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Determination of the stable conformation of $GABA_A$-benzodiazepine receptor bivalent ligands by low temperature NMR and X-ray analysis

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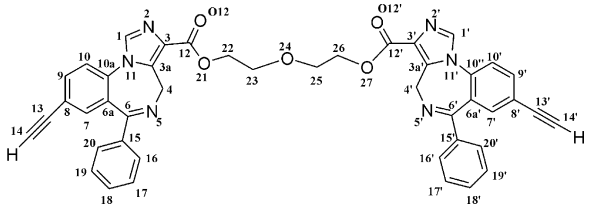
Abstract—The stable conformations of $GABA_A$-benzodiazepine receptor bivalent ligands **2** and **3** were determined by low temperature NMR spectroscopy and confirmed by single crystal X-ray analysis. The linear conformation was important for these dimers to access the binding site and exhibit potent in vitro affinity as illustrated for α5 subtype selective ligand **2** (15 nM). Bivalent ligand **3** with the 5 atom linker folded back upon itself both in solution and in the solid state, moreover, it did not bind to Bz receptors.

The $GABA_A$/BzR complex contains a chloride ion channel which comprises part of the major inhibitory neurotransmitter system in the CNS.[1] It has been proposed that the $GABA_A$/BzR chloride channel is a membrane-bound pentameric protein polymer, comprised principally of α, β, and γ subunits. Recombinant receptors containing these subunits most closely mimic the biological, electrophysiological and pharmacological properties of native $GABA_A$ receptors.[2]

In general, 1,4-benzodiazepines are nonselective ligands[3] that bind to all Bz receptor isoforms that are diazepam-sensitive. As a result, most of the 1,4-benzodiazepines display a wide range of pharmacological activities, such as anxiolytic/anticonvulsant, sedative/hypnotic, ataxic/myorelaxant, and amnesiac. Now it is believed that agents selective for specific BzR subtypes may permit one to separate out the pharmacological activities of these different isoforms.[4] This is a goal of paramount importance in the search for new anxiolytic agents and new anticonvulsant compounds.[5]

Bivalent ligands have been termed as compounds that contain two pharmacophores joined through a connecting unit (spacer). The general structure for bivalent ligands is P-X-P (P: pharmacophore; X: spanner).[6] They may exhibit enhanced selectivity and potency relative to their monovalent analogues P-X when a suitable spanner X is employed. The study by Portoghese et al. on bivalent ligands that bind to different opioid receptor subtypes is among one of the most successful reported to date.[6]

Recent studies on the binding selectivity of the inverse agonist RY-80 (**1**) indicated preferential binding to α5 BzR/$GABA_A$ subtypes (see Table 1). Since these subtypes are found primarily in the hippocampus and may be involved in the amnesic effects of diazepam,[2–5] it was decided to incorporate the pharmacophore of **1** into bivalent ligand **2**.[7] This ligand bound with selective efficacy for the α5 subtype and behaved as a selective antagonist of the effect of diazepam in oocytes.[7]



dimer A, **3**

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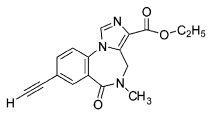
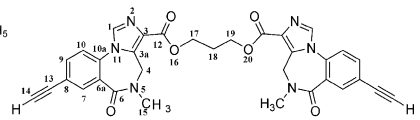
Table 1. In vitro binding data of RY-80 (**1**) and Xli-093 (**2**) at $GABA_A$/BzR subtypes ($\alpha\times\beta3\gamma2$)



RY-80, **1**



Xli-093, **2**

Ligand	K_i(nM)[a]					
	α1	α2	α3	α4	α5	α6
1	28.4	21.4	25.8	53	0.49	28.8
2	>1000	>1000	858	1550	15	>2000

[a] K_i values represent the mean of two determinations which differed by less than 10%. Data were generated at 4 °C[5a] using Ltk$^-$ cell membranes expressing human αxβ3γ2 receptors. 1.8 nM [^{3}H]Ro 15-1788 and 8 nM [^{3}H] Ro 15-4513 (for cells expressing α4β3γ2 and α6β3γ2) were used as radioligands.

Encouraged by the selectivity of **2**, a new series of bivalent ligands was designed and prepared. It was hoped these new dimers might exhibit enhanced selectivity as compared to the monomers. Among these, dimer A (**3**) was studied extensively.

It was important to determine the conformation of bivalent ligands with 3 to 5 atom linkers in solution since the steric requirements for affinity to the Bz pharmacophore/receptor model must be satisfied.[7,8]

In this vein, the crystal structure of the active dimer **2** (CCDC 222395)[9] indicated it existed in a linear conformation in the solid state while the 5 atom linked ligand **3** (CCDC 222396)[9] folded back upon itself (Fig. 1).

Since **2** adopted a linear arrangement in the solid state, it was important to establish whether the preferred conformation in solution paralleled that in the solid state. This is required to correlate the solution structure with biological activity in order to design better subtype selective bivalent ligands for other subtypes (α1, α2, and α3). Consequently, low temperature NMR studies were employed to determine the stable conformation of the dimers in solution to compare them with those in the crystal structure.

At room temperature, the proton NMR spectrum of 5 atom linked dimer **3** exhibited only one set of signals for the two domains of the molecule. Such a spectrum might be consistent with either a symmetrical linear structure or a folded structure in which fast conformational exchange results in an equal average environment for the two domains. As the temperature of the spectrometer was decreased, most of the signals for **3** split into two sets (Fig. 2). For example, the signal at H-1 (7.92 ppm at rt) split into two peaks at 7.91 and 7.88 ppm, respectively, at 193 K. Similar results were observed in the ^{13}C spectrum wherein C1 (134.9 ppm at rt) split into two signals at 135.3 and 135.4 ppm at 198 K.

This trend was also observed for other proton and carbon signals of **3** and was verified for overlapping

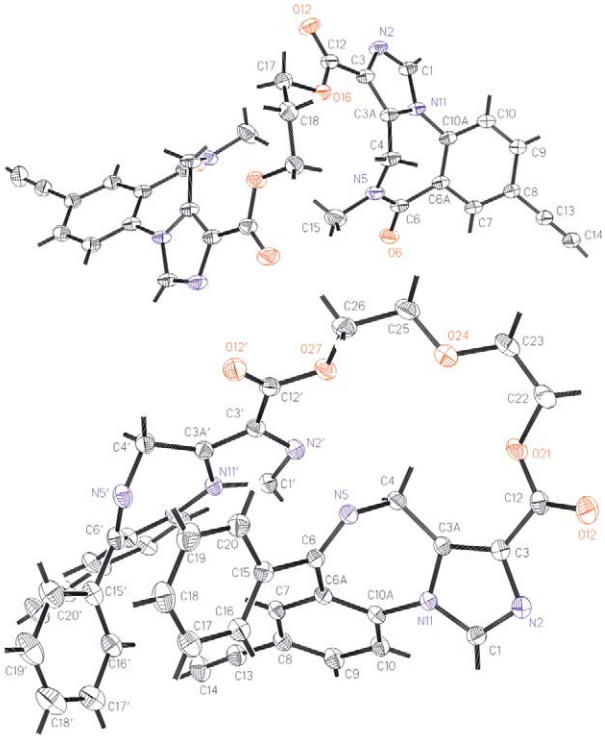


Figure 1. Crystal structures of **2** (top) and **3** (bottom).

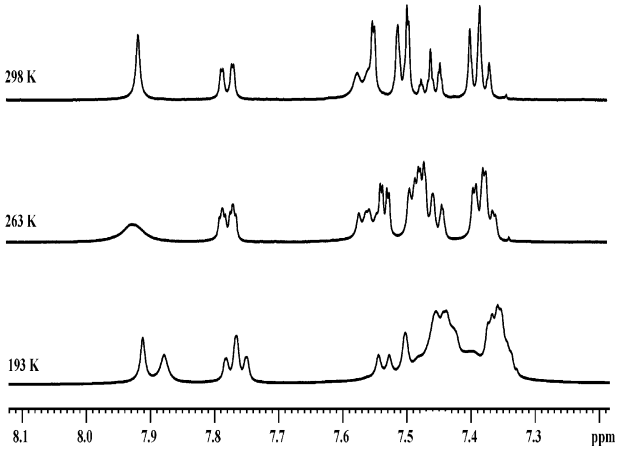


Figure 2. Aromatic region of ^{1}HNMR spectra of **3** in CD_2Cl_2 at variable temperatures.

parts of the proton spectrum by a ^{1}H{^{13}C}-HSQC experiment at 198 K. The two sets of signals appeared in a 1:1 ratio. The doubling of the signals would be consistent with a disruption of the symmetry between the two domains of the molecule as expected if **3** adopted a static folded structure similar to the crystalline state.

However, the possibility could not be ruled out that the split in the signals was caused by slowing a dynamic process within each domain, such as inversion of N11 or conformational interconversion of the seven-membered ring. In order to test this possibility, the NMR spectra of the monomer at low temperature were run as well. At temperatures as low as 173 K, only one set of signals

was observed in both the ^{1}H and ^{13}C NMR spectra of the monomer of **3** (Fig. 3). While at room temperature, the spectra of the monomer were virtually indistinguishable from those of **3**, a split of the signals of the monomer of **3** was not observed at lower temperatures. This was quite different from that observed with **3**. Some additional line broadening of some of the aromatic signals was observed at the lowest temperature in both the monomer and in **3**. This was, presumably, due to one of the conformational processes mentioned above. However, only in the case of the dimer **3** were two sets of signals observed.

Analysis of these data indicated that the stable conformation of **3** was asymmetric at low temperature. Certainly an interdomain interaction existed between the two monomeric heterocyclic units of **3**. The internal rotation of the molecule was decreased when the temperature was lowered which permitted observation of the two sets of signals of **3** on the NMR time scale.

A similar picture emerged upon study of the linker between the two domains. At room temperature, the four protons (22a, 22b, 26a, and 26b) in the 5 atom linker of **3** gave rise to two broad peaks in the ^{1}H NMR spectrum. At 263 K, the diastereotopic protons at $C(26)H_2$ and $C(22)H_2$ were separated and observed at δ 4.51–4.59 and 4.41–4.50 ppm, moreover, a detailed analysis of the P.E. COSY spectrum revealed different sets of signals for protons at CH_2-22 and CH_2-26 (Fig. 4), respectively. This indicated that the symmetry of the linker had been broken at the lower temperature.

In an attempt to establish a spatial proximity between the two domains, NOESY spectra were acquired for **3** at 183 K. While no clear cross peak was observed, which could unequivocally be assigned to an interdomain interaction, there were additional exchange cross peaks of H1/H1′ and H10/H10′ with a minor species (<2%) at δ 10.57/10.80 and 8.50 ppm, respectively. Although the origin of these cross peaks was not clear, they were not observed in the case of the monomer, again suggesting that there was some relatively strong interdomain interaction in **3**. Thus, it can be concluded

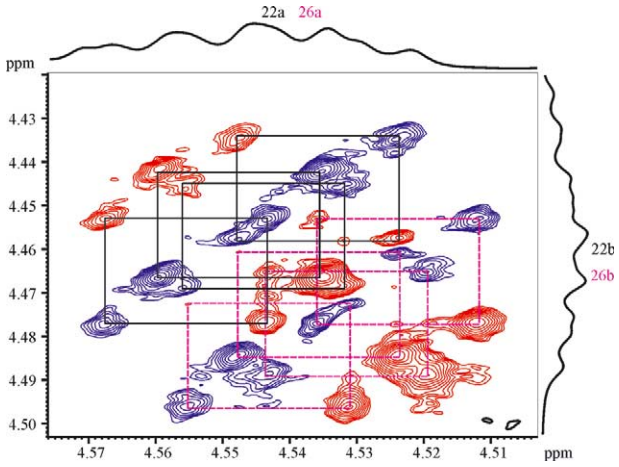


Figure 4. P.E. COSY spectrum of **3** at 263 K. H22a and H22b (black squares) could be separated from H26a and H26b (red dashed squares) in the spectrum.

that the stable conformation of the 5 atom linked **3** in solution was in good agreement with that in the crystal structure (see Fig. 1).

These conclusions are further supported on analysis of the spectra of the 3 atom linked dimer **2**. Analysis of the crystal structure of **2** indicated this molecule existed in the linear conformation in the solid state. The NMR spectra of this dimer were run at low temperature as well. Only a small splitting of about 3 Hz was observed for some of the aromatic protons at 253–233 K (Fig. 5).

Homonuclear decoupling experiments confirmed this splitting did not originate from a long range coupling, but the separation was much less substantial than that observed for **3**.

In addition, the exchange peaks observed in **3** were not seen in the spectrum of **2**. It became apparent from the study that the interdomain interaction in **2** was much weaker than that in **3**. It appeared that the folded conformation was less stable for **2** in solution, in agreement with that in the solid state.

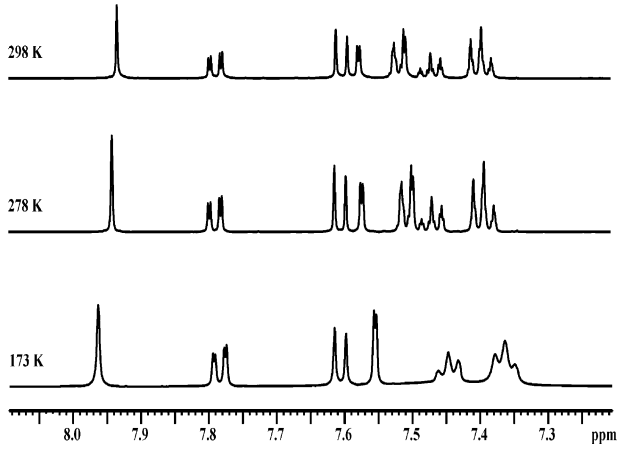


Figure 3. ^{1}H NMR spectra of monomer of **3** in CD_2Cl_2 at low temperatures.

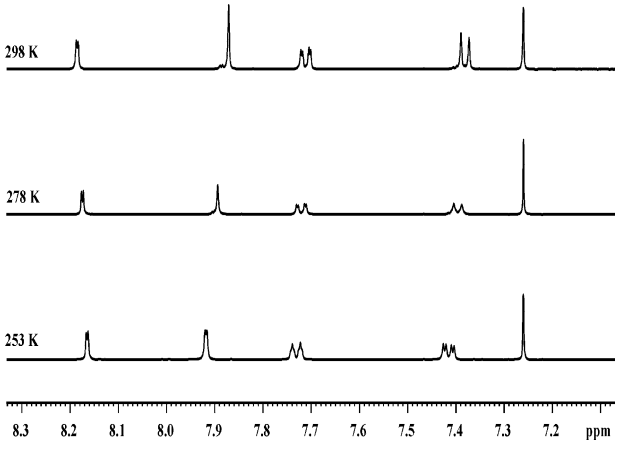


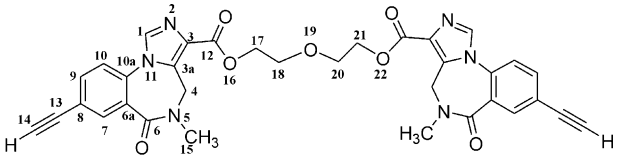
Figure 5. ^{1}H NMR spectra of **2** in $CDCl_3$ at low temperatures.

The preference of **2** to exist in a linear conformation makes it more able to access the receptor binding site to provide the potent α5 subtype selective affinity (15 nM).[7,8] It is the structural basis for the bioactivity. The linear **2** fits very well in the pharmacophore/receptor model.

Bivalent ligand **3**, presumably, was too hindered to access the receptor binding site in its folded conformation. The receptor binding data indeed supported this presumption. It was determined that the K_i value for **3** at [^{3}H]Ro 15-1788-labeled BZP sites was greater than 1000 nM, using the IC_{50} value for inhibition of radioligand binding and the Cheng–Prusoff approximation.

Additional studies in a more polar solvent (CH_3OH) capable of H-bonding indicated that several peaks from protons of **3** were split even at room temperature, while **2** still remained as one set of signals. These results further support the assumption that **3** exhibited a higher tendency to assume a folded structure than does **2**.

Furthermore, the low temperature NMR spectra of the O-containing 5 atom linked analogue of **2**, Xli-347 (**4**), were also studied. At 243 K, two clearly separated sets of proton signals were observed (Fig. 6). Two sets of carbon signals were also observed at low temperature in the ^{13}C spectra of **4**. These findings indicated that **4** as well as **3**, adopted a folded conformation as the most stable conformation in solution.



Xli-347, **4**

In summary, the more stable conformations of 3 and 5 atom linked bivalent $GABA_A$/BzR ligands were studied in solution by low temperature NMR spectroscopy and confirmed by X-ray analysis. Five atom linked dimer **3** as well as **4** exhibited more tendency to fold back onto itself in solution than **2**. The stable conformations for these dimers in solution are in agreement with those in the solid state as well as with the receptor binding affinities. The corresponding 5 carbon atom linked dimer related to **4** was also present, principally, in the linear conformation.

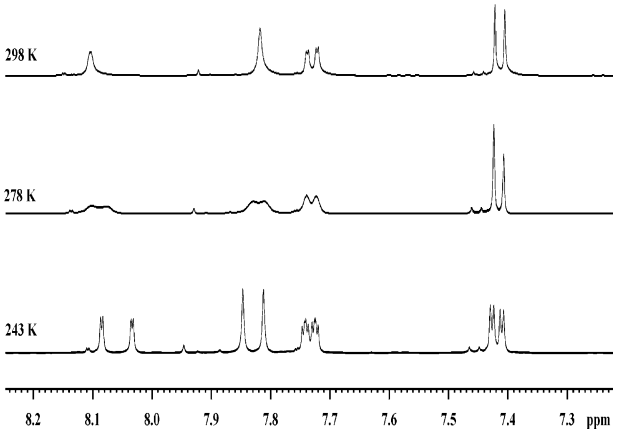


Figure 6. ^{1}HNMR spectra of **4** in CD_2Cl_2 at variable temperatures.

This provides some insight for the structural basis for receptor binding activity of **2** when compared to the lack of activity of **3**. Linear conformations for $GABA_A$/benzodiazepine receptor bivalent ligands are important for access to the receptor binding site in order to exhibit potent binding affinity. This phenomenon provides important information for the future design of bivalent ligands. It also confirms that low temperature NMR spectroscopy may be used to evaluate potential bivalent Bz receptor ligands for their bioactivity and rapidly establish the acceptable linker for action at BzR/$GABA_A$ receptors.

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9. Crystallographic data (excluding structure factors) for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 222395 and 222396. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK [fax: +44-1223-336033 or e-mail: deposit@ccdc.cam.ac.ck].