g, 11 mmol) dissolved in 10 mL CH₂Cl₂ was slowly added. After 12 h at room temperature, the mixture was poured into 20 mL 1 M hydrochloric acid. The organic layer was washed with 20 mL 2 M hydrochloric acid, neutralized with sodium hydrogencarbonate solution, dried and evaporated. The residue was dissolved in 40 mL methanol and 10% Pd on carbon (200 mg) were added. The hydrogenation was performed at room temperature (4 h/2 bar H₂). The catalyst was filtered off, the solvents evaporated and purification performed by flash chromatography on silica gel (dichloromethane/diethyl ether 9:1). The product was crystallized from petroleum ether (bp 40–60 °C). Yield: 82% of yellow crystals (mp 76–78 °C).

β -*N*-tert-Butyloxycarbonyl-(4-aminophenyl)ethylamine

2-(4-Aminophenyl)-ethylamine (1.36 g, 19 mmol) was dissolved in 20 mL dry dichloromethane under addition of 10 mL dry triethylamine. To this solution di-*tert*-butyldicarbonate (2.18 g, 10 mmol) dissolved in 10 mL dry dichloromethane was added in small portions. After 12 h at room temperature the reaction mixture was poured into 20 mL 0.01 M hydrochloric acid. After separation the organic layer was again washed with 0.01 M hydrochloric acid, neutralized with saturated hydrogencarbonate, dried and evaporated to dryness under reduced pressure. The product was crystallized from petroleum ether (bp 40–60 °C). Yield: 90% of a yellow powder (mp 83–85 °C).

γ -*N*-tert-Butyloxycarbonyl-(4-aminophenyl)propylamine

3-Phenylpropyl bromide (20 g, 0.1 mol) was dissolved in 50 mL dry dichloromethane and ammonium nitrate (8 g, 0.1 mol) was added. Tri-fluoroacetic anhydride (TFAA) (10 mL) was dropped to the solution under vigorous stirring at 10 °C. Stirring was continued at room temperature until the inorganic salt went into solution. The reaction mixture was then poured on ice, the organic layer separated and the aqueous solution extracted with 100 mL dichloromethane. The combined organic layers were washed with saturated sodium hydrogencarbonate solution, dried and evaporated. The resulting yellow oil was dissolved in 100 mL dimethylformamide and potassium phthalimide (23 g, 0.12 mol) was added. The reaction was heated for 2 h under reflux, cooled to room temperature and poured into 200 mL of a saturated ammonium chloride solution. The product was extracted with ethyl acetate/diethyl ether 1:1 (3 × 150 mL) and the organic layers were washed with 5% hydrochloric acid, saturated sodium hydrogencarbonate, brine and afterwards dried and evaporated. The crude intermediate was redissolved in 100 mL ethanol, boiled for a short period of time and cooled. The resulting crystals were composed of 3-(4-nitrophenyl)-propylphthalimide. Yield: 50%. Crude 3-(4-nitrophenyl)-propylphthalimide (0.05 mol) was dissolved in ethanol under addition of hydrazine hydrate (3 g). The mixture was refluxed for 6 h, cooled, acidified with 20 mL concentrated hydrochloric acid and refluxed for another 2 h. After cooling, the precipitate composed of phthalhydrazide was separated and the acidic solution was evaporated under reduced pressure to dryness. The residue was poured into 100 mL water, adjusted to pH 13–14 using sodium hydroxide and extracted with diethyl ether (3 × 100 mL). The dried organic layers were evaporated to a small volume and purified by flash chromatography on silica gel using

dichloromethane/methanol 9:1 (saturated with concentrated ammonia) as eluent. The isolated brown oil was Boc-protected and hydrogenated as described for *N*-tert-butyloxycarbonyl-(4-aminophenyl)-methylamine. Flash chromatography on silica gel and crystallization from petroleum ether yielded a yellow crystal powder (mp 76–78 °C).

N-tert-Butyloxycarbonyl-(*N'*-4-aminophenyl)-piperazine

The synthesis was performed as described for *N*-tert-butyloxycarbonyl-(4-aminophenyl)-methylamine using 1-(4-nitrophenyl)-piperazine as educt.

N-tert-Butyloxycarbonyl-(*N'*-4-aminobenzyl)-piperazine

4-Nitrobenzyl bromide (5 g, 0.023 mol) was dissolved in 50 mL dry ethanol under addition of three equivalents (0.7 mol) anhydrous piperazine. The reaction mixture was heated under reflux conditions for 1 h and then evaporated to dryness. The residue was purified by flash chromatography on silica gel using dichloromethane/methanol 9:1 (saturated with concentrated ammonia) as eluent. The main fraction, which turned with iodoplatinate reagent into purple, was collected and crystallized from 10 mL diisopropyl ether as yellow solid suitable for use in the following step.

The yellow solid was now suspended in 50 mL dry dichloromethane. After the addition of 10 mL anhydrous triethylamine, the suspension turned into a clear solution. Di-*tert*-butyl-dicarbonate (0.02 mol) was dissolved in 10 mL dichloromethane and added dropwise. The reaction and workup were performed as described for *N*-tert-butyloxycarbonyl-(4-aminophenyl)methylamine. The residue was dissolved in 40 mL methanol under addition of 10% Pd on carbon (500 mg) and hydrogenated for 4 h without pressure. The catalyst was filtered off, the filtrate evaporated under reduced pressure and purified by flash chromatography on silica gel (dichloromethane/diethyl ether 9:1). The product was crystallized from petroleum ether (bp 40–60 °C) as a yellow crystal powder (mp 95–97 °C). Yield: 55%.

Pharmacology

Tissue Preparation

Tissue preparation was performed according to Foster and Wong^[8]. Male Sprague-Dawley rats (200–250 g) were decapitated and their brains were removed rapidly. The cortex was dissected and homogenised in 20 volumes of ice-cold 0.32 M sucrose using a glass-Teflon homogeniser. The homogenate was centrifuged at 1000×g for 10 min. The pellet was discarded and the supernatant centrifuged at 20,000×g for 10 min. The resulting pellet was resuspended in 20 volumes of distilled water and centrifuged for 20 min at 8000×g. Then the supernatant and the buffy coat were centrifuged three times (48,000×g for 20 min) in the presence of 50 mM Tris-HCl, pH 8.0. All centrifugation steps were carried out at 4 °C. After resuspension in 5 volumes of 50 mM Tris-HCl, pH 8.0 the membrane suspension was frozen rapidly at –80 °C. On the day of assay the membranes were thawed and washed four times by resuspension in 50 mM Tris-HCl, pH 8.0 and centrifugation at 48,000×g for 20 min. The final pellet was suspended in assay buffer. The amount of protein in the final membrane preparation was determined according to the method of Lowry^[9] with some modifications of Hartree^[10]. The final protein concentration used for the studies was between 250–500 µg/ml.

[³H]-5,7-DCKA Binding Assay

Incubations were performed according to the methods modified from previous groups (Yoneda et al.,^[11]). Membranes were suspended and incubated in 50 mM Tris-HCl, pH 8.0 for 45 min at 4 °C with a fixed [³H]-5,7-DCKA concentration of 10 nM. Non-specific binding was defined by the addition of unlabeled glycine at 1 mM. Incubations were terminated using a Millipore filter system. The samples, all in duplicate, were rinsed three times with 2.5 mL ice cold assay buffer over glass fibre filters obtained from Schleicher & Schuell under a constant vacuum. Filtration was performed as rapidly as possible. Following separation and rinse the filters were placed into scintillation liquid (5 mL; Ultima Gold) and radioactivity retained on the filters was determined with a conventional liquid scintillation counter (Hewlett Packard, Liquid scintillation Analyser).

Patch Clamp Studies

Patch clamp studies were performed with polished glass electrodes (4–6 mΩ) in the whole cell mode at room temperature (20–22 °C). The contents of the intracellular solution were as follows (mM): CsCl (120), TEACl (20), EGTA (10), MgCl₂ (1), CaCl₂ (0.2), glucose (10), ATP (2), cAMP (0.25); pH was adjusted to 7.3 with CsOH or HCl. The extracellular solutions had the following basic composition (mM): NaCl (140), KCl (3), CaCl₂ (0.2), glucose (10), HEPES (10), sucrose (4.5), tetrodotoxin (TTX 3×10⁻⁴).

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