Rapid Evaluation of Suitable Substrates with High Affinity to Artificial Caffeine Receptors by MS Based Techniques

Daniela Mirk[a,b], Heinrich Luftmann[b], and Siegfried R. Waldvogel[a,b]

[a] Kekulé-Institut für Organische Chemie und Biochemie, Rheinische Friedrich Wilhelms-Universität Bonn, Gerhard-Domagk-Str. 1, D-53121 Bonn, Germany
[b] Westfälische Wilhelms-Universität Münster, Organisch-Chemisches Institut, Corrensstr. 40, D-48149 Münster, Germany

Reprint requests to Prof. Dr. S. R. Waldvogel. Fax: +49(0) 228-73 9608.
E-mail: waldvogel@uni-bonn.de

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A modification of our triphenylene ketal based receptor facilitates electrospray tandem mass spectrometry investigations. Binding affinities of eleven potential substrates, *e.g.* caffeine and other xanthine alkaloids, are probed in the gas phase with collision induced dissociation. The relative stabilities of the substrate-receptor complexes are rapidly determined and the findings are correlated with the corresponding results in solution.

Key words: Supramolecular Chemistry, Mass Spectrometry, Host-Guest Complexes, Receptors

Introduction

The detection of small, biologically relevant molecules is of particular interest due to their omnipresence in everyday life. Alkylated oxopurines, *e.g.* caffeine (**13**), belong to the most frequently consumed alkaloids [1, 2]. Many of those target molecules exhibit a C_3 or *pseudo*-C_3 symmetry, therefore receptors with a complementary scaffold are popular [3]. Based on functionalized triphenylene ketals [4], the first artificial caffeine receptors were established by us, recently [5]. These contain an unusual large and rigid backbone providing concave and convergent preorganized functional groups for the supramolecular interaction. A chiral modification of the receptors allowed the first enantiofacial discrimination on single heterocyclic guest molecules [6]. The binding modes for such highly dynamic supramolecular aggregates were studied by different spectroscopic means creating a highly consistent picture in solution and for the solid state [7]. A detailed protocol for the synthesis of the scaffold and the corresponding receptors was recently reported [8].

Herein, we present a novel approach for the detection of caffeine and other xanthine alkaloids by tandem mass spectrometry. Exciting advances in mass spectrometry have made gas phase studies of organic supramolecular aggregates possible [9, 10], *e.g.* the enantiomer discrimination of peptides [11, 12]. With the development of soft ionization techniques, in particular electrospray ionization (ESI) [13 – 15], the delicate transfer of large molecules from solution to the gas phase including the mass spectrometric investigation of their non-covalent interactions with appropriate binding partners became feasible. Mass spectrometry offers a rapid screening process wherein only very small samples are required. Thus, the presented fast evaluation method will open up for further applications, *e.g.* for doping control analysis [16].

Results and Discussion

For a mass spectrometric investigation the construction of the modified receptor **1** (Fig. 1) was required. **1** exhibits a *N*,*N*-dibenzylamino substituent on one of the three receptor arms, which is easily protonated and facilitates the detection by ESI-MS. The tertiary amino group or the corresponding ammonium moiety should not influence the host-guest interaction. The three urea moieties are capable of hydrogen bonding with the considered substrates and exhibit the same binding site as our first artificial caffeine receptor [5]. Therefore, the results should be comparable with earlier findings [5, 7].

For the synthesis of **1** trisamine **2** was treated with a 5:1 mixture of hexyl isocyanate and 3-(*N*,*N*-dibenzylamino)propyl isocyanate (**8**) yielding symmetric receptor **3** as major product and the desired receptor **1** (Scheme 1). Separation was achieved *via*

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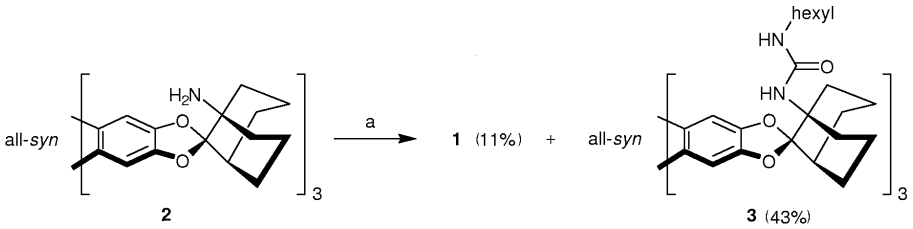
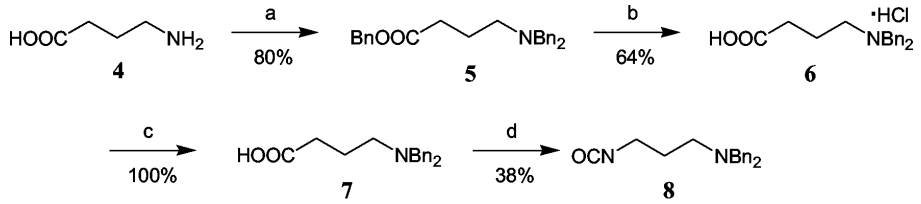


a) Hexyl isocyanate and R-NCO **8** (5:1), NEt_3, CH_2Cl_2, 16 h, 0 °C to r. t.

Scheme 1. Synthesis of receptor **1**.



a) Benzyl bromide, K_2CO_3, NaOH (2 N), CH_2Cl_2, 16 h, reflux; b) 1. aq. KOH (20%), 16 h, reflux; 2. HCl; c) aq. NaOH (20%), pH = 7; d) 1. DPPA, NEt_3, toluene (anhydr.), 3 d, r. t.; 2. reflux

Scheme 2. Synthesis of amino-substituted isocyanate **8**.

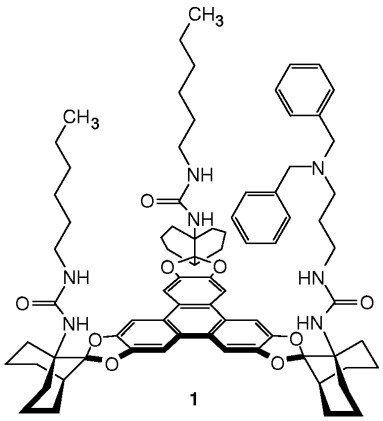


Fig. 1. Modified receptor **1** for MS investigations.

column chromatography. Choosing the appropriate ratio of isocyanates, the formation of receptors involving more than one protected amino function was suppressed and therefore mono-amino substituted **1** could be obtained in reasonable yields. Due to the hydrogen bonding capacity of the receptor, the empty receptor binds also solvent molecules and consequently the microanalysis was slightly deviated, whereas the structure was verified by the usual spectroscopic means.

The preparation of all-*syn* trisamine **2** as well as the following urea formation by treatment with isocyanates is well elaborated and has been reported in detail [8]. Amino-substituted isocyanate **8** was obtained in a multi-step sequence (Scheme 2). Starting from commercially available γ-aminobutyric acid (**4**) standard procedures yielded the known 4-(*N*,*N*-dibenzylamino)butyric acid (**7**). The last step, the conversion into the carboxylic acid azide intermediate followed by *Curtius* rearrangement leading to the corresponding isocyanate **8** was performed using diphenylphosphoryl azide (DPPA) as mild azide transfer reagent. DPPA was introduced in organic synthesis by *Shioiri* [17] and is easily available in large scale [18]. However, treatment of **7** with thionyl chloride led to a five-membered lactam by intramolecular attack of the free electron pair of the amino group on the highly reactive carbonyl carbon of the carboxylic acid chloride. This was accompanied by the loss of benzyl chloride. Due to the moisture sensitivity of **8**, a good match of microanalysis or IR spectra could not be obtained, but the nature of isocyanate **8** was proven by treatment with a primary amine followed by structural confirmation of the resulting product.

The collision-induced dissociation (CID) behavior of receptor **1** with different substrates **9 – 19** (Fig. 2) was studied in the gas phase [19]. Mixtures of **1**, a ninefold excess of the specific substrate, catalytic amounts of fumaric acid, and methanol in THF were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS). Surprisingly, not the monocation but

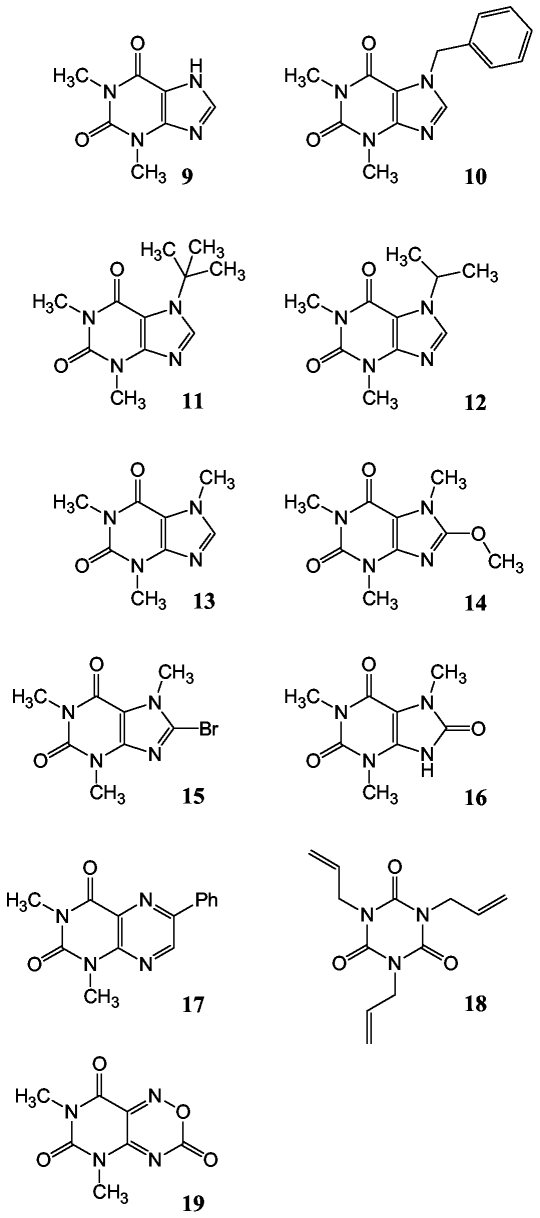


Fig. 2. Investigated substrates.

the doubly charged system consisting of receptor **1**, the corresponding substrate and two protons was detected. Employing the non-functionalized receptor **3** gave no reliable cationic species under the same conditions. Cone voltage was kept at a maximum of 14 V to prevent the dissociation of the complexes before they enter the quadrupole analyzer. Separation of a specific [M^{2+}] cation, acceleration and subsequent collision with argon enabled us to compare the association

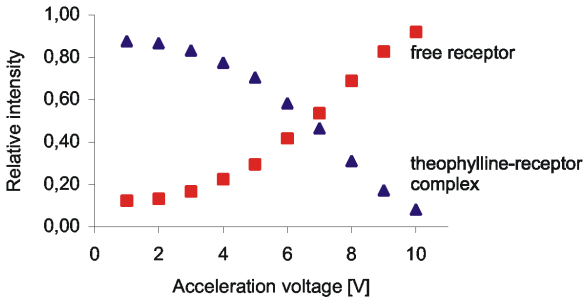


Fig. 3. Relative intensities of theophylline-**1** complex and free receptor in dependence on acceleration voltage.

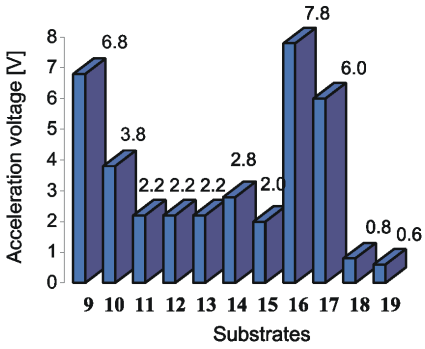


Fig. 4. Points of intersection for substrates **9** – **19**.

quality of different substrates. Increasing the acceleration voltage stepwise led to the dissociation of the substrate-receptor complex and therefore, the detected intensity of the complex decreased while the intensity of the empty receptor increased.

Figure 3 illustrates these findings with theophylline (**9**). The relative intensities of the complex and the empty receptor **1** are depicted in dependence on the acceleration voltage. The point of intersection (for **9** at 6.8 V), wherein the relative intensities for the free receptor as well as for the complex are 0.5, is a specific criterion for the individual substrate and indicates the stability of the corresponding receptor complex. These data were determined for each substrate and the individual results are summarized in Fig. 4.

The investigations do not allow the quantification of association constants, but the relative stabilities of different substrate-receptor complexes can be clearly assigned. A higher voltage at the point of intersection corresponds to a higher acceleration voltage of the cations and therefore, a higher stability of the particular substrate-receptor aggregate is revealed.

First, theophylline (**9**) and its derivatives 7-benzyltheophylline (**10**), 7-*tert*-butyltheophylline (**11**), and 7-*iso*-propyltheophylline (**12**) are compared with each other. Those substrates are able to form hydro-

gen bonds with receptor **1** using two carbonyl moieties and nitrogen N^9. The alkyl substituents on N^1 and N^3 are pointing between the receptor arms. The intersection point of theophylline is relative high, indicating that the affinity of theophylline towards the receptor is higher than the affinity for most of the other substrates. That can be explained by comparing the steric demand of derivatives **9 – 12**. The theophylline-receptor complex has the highest stability among these substrates. Exchanging hydrogen in position 7 to benzyl (**10**) leads to a higher steric demand of the substrate and therefore the stability of the substrate-receptor complex decreases. But the affinity of **10** increases compared to **11** and **12**, due to the fact that the benzyl group is able to turn vertical to the receptor arms, whereas the bulky *iso*-propyl and *tert*-butyl groups require more space, leading to significant repulsive interactions.

Similar affinities were found for caffeine (**13**) and its methoxy (**14**) and bromo derivatives (**15**), respectively. Their points of intersection are in the same range. NMR spectroscopic determination of the binding constant to receptor **3** in solution resulted in $35600 \pm 2000\ M^{-1}$ for caffeine and $9240 \pm 220\ M^{-1}$ for 8-methoxycaffeine (**14**) [5], seemingly the opposite result to the MS-MS screening experiments. But since the acceleration voltage varies in a very small range (0 – 10 V), the dissociation of the complexes takes place at very low tension. Keeping in mind that the decay is a gas phase process and a second proton may interfere with the supramolecular interaction, still a good consistency in the general course for the complex formation to suitable substrates was found. Furthermore, binding constants of 9240 or 35600 M^{-1}, respectively, are very low compared to the binding constant of 1,3,7-trimethyluric acid (**16**) in receptor **3**, which could not be assigned up to now, but was estimated to be > 200000. Comparing those numbers legitimates qualitative conclusions, since **16** leads clearly to the highest voltage at the point of intersection, indicating that the 1,3,7-trimethyluric acid-receptor complex is the most stable among the investigated complexes.

6-Phenyl-lumazin (**17**) forms a stable complex as well, whereas triallyltrizintrione **18** and oxadiazintrione **19** only represent weak binding partners. For the latter substrate a binding constant in receptor **3** measured by NMR spectroscopy in dichloromethane is also available [5]. This represents with $1240 \pm 115\ M^{-1}$ a very small value, which clearly correlates with the results of our MS screening experiments.

Conclusion

The synthesis of *N,N*-dibenzylamino-substituted receptor **1** provided a tool for the fast screening of various substrates using mass spectrometric analysis. We could demonstrate that our investigations furnished reasonable results, which allowed qualitative conclusions concerning the stability of different substrates in receptor **1**. These results were mostly consistent with earlier findings and facilitate new insights, whereas NMR spectroscopy could not be applied. Based on these findings, we are currently ameliorating the receptor's qualities, allowing a competition between substrates and the simultaneous determination of association constants. The results will be presented in due course.

Experimental Section

All reagents were used in analytical grades. Solvents were desiccated if necessary by standard protocols. Column chromatography was performed on silica gel (particle size 63 – 200 μm, Merck, Darmstadt, Germany) using mixtures of cyclohexane with ethyl acetate as eluents. TLC was done on silica gel 60 F_{254} on glass (Merck, Darmstadt, Germany). Melting points were determined on a Melting Point Apparatus SMP3 (Stuart Scientific, Watford Herts, UK) and were uncorrected. NMR spectra were recorded on a Bruker ARX 300 (Analytische Messtechnik, Karlsruhe, Germany) using TMS as internal standard or $CDCl_3$ with $\delta = 7.26$ ppm for ^{1}H NMR, and $\delta = 77.0$ ppm for ^{13}C NMR. EI mass spectra were obtained on a MAT8200 (Finnigan-MAT, Bremen, Germany) and ESI on a QUATTRO LCZ (Waters-Micromass, Manchester, UK).

General procedure for the MS-screening experiments

Solutions (1 mmol/l each) of receptor **1** and substrates **9 – 19** were prepared by dissolving appropriate amounts in tetrahydrofuran. Samples were prepared by mixing 100 μl of receptor solution, 900 μl of the respective substrate solution, 200 μl methanol, and 1 μl fumaric acid. The MS-screening experiments were acquired on a QUATTRO LCZ (Waters-Micromass, Manchester, UK). Samples were introduced by using a standard electrospray source with a syringe pump (flow rate: 1 ml/h). Applied parameters: capillary voltage 3.3 kV, cone voltage 27 V, unit resolution, collision gas 1.3×10^{-3} mbar argon.

N,N-Dibenzylamino-substituted receptor **1**

All-*syn* trisamine **2** (600 mg, 0.82 mmol) was dissolved in dichloromethane (20 ml) and chilled to 0 °C. Triethylamine (0.35 ml, 2.50 mmol, 3.0 eq.) was added, followed by the addition of a mixture of hexyl isocyanate (4.5 ml, 3.09 mmol,

3.8 eq.) and 3-(*N*,*N*-dibenzylamino)propyl isocyanate (**8**) (174 mg, 0.62 mmol, 0.76 eq.) in dichloromethane (5 ml). The reaction mixture was allowed to warm to ambient temperature, stirred overnight, and then diluted with ethyl acetate (125 ml). The organic phase was subsequently washed with 0.1 M hydrochloric acid (75 ml) at 5 °C and brine (3 × 75 ml), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. For the separation of the products the crude residue was adsorbed on silica and subjected to column chromatography (cyclohexane:ethyl acetate = 50:50) yielding receptor **3** [5] (394 mg, 0.35 mmol, 43%) (R_F (CH:EE = 50:50): 0.46) and **1** (115 mg (0.09 mmol, 11%) (R_F (CH:EE = 50:50): 0.24) as an off-white foam.

1: ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.66 - 3.20$ (m, 75H, bicyclo-H, CH_2, CH_3), 7.21 – 7.56 (m, 10H, phenyl-H), 7.57 – 7.94 (m, 6H, triphenylene-H). – ^{13}C NMR (75 MHz, $CDCl_3$) (selected signals of 77 detected): $\delta =$ 13.96 (CH_3), 39.88 (NHCH_2), 101.18 (spiro-C), 122.08, 124.48 (triphenylene-C), 129.25, 129.31 (phenyl-C), 147.74 (triphenylene-C). – MS (ESI (ES+)): *m/z* (%) = 1267.8 (10), 1266.9 (33), 1265.9 (84), 1264.9 (87) [M+H$^+$].

*Benzyl 4-(N,N-dibenzylamino)butyrate (**5**)*

4-Aminobutyric acid (39.95 g, 0.387 mol) was dissolved in dichloromethane (250 ml), potassium carbonate (72 g, 0.514 mol, 1.3 eq.) and 2 N aqueous sodium hydroxide solution (100 ml) were added. The biphasic mixture was heated at reflux conditions and benzyl bromide (170 ml, 1.40 mol, 3.6 eq.) was added drop by drop within 15 min. into the hot mixture. After 16 h, the mixture was brought to ambient temperature and the aqueous phase was extracted with diethyl ether (150 ml). The combined organic phases were washed with brine (2 × 150 ml), dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Excess benzyl bromide was removed by distillation, **5** (115.55 g, 0.309 mol, 80%) remained as yellow oil and was used without further purification.

^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.86$ (tt, $^3J = 6.9$ Hz, 6.9 Hz, 2H, 3-H), 2.37, 2.47 (2 × t, $^3J = 6.9$ Hz, 4H, 2-H, 4-H), 3.56 (s, 4H, N-benzyl-CH_2), 5.05 (s, 2H, O-benzyl-CH_2), 7.20 – 7.37 (m, 15H, arom. CH). – ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 22.32$ (C-3), 31.88 (C-2), 52.42 (C-4), 58.24 (N-benzyl-CH_2), 70.74 (O-benzyl-CH_2), 126.84, 126.94, 127.57, 127.73, 128.09, 128.13, 128.16, 128.25, 128.35, 128.49, 128.61, 128.65, 128.74, 128.80, 128.98 (arom. CH), 133.29, 136.09 (quat. C), 173.36 (CO). – MS (EI, 70 eV): *m/z* (%) = 373.3 (3) [M$^+$], 282.2 (9) [M$^+$ – Bn], 210.1 (85) [$CH_2NBn_2^+$], 91.0 (100) [$C_7H_7^+$].

*4-(N,N-Dibenzylamino)butyric acid hydrochloride (**6**) [20]*

A solution of benzyl-4-(*N*,*N*-dibenzylamino)-butyrate (**5**) (101.76 g, 0.272 mol) in aqueous potassium hydroxide solution (20%, 80 ml) was heated at reflux conditions over night. Subsequently, 6 N hydrochloric acid (100 ml) was added at ambient temperature and the solution was concentrated *in vacuo*. The remaining residue was crystallized twice from acetic acid (50 ml) and hydrochloride **6** was obtained in colorless crystals (55.72 g, 0.174 mol, 64%).

M. p. 146 – 147 °C. – ^{1}H NMR (300 MHz, D_2O): $\delta =$ 2.10 – 2.18 (m, 2H, 3-H), 2.46 (t,$^3J = 6.9$ Hz, 2H, 2-H), 3.22 – 3.28 (m, 2H, 4-H), 4.48 (s, 4H, N-benzyl-CH_2), 7.53 – 7.63 (m, 10H, arom. CH). – ^{13}C NMR (75 MHz, D_2O): $\delta = 19.15$ (C-3), 31.12 (C-2), 51.88 (C-4), 57.74 (N-benzyl-CH_2), 129.45, 129.86, 130.67, 131.50 (arom. C), 177.15 (CO).

*4-(N,N-Dibenzylamino)butyric acid (**7**) [20]*

4-(*N*,*N*-Dibenzylamino)butyric acid hydrochloride (**6**) (11.00 g, 34.4 mmol) was dissolved in water (250 ml) and neutralized with aqueous sodium hydroxide solution (20%). After removal of water under reduced pressure, the residue was dissolved in chloroform (150 ml) at reflux conditions, filtered hot and washed with chloroform (3 × 20 ml). Evaporation of the volatiles yielded a colorless, viscous oil (9.78 g, 34.4 mmol, 100%).

^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.75$ (tt,$^3J = 6.6$ Hz, 6.6 Hz, 2H, 3-H), 2.20 (t, $^3J = 6.6$ Hz, 2H, 2-H), 2.12 (t, $^3J =$ 6.6 Hz, 2H, 4-H), 3.60 (s, 4H, N-benzyl-CH_2), 7.20 – 7.33 (m, 10H, arom. CH), 12.04 (bs, 1H, COOH). – ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 21.82$ (C-3), 34.47 (C-2), 53.05 (C-4), 58.03 (N-benzyl-CH_2), 127.49, 128.42, 129.41 (arom. CH), 136.94 (quat. C), 177.93 (CO). – MS (EI, 70 eV): *m/z* (%) = 283.1 (7) [M$^+$], 210.1 (74) [$CH_2NBn_2^+$], 91.0 (100) [$C_7H_7^+$].

*3-(N,N-Dibenzylamino)propyl isocyanate (**8**)*

4-(*N*,*N*-Dibenzylamino)butyric acid (**7**) (4.77 g, 16.8 mmol) was dissolved in dry toluene (125 ml), treated with triethylamine (2.8 ml, 20.2 mmol, 1.2 eq.), diphenylphosphoryl azide (DPPA) (4.4 ml, 20.2 mmol, 1.2 eq.) and stirred at ambient temperature for 3 d, followed by refluxing until gas evaporation was no longer observed. Upon removal of the solvents under reduced pressure the remaining oil was purified by distillation in high vacuum yielding **8** as yellow oil (1.78 g, 6.3 mmol, 38%).

B. p. 154 – 156 °C/5 × 10^{-5} mbar. – ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.81 - 1.89$ (m, 2H, 3-H), 3.12 – 3.19, 3.23 – 3.29 (m, 4H, 2-H, 4-H), 4.55, 4.61 (s, 4H, N-benzyl-CH_2), 7.21 – 7.33 (m, 10H, arom. CH). – ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 21.82$ (C-3), 34.47 (C-2), 53.05 (C-4), 58.03 (N-benzyl-CH_2), 127.49, 128.42, 129.41 (arom. C), 136.94 (quat.-C), 177.93 (CO). – MS (EI, 70 eV): *m/z* (%) = 280.2 (67) [M$^+$], 251.1 (5) [M$^+$–CO], 189.1 (62) [M$^+$–C_7H_7], 91.0 (100) [$C_7H_7^+$], 77.0 (13) [$C_6H_5^+$].

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