Bicyclic σ receptor ligands by stereoselective Dieckmann analogous cyclization of piperazinebutyrate†

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Starting from racemic 2-aminoadipic acid (**6**) the piperazinedione **10** with a butyrate side chain was synthesized in four reaction steps. The four-carbon bridge was established upon deprotonation of **10** with LHMDS and subsequent trapping of the lithium alcoholate with Me_3SiCl to give diastereoselectively the mixed methyl silyl ketal **12** in 94% yield. The relative configuration of the new center of chirality was determined by X-ray crystal structure analysis of **12**. The high diastereoselectivity during the conversion of the butyrate **10** into the mixed methyl silyl ketal **12** supports the formation of the lithium alcoholate **11** as central intermediate. Stereoselective reduction of the ketone **13** with $LiBH_4$ led to the alcohol **14,** which was benzylated and reduced to provide the final bicyclic products **16** and **17**. Whereas the alcohol **16** shows only moderate affinity to both σ receptor subtypes, the benzyl ether **17** represents a potent and selective σ_1 receptor ligand ($K_i = 47$ nM). Comparison of the σ receptor affinities of **16** and **17** with the smaller homologues **5a** and **5c** clearly indicates that the size of the bridge and the O-substituent determines subtype selectivity.

Introduction

The restriction of conformational flexibility of molecules is a general strategy for the improvement of affinity and selectivity during the development of novel drugs. The energy, which is produced during the interaction of a ligand with its target protein, is considerably increased with conformationally constrained ligands. This phenomenon originates from the entropy, since flexible ligands lose freedom during interaction with target proteins. Therefore, a ligand, which is forced by conformational constraints into the bioactive conformation of a related flexible ligand, shows considerably higher affinity than the more flexible analogue.[1]

We are interested in conformationally constrained piperazine derivatives, which will be developed as selective κ-opioid receptor agonists and σ ligands, since the piperazine scaffold is a common structural element of some very potent κ agonists[2,3] and σ ligands.[4,5] In example the 1,4-disubstituted piperazine derivatives **1**[4] and **2**[5] represent very potent ligands for the σ_1 receptor. (Fig. 1)

Very recently we have reported on the synthesis and κ receptor affinity of piperazine derivatives with an additional three-carbon-bridge. In the resulting bicyclic systems the pharmacophoric elements are fixed in a definite orientation to each other.[6,7] In the field of σ receptor ligands the flexible hydroxymethyl group of the potent σ_1 receptor ligands **3**[8] was also incorporated into a conformationally constrained bicyclic system. Depending on the stereochemistry and the substitution pattern **4** and **5** represent potent σ_1 and/or σ_2 ligands.[9,10]

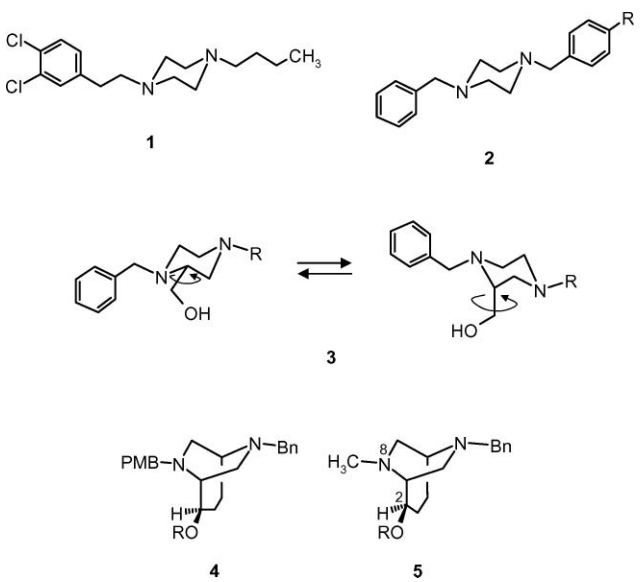

Fig. 1 Piperazines without and with conformational restriction showing high σ receptor affinity.

Whereas the alcohol **4a** (R = H) and the methyl ether **4b** (R = CH_3) with (1*R*,2*R*,5*S*)-configuration are selective σ_1 receptor ligands with K_i values of 6.5 nM and 26 nM ($\sigma_1 : \sigma_2 = 124$ and 22)[9] the corresponding alcohol **5a** (R = H) with (1*R*,2*R*,5*S*)-configuration and a N-8 methyl moiety does not interact significantly with both σ receptor subtypes. The methyl ether **5b** (R = CH_3) prefers the σ_2 subtype (K_i (σ_2) = 1350 nM; (K_i (σ_2) = 327 nM) and after introduction of a benzyl group (**5c**, R = Bn) the affinity to both subtypes was dramatically increased leading to a potent but

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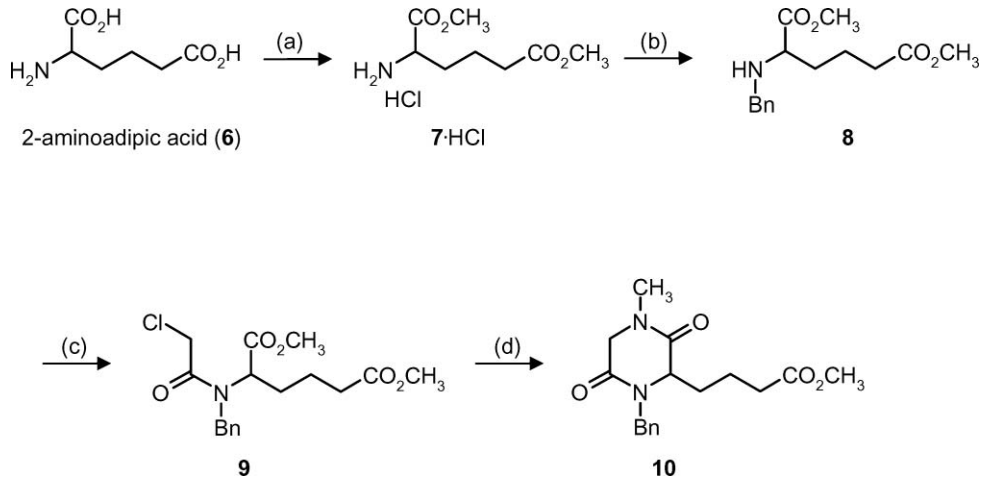


Scheme 1 Synthesis of the dioxopiperazine **10** with butyrate side chain. *Reagents and conditions*: (a) Me_3SiCl, MeOH, 18 h, rt, 99%. (b) 1. C_6H_5CHO, CH_2Cl_2, NEt_3, $MgSO_4$, rt, 3.5 h; 2. $NaBH_4$, MeOH, rt, 4 h. (c) $ClCOCH_2Cl$, NEt_3, CH_2Cl_2, rt, 4 h, 51% (over three steps). (d) H_3CNH_2, CH_3CN, rt, 16 h, 55%.

unselective σ ligand ((K_i (σ_1) = 16 nM; (K_i (σ_2) = 30 nM) (compare Table 1).[10]

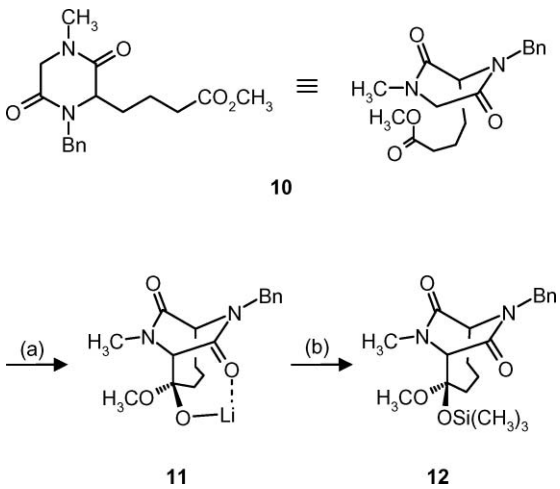
Herein we report on the expansion of the three-carbon bridge of the 6,8-diazabicyclo[3.2.2]nonane framework of **4** and **5** to a four-carbon-bridge and the σ_1 and σ_2 receptor affinities of the resulting 7,9-diazabicyclo[4.2.2]decanes. The influence of this bridge expansion on the σ_1 and σ_2 receptor affinity is initially investigated with racemic mixtures. Racemic mixtures contain both enantiomers and so all stereoisomers will be included in the study. After detection of potent ligands in a new compound class (*e.g.* 7,9-diazabicyclo[4.2.2]decanes), the particular enantiomers will be synthesized separately in a second optimization step.

Results and discussion

Chemistry

The synthesis of the novel 7,9-diazabicyclo[4.2.2]decane ring system started with esterification of racemic 2-aminoadipic acid (**6**) with methanol and chlorotrimethylsilane to give HCl salt of diester **7**.[11] Reaction of primary amine **7** with benzaldehyde afforded an imine which was subsequently reduced with $NaBH_4$ to yield the monobenzylamine **8**. Acylation of **8** with chloroacetyl chloride led to chloroacetamide **9**. Heating of chloroacetamide **9** with methylamine provided in a one-pot procedure the dioxopiperazinebutyrate **10** in 55% yield. In the first step an S_N2 reaction of **9** with methylamine led to a secondary amine, which upon intramolecular aminolysis formed the dioxopiperazinebutyrate **10**. (Scheme 1)

The classical Dieckmann cyclization of the corresponding propionates[12,13] and acetates[14] of dioxopiperazines was not successful, since the corresponding enolates of the resulting β-dicarbonyl products contain a *trans*-configured double bond in a small cyclic substructure, which is forbidden according to Bredt's rule.[15,16] Similarly butyrate **10** should not cyclize under normal Dieckmann cyclization conditions, although the *trans*-configured enolate of the corresponding bicyclic β-dicarbonyl compound **13** is located within an eight-membered ring, which is allowed according to Bredt's rule. Nevertheless, the recently described variation of the Dieckmann cyclization,[12-14] which makes use of trapping the first intermediate hemiketal lithium salt **11** with chlorotrimethylsilane (TMSCl) was applied, because theoretical calculations have shown that piperazinediones with a four-carbon bridge are almost as stable as piperazinediones with a three-carbon bridge.[14] (Scheme 2)



Scheme 2 Dieckmann analogous cyclization of **10**: *Reagents and conditions*: (a) LHMDS, THF, 0.5 h, –78 °C. (b) Me_3SiCl, 2 h, –78 °C, warmed to rt, 1 h, 94%.

For this purpose, the piperazinebutyrate **10** was treated with LHMDS at –78 °C and after 30 min TMSCl was added. After flash chromatography purification the mixed methyl silyl ketal **12** was isolated in 94% yield as single diastereomer. Since the relative configuration of the newly formed center of chirality could not be assigned unambiguously by NMR spectroscopy an X-ray crystal structure analysis of the crystalline ketal **12** was performed after recrystallization with iPr_2O.

In Fig. 2 the X-ray crystal structure of **12** is displayed. It is clearly shown that the siloxy group is directed to the carbonyl moiety in

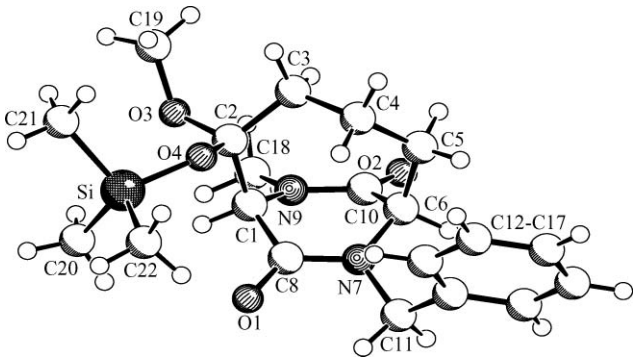
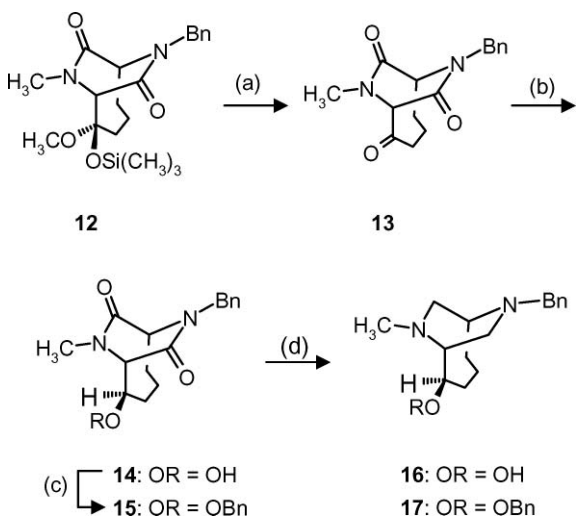


Fig. 2 X-ray crystal structure analysis of the mixed methyl silyl ketal **12**.

position 8. This configuration supports our hypothesized reaction path, which postulates the Li-chelate **11** as driving force for this cyclization. (Scheme 2) A coordination of Li^+ with the other carbonyl O-atom in position 10 would result in a less favorable seven-membered ring.

Since the novel 7,9-diazabicyclo[4.2.2]decane scaffold of **12** represents an interesting system for the synthesis of conformationally constrained σ receptor ligands the mixed methyl silyl ketal **12** was further preceded. At first the ketal **12** was hydrolyzed with diluted HCl to provide the ketone **13** in 97% yield. (Scheme 3) Reduction of the ketone **13** with $LiBH_4$ at −78 °C took place with high diastereoselectivity giving exclusively the alcohol **14**. Comparison of NMR data, tlc behavior and reaction path with those of the corresponding smaller homologues[9,10] proved unequivocally the relative configuration of the alcohol **14**. Benzylation of **14** with benzyl bromide led to the benzyl ether **15** and final $LiAlH_4$ reduction of the alcohol **14** and the benzyl ether **15** provided the basic bicyclic systems **16** and **17**, respectively.



Scheme 3 *Reagents and conditions*: (a) HCl, THF–H_2O, rt, 17.5 h, 97%. (b) $LiBH_4$, THF, −78 °C, 3 h, 99%. (c) NaH, BnBr, DMF, rt, 1 h, 82%. (d) $LiAlH_4$, THF, reflux, 15–16 h, 67% (**16**), 59% (**17**).

σ Receptor binding studies

Since σ receptor ligands have a great potential in the treatment of neuropsychiatric disorders[17,18] and in the diagnosis and therapy of tumors[19,20] the σ_1 and σ_2 affinities of **16** and **17** were evaluated in receptor binding studies with radioligands.

Table 1 Comparison of σ affinities of piperazines with a three-carbon bridge (**5**) and with a four-carbon bridge (**16**, **17**)

		$K_i \pm$ SEM/nM ($n = 3$)		
		σ_1	σ_2	
Compound	R	(^{3}[H]-(+)-pentazocine)	(^{3}[H]-di-*o*-tolylguanidine)	σ_1/σ_2 selectivity
5a[10]	H	>2 000	>2 000	—
5c[10]	$CH_2C_6H_5$	16 ± 5.6	30 ± 3.6	2
16	H	2 000	644 ± 23 nM	0.33
17	$CH_2C_6H_5$	47 ± 3.2 nM	754 ± 43 nM	16
(+)-pentazocine		42 ± 1.1	—	—
di-*o*-tolylguanidine		61 ± 18	42 ± 17	0.7

In the σ_1 assay membrane preparations of guinea pig brains were used as receptor material and [^{3}H]-(+)-pentazocine as radioligand. The non-specific binding was determined in the presence of a large excess of non-tritiated (+)-pentazocine. Homogenates of rat liver served as source for σ_2 receptors in the σ_2 assay. Since a σ_2 selective radioligand is not commercially available, the non-selective radioligand [^{3}H]-di-*o*-tolylguanidine was employed in the presence of an excess of non-radiolabeled (+)-pentazocine for selective masking of σ_1 receptors. An excess of non-tritiated di-*o*-tolylguanidine was used for determination of the non-specific binding.[21,22]

In Table 1 the σ_1 and σ_2 receptor affinities of alcohol **16** and benzyl ether **17** are compared with the σ affinities of the lead compounds **5a** and **5c**. The alcohol **16** shows low affinity at both σ_1 and σ_2 receptors with a slight preference for the σ_2 subtype. However, the σ affinities of the alcohol **16** are higher than the σ affinities of the smaller homologue **5a**, which is almost inactive at both σ receptor subtypes. Introduction of an O-benzyl group in **17** increases the σ_1 receptor affinity enormously (K_i = 47 nM) but does not change the σ_2 affinity. This result indicates that the O-substituent influences the subtype selectivity. A similar observation was made with the smaller homologue **5c**. Here the introduction of the O-benzyl group leads to a very potent σ_1 and σ_2 receptor ligand. In contrast to the bicyclic benzyl ether **5c** with a three-carbon bridge, which shows a slight preference for the σ_1 subtype, the homologous benzyl ether **17** with a four-carbon bridge displays remarkable σ_1 selectivity ($\sigma_1 : \sigma_2 = 16$). Obviously the size of the bridge controls the subtype selectivity together with the O-substituent.

Conclusion

The stepwise Dieckmann cyclization making use of trapping of the first intermediate lithium alcoholate **11** with Me_3SiCl was successfully applied on the synthesis of piperazine derivatives with a four-membered bridge. The increased conformational freedom of the butyrate side chain compared with propionate and acetate side chains does not inhibit this bridging reaction of the piperazinedione scaffold. The configuration of the resulting mixed methyl silyl ketal **12**, which was unambiguously proved by an X-ray crystal structure analysis, supports the hypothesis of the lithium chelate **11** being the driving force of this Dieckmann analogous cyclization. Whereas introduction of an O-benzyl group

into piperazine derivatives with a three-carbon bridge (**5c**) resulted in a very potent but unselective σ ligand, the corresponding benzyl ether **17** with a four-carbon bridge represents a potent and selective σ_1 receptor ligand. Obviously the size of the bridge has a considerable influence on the σ receptor subtype selectivity.

Experimental

General Chemistry

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: eluent, diameter of the column, height of the column packed, fraction size, R_f value. Melting point: Melting point apparatus SMP 3 (Stuart Scientific), uncorrected. MS: MAT GCQ (Thermo-Finnigan), EI (electron impact); Thermo Finnigan LCQ® ion trap mass spectrometer with an ESI (electrospray ionization) interface, Exact mass (ESI): MicroTof (Bruker Daltonics) Finnigan MAT 4200s. IR: IR spectrophotometer 480Plus FT-ATR-IR (Jasco). ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz): Mercury-400BB spectrometer (Varian); δ in ppm related to tetramethylsilane; coupling constants are given with 0.5 Hz resolution. The assignment of ^{1}H and ^{13}CNMR signals was supported by various 2D techniques including H/H-COSY and GHSQC two dimensional NMR techniques. HPLC: Merck Hitachi Equipment; UV detector: L-7400; autosampler: L-7200; pump: L-7100; degasser: L-7614; Method A: column: LiChrospher® 60 RP-select B (5 μm), 250-4 mm; flow rate: 1.00 mL min^{-1}; injection volume: 5.0 μL; detection at λ = 210 nm; solvents: A: water with 0.05% (v/v) trifluoroacetic acid; B: acetonitrile with 0.05% (v/v) trifluoroacetic acid: gradient elution: (A%): 0–4 min: 90%, 4–29 min: 90% to 0%, 29–31 min: 0%, 31–31.5 min: 0% to 90%, 31.5–40 min: 90%. Method B: LiChrospher® 60 RP-select B (5 μm), 250-4 mm; flow rate: 1.00 mL min^{-1}; injection volume: 5.0 μL; detection at λ = 210 nm; solvents: A: water with 0.05% (v/v) trifluoroacetic acid, B: methanol with 0.05% (v/v) trifluoroacetic acid: gradient elution: (A%): 0–1 min: 80%, 1–22 min: 80% to 0%, 22–30 min: 0%, 30–31.5 min: 0% to 80%, 31.5–40 min: 80%.

Dimethyl (*RS*)-2-aminohexanedioate hydrochloride (7·HCl)[11]. Chlorotrimethylsilane (51 mL, 652 mmol) was slowly added over a period of 30 min using a dropping funnel to a solution of 2-aminoadipic acid (**6**, 30.0 g, 186 mmol) in methanol (370 mL) under ice cooling. The mixture was stirred at rt for 18 h. The solvent was evaporated in vacuum. Et_2O (300 mL) was added to the crude viscous residue, the mixture was stirred intensively and concentrated in vacuum. This procedure was repeated four times to get yellow viscous oil. Yield 41.6 g (99%). $C_8H_{16}ClNO_4$ (225.7). ^{1}H NMR (D_2O): δ (ppm) = 1.47–1.67 (m, 2H, $CH_2CH_2CH_2CO_2CH_3$), 1.73–1.91 (m, 2H, $CH_2CH_2CH_2CO_2CH_3$), 2.31 (t, J = 7.2 Hz, 2H, $CH_2CH_2CH_2CO_2CH_3$), 3.54 (s, 3H, $CH_2CH_2CH_2CO_2CH_3$), 3.69 (s, 3H, $CHCO_2CH_3$), 4.01 (t, J = 6.4 Hz, 1H, $^+NH_3CHCO_2CH_3$). ^{13}C NMR (D_2O): δ (ppm) = 19.9 (1C, $CH_2CH_2CH_2CO_2CH_3$), 29.1 (1C, $CH_2CH_2CH_2CO_2CH_3$), 33.0 (1C, $CH_2CH_2CH_2CO_2CH_3$), 52.8 (1C, $^+NH_3CHCO_2CH_3$), 52.9 (1C, $CH_2CH_2CH_2CO_2CH_3$), 53.8 (1C, $CHCO_2CH_3$), 170.6 (1C, $CH_2CH_2CH_2CO_2CH_3$), 177.7 (1C, $CHCO_2CH_3$). MS (EI): m/z (%) = 190 [MH^+ – Cl, 100]. IR (neat): ν/cm^{-1} = 3392 (N–H), 1730 (C=O), 1225 (C–O).

Dimethyl (*RS*)-2-[*N*-benzyl-*N*-(2-chloroacetyl)amino]hexanedioate (9). Benzaldehyde (10.3 mL, 102 mmol), triethylamine (14.1 mL, 120 mmol) and anhydrous $MgSO_4$ (5.6 g, 46.5 mmol) were added to a solution of **7**.HCl (21 g, 93 mmol) in dry CH_2Cl_2 (165 mL) under ice-cooling. The mixture was stirred at rt for 3.5 h. The solvent was evaporated in vacuum. Et_2O (600 mL) was added to the residue, the mixture was filtered and concentrated in vacuum to obtain the corresponding imine as pale yellow oil. The oily residue (21.5 g) was dissolved in dry CH_3OH (210 mL) and $NaBH_4$ (6.2 g, 163 mmol) was added slowly over a period of 1 h under ice-cooling. The mixture was then stirred at rt for 4 h. The solvent was evaporated slowly in vacuum, H_2O (100 mL) was added to the residue and the aqueous layer was extracted with CH_2Cl_2 (6 × 100 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated in vacuum to obtain amine **8** as a pale yellow oil. The crude oil (**8**, 15.8 g) was dissolved in CH_2Cl_2 (130 mL), chloroacetyl chloride (5.7 mL, 71.5 mmol) and triethylamine (5.0 mL, 35.7 mmol) were added slowly under ice-cooling and the mixture was stirred at rt for 4 h. The solvent was evaporated in vacuum, Et_2O (100 mL) was added to the residue and the mixture was filtered. The filtrate was concentrated in vacuum to obtain a pale yellow viscous oil. This process was repeated three times with Et_2O. The crude oil was purified by fc (cyclohexane/ethyl acetate = 7 : 3, 8 cm, 25 cm, 100 mL, R_f 0.27) to obtain a colorless viscous oil. Yield 10.5 g (51%) (over three steps). $C_{17}H_{22}ClNO_5$ (355.8). Purity (HPLC, method A): 94.6%, t_R = 19.3 min. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.50 (tt, J = 6.8/5.8 Hz, 0.3H, $CH_2CH_2CH_2CO_2CH_3^{min}$), 1.61 (tt, J = 7.7/7.4 Hz, 2 × 0.7H, $CH_2CH_2CH_2CO_2CH_3^{maj}$), 1.69 (s broad, 0.3H, $CH_2CH_2CH_2CO_2CH_3^{min}$), 1.76–1.85 (m, 1H, $CH_2CH_2CH_2CO_2CH_3^{maj+min}$), 1.98–2.08 (m, 1H, $CH_2CH_2CH_2CO_2CH_3^{maj+min}$), 2.14–2.18 (m, 2 × 0.3H, $CH_2CH_2CH_2CO_2CH_3^{min}$), 2.25 (2 × t, J = 7.3 Hz, 2 × 0.7H, $CH_2CH_2CH_2CO_2CH_3^{maj}$), 3.46 (s, 3 × 0.3H, $CHCO_2CH_3^{min}$), 3.60 (s, 3 × 0.7H, $CO_2CH_3^{maj}$), 3.63 (s, 3H, $CH_2CO_2CH_3^{maj+min}$), 4.02 (d, J = 12.7 Hz, 0.7H, $NCH_2C_6H_4OCH_3^{maj}$), 4.07 (d, J = 12.6 Hz, 0.7H, $NCH_2C_6H_5^{maj}$), 4.18 (d, J = 12.4 Hz, 0.3H, $NCH_2C_6H_5^{min}$), 4.32 (d, J = 12.4 Hz, 0.3H, $NCH_2C_6H_4OCH_3^{min}$), 4.42 (t, J = 7.3 Hz, 0.3H, $CHCO_2CH_3^{min}$), 4.54 (t, J = 7.5 Hz, 0.7H, $CHCO_2CH_3^{maj}$), 4.58–4.62 (m, 0.7H + 2 × 0.3H, $COCH_2Cl^{maj}$, $COCH_2Cl^{min}$), 4.69 (d, J = 17.2 Hz, 0.7H, $COCH_2Cl^{maj}$), 7.21–7.38 (m, 5H, 2-H, 3-H, 4-H, 5-H, 6-H_{benzyl}). ^{13}C NMR ($CDCl_3$): δ (ppm) = 21.6 ($CH_2CH_2CH_2CO_2CH_3^{min}$), 22.0 ($CH_2CH_2CH_2CO_2CH_3^{maj}$), 28.8 ($CH_2CH_2CH_2CO_2CH_3^{maj}$), 29.9 ($CH_2CH_2CH_2CO_2CH_3^{min}$), 33.4 ($CH_2CH_2CH_2CO_2CH_3^{min}$), 33.6 ($CH_2CH_2CH_2CO_2CH_3^{maj}$), 41.8 ($NCH_2C_6H_5^{maj}$), 46.8 ($COCH_2Cl^{min}$), 51.2 ($COCH_2Cl^{maj}$), 51.8 ($CH_2CO_2CH_3^{maj}$), 52.4 ($CHCO_2CH_3^{maj}$), 52.7 (2C, $CH_2CO_2CH_3^{maj+min}$), 58.8 ($CHCO_2CH_3^{maj}$), 60.1 ($CHCO_2CH_3^{min}$), 127.1 (2C, C-2, C-6$_{benzyl}^{maj}$), 127.6 (2C, C-2, C-6$_{benzyl}^{min}$), 128.3 (1C, C-1$_{benzyl}^{maj}$), 128.6 (1C, C-1$_{benzyl}^{min}$), 129.2 (4C, C-3, C-5$_{benzyl}^{maj+min}$), 135.9 (1C, C-4$_{benzyl}^{maj}$), 137.2 (1C, C-4$_{benzyl}^{min}$), 167.9 (1C, N-*C*=O^{maj}), 168.3 (1C, N^+=*C*-O^{-min}), 170.6 (1C, $CHCO_2CH_3^{min}$), 171.0 (1C, $CHCO_2CH_3^{maj}$), 173.5 (1C, $CH_2CO_2CH_3^{min}$), 173.7 (1C, $CH_2CO_2CH_3^{maj}$). MS (EI): m/z (%) = 278 [$(M–COCH_2Cl)^+$,

100], 106 [$(C_6H_5CH_2NH)^+$, 21], 91 [(benzyl)$^+$, 50]. IR (neat): ν/cm^{-1} = 1731 (C=O, ester), 1656 (C=O, amide), 1200 (C–O, ester), 1171 (C–O, ester).

Methyl (*RS*)-4-[1-benzyl-4-methyl-3,6-dioxopiperazin-2-yl]-butyrate (10). Under ice-cooling NEt_3 (1.0 mL, 7.5 mmol) and methylamine (2.0 M in THF, 3.8 mL, 7.5 mmol) were added slowly to a solution of **9** (2.3 g, 6.6 mmol) in CH_3CN (12 mL) over a period of 10 min. The mixture was warmed to rt and stirred for 16 h. Then the solvent was evaporated in vacuum, Et_2O (150 mL) was added to the residue in portions, the mixture was filtered and concentrated in vacuum. The residue was purified by fc (acetone/dichloromethane = 1 : 9 + 1% *N*,*N*-dimethylethylamine, 4 cm, 15 cm, 20 mL, R_f 0.26) to yield a colorless viscous oil. Yield 1.15 g (55%). $C_{17}H_{22}N_2O_4$ (318.1). Purity (HPLC, method B): 98.5%, t_R = 14.3 min. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.47–1.59 (m, 1H, $CH_2CH_2CH_2CO_2CH_3$), 1.62–1.73 (m, 1H, $CH_2CH_2CH_2CO_2CH_3$), 1.78–1.94 (m, 2H, $CH_2CH_2CH_2CO_2CH_3$), 2.18–2.34 (m, 2H, $CH_2CH_2CH_2CO_2CH_3$), 2.97 (s, 3H, NCH_3), 3.66 (s, 3H, OCH_3), 3.88 (t, J = 3.6 Hz, 1H, NCHCO), 3.93 (d, J = 17.5 Hz, 1H, NCH_2CO), 3.98 (d, J = 14.8 Hz, 1H, $NCH_2C_6H_5$), 4.17 (d, J = 17.5 Hz, 1H, NCH_2CO), 5.31 (d, J = 14.8 Hz, 1H, $NCH_2C_6H_5$), 7.23–7.26 (m, 2H, 3-H, 5-H$_{benzyl}$), 7.27–7.32 (m, 3H, 2-H, 4-H, 6-H$_{benzyl}$). ^{13}C NMR ($CDCl_3$): δ (ppm) = 19.8 (1C, $CH_2CH_2CH_2CO_2CH_3$), 31.1 (1C, $CH_2CH_2CH_2CO_2CH_3$), 33.3 (1C, $CH_2CH_2CH_2CO_2CH_3$), 33.7 (1C, NCH_3), 47.1 (1C, $NCH_2C_6H_5$), 51.8 (1C, NCH_2CO), 51.9 (1C, OCH_3), 58.8 (1C, NCHCO), 128.3 (1C, C-4$_{benzyl}$), 128.6 (2C, C-2, C-6$_{benzyl}$), 129.1 (2C, C-3, C-5$_{benzyl}$), 135.5 (1C, C-1$_{benzyl}$), 163.9 (1C, N–C=O), 166.1 (1C, N–C=O), 173.3 (1C, C-1$_{ester\ carbonyl}$). Exact mass (ESI): m/z = calculated for $C_{17}H_{22}N_2O_4Na^+$ 341.1477, found 341.1472. IR (neat): ν/cm^{-1} = 1732 (C=O ester), 1656 (C=O amide), 1173 (C–O ester).

(1*RS*,2*SR*,6*RS*)-7-Benzyl-2-methoxy-9-methyl-2-(trimethylsilyloxy)-7,9-diazabicyclo[4.2.2]decane-8,10-dione (12). Lithium hexamethyldisilazide (LHMDS), freshly prepared from *n*-BuLi (1.3 mL, 1.5 mmol) and hexamethyldisilazane (0.34 mL, 1.5 mmol) in dry THF (5 mL) at 0 °C, was slowly added to a solution of **10** (0.37 g, 1.2 mmol) in dry THF (10 mL) at −78 °C. The mixture was stirred for 0.5 h at −78 °C, chlorotrimethylsilane $(CH_3)_3SiCl$ (0.54 mL, 4.1 mmol) was slowly added to the solution, the mixture was stirred at −78 °C for 2 h and then warmed to rt and stirred for 1 h. The solvent was evaporated to half of the original volume and ethyl acetate (10 mL) was added. The mixture was washed with H_2O (50 mL) and the aqueous layer was extracted with CH_2Cl_2 (5 × 50 mL). The combined organic layers were washed with brine and dried over Na_2SO_4. The solvent was evaporated in vacuum to obtain a colorless solid which was purified by fc (cyclohexane/ethyl acetate = 3 : 7, 3 cm, 16 cm, 10 mL, R_f 0.46) to obtain colorless crystals, mp 118–121 °C. Yield 0.43 g (94%). $C_{20}H_{30}N_2O_4Si$ (390.3). Purity (HPLC, method A): 100%, t_R = 19.7 min. ^{1}H NMR ($CDCl_3$): δ (ppm) = 0.00 (s, 9H, $(CH_3)_3Si$), 1.11–1.26 (m, 3H, 3-H, 4-H), 1.51–1.55 (m, 1H, 5-H), 1.56–1.67 (m, 2H, 3-H, 5-H), 2.72 (s, 3H, NCH_3), 3.00 (s, 3H, OCH_3), 3.74 (dd, J = 6.4/1.8 Hz, 1H, 6-H), 3.79 (d, J = 0.9 Hz, 1H, 1-H), 4.09 (d, J = 14.6 Hz, 1H, $NCH_2C_6H_5$), 4.44 (d, J = 14.6 Hz, 1H, $NCH_2C_6H_5$), 6.99–7.05 (m, 5H, 2-H, 3-H, 4-H, 5-H, 6-H$_{benzyl}$). ^{13}C NMR ($CDCl_3$): δ (ppm) = 0.0 (3C, $(CH_3)_3Si$), 15.5 (1C, C-4), 27.9 (1C, C-5), 31.9 (1C, C-3), 32.9 (1C, NCH_3), 46.4 (1C, $NCH_2C_6H_5$), 47.1 (1C, OCH_3), 57.7 (1C, C-6), 66.3 (1C, C-1), 103.5 (1C, C-2), 126.2 (1C, C-4$_{benzyl}$), 126.7 (2C, C-2, C-6$_{benzyl}$), 127.0 (2C, C-3, C-5$_{benzyl}$), 134.3 (1C, C-1$_{benzyl}$), 162.6 (1C, N–C=O), 189.2 (1C, N–C=O). Exact mass (ESI): m/z = calculated for $C_{20}H_{30}N_2O_4SiNa^+$ 413.1873, found 413.1867. IR (neat): ν/cm^{-1} = 1654 (C=O amide), 1472 (C–N amide), 1255 (C–O ketal), 836 (Si–O ketal).

X-ray crystal structure analysis of 12. A sample of mixed methyl silyl ketal **12** was recrystallized from iPr_2O to get crystals, which were suitable for X-ray crystal structure analysis.

Formula $C_{20}H_{30}N_2O_4Si$, M = 390.55, colorless crystal 0.40 × 0.05 × 0.03 mm, a = 8.9326(1), b = 10.1193(1), c = 11.8959(1) Å, α = 82.230(1), β = 80.314(1), γ = 86.226(1)° V = 1049.32(2) Å^3, ρ_c = 1.236 g cm^{-3}, μ = 1.210 mm^{-1}, empirical absorption correction (0.643 SYMBOL 163 \f "Symbol" T SYMBOL 163 \f "Symbol" 0.965), Z = 2, triclinic, space group $P\bar{1}$ (No. 2), λ = 1.54178 Å, T = 223(2) K, ω and φ scans, 12 315 reflections collected (SYMBOL 177 \f "Symbol"h, SYMBOL 177 \f "Symbol"k, SYMBOL 177 \f "Symbol"l), [(sinθ)/λ] = 0.60 Å^{-1}, 3630 independent (R_{int} = 0.057) and 3025 observed reflections [I SYMBOL 179 \f "Symbol" 2 SYMBOL 115 \f "Symbol"(I)], 249 refined parameters, R = 0.048, wR^2 = 0.127, max. (min.) residual electron density 0.26 (–0.23) e Å^{-3}, hydrogen atoms calculated and refined as riding atoms.

Data set was collected with a Nonius KappaCCD diffractometer. Programs used: data collection COLLECT (Nonius B.V., 1998), data reduction Denzo-SMN,[28] absorption correction Denzo,[29] structure solution SHELXS-97,[30] structure refinement SHELXL-97,[31] graphics SCHAKAL.[32]

CCDC 766603 contains the supplementary crystallographic data for this paper.† These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; Fax: +44 1223 336033, E-mail: deposit@ccdc.cam.ac.uk).

(1*RS*,6*RS*)-7-Benzyl-9-methyl-7,9-diazabicyclo[4.2.2]decane-2,8,10-trione (13). The ketal **12** (0.78 g, 2.0 mmol) was dissolved in a mixture of THF (9 mL) and H_2O (9 mL). 1 M HCl (3.5 mL) was added to the mixture and the mixture was stirred for 17.5 h at rt. The solvent was evaporated to half of the original volume, washed with saturated $NaHCO_3$ solution (30 mL) and the aqueous layer was extracted with CH_2Cl_2 (6 × 20 mL). The combined organic layers were washed with brine, dried over Na_2SO_4 and the solvent was evaporated in vacuum to obtain a colorless solid. Purification by fc (acetone/dichloromethane = 1 : 9, 4 cm, 14 cm, 30 mL, R_f 0.22) yielded colorless crystals, mp 193–198 °C. Yield 0.56 g (97%). $C_{16}H_{18}N_2O_3$ (286.1). Purity (HPLC, method B): 99.4%, t_R = 11.9 min. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.24–1.35 (m, 1H, 4-H), 1.73–1.81 (m, 1H, 4-H), 1.95–1.99 (m, 2H, 5-H), 2.34 (ddd, J = 14.2/9.1/0.9 Hz, 1H, 3-H), 2.51 (dd, J = 14.2/9.2 Hz, 1H, 3-H), 2.88 (s, 3H, NCH_3), 4.16 (dd, J = 5.8/2.8 Hz, 1H, 6-H), 4.18 (d, J = 14.6 Hz, 1H, $NCH_2C_6H_5$), 4.41 (s, 1H, 1-H), 4.86 (d, J = 14.6 Hz, 1H, $NCH_2C_6H_5$), 7.19–7.29 (m, 5H, 2-H, 3-H, 4-H, 5-H, 6-H$_{benzyl}$). ^{13}C NMR ($CDCl_3$): δ (ppm) = 19.3 (1C, C-4), 31.3 (1C, C-5), 33.5 (1C, C-3), 39.0 (1C, NCH_3), 48.6 (1C, $NCH_2C_6H_5$), 59.1 (1C, C-6), 73.6 (1C, C-1), 128.7 (1C, C-4$_{benzyl}$), 128.8 (2C, C-2, C-6$_{benzyl}$), 129.3 (2C, C-3, C-5$_{benzyl}$), 135.1 (1C, C-1$_{benzyl}$), 161.2

(1C, N–C=O), 167.4 (1C, N–C=O), 203.1 (1C, C-2). Exact mass (ESI): m/z = calculated for $C_{16}H_{18}N_2O_3Na^+$ 309.1215, found 309.1210. IR (neat): ν/cm^{-1} = 1715 (C=O ketone), 1666 (C=O amide), 1448 (C–N amide).

(1*RS*,2*SR*,6*RS*)-7-Benzyl-2-hydroxy-9-methyl-7,9-diazabicyclo[4.2.2]decane-8,10-dione (14). Under N_2 atmosphere $LiBH_4$ (0.09 g) was slowly added to a solution of **13** (0.42 g, 1.4 mmol) in dry THF (30 mL) at −78 °C and the mixture was stirred for 3 h. The excess $LiBH_4$ was destroyed with 1 M HCl (pH 4) then 1 M NaOH was added until the solution attained pH 9 and the mixture was stirred for 0.5 h at rt. The aqueous layer was extracted with ethyl acetate (5 × 15 mL). The combined organic layers were washed with brine, dried (Na_2SO_4) and the solvent was evaporated in vacuum to obtain a colorless sponge. The residue was purified by fc (cyclohexane/ethyl acetate = 1 : 9, 3 cm, 15 cm, 20 mL, R_f 0.05) to obtain a colorless solid, mp 115–120 °C. Yield 0.42 g (99%). $C_{16}H_{20}N_2O_3$ (288.1). Purity (HPLC, method A): 97.7%, t_R = 11.7 min. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.27–1.36 (m, 1H, 3-H), 1.55–1.72 (m, 3H, 3-H, 4-H), 1.76–1.83 (m, 1H, 5-H), 1.88–1.96 (m, 1H, 5-H), 2.94 (s, 3H, NCH_3), 3.07 (s, 1H, OH), 3.95 (t, J = 3.6 Hz, 1H, 6-H), 4.01 (d, J = 14.7 Hz, 1H, NCH_2C$_6$H$_5$), 4.10 (s broad, 2H, 1-H, 2-H), 5.04 (d, J = 14.7 Hz, 1H, NCH_2C$_6$H$_5$), 7.19–7.29 (m, 5H, 2-H, 3-H, 4-H, 5-H, 6-H$_{benzyl}$). ^{13}C NMR ($CDCl_3$): δ (ppm) = 17.6 (1C, C-3), 32.1 (1C, C-4), 32.3 (1C, C-5), 32.7 (1C, NCH_3), 47.6 (1C, NCH_2C$_6$H$_5$), 59.1 (1C, C-6), 66.0 (1C, C-1), 71.7 (1C, C-2), 128.5 (1C, C-4$_{benzyl}$), 128.7 (2C, C-2, C-6$_{benzyl}$), 129.2 (2C, C-3, C-5$_{benzyl}$), 135.3 (1C, C-1$_{benzyl}$), 167.1 (1C, N–C=O), 167.8 (1C, N–C=O). Exact mass (ESI): m/z = calculated for $C_{16}H_{20}N_2O_3Na^+$ 311.1372, found 311.1366. IR (neat): ν/cm^{-1} = 3293 (O–H), 1661, 1634 (C=O), 1474, 1456 (C–N), 1132 (C–O).

(1*RS*,2*SR*,6*RS*)-7-Benzyl-2-benzyloxy-9-methyl-7,9-diazabicyclo[4.2.2]decane-8,10-dione (15). Under N_2 atmosphere NaH (0.05 g, 0.8 mmol, 60% dispersion in paraffin oil) was slowly added to a solution of **14** (0.05 g, 0.18 mmol) and $C_6H_5CH_2Br$ (0.04 mL, 0.3 mmol) in dry DMF (4 mL) under ice-cooling. The reaction mixture was warmed to rt and stirred for 1 h. The excess NaH was destroyed with H_2O (5 mL) under ice-cooling. The aqueous layer was extracted with ethyl acetate (5 × 10 mL). The combined organic layers were washed with brine, dried (Na_2SO_4) and the solvent was evaporated in vacuum. The crude solid was purified by fc (ethyl acetate/cyclohexane = 7 : 3, 2 cm, 15 cm, 10 mL, R_f 0.18). to obtain colorless crystals, mp 126–130 °C. Yield 0.057 g (82%). $C_{23}H_{26}N_2O_3$ (378.2). Purity (HPLC, method A): 97.2%, t_R = 18.6 min. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.23–1.32 (m, 1H, 4-H), 1.55 (dt, J = 14.4/2.9 Hz, 1H, 3-H), 1.67 (dquint, J = 14.6/4.6 Hz, 1H, 4-H), 1.74–1.82 (m, 2H, 3-H, 5-H), 1.84–1.92 (m, 1H, 5-H), 2.78 (s, 3H, NCH_3), 3.74–3.78 (m, 1H, 2-H), 3.92 (dd, J = 4.9/2.9 Hz, 1H, 6-H), 4.06 (d, J = 14.7 Hz, 1H, NCH_2C$_6$H$_5$), 4.19 (d, J = 5.0 Hz, 1H, 1-H), 4.60 (d, J = 12.2 Hz, 1H, OCH_2C$_6$H$_5$), 4.74 (d, J = 12.2 Hz, 1H, OCH_2C$_6$H$_5$), 5.00 (d, J = 14.7 Hz, 1H, NCH_2C$_6$H$_5$), 7.19–7.25 (m, 6H, 2-H, 3-H, 4-H, 5-H, 6-H$_{N\text{-benzyl}}$, 4-H$_{O\text{-benzyl}}$), 7.29 (t, J = 7.3 Hz, 2H, 3-H, 5-H$_{O\text{-benzyl}}$), 7.36 (d, J = 6.9 Hz, 2H, 2-H, 6-H$_{O\text{-benzyl}}$). ^{13}C NMR ($CDCl_3$): δ (ppm) = 17.7 (1C, C-4), 29.1 (1C, C-3), 32.1 (1C, C-5), 32.9 (1C, NCH_3), 47.7 (1C, NCH_2C$_6$H$_5$), 59.2 (1C, C-6), 63.9 (1C, C-1), 71.5 (1C, OCH_2C$_6$H$_5$), 77.9 (1C, C-2), 128.0 (2C, C-2, C-6$_{O\text{-benzyl}}$), 128.1 (1C, C-4$_{N\text{-benzyl}}$), 128.2 (1C, C-4$_{O\text{-benzyl}}$), 128.6 (2C, C-2, C-6$_{N\text{-benzyl}}$), 128.7 (2C, C-3, C-5$_{N\text{-benzyl}}$), 129.1 (2C, C-3, C-5$_{O\text{-benzyl}}$), 136.0 (1C, C-1$_{N\text{-benzyl}}$), 138.0 (1C, C-1$_{O\text{-benzyl}}$), 165.3 (1C, N–C=O, 169.0 (1C, N–C=O). Exact mass (ESI): m/z = calculated for $C_{23}H_{26}N_2O_3Na^+$ 401.1841, found 401.1836. IR (neat): ν/cm^{-1} = 1654 (C=O amide), 723, 696 (aromatic out-of-plane bend).

(1*RS*,2*RS*,6*SR*)-7-Benzyl-9-methyl-7,9-diazabicyclo[4.2.2]-decan-2-ol (16). Under N_2 atmosphere $LiAlH_4$ (0.9 mL, 0.86 mmol, 1 M solution in THF) was added dropwise under ice cooling to a solution of **14** (0.05 g, 0.17 mmol) in dry THF (10 mL). The mixture was warmed to rt and stirred under reflux for 16 h. The excess $LiAlH_4$ was destroyed with H_2O (1 mL) under ice-cooling. The mixture was again stirred under reflux for 1 h, cooled to rt and filtered. The precipitate was washed with CH_2Cl_2 (20 mL) and the solvent was evaporated in vacuum. The crude viscous oil was purified by fc (ethyl acetate/cyclohexane = 8.5 : 1.5 + 0.5% *N*,*N*-dimethylethylamine, 2 cm, 15 cm, 10 mL, R_f 0.19) to obtain a colorless viscous oil. Yield 0.79 g (67%). $C_{16}H_{24}N_2O$ (260.2). Purity (HPLC, method A): 97.3%, t_R = 11.0 min. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.30–1.36 (m, 1H, 5-H), 1.43–1.50 (m, 1H, 5-H), 1.54–1.62 (m, 1H, 4-H), 1.92 (ddd, J = 12.6/9.9/5.7 Hz, 1H, 3-H), 2.06 (ddd, J = 15.0/11.7/4.9 Hz, 1H, 4-H), 2.28 (s, 3H, NCH_3), 2.34 (t, J = 10.8 Hz, 1H, 3-H), 2.54 (s broad, 1H, 1-H), 2.57 (s broad, 1H, 10-H), 2.73 (dd, J = 11.6/3.9 Hz, 1H, 8-H), 2.80 (s broad, 1H, 6-H), 2.86 (d broad, J = 11.0 Hz, 1H, 8-H), 2.95 (ddd, J = 11.0/4.1/1.7 Hz, 1H, 10-H), 3.50 (d, J = 12.8 Hz, 1H, NCH_2C$_6$H$_5$), 3.57 (d, J = 12.8 Hz, 1H, NCH_2C$_6$H$_5$), 3.67 (dt, J = 10.2/5.0 Hz, 1H, 2-H), 7.16 (t, J = 7.2 Hz, 1H, 4-H$_{N\text{-benzyl}}$), 7.23 (t, J = 7.3 Hz, 2H, 3-H, 5-H$_{N\text{-benzyl}}$), 7.28 (d, J = 7.1 Hz, 2H, 2-H, 6-H$_{N\text{-benzyl}}$). A signal for the OH proton is not seen in the spectrum. ^{13}C NMR ($CDCl_3$): δ (ppm) = 21.6 (1C, C-4), 33.9 (1C, C-3), 36.2 (1C, C-5), 43.9 (1C, C-8), 46.3 (1C, NCH_3), 52.7 (1C, C-10), 57.9 (1C, C-6), 63.4 (1C, NCH_2C$_6$H$_5$), 65.5 (1C, C-1), 74.1 (1C, C-2), 127.1 (1C, C-4$_{N\text{-benzyl}}$), 128.3 (2C, C-2, C-6$_{N\text{-benzyl}}$), 129.2 (2C, C-3, C-5$_{N\text{-benzyl}}$), 139.9 (1C, C-1$_{N\text{-benzyl}}$). Exact mass (ESI): m/z = calculated for $C_{16}H_{24}N_2OH^+$ 261.1967, found 261.1961. IR (neat): ν/cm^{-1} = 3361 (O–H), 2776 (C–H), 717, 696 (aromatic out-of-plane bend).

(1*RS*,2*RS*,6*SR*)-7-Benzyl-2-benzyloxy-9-methyl-7,9-diazabicyclo[4.2.2]decane (17). Under N_2 atmosphere $LiAlH_4$ (0.03, 0.7 mmol) was added slowly to a solution of **15** (0.05 g, 0.14 mmol) in dry THF (10 mL) under ice-cooling. The mixture was warmed to rt and then stirred under reflux for 15 h. Excess $LiAlH_4$ was destroyed with H_2O (1 mL) under ice-cooling. The mixture was again stirred under reflux for 1 h. The solution was cooled to rt and filtered through a sintering funnel. The precipitate was washed with CH_2Cl_2 (20 mL). The solvent was evaporated in vacuum. The crude oil was purified by fc (petroleum ether/ethyl acetate = 9 : 1, 2 cm, 13 cm, 10 mL, R_f 0.42 (ethyl acetate/cyclohexane = 3 : 7)) to obtain a colorless viscous oil. Yield 0.03 g (59%). $C_{23}H_{30}N_2O$ (350.2). Purity (HPLC, method A): 94.9%, t_R = 19.2 min. ^{1}H NMR ($CDCl_3$): δ (ppm) = 1.26–1.31 (m, 1H, 5-H), 1.40–1.48 (m, 1H, 5-H), 1.54–1.62 (m, 1H, 4-H), 1.96–2.06 (m, 2H, 3-H, 4-H), 2.23 (s, 3H, NCH_3), 2.39 (q, J = 11.0 Hz, 1H, 3-H), 2.54 (s broad, 1H, 1-H), 2.67 (s broad, 1H, 6-H), 2.77 (dd, J = 11.1/4.1 Hz, 2H, 8-H, 10-H), 2.93–3.00 (m, 2H, 8-H, 10-H), 3.35 (s broad, 1H, 2-H), 3.48 (d, J = 13.0 Hz, 1H, NCH_2C$_6$H$_5$), 3.60 (d, J = 13.0 Hz, 1H, NCH_2C$_6$H$_5$), 4.42 (d, J = 12.2 Hz, 1H, OCH_2C$_6$H$_5$), 4.49 (d, J = 12.2, 1H, OCH_2C$_6$H$_5$), 7.13–7.24 (m, 8H, 2-H, 3-H, 4-H, 5-H, 6-H$_{N\text{-benzyl}}$, 3-H, 4-H, 5-H$_{O\text{-benzyl}}$), 7.30 (d, J = 7.3 Hz, 2H, 2-H, 6-H$_{O\text{-benzyl}}$). ^{13}C NMR ($CDCl_3$): δ (ppm) = 21.7 (1C, C-4), 32.1 (1C,

C-3), 36.1 (1C, C-5), 44.9 (1C, C-8), 46.2 (1C, NCH_3), 52.7 (1C, C-10), 57.3 (1C, C-6), 63.1 (1C, N$CH_2C_6H_5$), 71.0 (2C, C-1, C-2), 77.4 (1C, O$CH_2C_6H_5$), 127.0 (2C, C-2, C-6$_{O\text{-benzyl}}$), 127.5(1C, C-4$_{N\text{-benzyl}}$), 127.6 (1C, C-4$_{O\text{-benzyl}}$), 128.2(2C, C-2, C-6$_{N\text{-benzyl}}$), 128.4 (2C, C-3, C-5$_{N\text{-benzyl}}$), 129.3 (2C, C-3, C-5$_{O\text{-benzyl}}$), 131.1 (1C, C-1$_{N\text{-benzyl}}$), 139.5 (1C, C-1$_{O\text{-benzyl}}$). Exact mass (ESI): m/z = calculated for $C_{23}H_{30}N_2OH^+$ 351.2436, found 351.2431. I.R. (neat): ν/cm^{-1} = 2922 (C–H), 1509 (C=C aromatic), 1246 (C–O), 731 (C=C aromatic out-of-plane bend), 698 (C=C aromatic out-of-plane bend).

Receptor binding studies

Materials and general procedures. The guinea pig brains and rat livers were commercially available (Harlan-Winkelmann, Borchen, Germany). The pig brains were a donation of the local slaughterhouse (Coesfeld, Germany). Homogenizer: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany). Centrifuge: High-speed cooling centrifuge model Sorvall RC-5C plus (Thermo Fisher Scientific, Langenselbold, Germany). Filter: Printed Filtermat Typ A and B (Perkin Elmer LAS, Rodgau-Jügesheim, Germany), presoaked in 0.5% aqueous polyethylenimine for 2 h at room temperature before use. The filtration was carried out with a MicroBeta FilterMate-96 Harvester (Perkin Elmer). The scintillation analysis was performed using Meltilex (Typ A or B) solid scintillator (Perkin Elmer). The solid scintillator was melted on the filtermat at a temperature of 95 °C for 5 min. After solidifying of the scintillator at room temperature, the scintillation was measured using a MicroBeta Trilux scintillation analyzer (Perkin Elmer). The counting efficiency was 40%. All experiments were carried out in triplicates using standard 96-well-multiplates (Diagonal, Muenster, Germany). The IC_{50}-values were determined in competition experiments with at least six concentrations of the test compounds and were calculated with the program GraphPad Prism® 3.0 (GraphPad Software, San Diego, CA, USA) by non-linear regression analysis. The K_i-values were calculated according to the formula of Cheng and Prusoff.[21] The K_i-values are given as mean value ± SEM from three independent experiments.

Membrane preparation for the σ_1 assay[22–24]. Five guinea pig brains were homogenized with the potter (500–800 rpm, 10 up-and-down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1200 × g for 10 min at 4 °C. The supernatant was separated and centrifuged at 23 500 × g for 20 min at 4 °C. The pellet was resuspended in 5–6 volumes of buffer (50 mM TRIS, pH 7.4) and centrifuged again at 23 500 × g (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5–6 volumes of buffer, the protein concentration was determined according to the method of Bradford[25] using bovine serum albumin as standard, and subsequently the preparation was frozen (–80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

Performing of the σ_1 assay[22-24]. The test was performed with the radioligand [^{3}H]-(+)-pentazocine (32.2 Ci/mmol; Perkin Elmer LAS). The thawed membrane preparation (about 75 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [^{3}H]-(+)-pentazocine, and buffer (50 mM TRIS, pH 7.4) in a total volume of 200 µL for 150 min at 37 °C. The incubation was terminated by rapid filtration through the presoaked filtermats using a cell harvester. After washing each well five times with 300 µl of water, the filtermats were dried at 95 °C. Subsequently, the solid scintillator was placed on the filtermat and melted at 95 °C. After 5 min, the solid scintillator was allowed to solidify at room temperature. The bound radioactivity trapped on the filters was counted in the scintillation analyzer. The non-specific binding was determined with 10 µM unlabeled (+)-pentazocine. The K_d-value of (+)-pentazocine is 2.9 nM.[26]

Membrane preparation for the σ_2 assay[22–24]. Two rat livers were cut into smaller 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 1200 × g for 10 min at 4 °C. The supernatant was separated and centrifuged at 31 000 × g for 20 min at 4 °C. The pellet was resuspended in 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 × g for 20 min at 4 °C. The final pellet was resuspended in 5–6 volumes of buffer, the protein concentration was determined according to the method of Bradford[25] using bovine serum albumin as standard, and subsequently the preparation was frozen (–80 °C) in 1.5 mL portions containing about 2 mg protein/mL.

Performing of the σ_2 assay[22–24]. The test was performed with the radioligand [^{3}H]-ditolylguanidine (50 Ci/mmol; ARC, St. Louis, MO, USA). The thawed membrane preparation (about 100 µg of the protein) was incubated with various concentrations of test compounds, 3 nM [^{3}H]-ditolylguanidine, and buffer containing (+)-pentazocine (2 µM (+)-pentazocine in 50 mM TRIS, pH 8.0) in a total volume of 200 µL for 150 min at room temperature. The incubation was terminated by rapid filtration through the presoaked filtermats using a cell harvester. After washing each well five times with 300 µL of water, the filtermats were dried at 95 °C. Subsequently, the solid scintillator was placed on the filtermat and melted at 95 °C. After 5 min, the solid scintillator was allowed to solidify at room temperature. The bound radioactivity trapped on the filters was counted in the scintillation analyzer. The non-specific binding was determined with 10 µM unlabeled ditolylguanidine. The K_d-value of ditolylguanidine is 17.9 nM.[27]

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