^{1}H-NMR Investigations on the Hydrogen Bond Formation between the Tranquilizers Diazepam and Nitrazepam and Some Nucleobases *

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The formation of hydrogen bonds between the minor tranquilizers diazepam and nitrazepam and a few nucleobases was studied in deuterochloroform solution by means of proton magnetic resonance spectroscopy. The thermodynamic and spectroscopic data of the associations were evaluated on the basis of a dimer model, using the concentration dependent shifts of the protons involved in hydrogen bonds. The interactions of nitrazepam ($\Delta H^0 = -10$ to -21 kJ/mol; ΔG^0_{25} -0.2 to -7.4 kJ/mol) were found to be stronger than those of diazepam ($\Delta H^0 = -10$ to -13 kJ/mol; $\Delta G^0_{25} = 6.0$ to 6.4 kJ/mol). The various binding sites of the benzodiazepines for hydrogen bonds are discussed.

Introduction

Since the discovery of Librium in 1955, 1,4-benzodiazepines have reached an immense importance as minor tranquilizers. Because of their excessive use it is highly desirable to understand the molecular basis of their phamacological activity and of possible side effects. Recently, the existence of a brain specific benzodiazepine receptor has been reported [1–3], but up to now, there is only little information available about the nature of the interaction and about the binding sites of receptor and drug as well. Concerning the anomalies of human chromosomes, as observed under the influence of diazepam *in vitro* and *in vivo* [4, 5], the molecular mechanism is also not known yet.

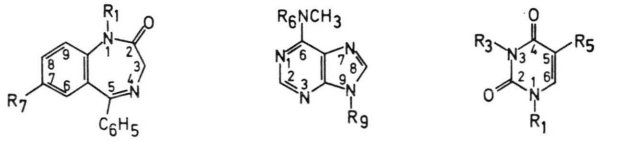
This paper represents an investigation about the ability for hydrogen bond interactions of diazepam and nitrazepam, two clinically used 1,4-benzodiazepines. For these studies derivatives of the nucleic acid bases adenine and uracil served as interacting partners. The experiments were carried out by means of proton magnetic resonance spectroscopy (^{1}H-NMR) using chloroform as solvent.

Materials and Methods

1,3-Dihydro-7-nitro-5-phenyl-2 H-1,4-benzodiazepin-2-one (nitrazepam, Mogadan®, Mogadon®) and 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2 H-1,4-benzodiazepin-2-one (diazepam, Valium®) were kindly donated by Hoffmann-La Roche, Grenzach-Wyhlen.

6-Methylamino-9-methylpurine (m^6m^9Ade), 6-dimethylamino-9-ethylpurine ($m_2^6e^9$Ade) and 1-ethyl-2,4-dihydroxypyrimidine (e^1Thy) were purchased from Cyclo Biochemicals, Tucson, USA. 2,4-Dihydroxy-1,3-dimethylpyrimidine (m^1m^3Ura) was obtained from Fluka, Buchs, Switzerland. The molecular structure of the substances used is shown in Fig. 1. Deuterated chloroform (Sharp & Dohme, München) was kept on molecular sieves (4 Å, Riedel-de Haën, Seelze-Hannover) in order to eliminate water and polar impurities. All substances were used without further purification.

The proton magnetic resonance spectra were recorded on a Varian HA-100 spectrometer, locked on an internal reference of 3% Tetramethylsilan. Chemical shifts were determined to an accuracy of ± 0.002 ppm. The sample temperature was regulated with an accuracy of ± 1 °C by a Varian variable



1,4-Benzodiazepines

R_1	R_7	
H	NO_2	Nitrazepam
CH_3	Cl	Diazepam

Adenine-derivatives

R_6	R_9	
H	CH_3	m^6m^9Ade
CH_3	C_2H_5	$m_2^6e^9$Ade

Uracil-derivatives

R_1	R_3	R_5	
C_2H_5	H	CH_3	e^1Thy
CH_3	CH_3	H	m^1m^3Ura

Fig. 1. Chemical structure of the substances used in this study.

* Part of the MS thesis of Hans-Helmut Paul.

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temperature system. For its calculation the methanol chemical shift of a seperate sample was used [6].

The spectroscopic changes due to the molecular associations studied were analysed using the BMDo7R nonlinear least square fit program of the Health Computing Facility, University of California, Los Angeles [7]. The errors of the quantities evaluated (s. Tables I and II) are identical with the asymptotic standard deviations computed by the BMDo7R program. All calculations were carried out on the CD3300 computer of the Hochschulrechenzentrum der Universität Giessen, Giessen.

Results

Self-association

Before associations between 1,4-benzodiazepines and derivatives of nucleic acid bases can be evaluated, the self-association of each substance used has to be determined. It can be assumed that m^1m^3Ura, $m_2{}^6e^9$Ade, as well as diazepam do not self-associate in chloroform, since these compounds have no donor sites for hydrogen bonds. Indeed, all proton chemical shifts of these substances remained unchanged with increasing concentrations.

Nitrazepam and the nucleobases e^1Thy and m^6m^9Ade, however, self-associate via hydrogen bonding, as can be seen from the downfield shifts of the corresponding protons.

The concentration dependence of the chemical shifts observed was analyzed by means of a dimer model, assuming that dimer complexes form in solution. In this case, self-association of molecules A occurs according to reaction (1), with $K_2{}^A$ as equilibrium constant.

$$A + A \overset{K_2^A}{\rightleftharpoons} A_2 . \tag{1}$$

The concentration dependence of the chemical shift of a proton of substance A is given then by Eq. (2) [8]

$$\delta_{obs} = \delta_m + \left(1 - \frac{\sqrt{1 + 8\,K_2{}^A \cdot a_0}}{4\,K_2{}^A \cdot a_0}\right) \Delta_2 . \tag{2}$$

δ_{obs} is the chemical shift observed, δ_m the monomer shift, Δ_2 the chemical shift of the proton in the dimer complex relative to δ_m (dimer shift), and a_0 is the initial concentration of A. Concentrations up to 0.5 M were used, if not limited by the solubility of the substance. The temperature dependence of the equilibrium constant is given by the Vant' Hoff equation, which can be written as

$$K_2{}^A = \exp\left(-\frac{\Delta G^0}{RT}\right) = \exp\left(\frac{\Delta S^0}{R} - \frac{\Delta H^0}{RT}\right), \tag{3}$$

where ΔG^0, ΔH^0, and ΔS^0 are the standard free enthalpy, standard enthalpy and standard entropy of the reaction, and T is the absolute temperature.

The spectroscopic parameters δ_m and Δ_2 are, in general, temperature dependent [9] and can be described as follows:

$$\begin{aligned} \delta_m &= \delta_{m0} + \delta_{mt} \cdot t \\ \Delta_2 &= \Delta_{20} + \Delta_{2t} \cdot t , \end{aligned} \tag{4}$$

where t is the temperature in °C, δ_{mo} the monomer and Δ_{20} the dimer shift at 0 °C; δ_{mt} and Δ_{2t} are the corresponding temperature coefficients. They were found to be $10^{-2} - 10^{-3}$ ppm/deg. Thus δ_{obs}, the chemical shift observed, can be expressed by inserting Eqns (3) and (4) into Eqn (2):

$$\delta_{obs} = (\delta_{m0} + \delta_{mt} \cdot t) + \left(1 - \frac{\sqrt{1 + 8\,a_0 \cdot \exp\left(\frac{\Delta S^0}{R} - \frac{\Delta H^0}{RT}\right)}}{4\,a_0 \cdot \exp\left(\frac{\Delta S^0}{R} - \frac{\Delta H^0}{RT}\right)}\right) \cdot (\Delta_{20} + \Delta_{2t}\, t) . \tag{5}$$

The parameters ΔH^0, ΔS^0, δ_{mo}, δm_t, Δ_{20}, and Δ_{2t} were varied simultaneously in a nonlinear least square fit. Their final values were determined by the minimum of the root mean square deviation of the experimental δ_{obs}-values from the calculated ones, obtained from Eqn (5). This method of fitting directly the experimental data and considering simultaneously the temperature- and concentration-dependences should result in the best possible values of the parameters computed [10]. The data of self-association obtained in this way for nitrazepam, m^6m^9Ade, and e^1Thy are represented in Table I.

Mixed associations

For mixed associations, the dimer model is based on the following set of competitive reactions:

$$\begin{aligned} A + A &\overset{K_2^A}{\rightleftharpoons} A_2 \\ B + B &\overset{K_2^B}{\rightleftharpoons} B_2 \\ A + B &\overset{K_c}{\rightleftharpoons} AB \equiv C . \end{aligned} \tag{6}$$

Substance	$-\Delta H^0$ [kJ/mol]	$-\Delta G_0^{25}$ [kJ/mol]	δ_{mo} [ppm]	Δ_{20} [ppm]
Nitrazepam	20.6 ± 1.5	9.2 ± 0.9	8.32 ± 0.14	2.95 ± 0.12
m^6m^9Ade	15.7 ± 1.2	−1.0 ± 0.1	5.76 ± 0.02	3.08 ± 0.16
e^1Thy	16.4 ± 0.9	3.5 ± 0.2	7.97 ± 0.06	3.55 ± 0.06

Table I. The evaluated thermodynamic and NMR parameters of self-association of the substances used. For diazepam, m^1m^3Ura and $m_2{}^6e^9$Ade no self-association could be observed. Abbreviations see text and Fig. 1.

$K_2{}^A$, $K_2{}^B$ and K_C are the equilibrium constants of self-associations and of mixed association, respectively. The initial concentrations a_0 and b_0 and the monomer concentrations a_1 and b_1 of molecules A and B, resp., and the concentration c of the complex C are linked by the law of mass action, from which the following set of two nonlinear equations is derived:

$$a_0 = a_1 + 2\,K_2{}^A \cdot a_1{}^2 + K_c \cdot a_1 \cdot b_1$$
$$b_0 = b_1 + 2\,K_2{}^B \cdot b_1{}^2 + K_c \cdot a_1 \cdot b_1\,. \qquad (7)$$

From Eqn (7) the unknown monomer concentrations a_1 and b_1 were calculated numerically by a Newton iteration method. Using the values obtained, the chemical shift observed for a proton of substance B, *e. g.*, is given by

$$\delta_{obs} = \delta_m + \frac{2\,K_2{}^B \cdot b_1{}^2}{b_0} \cdot \Delta_2 + \frac{K_c \cdot a_1 \cdot b_1}{b_0} \cdot \Delta_c\,, \qquad (8)$$

where Δ_c is the chemical shift of the proton in the mixed complex relative to its monomer shift. Substance B has conventionally been chosen to be the proton donor of a mixed association. If both substances posses donor and acceptor sites, two titrations are possible, so that each of the substances can be A or B, depending on which proton is observed.

The temperature dependence of the self-association constants, the monomer shifts, as well as K_c and Δ_c are given by Eqns (3) and (4). Using a numerical combination of Eqns (3), (4), (7) and (8), δ_{obs} is represented as a function of the initial concentrations a_0 and b_0, of 6 thermodynamic, and of 6 spectroscopic parameters. If the parameters of self-association of both interacting substances are known, ΔH^0, $\Delta G^0{}_{25}$, Δ_{co}, and Δ_{ct} of the mixed associations can be calculated from a least square fit with respect to the chemical shift observed.

Mixed associations of nitrazepam

In the case of mixed associations between nitrazepam and e^1Thy, m^6m^9Ade or $m_2{}^6e^9$Ade the resonance lines of the corresponding NH-protons were shifted downfield with increasing concentrations of the interacting substance, indicating hydrogen bond formation [11]. This behaviour is illustrated for the H-1 proton of nitrazepam in the system nitrazepam-e^1Thy in Fig. 2. For the CH resonance lines no concentration dependent shifts were observed.

In the case of mixed associations between nitrazepam and m^1m^3Ura, however, an unexpected effect was observed (Fig. 3). The resonance line of H-1 (nitrazepam) shifted either upfield or downfield by the addition of m^1m^3Ura. The direction of the shift depended on the temperature of the sample and on the initial concentration of nitrazepam. This result, especially the upfield shift of a proton involved in hydrogen bonds, can be understood from an analysis of the functional dependence of δ_{obs} given by Eqns (7) and (8). Calculations show, that upfield shifts may be observed, if $\Delta_c < \Delta_2$ and if the self-association of the proton donor is strong enough. As an example of a model calculation this is illustrated in Fig. 4, where δ_{obs} is represented as a function of the acceptor concentration a_0. For this

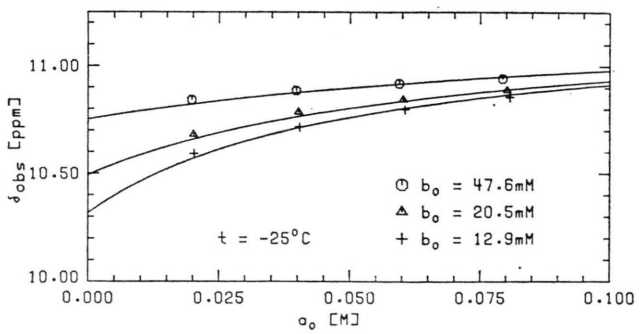

Fig. 2. Concentration dependence of the H-1 chemical shift of nitrazepam (concentration b_0) in the case of mixed association with e^1Thy (concentration a_0) at −25 °C.

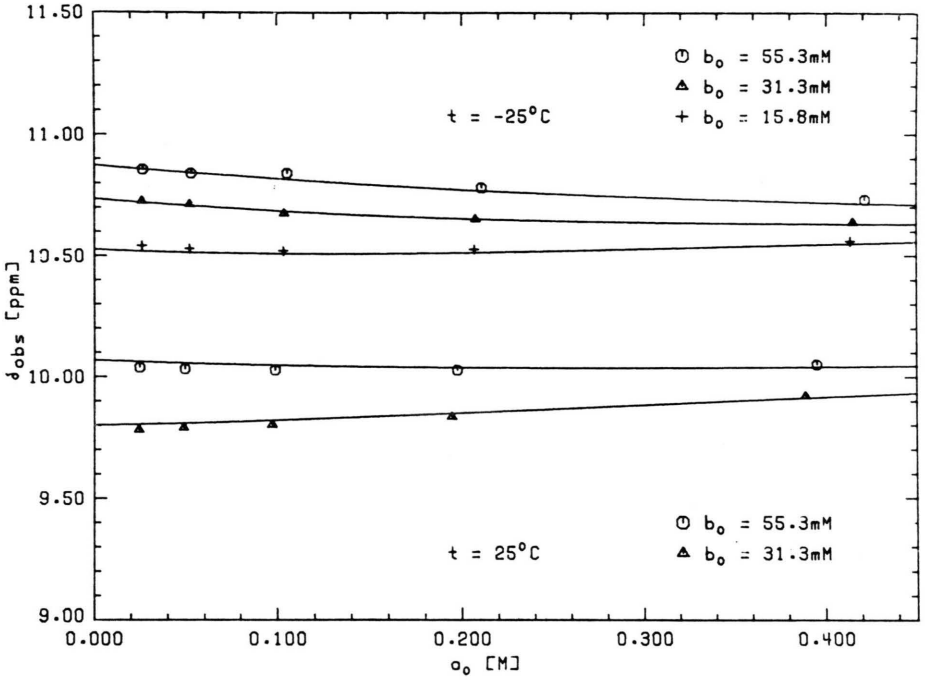

Fig. 3. Concentration dependence of the H-1 chemical shift of nitrazepam (concentration b_0) due to the binding to m^1m^3Ura (concentration a_0) at −25 °C and 25 °C.

calculation the parameters K_2^A, K_2^B, K_c, Δ_2 and Δ_c were kept constant, whereas the donor concentration b_0 was varied. It can be seen, that δ_{obs} is shifted to lower field with increasing acceptor concentration at low donor concentrations, but is shifted to higher field at larger donor concentrations. At an intermediate range of b_0 only slight variations of the chemical shift can be observed, although mixed complexes are formed.

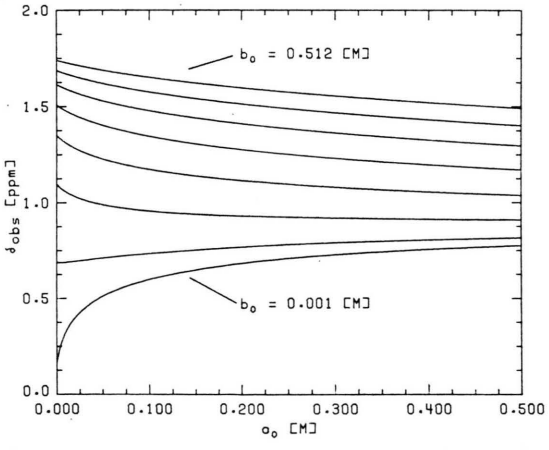

Fig. 4. Model calculations showing the influence of the donor concentration b_0 on the functional dependence of δ_{obs} on the acceptor concentrations a_0 in case of $\Delta_c < \Delta_2$. The following parameters were used for the calculations: $K_2^A = K_2^B = K_c = 50\ M^{-1}$; $\Delta_c = 1$ ppm; $\Delta_2 = 2$ ppm; $\delta_m = 0$ ppm; $b_0 = 0.001, 0.008, 0.027, \ldots, 0.512$ M;

Some more detailed information about the associations was obtained from the evaluation of the thermodynamic and spectroscopic parameters by means of a least square fit of the chemical shifts observed of the protons involved in H-bonds. The parameters evaluated are given in Table II. The temperature dependences of the association constants are shown as Vant' Hoff plot in Fig. 5. It can be seen that the standard enthalpy is nearly the same for the self-association of nitrazepam as well as for the associations of nitrazepam with e^1Thy and m^6m^9Ade, and is about twice as large for the associations with m^1m^3Ura and $m_2{}^6e^9$Ade. Furthermore, the difference between the self-association of nitrazepam and its association with m^1m^3Ura is more pronounced at lower temperatures. This explains why the upfield shift of the H-1 proton of nitrazepam was observed especially at low temperatures.

Mixed associations of diazepam

When $m_2{}^6e^9$Ade or m^1m^3Ura were added to solutions of diazepam, changes of the proton chemical

Association	Proton observed	$-\Delta H^0$ [kJ/mol]	$-\Delta G^0_{25}$ [kJ/mol]	Δ_{CO} [ppm]
Nitrazepam-$m_2^6e^9$Ade	(N-1)-H Nitr.	10.8±0.3	0.2±0.1	4.40±0.34
Nitrazepam-m^1m^3Ura	(N-1)-H Nitr.	10.4±0.9	4.2±0.4	2.21±0.01
Nitrazepam-m^6m^9Ade	(N-1)-H Nitr. NH-6 m^6m^9Ade	21.1±2.6	5.0±2.1	4.64±0.20 2.62±0.11
Nitrazepam-e^1Thy	(N-1)-H Nitr. (N-3)-H e^1Thy	18.7±0.8	7.4±0.7	3.14±0.03 3.64±0.09
Diazepam-m^6m^9Ade	NH-6 m^6m^9Ade	13.0±1.8	−6.4±1.9	3.0 [a]
Diazepam-e^1Thy	(N-3)-H e^1Thy	10.5±3.6	−6.0±1.7	4.0 [a]

[a] See text.

Table II. The evaluated thermodynamic and NMR parameters of mixed associations of the 1,4-benzodiazepines with nucleobases. Abbreviations see text and Fig. 1.

shifts could not be observed. On the other hand, the resonances of the H-3 and the H-6 proton of e^1Thy and m^6m^9Ade, resp., were shifted slightly downfield by the addition of diazepam, indicating a small interaction. The shifts, corresponding to saturation fractions smaller than 0.2 [10], were too small in order to calculate thermodynamic and spectroscopic parameters separately. Therefore, the complex shifts Δ_c were not varied in a least square fit. They were estimated to be 3 or 4 ppm, which is about the same value as has been obtained for the associations of these substances with nitrazepam. The thermodynamic parameters seem to be almost independent of the exact value of Δ_c. Their final values are represented in Table II.

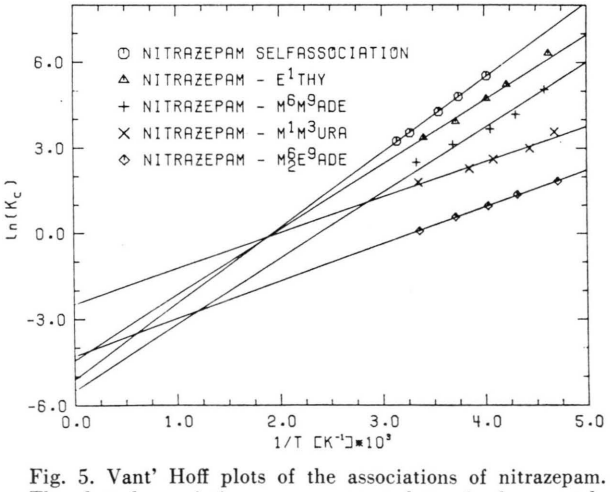

Fig. 5. Vant' Hoff plots of the associations of nitrazepam. The plotted association constants were determined separately at fixed temperatures, whereas the corresponding thermodynamic parameters were taken from a temperature dependent least square fit.

Discussion

From Tables I and II it can be seen that the obtained enthalpies ΔH^0 are in the range of −10 to −13 kJ/mol for the associations of diazepam with m^6m^9Ade and e^1Thy and of nitrazepam with $m_2^6e^9$Ade and m^1m^3Ura. For the associations of nitrazepam with m^6m^9Ade and e^1Thy and for the self-association of nitrazepam the ΔH^0-values are about twice as large. A ΔH-value of about −12 kJ/mol seems to correspond to complexes involving only one hydrogen bond, whereas a ΔH-value of about −20 kJ/mol may indicate complexes bound by two hydrogen bonds (cyclic dimers). The 4-nitrogen as well as the 2-carbonyl group are possible H-acceptor sites of the 1,4-benzodiazepines, whereas the (N-1)-H of nitrazepam can serve as a proton donor. Sterical considerations show, however, that nitrazepam can form cyclic dimers only by using H-1 and (C-2) = 0 as binding sites. Concerning the single bonded complexes of diazepam, it cannot be decided from the experimental results obtained whether N-4 or (C-2) is the most favoured acceptor site. As has been shown, N-4 is the preferred protonation site of the 1,4-benzodiazepines [12, 13] and should be, therefore, the preferred acceptor site of hydrogen bonds. On the other hand, a sterical hindrance due to the 5-phenyl group should diminish the acceptor function of this site.

The free standard enthalpies ΔG^0_{25} (at 25 °C) of the interactions of diazepam are positive, corresponding to association constants <1 whereas the ΔG-values of the complexes formed by nitrazepam are negative. Their absolute values decrease in the sequence $e^1Thy > m^6m^9Ade > m^1m^3Ura > m_2^6e^9Ade$. From this it might be concluded, that the associa-

tions via one or via two hydrogen bonds involving uracil derivatives are stronger than the corresponding complexes involving adenine-derivatives. This might be caused in part by a better acceptor function of the carbonyl oxygens of the uracils in comparison with the nitrogen atoms of the adenines. A competitive solvent interaction being stronger in the case of adenine than in the case of uracil derivatives might also be responsible for these differences in the ΔG-values.

The complex shifts evaluated for the associations of nitrazepam are between 2 and 5 ppm and are more or less temperature dependent. The H-1 complex shifts in the case of the interactions with the adenine derivatives $m_2{}^6e^9$Ade and m^6m^9Ade seem to be larger than the ones of the interactions with the uracil derivatives e^1Thy and m^1m^3Ura. The reason for this may be an additional downfield shift of the proton involved in hydrogen bonds due to the ring current effect of the purine ring system of the adenines. In the case of m^1m^3Ura a sterical hindrance of the association due to the CH_3-substitution at N-3 may be responsible for the small value of Δ_c.

The hydrogen bonds involving (N-1)-H, (C-2) = 0, or N-4 might be only one of several molecular interaction abilities of the 1,4-benzo-diazepines. Concerning the chromosomal anomalies, observed under the influence of diazepam [4, 5], they could be assumed as caused by an influence of the drugs on the DNA interstrand hydrogen bonding. However, this does not prove to be very probable, since the observed interaction of the drugs with the nucleobases used is weaker than that of base pairing [14]. Nevertheless, from the results reported, it can be seen, that the amide group can form hydrogen bonds strong enough, at least for nitrazepam, that they can be considered to be an essential component of the specific molecular action of the drugs at their receptor, especially if its binding site posses a stronger donor or acceptor ability for hydrogen bonds than the nucleobases. In regard to both, the pharmacological activities and the side effects of the benzodiazepines, however, the metabolism of the drugs has to be taken into account. It has been shown, that many of the benzodiazepines of interest are metabolized to pharmacologically active derivatives containing the amide structure [15], and it has been suggested, that this structure is an absolute requirement for activity [16]. This suggestion might be supported by the results reported above, indicating a favoured interaction via hydrogen bonds due to the amide group.

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