JOURNAL OF MAGNETIC RESONANCE **130,** 281–286 (1998)
ARTICLE NO. MN971301

^{1}H NMR Studies on the Interaction of β-Carboline Derivatives with Human Serum Albumin

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Received February 20, 1997; revised September 29, 1997

^{1}H NMR studies were performed on two β-carboline derivatives interacting with human serum albumin. The spin–lattice relaxation rates of the two derivatives, having side chains of different length and polarity, were used to demonstrate a diverse motional behavior in solution together with slightly different relaxation pathways. Single- and double-selective excitation made it possible to evaluate dynamics in the free and protein-bound states. Occurrence of a relatively long hydrophilic chain interacting with the proton-acceptor nitrogen of the β-carboline moiety was shown to yield lower association constants, slower dissociation rates, and diverse interacting modes with the indole hydrophobic site of the protein.

INTRODUCTION

β-Carbolines (β-CB) (Fig. 1) are long known to interact with $GABA_A$ receptors that are ligand-gated chloride channels modulated by allosteric binding sites for benzodiazepines, barbiturates, and other drugs (*1*).

Among β-CB, a particularly well-studied class is that of the 3-carboxy-β-carbolines such as β-CCM (R_1 = OCH_3, R_2 = R_3 = R_4 = H) (*2–7*). These compounds, however, behave as inverse agonists by acting *in vivo* in a way opposite to that of the clinically useful benzodiazepines, β-CCM being anxiogenic and convulsant in animal and man.

It is therefore agreed that, although both classes of compounds mutually and competitively displace each other from their binding site on the receptor, β-CB bind to a site distinct from, although possibly overlapping with, that of benzodiazepines (*8–11*).

Pharmacological investigations (*12–14*) made it possible to develop a model that implies interaction of both nitrogen atoms (Fig. 1) with hydrogen-bonding acceptor and donor sites located inside a hydrophobic pocket of the receptor. However the search for new agonist β-CB (*15–17*), as well as for photoaffinity labels of the $GABA_A$ receptor (*18*), requires further delineation of structural features at the bound site.

β-CB have been also shown to interact with human serum albumin (HSA) at the indole site (*19*). Although β-CB–HSA dissociation constants are much higher than those from the $GABA_A$ receptor, screening tests with a number of derivatives have provided a kind of similarity as for the order of affinities (*20, 21*). As a consequence dynamic solution studies can be afforded with HSA that take advantage of faster exchange rates from the bound state and that may be somehow related to the interaction mode with the receptor.

Here we present a NMR study on the β-CB–HSA interaction by extensively using selective excitation techniques in order to gain relevant information on the binding process (*22–24*). Two recently synthesised derivatives (**1a**, R_1 = OCH_3, R_2 = R_3 = H, R_4 = NH_2, and **1b,** R_1 = OCH(OH)-CH(OH)CH_2OH, R_2 = R_3 = H, R_4 = NH_2) (*25*) have been tested with the aim of relating NMR parameters with biological activities.

MATERIALS AND METHODS

The two derivatives were obtained as reported elsewhere (*25*). Due to the very low solubility at pH $\geq$ 6.9, all NMR samples were prepared in deuterium oxide buffered at pH 5.5 with citric acid/sodium citrate (from Sigma Chemical Co.), 100 mmol dm^{-3}. Human serum albumin was obtained from Sigma and used without further purification. Solutions were made in the same buffer, and the pH was adjusted to 5.5 with either DCl or NaOD. All solutions were carefully deoxygenated by sealing off the sample after a few freezing–vacuum pumping–thawing cycles.

All NMR experiments were carried out on a Bruker AM 500 spectrometer at the controlled temperature of 300 ± 1 K. Chemical shifts were referenced to internal [$^2H_4$]-TSP (trimethylsilylpropansulfonic acid sodium salt). Spin–lattice relaxation rates (R_1) were measured with the inversion recovery pulse sequence. R_1 values were calculated with an exponential regression analysis of the recovery curves of longitudinal magnetization components. Single- and double-selective proton spin–lattice relaxation rates were measured with inversion recovery pulse sequences modified so as to obtain single- or double-selective inversion of the desired proton resonances, as reported

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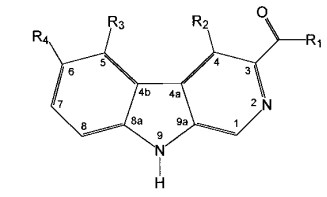

1a $R_1 = \overset{12}{OCH_3}$, $R_2 = R_3 = H$, $R_4 = NH_2$

1b $R_1 = \overset{12}{OCH}(OH)\overset{13}{CH}(OH)\overset{14}{CH_2}OH$
$R_2 = R_3 = H$ $R_4 = NH_2$

FIG. 1. Molecular structures of synthesized β-carboline derivatives.

elsewhere (*26, 27*). All proton R_1 values were calculated in the initial rate approximation (*26*).

Molecular structures were generated by using the HYPERCHEM software package implemented on a Pentium-120 MHz PC.

RESULTS AND DISCUSSION

The ^{1}H NMR parameters of the two β-CB analogues, **1a** and **1b,** are summarized in Table 1, whereas the concentration dependence of the ^{1}H NMR chemical shift of H_1 in **1b** at 300 K is shown in Fig. 2, all other aromatic protons of both derivatives behaving in a very similar way. The observation that aromatic protons are exponentially shifted upfield by raising the concentration is consistent with the occurrence of co-operative auto-aggregation of solute molecules, leading to self-stacked adducts $(\beta\text{-CB})_n$ $(n \geq 2)$. It is also evident from Fig. 2 that such self-association phenomena can be neglected for $[\beta\text{-CB}] \leq 0.001$ mol dm^{-3}.

As shown in Table 2, the two analogues display similar NMR spectra consistent with the diverse electron-withdrawing properties of the alkyl-ester chain. In contrast, the proton spin–lattice relaxation rates, measured upon either nonselective or single-selective excitation modes, are somehow faster for protons in **1b** than in **1a** in any case.

The nonselective, R^{ns}, and single-selective, R^s, proton spin–lattice relaxation rates usually refer to a sum of uncorrelated pairwise ^{1}H–^{1}H dipole–dipole interactions, and the eventual contributions by other relaxation mechanisms are grouped into an extra term, ρ_i^* (*29*),

$$R_i^{ns} = \sum_{j \neq i} \rho_{ij} + \sum_{j \neq i} \sigma_{ij} + \rho_i^* \quad [1]$$

$$R_i^{s} = \sum_{j \neq i} \rho_{ij} + \rho_i^*, \quad [2]$$

where ρ_{ij} and σ_{ij} are the direct- and cross-relaxation rates for any H_i–H_j interaction and the sum is extended to all the dipolarly connected protons. ρ_{ij} and σ_{ij} are calculated differently by the single-, double-, and zero-quantum relaxation transition probabilities such that the following equations can be given in the case of time-independent proton–proton distances (*29*),

$$\rho_{ij} = \frac{1}{10} \frac{\gamma^4 \hbar^2}{r_{ij}^6} \left\{ \frac{3\tau_{ij}}{1 + \omega^2 \tau_{ij}^2} + \frac{6\tau_{ij}}{1 + 4\omega^2 \tau_{ij}^2} + \tau_{ij} \right\} \quad [3]$$

$$\sigma_{ij} = \frac{1}{10} \frac{\gamma^4 \hbar^2}{r_{ij}^6} \left\{ \frac{6\tau_{ij}}{1 + 4\omega^2 \tau_{ij}^2} - \tau_{ij} \right\} \quad [4]$$

where $\hbar$ is the reduced Planck constant ($= 1.0545887 \times 10^{-27}$ erg s rad^{-1}), γ is the proton magnetogyric ratio ($=$ 26,753 rad s^{-1} G^{-1}), ω is the proton Larmor frequency ($=$ 3.1416×10^9 rad s^{-1} in a magnetic field of 117,400 G), and τ_{ij} is the correlation time of the motion that modulates the reorientation of the H_i–H_j internuclear vector, where the two protons are at the time-independent distance r_{ij}. Equations [3] and [4] can also be modified in order to take time-dependent distances into account as well (*30*).

As a consequence, the R^{ns}/R^s ratio (also shown in Table

TABLE 1
500 MHz ^{1}H NMR Parameters of 1a (0.75 mmol dm^{-3}) and 1b (0.5 mmol dm^{-3}) in Deuterium Oxide Buffered at pH 5.5, T = 300 ± 1K

	1a				1b			
	δ (ppm)	R^{ns} (s^{-1})	R^s (s^{-1})	R^{ns}/R^s	δ (ppm)	R^{ns} (s^{-1})	R^s (s^{-1})	R^{ns}/R^s
H_1	8.55	0.48 ± 0.04	0.53 ± 0.04	0.91 ± 0.15	8.58	1.28 ± 0.09	1.09 ± 0.08	1.17 ± 0.14
H_4	8.38	0.39 ± 0.03	0.42 ± 0.03	0.93 ± 0.15	8.44	0.78 ± 0.06	0.85 ± 0.07	0.92 ± 0.16
H_5	7.68	0.72 ± 0.06	1.05 ± 0.08	0.69 ± 0.11	7.69	1.98 ± 0.14	1.96 ± 0.13	1.01 ± 0.15
H_7	7.30	0.84 ± 0.07	1.06 ± 0.08	0.79 ± 0.14	7.31	1.72 ± 0.13	1.80 ± 0.13	0.95 ± 0.16
H_8	7.43	0.72 ± 0.04	0.70 ± 0.05	1.03 ± 0.14	7.44	1.71 ± 0.12	1.79 ± 0.11	0.95 ± 0.14
H_{12}	3.93	0.98 ± 0.07	1.23 ± 0.09	0.80 ± 0.12	4.50	1.75 ± 0.13	1.51 ± 0.10	1.16 ± 0.17
H_{13}	—	—	—	—	4.13	0.93 ± 0.06	0.76 ± 0.04	1.22 ± 0.16
H_{14}	—	—	—	—	3.80	1.67 ± 0.09	1.54 ± 0.09	1.08 ± 0.13

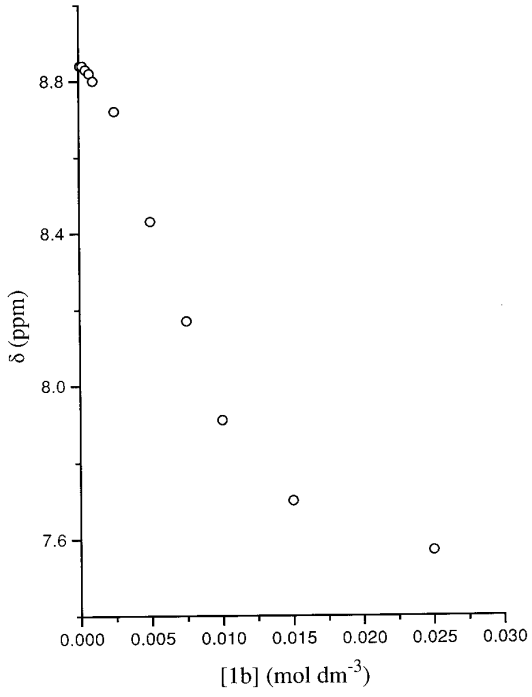

FIG. 2. Dependence of the ^{1}H NMR chemical shift (ppm) of H_1 of the **1b** derivative upon concentration in deuterium oxide buffered at pH 5.5, $T = 300 \pm 1$ K.

2), as first suggested by Freeman *et al.* (*26*), provides a means of evaluating, alternatively, the motional correlation time or the relevance of the dipole–dipole interaction in determining the relaxation pathway since one or the other yields reduction from $R^{ns}/R^{s} = 1.50$ which is measured in the case of a pure dipolar relaxation mechanism within the extreme narrowing region (*26*).

The data in Table 2 show that corresponding protons in the two b-CB derivatives display different relaxation rates, but similar R^{ns}/R^{s} values quite smaller than 1.50.

An estimate of the motional correlation time was obtained by evaluating the absolute value of the H_7–H_8 dipole–dipole interaction energy by measuring the proton spin–lattice relaxation rates following double-selective excitation of the two involved proton resonances (*27*) and by considering $r_{7,8} = 0.243$ nm. The following equation was then applied,

$$\sigma_{7,8} = R_7^{7,8} - R_7^{s} = R_8^{7,8} - R_8^{s} = \frac{1}{10}\frac{\gamma^4\hbar^2}{r_{7,8}^6}\left\{\frac{6\tau_{7,8}}{1 + 4\omega^2\tau_{7,8}^2} - \tau_{7,8}\right\}, \qquad [5]$$

where R_i^{ij} stands for the spin–lattice relaxation rate of proton i after double-selective excitation of protons i and j. Quite interestingly the same value of $\sigma_{7,8}$ was obtained for **1a** and **1b** ($\sigma_{7,8} = \sigma_{8,7} = 0.07 \pm 0.03$ s^{-1} was calculated in both cases) yielding $\tau_{7,8} = 0.2 \pm 0.1$ ns at 300 ± 1 K.

This correlation time cannot be identified with the molecular reorientational time since the β-carboline moiety is very likely to undergo anisotropic reorientation and the considered internuclear vector may be rather affected by internal motions. However $\tau_c = 0.2$ ns is outside the $\omega\tau_c < 1$ region and would provide $R^{ns}/R^{s} = 1.24$. The lower observed ratios can be ascribed to contributions by relaxation mechanisms *other* than the ^{1}H–^{1}H dipole–dipole, especially the ^{1}H–^{14}N dipole–dipole interaction. It can in fact be noticed that protons within the *proton-rich* environment of the side chain of **1b** display R^{ns}/R^{s} ratios quite close to 1.24.

Since the two molecules have the same value of $\tau_{7,8}$ and yield similar R^{ns}/R^{s} ratios, the difference in proton relaxation rates can mainly be explained by additional ^{1}H–^{1}H dipolar interactions contributing to the relaxation of protons in **1b** in respect to the corresponding protons in **1a.** A certain contribution from a diverse motional anisotropy can also be devised, since it is known that similar aromatic molecules as 1,2-dichloro-benzene and 1,2,3-trichloro-benzene have different values for the motional correlation times along the three Cartesian axes (*31*).

Supporting evidence for the above conclusions was found by considering that protons in the side chain in **1b** do not show features typical of segmental motion, but display R^{ns}/R^{s} ratios very similar to those of ring protons and not very far from the value of 1.24 consistent with a motional correlation time of 0.2 ns. It may be suggested that the chain folds back toward the ring, yielding a spheroid which is likely to reorient more isotropically than the **1a** ellipsoid. As a matter of fact, when minimizing the conformational energy of **1b** in computer programs of molecular mechanics, two folded structures (shown in Fig. 3) with the terminal hydroxyl

TABLE 2

Chemical Shift Changes ($\Delta\delta$, ppm), ^{1}H NMR Spin–Lattice Relaxation Rate Enhancements (ΔR^{ns} and ΔR^{s}, s^{-1}) and R^{ns}/R^{s} Ratios Measured for 1a (0.75 mmol dm^{-3}) and 1b (0.50 mmol dm^{-3}) in Deuterium Oxide Buffered at pH 5.5 in the Presence of Human Serum Albumin at [protein]/[ligand] = 0.07, $T = 300 \pm 1$ K

	1a	1b	1a			1b		
	$\Delta\delta^a$ (ppm)	$\Delta\delta^a$ (ppm)	ΔR^{ns} (s^{-1})	ΔR^{s} (s^{-1})	R^{ns}/R^{s}	ΔR^{ns} (s^{-1})	ΔR^{s} (s^{-1})	R^{ns}/R^{s}
H_1	0.03	0.02	0.50	1.19	0.53	0.68	1.20	0.82
H_4	0.03	0.03	0.23	0.57	0.63	0.33	0.85	0.65
H_5	0.01	0.00	0.70	0.89	0.71	0.99	1.46	0.87
H_7	0.00	0.00	0.71	1.13	0.67	0.86	1.29	0.83
H_8	0.01	0.01	0.48	1.24	0.36	0.85	0.96	0.93
H_{12}	0.01	−0.11	0.08	0.08	0.68	nd	nd	nd
H_{13}	—	0.00	—	—	—	0.14	0.57	0.80
H_{14}	—	0.00	—	—	—	nd	nd	nd

[a] Positive values indicate downfield shifts.

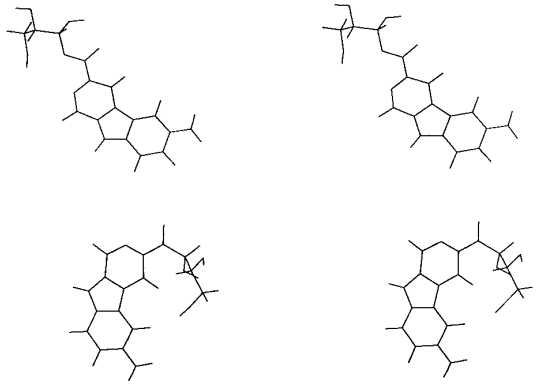

FIG. 3. Stereo views of the molecular models of the **1b** derivative as obtained by minimizing the conformational energy with the MM+ force field parameters.

alternatively facing N_2 or the NH_2 were obtained, thus suggesting that the ester chain moves between two minimum energy states that enrich the dipolar environment of ring protons.

Upon addition of HSA the following phenomena were observed:

(a) All chemical shifts, but that of H_{12} of **1b,** were almost unaffected (Table 2). H_1, H_4, H_5, and H_8 of both **1a** and **1b** experienced a very slight downfield shift, whereas H_{12} of **1b** was moved 0.11 ppm upfield.

(b) The nonselective and single-selective spin–lattice relaxation rates were differently enhanced (Table 2) such that the R^{ns}/R^{s} ratio was lower in respect to that of the free state in solution. As already shown in several binding studies (*22, 24*), the selective relaxation rate enhancement was larger than the nonselective one. The most affected protons were $H_8 > H_1 > H_7$ in **1a** and $H_5 > H_7 > H_1$ in **1b,** thus very likely indicating a diverse interaction mode.

The NMR experiments carried out in solutions of β-CB in the presence of HSA at low protein:ligand ratios must take the exchange process of the ligand between free and bound environments into account. In the absence of any exchange process each site would contribute to the observed NMR behavior with its own parameters proportional to the fractional existential probabilities, p_f (f ≡ free) or p_b (b ≡ bound).

As for the bound site itself we may expect, besides changes in chemical shifts and coupling constants, that relaxation rates will be affected by the slowing down of molecular motions as well as by the occurrence of intermolecular interactions with protons in the protein core. While this last event causes enhancement of both relaxation rates, the first yields $R^{s}_{ib} > R^{s}_{if}$ but $R^{ns}_{ib} < R^{ns}_{if}$.

The exchange process can be schematized as

$$\begin{array}{c} \beta\text{CB} \\ k_{off} \uparrow\downarrow k_{on} \\ \beta\text{CB}-\text{HSA} \end{array}$$

where k_{on} and k_{off} are the rate constants for association and dissociation of β-CB to and from the bound site respectively. The association process is diffusion controlled and is usually much faster than any relaxation rate in the free state, whereas the disassociation process reflects the strength of the binding interaction which may result in rapid, intermediate, or slow exchange conditions in the time scale of proton relaxation rates.

Slow exchange ($k_{off} \ll R_i^{ns}, R_i^{s}$) of the ligand leaves the two sites to separately behave as if in the absence of each other. However, since the bound site has a very low existential probability at the used [protein]/[ligand] ratios, there is in practice no chance left to detect features of the bound site.

Rapid exchange ($k_{off} \gg R_i^{ns}, R_i^{s}$), in contrast, yields weighted average parameters and makes it possible to detect features of the bound site while measuring NMR parameters of the exceeding free site:

$$R_{iobs} = p_f R_{if} + p_b R_{ib}. \qquad [6]$$

Is this why the single-selective, and not the nonselective, proton spin–lattice relaxation rates were suggested as the method of choice for NMR detection of relatively weak binding interactions (*22*)? The different dependence on $\omega\tau$, in fact, results in large contributions of the bound site in one case and not in the other.

In the intermediate exchange regime ($k_{off} \approx R_i^{ns}, R_i^{s}$), it is the rate constant itself that enters the relaxation equations, providing a generalized increase of all relaxation rates of the free ligand.

The results obtained with **1a** and **1b** (Table 2) give evidence that the two β-CB derivatives interact with HSA in different ways.

The first observation is that higher R^{ns}/R^{s} ratios are measured for protons of **1b** than for those of **1a** and that this mainly arises from larger nonselective relaxation rate enhancements. Enhancements of selective relaxation rates are, in contrast, rather similar to each other with the exclusion of H_4, H_5, and H_8. Since, as already stated, binding to a macromolecular site is not expected to yield large enhancements of R^{ns}, especially at high Larmor frequencies, one can conclude that, most probably, intermediate exchange conditions apply to the nonselective relaxation rate, and therefore, that the out-rate constant, k_{off}, is larger for **1b** than for **1a.** The same does not evidently hold for R^{s} since

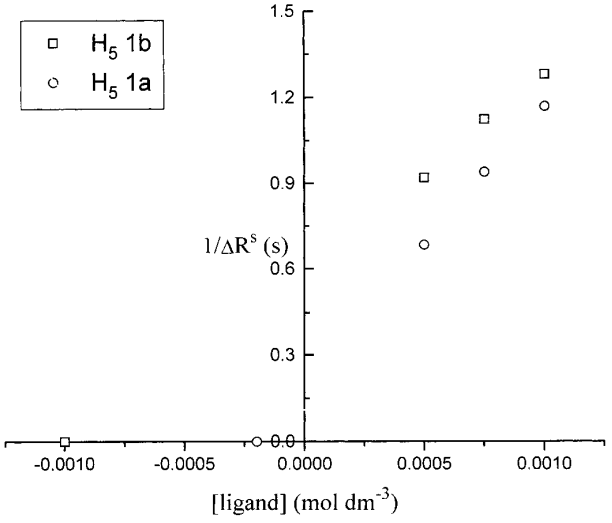

FIG. 4. Titration of $1/\Delta R^s$ of the H_5 proton of **1a** and **1b** as a function of the ligand concentration in the presence of 35 μmol dm^{-3} HSA in deuterium oxide buffered at pH 5.5 at $T = 300 \pm 1$ K.

ΔR_5^s (**1b**) $>$ ΔR_5^s (**1a**) but ΔR_8^s (**1b**) $<$ ΔR_8^s (**1a**). It is therefore concluded that the enhancements of selective relaxation rates reflect different binding modes, yielding different dipole–dipole intermolecular interactions contributing to the relaxation mechanism of bound β-CB.

In order to ascertain this last inference, the association constants of the two derivatives with HSA were evaluated by titrating the selective relaxation rate enhancement, as stated elsewhere (*32*). The plots, shown in Fig. 4, made it possible to extrapolate the values of K_{ass} since $1/\Delta R^s = 0$ at $[\text{ligand}] = -1/K_{ass}$. It is evident that the binding constant of **1a** ($K_{ass} \sim 5 \times 10^3$) is larger than that of **1b** ($K_{ass} \sim 10^3$), thus indicating that, at the used concentrations of β-CB and HSA, the protein is saturated in both cases and supporting the inference of a faster k_{off} for **1b.**

A further proof of the reached conclusion was obtained by measuring H_7–H_8 dipolar cross-relaxation rates in the presence of HSA, in the same way as they were measured in the free state. The reasonable assumption of fast exchange conditions ($k_{off} \gg \sigma_{7,8}$ in any environment) brought us to the equation

$$\sigma_{7,8}^{(obs)} = p_f \sigma_{7,8}^{(f)} + p_b \sigma_{7,8}^{(b)}, \qquad [7]$$

which made it possible to evaluate $\sigma_{7,8}$ in the bound state, $\sigma_{7,8}^{(b)}$. Consistently with the slow reorientational motions experienced by β-CB in the protein-bound state, negative values were obtained but they were now different from each other ($\sigma_{7,8}^{(b)} = -0.08$ s^{-1} for **1b** and $\sigma_{7,8}^{(b)} = -0.23$ s^{-1} for **1a**). As a consequence, in respect of what was obtained in the free state, where $\tau_{7,8}$ was found at the same value for **1a** and **1b,** the following values were calculated within the assumption of a 1:1 interaction ($p_b = 0.07$): $\tau_{7,8} = 11 \pm 2$ ns for **1a** and 3.6 ± 0.6 ns for **1b.**

These findings clearly indicate that **1a** is tightly bound to HSA such that it reorients with almost the same motional correlation time of the protein, whereas **1b** retains some degree of motional flexibility as a consequence of a diverse interaction mode with the hydrophobic pocket in the protein. We have no evidence that the binding site in the protein framework is the same for the two β-carbolines. However if we assume that all β-carbolines interact at the same hydrophobic site of HSA, we reach the conclusions that the synthesized derivatives have different out-rate constants, are held differently at the bound site, and experience different intermolecular interactions with protein protons.

CONCLUSIONS

The knowledge of molecular parameters underlying steric and topographic requirements of the high affinity binding to the $GABA_A$ receptor may be expanded by investigations using HSA as the binding target. As a matter of fact the indole site of HSA has been shown to specifically bind diazepam, the most widely known agonist of the receptor. For this reason and also because of analogies in binding affinities, similar structural features have been hypothesized for the two (*20, 21*).

The indole site of HSA is a 1.2–1.6 nm deep, 0.6–0.8 nm wide hydrophobic cleft (*28*) that matches the planar hydrophobic moiety of β-CB. By the same way, binding studies with radio-labeled ligands and modulation of the affinity of several β-CB have strongly suggested the occurrence of a similar hydrophobic pocket in the $GABA_A$ receptor. In both cases, in fact, substituents in position 1, 7, or 9 or hydrogenation of aromatic rings of β-carboline greatly reduce the binding affinity, thus supporting the view that planarity and hydrophobicity play a major role in determining the interaction mode.

A third requirement, at least as important as these, involves N_2 as hydrogen-bond acceptor, which explains why substituents in position 3 of β-CB affect the binding constant through electronic, steric, or hydrophobic mechanisms. Electron-donor substituents increase the basicity of N_2 and, by emphasizing its H-bonding acceptor properties, enhance the binding constant. However, electron-withdrawing substituents do not inhibit binding provided they are made of relatively short hydrophobic chains.

Compounds **1a** and **1b** have been synthesized and investigated in order to gain further information on the effects of substituents in position 3 of β-CB.

From this last point of view the following inferences can be made.

1. H_4, H_5, and, especially, the methyl group of **1a** are somehow shielded by interactions with protons of the hy-

drophobic pocket, whereas all other protons experience relevant dipolar interactions which lead to relatively large enhancements of the corresponding selective relaxation rates.

2. H_4, H_8, and side chain protons of **1b** are the least affected by HSA while H_5 is the most, such that the most exposed region of **1b** to the protein surface is somehow rotated in respect to the **1a**–HSA interaction.

3. While the methyl group of **1a** is poorly, if any, affected by HSA, the selective relaxation rate of H_{13} of **1b** is rather enhanced by the protein, yielding evidence of a certain interaction which may be related also to the change in chemical shift experienced by H_{12}.

It may be finally speculated that **1a** and **1b** interact differently with HSA because the interaction between N_2 and the hydroxyl groups within the side chain of **1b** hinders the formation of the hydrogen bond involving a proton donor in the hydrophobic pocket. It is therefore possible to think of a modulation of activities of β-CB by suitably changing the steric hindrance and the hydrophilic power of the side chain.

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