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A way to manage the thermal flexibility of ligand candidates for bioassays

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Abstract—An original way to manage both stereochemistry and conformational constraints in ligand candidates for bioassays is presented with reference to a group of model *N*,*N*′-tetrasubstituted *o*-phthalamides and thioamides. The study shows that a scale of thermal flexibility in solution can be envisaged, the divisions of which are represented by compounds sharing quite similar geometrical features. NMR spectroscopy and powder X-ray analysis were used for the physical chemical investigation. An attempt to exploit the conformational instability of a model thioamide in the medium of a bioassay was also performed.

1. Introduction

Recently, we reported our interest in chemical libraries for broad screening purposes, designed using symmetry considerations to control the internal motions of the library members. The properties of certain derivatives of piperidine[1] and *o*-phthalic acid[2] (Fig. 1) were studied in the solid phase and computed in the gas phase. Nitrogen inversion and ring flipping characterize the internal motion of the model piperidine diol **1**. Nitrogen oxidation precludes inversion and unbiases the ring flipping equilibrium in a manner, which can be modulated by the choice of the substituents at positions 3 and 5. Symmetry is broken along the series of models **1**–**3** and other substituents can be imagined to become more or less restricted in their own local motion on the basis of their ability as hydrogen bond donors. As for compounds **4**–**7**, rotation around C_{Ar}–CO(S) and CO(S)–N bonds constituted the focus of the computational study. Experimentally, single crystal X-ray analysis was accomplished for compounds **6** and **7**, the melting behavior of which was also studied.

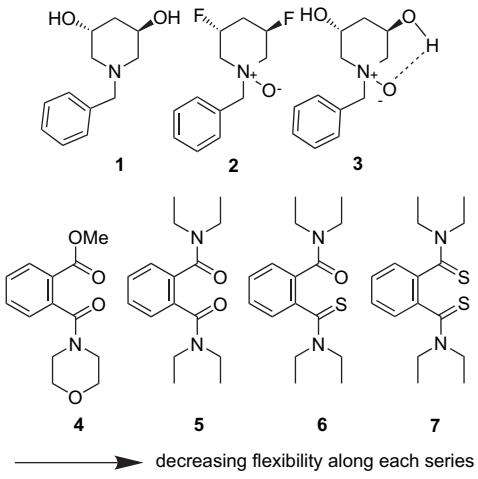

Figure 1.

Many compounds with general formula **I** (Fig. 2) had previously been found to possess nanomolar potency as antagonists of Neurokinin A at the human tachykinin NK-2 receptor.[3]

During this research we found,[4] in agreement with previous literature,[5,6] that amides of type **I** possess expanded features when compared with other *o*-substituted tertiary aromatic amides.[7] So, for instance, once obtained by double amidation of *o*-phthalic acid with the same achiral, differentially substituted, secondary amine, they exist in solution as mixtures of three conformational isomers, one asymmetric (**Ib**) and two dissymmetric (**Ia** and **Ic**). The amide groups are preferentially antiparallel to each other and their planes tend to be orthogonal to the aromatic ring. Interconversion occurs through rotation around the C_{Ar}–CO and CO–$N(CH_2R_1)CH_2R_2$ bonds. Taking the prominent biological

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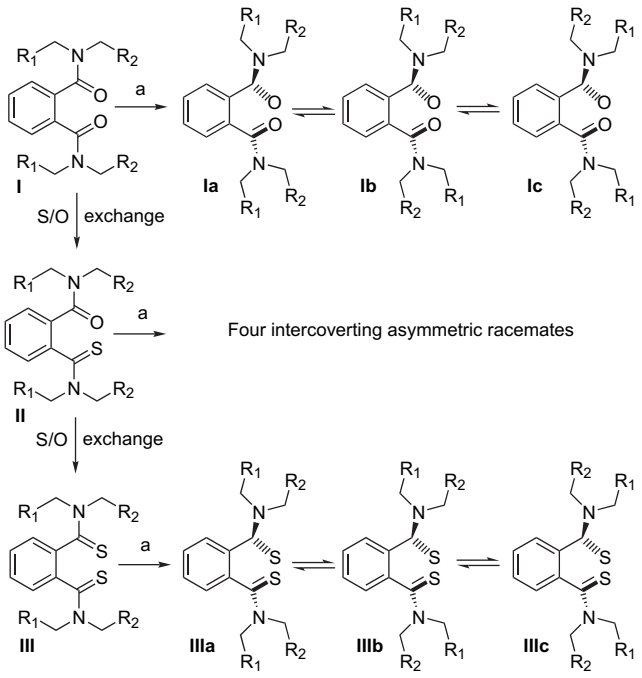
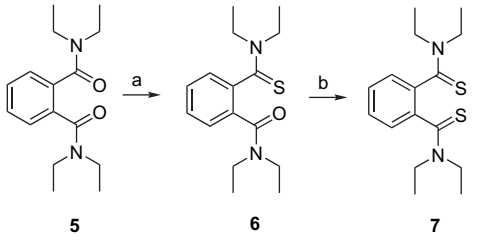


Figure 2. (a) Dissolution.

activity of many compounds of type **I** as a kind of validation, the idea of exploiting their internal motion to build a scale of thermal flexibility was boosted when we became aware that the thioamide replacement of *o*-substituted tertiary benzamides not only slows rotation around the CS–NR_1R_2 bond, but also around the C_{Ar}–CS bond, without major geometrical changes.[8] Indeed, the main difference between compounds **1**–**3** and compounds **4**–**7** (Fig. 1) is just that the piperidine derivatives are enantiomerically stable at any reasonable pharmaceutical and pharmacological time scale,[9] while the same is untrue for the *o*-phthalic acid derivatives. So, in principle, a library obtained by the proper decoration of compounds **4**–**7** could permit to observe the emergence of chirality along the corresponding flexibility scale and to investigate the role of absolute stereochemistry on the bio-performance of the library members.[10,11] On this basis, we wish to show here that compounds of general formulas **I**–**III** are suitable to define a scale of thermal flexibility in solution, the divisions of which are represented by compounds sharing very similar geometrical properties. The result was achieved by studying the behavior of compounds **5**–**7** and **9a**–**9c** in dilute solution by NMR spectroscopy. In addition, the practical conversion of the conformational mixture **9a**–**9c** into the sole isomer **9a** is also described and discussed. Eventually, we disclose that the double thionation of **8**, a tachykinin receptor ligand of type **I**, to give the corresponding compound of type **III** depletes the affinity toward the receptor. This information was obtained while assessing whether the instability of **9a**, after dissolution, could usefully match the bioassay time scale.

2. Results

The synthesis of compounds **5**–**7** is outlined in Scheme 1. Diethyl amine was chosen to yield models of compounds of general formulas **I**, **II**, and **III** in which R_1=R_2, suitable for the ^{1}H NMR spectroscopic investigation.



Scheme 1. (a) LR, 1.04 equiv, THF, rt overnight, then chromatography, 40%. (b) LR, 1.17 equiv, refluxing THF, 8 h, then chromatography, 61%.

We planned to study the conformational interconversion by observing the possible anisochronicity of the methylenic protons within each ethyl group. Phthalamide **5** was converted consecutively into the mono-thioamide **6** and into the bis-thioamide **7**, by means of Lawesson's reagent[12] (LR hereinafter) under two different reaction conditions. Compound **6** was obtained in 40% yield, after chromatographic separation, by reacting compound **5** with a slight molar excess of the thionating agent in THF at rt. This reaction also yielded 10% of compound **7**. This product in turn, was obtained from purified compound **6** in 61% yield after chromatography, by reacting the substrate with an overall excess of LR, added portionwise to the reaction mixture in refluxing THF.

2.1. NMR investigation on compounds 5–7

The rt ^{1}H NMR spectra in $CDCl_3$ at 600 MHz of compounds **5**, **6**, and **7** are stacked in Figure 3. The spectrum of compound **7** (upper spectrum) reveals the dissymmetry of the solute. The aromatic protons give rise to an AA′BB′ spin system. As for the aliphatic protons, the triplets resonating at 1.15 and 1.30 ppm, each integrating to 1.5 times the total area of the aromatic spin system, indicate that a sole cis/trans relationship concerns the amide groups. The eight methylenic protons resonate as four multiplets each integrating to half the total area of the aromatic spin system. The fact that the protons, which belong to the same methylenic group are anisochronous is proved by bidimensional analysis and reflects the existence of a stereogenic element. The fact that there are only four different chemical shifts for the methylenic protons suggests the existence of an internal binary axis of rotation. Aside from residual ethyl acetate, the spectrum of compound **6** is essentially the combination of two different copies (with different chemical shifts) of the spectrum of **7**, thus suggesting that the single O/S replacement solely removed the rotational symmetry from compound **7**. The two multiplets centered at 3.71 and 4.61 ppm in the spectrum of **7** show a slightly more complex fine structure than those centered at 3.46 and 3.30 ppm. A similar appearance also affects four out of eight multiplets in the spectrum of **6** (see insets in Fig. 3). To explain this evidence, we considered compound **7** and found, using the bidimensional analysis and computational simulation, that a long range ^4J of 1.3 Hz correlates two hydrogens at the extrema of each H–C(H)(Me)–N(CS)–C(Me)(H)–H fragment. Although initially surprised to observe such a correlation at rt for an acyclic spin system, we reasoned that the

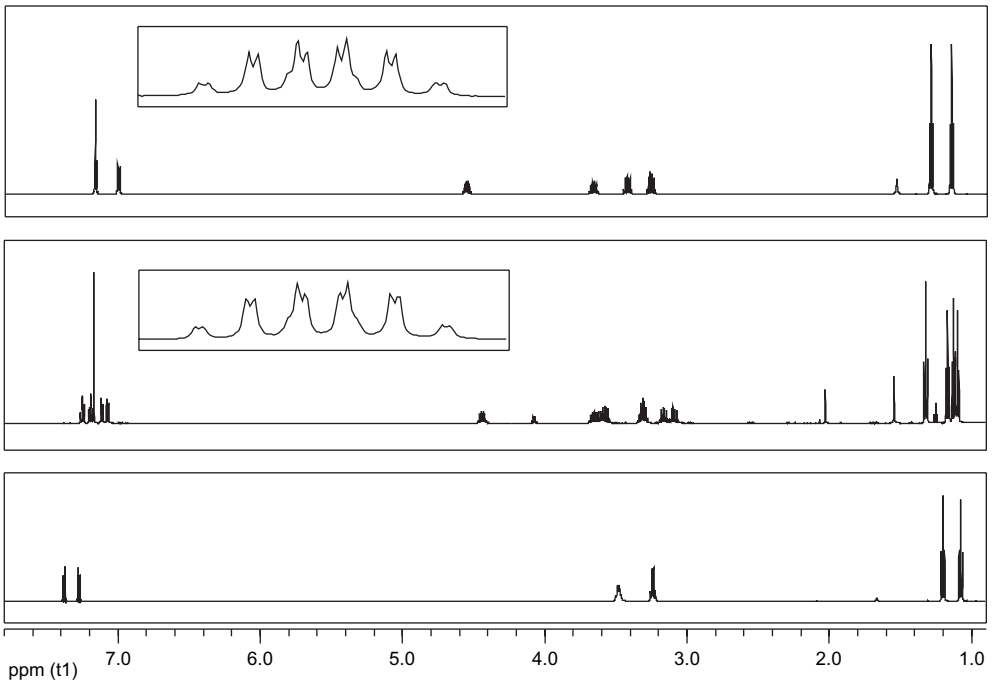


Figure 3. Upper spectrum: compound **7**, inset: multiplet centered at 4.61 ppm. Middle spectrum: compound **6**, inset: multiplet centered at 4.49 ppm. Lower spectrum: compound **5**.

conformational equilibrium related to the fast rotation around N–CH_2 bonds could privilege conformers possessing the proper W arrangement of atoms.[13] To this regard, it is worth noting that one such conformer had been effectively observed in the crystal, while the diasteroisomer obtainable from that by inverting both C_{Ar}–CS bonds was computed (ab initio) to be 5 kcal/mol higher in energy.[2] As for the survey of flexibility differences amongst compounds **5**–**7** in solution, the rt spectrum of compound **5** (lower spectrum in Fig. 3) serves well to introduce the discussion. In fact, the different broadening of the signals centered at 3.23 and 3.47 ppm indicates the geometrical similarity between this compound and compound **7**. At lower temperatures (Fig. 4), each methylenic signal splits into two signals. All four signals integrate to the same value and those originating from the low field signal show a higher difference of chemical shifts than those originating from the high field quartet. In other words, the spectrum of compound **5** at low temperature approaches that of compound **7** at rt. Actually, the rt spectrum of **5** reveals the expected fast interconversion of the enantiomeric conformers existing in the $CDCl_3$ solution. To estimate the rate of enantiomerization at different temperatures, we focussed our attention on the methylenic group resonating at 3.47 ppm (rt spectrum), while irradiating the corresponding methyl group at 1.20 ppm, and successfully simulated this dynamic behavior by computation.

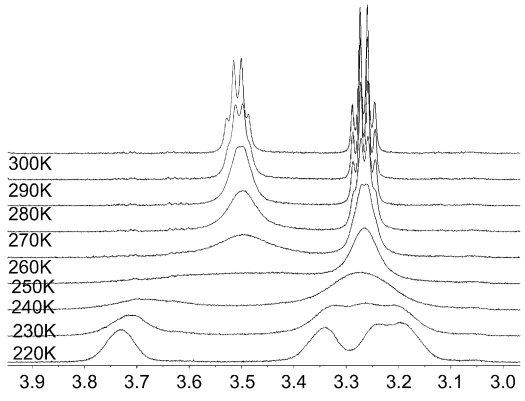


Figure 4.

Eventually, we extrapolated that interconversion occurs with a frequency of about 35,000 s^{-1} at rt, in $CDCl_3$.[6] Since the $CDCl_3$ spectra of **6** and **7** suggested that rotation around the bonds of our interest could be frozen, even C_{Ar}–CO rotation in **6**, we moved to DMSO-d_6 solutions to acquire spectra at higher values of temperature. While assigning the signals in this solvent, we found in the NOESY spectrum (mixing time=0.4 s) of compound **6**, that the two ethyl signals assigned to the oxygenated half of the molecule gave exchange correlation cross-peaks (same sign as diagonal peaks), while the other two ethyl signals (the thionated half) only had NOE type cross-peaks (opposite sign). An indication of the fact that the rotation around the CO–NEt_2 bond, but not that around CS–NEt_2 bond, takes place on the time scale of the NOESY experiment. The finding was confirmed by observing (Fig. 5) the selective broadening of the peaks originating from the oxygenated half of the molecule in the mono-dimensional spectrum of **6** acquired at 340 K. Eventually, for compound **7**, we tried to speed up the rotations under inspection by increasing the temperature to 380 K (DMSO-d_6) without however obtaining any hint of a dynamic process taking place. Compound **7** therefore seems to be the best candidate for attempting the separation and isolation of the enantiomers, a task, which is also in our schedule. By comparing the dynamic properties of compounds **5** and **6**, we can state that, at rt, rotation around CO–NEt_2 bonds is slow in both cases. As for the rotation around C_{Ar}–CO bonds, we have seen that enantiomerization is fast in compound **5**. For this isomerization to occur, it is

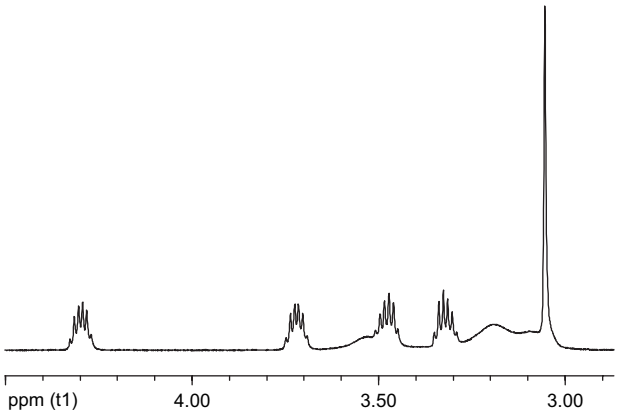
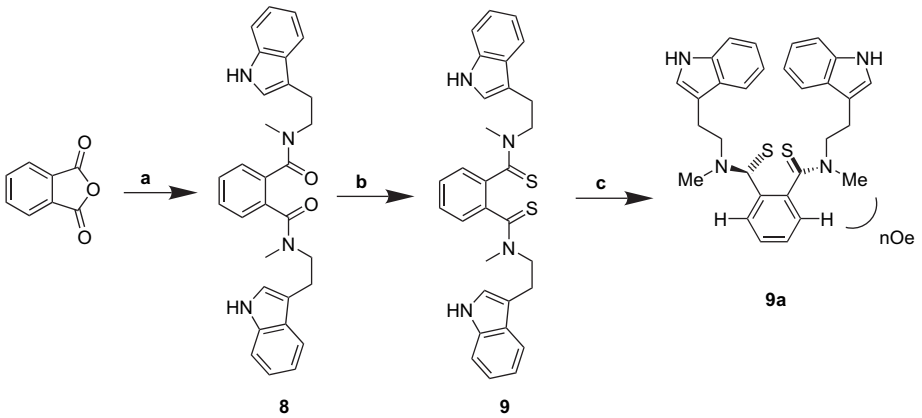


Figure 5. Selective broadening of amide signals in **6**.

necessary that both amide groups invert their orientation with respect to the aromatic plane. Albeit a single step mechanism suffices to interpret DNMR data for compound **5**, literature suggests the existence of a *syn*/*anti* conformational equilibrium strongly privileging the *anti* conformer.[5,6] In a previous study,[2] the stability differences between *syn* and *anti* conformers had been calculated ab initio to be 6.0 kcal/mol for compound **5** and 10.4 kcal/mol for compound **6**. Therefore, if the *syn*/*anti* oscillation occurred in **6** due to C_{Ar}–CO bond rotational lability, no consequence would be easily detected by conventional NMR spectroscopy. The stereochemical properties of the solute would be dictated by the thionated moiety, the rigidity of which is apparent when the spectrum acquired at 340 K is considered.

2.2. Synthesis of compounds 8–9a

The synthesis of compounds **8**–**9a** is outlined in Scheme 2. *N*-Methyltryptamine was chosen to give models of compounds of general formulas **I**, **II**, and **III** in which $R_1 \neq R_2$, suitable for managing different aspects of the problem. The use of an aromatic appending moiety was thought to be interesting in the perspective of the library generation, while the methyl group was chosen to facilitate the understanding of the ^{1}H NMR spectra. Indole was selected as the aromatic group, both for a possible investigation on the mode of binding of the corresponding tachykinin receptor ligands, or to make the thionations more easily followed by TLC analysis. In our experience, 3-indolylmethyl-containing products give violet spots on silica after exposure to *p*-anisaldehyde and heat. Moreover, indole itself is considered as a biologically validated sub-structure.[14] So, our study could furnish an example, maybe the first one, of double use of the indole group in a well defined, yet modulatable, tridimensional space. Compound **8** was obtained in 89% yield, after aqueous work-up, making phthalic anhydride react with a slight excess of *N*-methyltryptamine in DMF at rt, before the addition of EDCI, DIPEA, and HOBT. Compound **8** gave a single spot when analyzed by TLC on silica using 5% of methanol in ethyl acetate as eluent. The thionation of **8** by LR in refluxing toluene proved difficult to monitor by TLC, with the plates being crowded with many spots, soon after the beginning of substrate conversion. Aware that the use of LR usually results in the appearance of many spots on the TLC plate, we monitored the reaction progress using exposure to *p*-anisaldehyde to reveal the TLC spots, HPLC analysis, and MS spectrometry. The combination made us realize that the four diasteroisomers expected for the mono-thionated product and the three diasteroisomers expected for the bis-thionated derivatives could give spots of different R_f values. For the sake of simplicity, we decided to concentrate on the supposed bis-thionated products at this stage of the study. We therefore used an excess of thionating agent and focussed our attention only on the fast moving, indole revealing spots. When we decided that these spots had accumulated enough, we stopped heating and purified the crude material by chromatography. In a previous experience, we had observed that different chromatographic behavior on silica of similar labile diasteroisomers does not ensure their isolation.[4] Therefore, we decided to collect together the three bis-thionated products (overall yield of 49%), despite each one having a specific R_f value on silica, using 3% ethyl acetate in dichloromethane as eluent (R_f=0.32, 0.26, 0.17). This mixture could be readily evaluated in DMSO-d_6, but proved to be sparingly soluble in $CDCl_3$, an occurrence which enabled us to get an unexpected result. In practice, after the filtration of the $CDCl_3$ suspension, the DMSO-d_6 spectrum of the solid was essentially that of only one dissymmetric isomer whose configuration was later assessed as **9a** (Scheme 2) from the NOESY spectrum. The serendipitous isolation of **9a** had two consequences. First, as described below in the text, we were able to follow the kinetics of the conversion into the



Scheme 2. (a) *N*-Methyltryptamine, 2.3 equiv, DMF, rt 1 h, then DIPEA, 2.0 equiv, EDCI, 1.2 equiv, HOBT, 1.2 equiv, rt, overnight, 89%. (b) LR, 2.3 equiv, refluxing toluene, 1 h, then chromatography, 49%. (c) $CHCl_3$, 6.7 equiv, 40 °C, 8 h, 98%.

thermodynamic mixture in DMSO-d_6. Second, we succeeded to convert the mixture of the three isomeric bis-thionated products, as obtained from the chromatographic separation, directly into **9a** in quantitative yield. Indeed, after some attempts, we obtained 464 mg of essentially pure isomer **9a**, by heating 473 mg of dried eluate, containing less than 40% of the same compound, at 40 °C for 8 h in a corked HPLC vial with 500 μl of chloroform (molar ratio 1/6.7). Although serendipitous in origin, this transformation would be looked at as an example of phase transition driven reaction, namely a reaction the selectivity of which should be due to the stability difference amongst the possible isomers in the solid phase, while their interconversion occurs smoothly in the liquid, on the experimental time scale.[15,16]

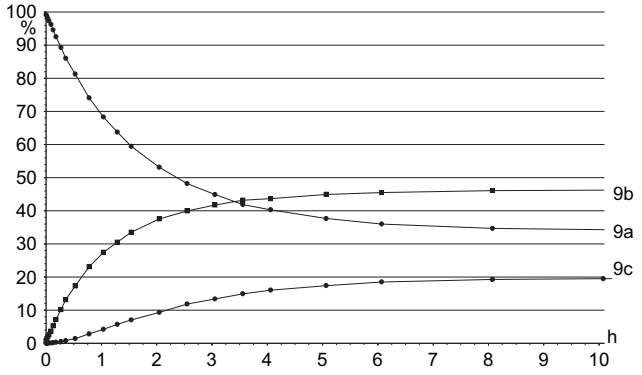


Figure 7.

2.3. Physical–chemical investigation on compound 9a

Compound **9a** was quite stable as a solid and could be stored at rt on the shelf for months. In order to ascertain this observation, we examined an aliquot of the crude reaction product by X-ray powder diffraction analysis. This allowed us to realize that **9a** had accumulated as a crystalline powder during the reaction and that its thermal stability as a solid is effectively high. After the rt analysis, we acquired spectra at 333 K every 2 h for a day without observing any change with respect to the first spectrum. In Figure 6, the first (a) and the eleventh (b) spectra are reported.

Nevertheless, the conversion into the mixture could be monitored by NMR at rt, after dissolution in DMSO-d_6 (Fig. 7). While peaks belonging to the asymmetric form **9b** appeared as soon as the solution was prepared and inserted into the magnet, the other symmetric conformer **9c** appeared, in traces, only after 4 min. The growing curve for these two conformers have exponential and sigmoidal shapes, respectively, supporting the idea by which each symmetric conformer can convert (in one step) only into asymmetric ones and vice versa. Calculating the percentage of each isomer at each stage was complicated by the mutual overlap of signals. We chose to monitor the intensity of the methyl singlets because of their higher sensitivity. Two singlets of interest fall just on top of some methylenic multiplets and we therefore adopted the following protocol.

We integrated each cluster of overlapping peaks as a whole and, separately, one or more other methylenic multiplets originating from the same isomers and comprising the same number of protons, so that the difference between the chosen integrals was equal to the area of the methyl singlet alone. To avoid any complication that may have arisen from the different saturation of different signals, each spectrum was acquired in a single scan. Plotting the calculated percentage, instead of the area, compensated for any change in instrumental sensitivity over the course of the experiment. What we discovered in this way is that, even at rt, the rotation around the thioamide bond is so slow that it takes hours before the equilibrium mixture is reached. A stationary state is achieved after 13 h (**9a**: 34.3%, **9b**: 46.2%, **9c**: 19.5%), while **9a** still accounts for about 60% of the mixture 90 min after dissolution.

2.4. Binding affinity tests

In the light of the pharmaceutical and pharmacological time scale definitions,[9] compound **9a** could be an example of a pharmaceutically stable substance, whose pharmacological stability would depend upon the consideration of either in vitro, or in vivo experiments. It is apparent that **9a**, independently from any consideration of its racemic nature, could not be looked at as a sole substance with respect to an in vivo experiment.[17] Conversely, its homogeneity could be challenged on the time scale of an in vitro assay. Before the present study, we had synthesized compound **8** during our efforts to obtain antagonists of Neurokinin A at the human tachykinin NK-2 receptor.[4] In Figure 8, it is shown that compound **8** inhibits the binding of iodinated NKA to human NK-2 receptor in concentration-dependent manner, with sub-micromolar potency.

With compound **9a** at hand and the knowledge of its behavior in DMSO-d_6 solution, we reasoned that there was the possibility to investigate the binding mode of **8** by using

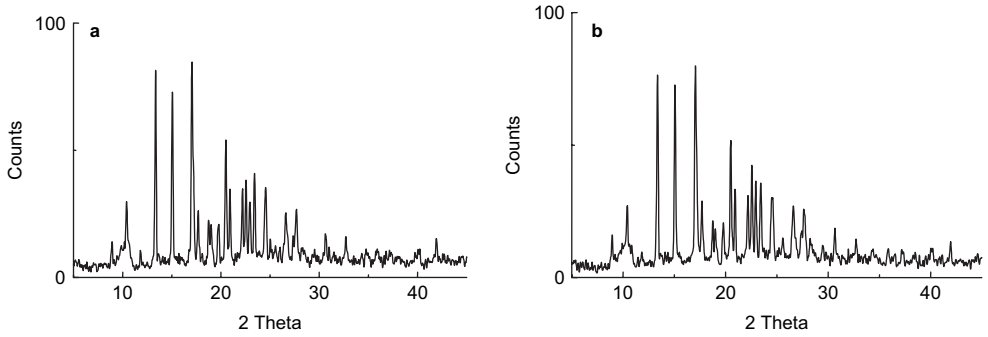


Figure 6. X-ray powder diffraction spectra of **9a** at 333 K. (a) Time zero. (b) After 22 h.

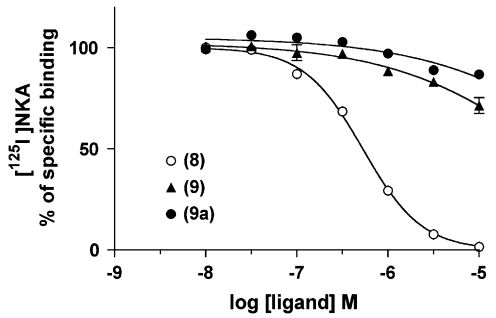


Figure 8.

separately compound **9a** and its equilibrium mixture. In principle, only the inspection of the preferred absolute stereochemistry would have been precluded, **9a** being reasonably a racemate. Unfortunately, the thionated mixture **9**, as well as compound **9a** was far less potent than **8** in the same assay (Fig. 8). Worst, the possibility to discriminate **9a** from **9** by the careful reduction of the equilibration time of the bioassay at higher concentrations of the thionated candidates was hampered by their insolubility in the medium of the bioassay. Overall, the only information we gained from these pharmacological experiments was that the double thionation of **8** provokes a marked affinity loss at the receptor. On the other hand, since the overall tridimensional shape is conserved when passing from **8** to the mixture **9a**–**9c**, it is not unreasonable to admit that the removal of at least one critically positioned hydrogen bond acceptor from **8** is responsible for affinity depletion. The interpretation would agree also with similar results obtained in a previous study with other ligands at the same receptor.

3. Conclusion

In this paper, we have shown that compounds of general formulas **I**–**III** can define a scale of thermal flexibility in solution, the divisions of which are represented by compounds sharing very similar geometrical properties. Although we have not shown the effective emergence of chirality along the series of the investigated model compounds, that of a homogenous and rt thermally stable substance from a conformational mixture was demonstrated. The resolution of compound **7** into its enantiomers and the assessment of their lifetimes is under investigation. In addition we are working to synthesize a pool of compound **8** congeners. The aim is to investigate whether the scope of the solidification driven conversion from **9a**–**9c** into **9a** is wider, and also to use the entire pool for challenging other biological targets than human NK-2 receptor. The results of these efforts will be reported in due course.[18]

4. Experimental

4.1. General

Anhydrous solvents were purchased from Fluka. TLC monitoring: Merck silica gel 60 F_{254} plates, detection by UV light or *p*-anisaldehyde and heat. HPLC monitoring: Waters 2690 Separation Module equipped with a Waters 996 photodiode array detector. Separations were obtained by a Symmetry300™ C_{18} 5 μm column (4.6×250 mm), eluting at a flow rate of 1 ml/min with a mobile phase consisting of A—water+0.1% TFA, B—acetonitrile+0.1% TFA. Gradient elutions were performed from 20% to 80% B in 20 min. Melting points were determined in capillary tubes and are uncorrected. IR spectra were collected with a Nicolet Avatar 360 FTIR E.S.P. spectrophotometer. ^{1}H NMR spectra were acquired at 500 or 600 MHz on Bruker Avance instruments and referenced against the residual solvent peak (DMSO-d_6 at 2.50 ppm and $CDCl_3$ at 7.25 ppm). For variable temperature studies, we assumed that the temperature of the probe (reported by the spectrometer) was the same as that of the solution. NOESY spectra were typically acquired with a mixing time of 0.4 s, 2K points along f_2 and 512 points along f_1, in the phase-sensitive mode according to the States-TPPI protocol. The f_1 dimension was extended four times with Linear Prediction. The baseplane of the transformed spectrum was further processed with polynomial corrections of the fifth order in both dimensions. ^{13}C NMR spectra were acquired on a Varian Gemini 200 spectrometer, at the operating frequency of 50 MHz. All the processing was performed using the program SwaN-MR.[19] Mass spectra were obtained with a Finnigan LCQ ion trap mass spectrometer, operated in positive-ion electrospray ionization. The samples were analyzed by full-scan MS and product ion MS/MS of the protonated quasi-molecular ions, at 30% relative collision energy, using helium as the collision gas. The high resolution mass spectrum of compound **8** was obtained with a Thermo Electron LTQ Orbitrap spectrometer, equipped with nano-electrospray ion source, operated in positive-Electrospray. The instrument was externally calibrated with a mixture of caffeine, MRFA, and Ultramark 1621. The sample (about 10 μg/mL in 1:1 acetonitrile/10 mM ammonium acetate) was introduced by infusion at 1 μL/min, and analyzed in full-scan MS mode at resolution=50,000. X-ray powder diffraction data were collected on a Bruker D8 Advance powder diffractometer, equipped with a Debye–Scherrer transmission θ–θ geometry, using the Cu Kα radiation. The Sol-X solid state Si(Li) detector was used. C/Ni Goebel–Spiegel mirrors in the incident beam were used as monochromator; 1.0 mm divergence, 0.2 scatter and 0.1 for the receiving slits were used. The sample was prepared by pressing the thin layer of the sample on a Pt–Rh fine foil. The sample was heated under high-temperature (HT) chamber (mri wide range) from rt to 333 K with a step of 0.2 °C/min. The spectra were recorded in the 2θ range 5–45 °C. Binding affinity experiments: Inhibition binding curves were performed with membrane preparations (150 μg/ml) of stably transfected CHO cells expressing the human tachykinin NK_2 receptor, using [^{125}I]NKA (0.13 nM) as radioligand.[20] Binding data were fitted by nonlinear regression using GraphPad Prism 4.0.

4.1.1. Synthesis of 2-diethylthiocarbamoyl-*N*,*N*-diethylbenzamide. Compound 6. LR (1.17 g, 2.88 mmol) was suspended in 10 ml of anhydrous THF under a nitrogen atmosphere. A solution of compound **5** (762 mg, 2.76 mmol) in anhydrous THF (12 ml) was added through a dropping funnel, while magnetically stirring. The reaction mixture was left under stirring overnight at rt. The volatiles were removed under reduced pressure and the residue (2.21 g as a yellow oil) was purified by Flash-Master chromatography.

The column was conditioned with *n*-hexane. Compound **7** was eluted first using 50% of CH_2Cl_2 in *n*-hexane (31 mg as a yellow solid; yield=10%). Compound **6** was then eluted using 20% of ethyl acetate in *n*-hexane (314 mg as a white solid; yield=40%).

4.1.1.1. 2-Diethylthiocarbamoyl-*N*,*N*-diethyl-benzamide. Compound 6. Mp: 81 °C–82 °C (*n*-hexane). R_f=0.1, CH_2Cl_2/*n*-hexane, 1:1. IR (KBr) ν (cm^{-1}): 2990, 2968, 2930, 2880, 2867, 1626, 1510, 1429. ESI$^+$-MS: *m*/*z*= 293 (MH^+), 220, 192, 164, 130, 121, 105. $C_{16}H_{24}N_2OS$ (292.44): calcd C, 65.71; H, 8.27; N, 9.58; found C 65.43, H 8.42, N 9.44. ^{1}H NMR (DMSO-d_6, 300 K, 600 MHz): 7.39 δ (1H, t), 7.33 δ (1H, t), 7.25 δ (1H, d), 7.15 δ (1H, d), 4.32 δ (1H, m), 3.61 δ (1H, m), 3.57 δ (1H, m), 3.43 δ (1H, m), 3.28 δ (1H, m), 3.16 δ (1H, m), 3.09 δ (1H, m), 3.01 δ (1H, m), 1.19 δ (3H, t), 1.07 δ (3H, t), 1.05 δ (3H, t), 1.04 δ (3H, t). ^{13}C NMR ($CDCl_3$) δ: 196.69, 169.38, 140.90, 132.76, 128.51, 127.23, 125.53, 124.81, 48.44, 45.46, 43.42, 38.81, 13.86, 13.45, 12.32, 10.82.

4.1.1.2. *N*,*N*,*N*′,*N*′-Tetraethyl-benzene-1,2-dicarbothioic acid. Compound 7. Mp: 118 °C–119 °C (ethyl acetate). R_f=0.1, ethyl acetate/*n*-hexane, 1:4. IR (KBr) ν (cm^{-1}): 3076, 3058, 3007, 2974, 2953, 2872, 1507, 1429. ESI$^+$-MS: *m*/*z*=309 (MH^+), 236, 208, 180. $C_{16}H_{24}N_2S_2$ (308.51): calcd C, 62.29; H, 7.84; N, 9.08; found C 62.50, H 7.99, N 9.01. ^{1}H NMR (DMSO-d_6, 300 K, 600 MHz): 7.25 δ (2H, m), 7.08 δ (2H, m), 4.60 δ (2H, m), 3.70 δ (2H, m), 3.46 δ (2H, m), 3.29 δ (2H, m), 1.29 δ (6H, t, *J*=7.1 Hz), 1.14 δ (6H, t, *J*=7.2 Hz). ^{13}C NMR ($CDCl_3$) δ: 196.27, 138.38, 127.52, 124.48, 48.96, 45.12, 13.52, 10.51.

4.1.2. Synthesis of compound 7 from compound 6. LR (476 mg, 1.17 mmol) and thioxoamide **6** (292 mg, 1.0 mmol) were suspended in 8 ml of anhydrous THF under magnetic stirring and a nitrogen atmosphere. The solvent was refluxed for 8 h, and then the mixture was left to stand overnight. LR (202 mg, 0.50 mmol) was added and the solvent was refluxed for 3 h. LR (203 mg, 0.50 mmol) was added and the solvent refluxed for 5 h, then the mixture was left on standing overnight. LR (203 mg, 0.50 mmol) was added and the solvent refluxed for 1 h. The volatiles were removed under reduced pressure and the residue (2.06 g) was purified by Flash-Master chromatography. Compound **7** (188 mg as a white solid, yield=61%) was eluted using 50% of CH_2Cl_2 in *n*-hexane.

4.1.3. Synthesis of *N*,*N*′-bis-[2-(1*H*-indol-3-yl)-ethyl]-*N*,*N*′-dimethyl-phthalamide. Compound 8. Phthalic anhydride (325 mg, 2.60 mmol) and *N*-methyltryptamine (1.00 g, 5.74 mmol) were dissolved in 8 ml of anhydrous DMF under a nitrogen atmosphere. The mixture was magnetically stirred for 1 h at rt. DIPEA (672 mg, 5.20 mmol), EDCI (598 mg, 3.12 mmol), and HOBt (422 mg, 3.12 mmol) were added and the mixture was left overnight under stirring. The solution was poured into 1 M aqueous HCl under vigorous stirring. The white precipitated material was collected by filtration, washed with water, and dried in the air. Compound **8** (1.11 g; yield=89%) was obtained as a white solid and used for thionation without further purification. R_f=0.31 (ethyl acetate as eluent). The sample for bioassays and analysis was purified by chromatography on silica eluting with ethyl acetate. Mp: 97–102 °C. ESI$^+$-MS: *m*/*z*=479 (MH^+), 305, 144. IR (KBr) ν (cm^{-1}): 3400, 3288, 3047, 2929, 2853, 1623. ^{1}H NMR (DMSO-d_6, 300 K, 500 MHz): 10.82 δ (1H, br s), 10.80 δ (1H, br s), 10.79 δ (1H, br s), 10.76 δ (1H, br s), 7.60 δ (1H, t), 7.44 δ (1H, dd), 7.42 δ (1H, t), 7.35–7.21 δ (5H, m), 7.09–6.91 δ (6H, m), 6.79 δ (1H, m), 3.66 δ (2H, m), 3.33 δ (2H, m), 2.99–2.91 δ (4H, m), 3.01, 3.00, 2.83 and 2.77 δ (6H, singlets). ^{13}C NMR (DMSO-d_6) δ: 169.26, 169.20, 168.84, 168.80, 136.42, 136.02, 135.16, 134.87, 134.72, 134.64, 128.41, 128.33, 128.25, 128.16, 127.08, 126.86, 126.34, 126.20, 125.99, 122.71, 120.75, 118.46, 118.00, 117.94, 111.28, 111.25, 111.18, 110.68, 51.56, 47.46, 47.26, 32.01, 23.60, 22.32. HRMS (MH^+) calcd for $C_{30}H_{31}N_4O_2$: 479.2442; found: 479.2419.

4.1.4. Synthesis of *N*,*N*′-bis-[2-(1*H*-indol-3-yl)-ethyl]-*N*,*N*′-dimethyl-benzene-1,2-dicarbothioic acid. Compound 9. LR (694 mg, 1.71 mmol) and amide **8** (362 mg, 0.75 mmol) were suspended in 11 ml of anhydrous toluene under magnetic stirring and a nitrogen atmosphere. The solvent was made to reflux for 1 h. During this time, a dark orange solution formed. The volatiles were removed under reduced pressure and the residue (1.45 g) was purified by Flash-Master chromatography using CH_2Cl_2 as eluent. Compound **9** was obtained (188 mg as a pale yellow solid, yield=49%) by collecting together the fractions corresponding to three spots on the TLC plate: R_f=0.17, 0.26, 0.32 (ethyl acetate/CH_2Cl_2, 3:97 as eluent). ESI$^+$-MS: *m*/*z*=511 (MH^+), 337, 144. $C_{30}H_{30}N_4S_2$ (510.72): calcd C, 70.55; H, 5.92; N, 10.97; found C 70.07, H 5.99, N 10.67. ^{1}H NMR (DMSO-d_6, 300 K, 600 MHz) of the major (*E*,*Z*) conformer: 10.86 δ (1H, br s), 10.83 δ (1H, br s), 7.73 δ (1H, d, *J*=8.0 Hz), 7.36–6.85 δ (13H, m), 4.45 δ (1H, m), 4.00 δ (1H, m), 3.81 δ (1H, m), 3.51 δ (3H, s), 3.50 δ (1H, m), 3.25–3.16 δ (2H, m), 3.12 δ (3H, s), 3.00 δ (1H, m). After the identification, **9** was renamed as mixture **9a**–**9c**. ^{13}C NMR (DMSO-d_6) δ: 196.66, 196.54, 196.31, 196.06, 139.21, 138.70, 138.65, 138.23, 136.12, 135.98, 127.41, 127.09, 127.00, 126.82, 126.78, 124.96, 124.74, 124.40, 124.27, 122.96, 122.78, 120.87, 118.43, 118.151, 111.26, 110.84, 110.74, 110.16, 110.10, 57.47, 57.34, 54.53, 54.07, 42.42, 42.38, 23.71, 20.52. The acquisition of ^{13}C NMR spectrum started several hours after compound **9a** dissolution and must be considered as the spectrum of the thermodynamic mixture **9a**–**9c** in DMSO-d_6.

4.1.5. Conversion to thioamide 9a. The mixture of thioamides **9a**–**9c**, obtained as described above (473 mg, 0.93 mmol) and $CHCl_3$ (500 µl, 6.2 mmol) were placed in a stoppered HPLC vial, which was heated at 40 °C for 8 h in a sand bath. The mixture was filtered with the help of few milliliters of pre-cooled $CHCl_3$. Compound **9a** (464 mg) was recovered as a white solid. R_f=0.32 (ethyl acetate/CH_2Cl_2, 3:97 as eluent). ESI$^+$-MS: *m*/*z*=511 (MH^+), 337, 144. The composition of this solid was not altered after standing for a few months on the shelf, at rt. On the other hand, it melted in a capillary tube, after 30 min at 130 °C. IR (KBr) ν (cm^{-1}): 3275, 3057, 3000, 2930, 2852, 1514, 1457. ^{1}H NMR (DMSO-d_6, 300 K, 600 MHz): 10.84 δ (1H, br s), 8.32 δ (1H, s), 7.73 δ (1H, d, *J*=7.9 Hz), 7.33 δ (4H, m), 7.22 δ (2H, s), 7.10 δ (2H, dd, *J*=5.6, 3.2 Hz),

7.05 δ (2H, t, J=7.6 Hz), 6.91 δ (2H, t, J=7.4 Hz), 4.61 δ (2H, td, J=11.6, 5.2 Hz), 3.96 δ (2H, td, J=11.6, 5.2 Hz), 3.29 δ (2H, m), 3.21 δ (6H, s), 3.08 δ (2H, m).

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