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Sugar-Based Enantiomeric and Conformationally Constrained Pyrrolo[2,1-*c*][1,4]-Benzodiazepines as Potential $GABA_A$ Ligands

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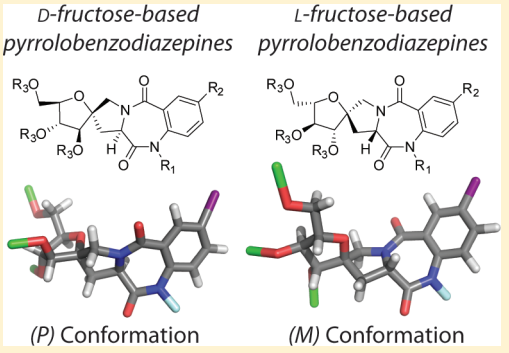
Supporting Information

ABSTRACT: Synthesis of a library of pyrrolo[2,1-*c*][1,4]-benzodiazepines derived from spiro bicyclic D- or L-proline analogues containing a D- or L-fructose moiety was developed. The L-fructose moiety was obtained by using a new synthetic pathway starting from L-arabinose through a six steps synthesis in 18% overall yield. Molecular modeling calculations and DNMR studies showed that D- and L-fructose-based pyrrolobenzodiazepines exhibit a rigid (*P*)- and (*M*)-helical conformation, respectively, in which the C-11a substituent was always pseudoequatorial. Additionally, pyrrolobenzodiazepines functionalized with a chloride, bromide, nitro, or amino group in the benzene ring, with or without *N*-methylation and with or without protection of sugar alcohol groups, allowed a relationship between the molecular structure and biological activity to be established. The conformation of the diazepam ring was not the sole key player influencing binding affinities, and the sugar moiety can in some cases increase the binding activity, possibly by participating in the binding event. Finally, these compounds have increased the understanding of the differential recognition of (*M*)-/(*P*)-helical benzodiazepines on $GABA_A$ receptor.



INTRODUCTION

Anxiety disorders are highly prevalent disabling disorders, which frequently turn into chronic clinical conditions.[1,2] Benzodiazepines are fast-acting and effective antianxiety agents[3] and the most commonly prescribed anxiolytics. However, their side effects such as sedation and, following chronic administration, development of tolerance, consecutive abuse liability, and withdrawal symptoms, render their use problematic in the long term treatment of anxiety disorders.[2] Hence, development of new anxiolytic agents which retain the rapid anxiolytic potential of benzodiazepines, but lack their unfavorable side effects, remains a challenge.

Most antianxiety drugs work by modulating neurotransmitters in the brain, and in particular, benzodiazepine derivatives act on the γ-amino butyric acid receptor type A ($GABA_A$ receptor), a ligand-gated chloride ion channel that mediates the effects of the major inhibitory neurotransmitter γ-amino butyric acid (GABA) in the central nervous system.[2,4,5] Furthermore, benzodiazepine anxiolytics, contrary to GABA, do not open directly the receptor channel gate but are known to induce a conformational change at the $GABA_A$ receptor.[6] Additionally, benzodiazepines exhibit an interesting conformational enantiomerism which influences their ability to bind to the benzodiazepine binding site.[7] In particular, the seven-membered ring of 1,4-benzodiazepine has a boat conformation, referred to as *P* (plus) or *M* (minus) on the basis

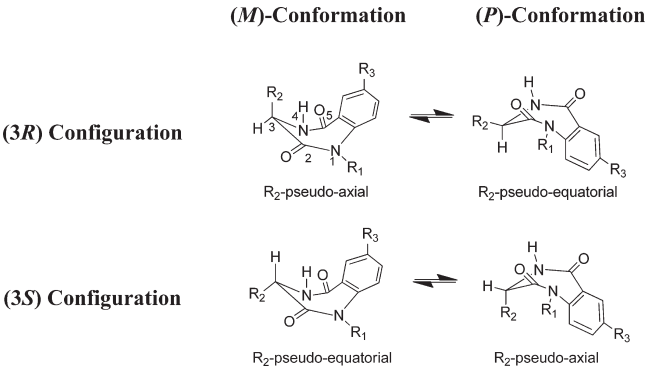


Figure 1. Conformational equilibrium in (3*R*)- and (3*S*)-1,4-benzodiazepine-2,5-dione.

of the sign of the torsion angle around C3−N,4[8] as illustrated in Figure 1 for (3*R*)- and (3*S*)-1,4-benzodiazepine-2,5-dione. The effect of the conformational changes on the binding ability has been investigated by several groups, which highlighted that (3*R*)- and (3*S*)-1,4-benzodiazepines have different binding affinities to the $GABA_A$ receptor.[7−9] From single crystal X-ray analysis[7] and computational studies of 1,4-benzodiazepines ring inversion,[10] it

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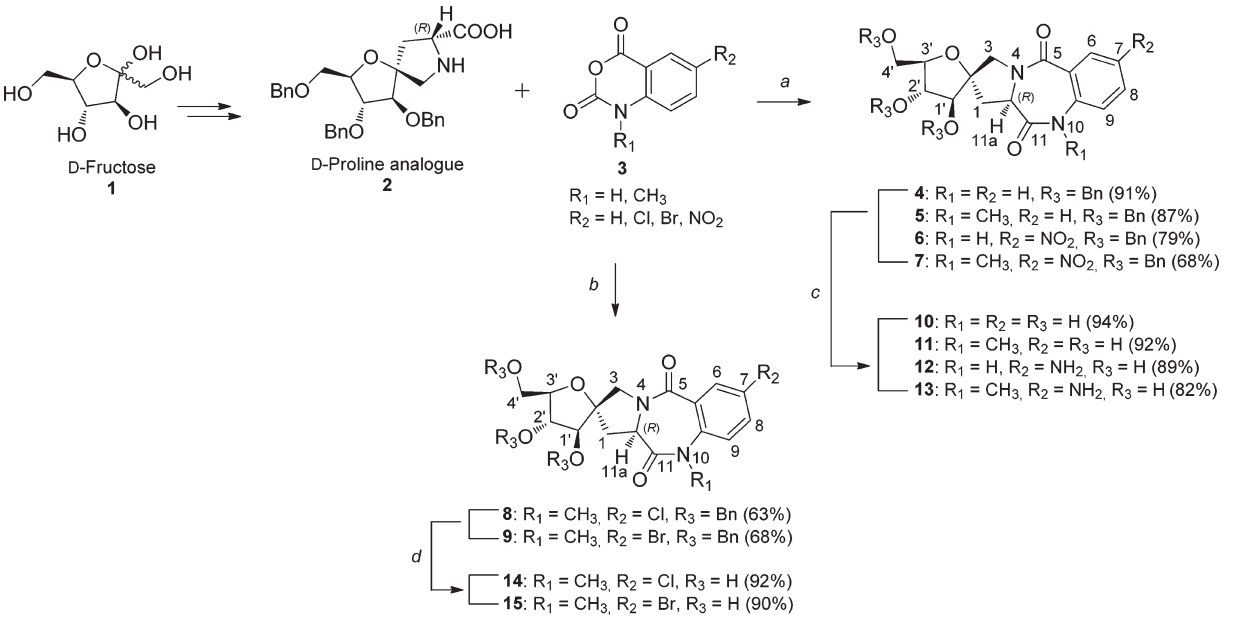
Scheme 1. Key Steps toward D-Fructose-Based Pyrrolobenzodiazepines[20−23] [a]



[a] Reagents and conditions: (a) dry DMF, reflux, 2 h; (b) (i) dry DMF, reflux, 2 h, (ii) CH_3I, Cs_2CO_3, dry DMF, rt, 2 h; (c) $Pd(OH)_2/C$ (10% w/w), CH_3OH/EtOAc, 24 h; (d) BCl_3 (1.0 M in CH_2Cl_2), rt, 1 h.

was observed that both enantiomers have a preferred conformation in which R_2 is pseudoequatorially oriented (Figure 1). In addition, the *S*-enantiomer with (*M*)-conformation was always more active than the corresponding *R*-enantiomer with (*P*)-conformation in [^{3}H]diazepam binding assays and *in vivo* pharmacological tests.[7−9] Therefore, it was suggested that the primary source of binding selectivity of 1,4-benzodiazepine enantiomers was the differential recognition of ligand conformations by the receptor, with a preferred recognition of (*M*)-1,4-benzodiazepine conformation.[8,9] Hence, benzodiazepine ring helical chirality and consequent stereospecific $GABA_A$ receptor binding is important for the development of new lead compounds. Consequently, great efforts have been devoted to the synthesis of conformationally constrained benzodiazepine derivatives.[10,11] An enantioselective synthesis of 1,4-benzodiazepine-2,5-diones derived from proline (pyrrolo[2,1-*c*][1,4]benzodiazepines) has been previously accomplished and afforded indeed rigid benzodiazepines.[12] Additionally, some hybrid benzodiazepine structures containing monosaccharides have also been reported by other research groups.[13−18] However, the ability of proline and monosaccharide derived benzodiazepines to bind to $GABA_A$ receptor and their behavior as anxiolytic agents has been scarcely studied so far.[7,19] Herein we describe the synthesis of a library of enantiomeric and conformationally constrained pyrrolo[2,1-*c*][1,4]-benzodiazepines spiro-linked to a five-membered ring carbohydrate and their preliminary evaluation as $GABA_A$ receptor ligands.

RESULTS AND DISCUSSION

1. Synthesis. Bicyclic spiro analogue D-proline **2** was obtained through a 15-step pathway in 40% overall yield starting from D-fructose **1**, in accordance with previously published material (Scheme 1).[20,21] Condensation of the D-proline analogue **2** with isatoic anhydrides type **3** afforded the D-fructose-based pyrrolobenzodiazepines **4**−**7** with (11a*R*) configuration in good yields. Subsequent hydrogenolysis with $Pd(OH)_2/C$ resulted in debenzylation and reduction of the nitro group, affording the sugar-based pyrrolo[2,1-*c*][1,4]-benzodiazepines **10**−**13**, according to Araújo et al.[22] D-Fructose-based pyrrolobenzodiazepines **8** and **9** were obtained in a two-step reaction by condensation of compound **2** with 5-chloro- or 5-bromoisatoic anhydride, followed by *N*-methylation with iodomethane and cesium carbonate in DMF. To avoid removal of the chloro/bromo substituent, debenzylation was accomplished with BCl_3 (1.0 M in CH_2Cl_2) to give compounds **14**−**15** in good yields.

Hence, since it is established that α-amino acids are the key starting materials that provide the configuration at C-11a of pyrrolo[2,1-*c*][1,4]benzodiazepines, it is desirable to synthesize the enantiomeric sugar-based pyrrolobenzodiazepines with (11a*S*) configuration, starting from the L-proline analogue **24** (Scheme 2). Following the synthetic route for the D-proline analogue **2**, compound **24** was originated from L-fructose derivative **22**. One of the possible approaches to obtain this compound relies on the use of L-fructose as starting material. However, because this is an expensive and unnatural sugar, alternative synthesis of L-fructose has been elaborated from other carbohydrates of the L-series, for example, by chain elongation of the corresponding arabinonic acid, arabinose or glyceraldehyde, or by inversion of configuration of sorbose.[24−28] We describe herein a new synthesis of the α/β-methyl 3,4,6-tri-*O*-benzyl-α,β-L-*arabino*-hex-2-ulo-2,5-furanoside (**22**), starting from L-arabinose **16** (Scheme 2) in 18% overall yield. This efficient approach took advantage of the stereochemistry of the arabinonolactone **17**, which was easily prepared by oxidation of the anomeric position of compound **16** with bromine in water.[29] Benzylation of lactone **17** using benzyl 2,2,2,-trichloroacetimidate under acidic conditions afforded compound **18**. Introduction of the formyl group was then accomplished via insertion of a dithiane and subsequent hydrolysis. Arabinonolactone **18** was reacted with

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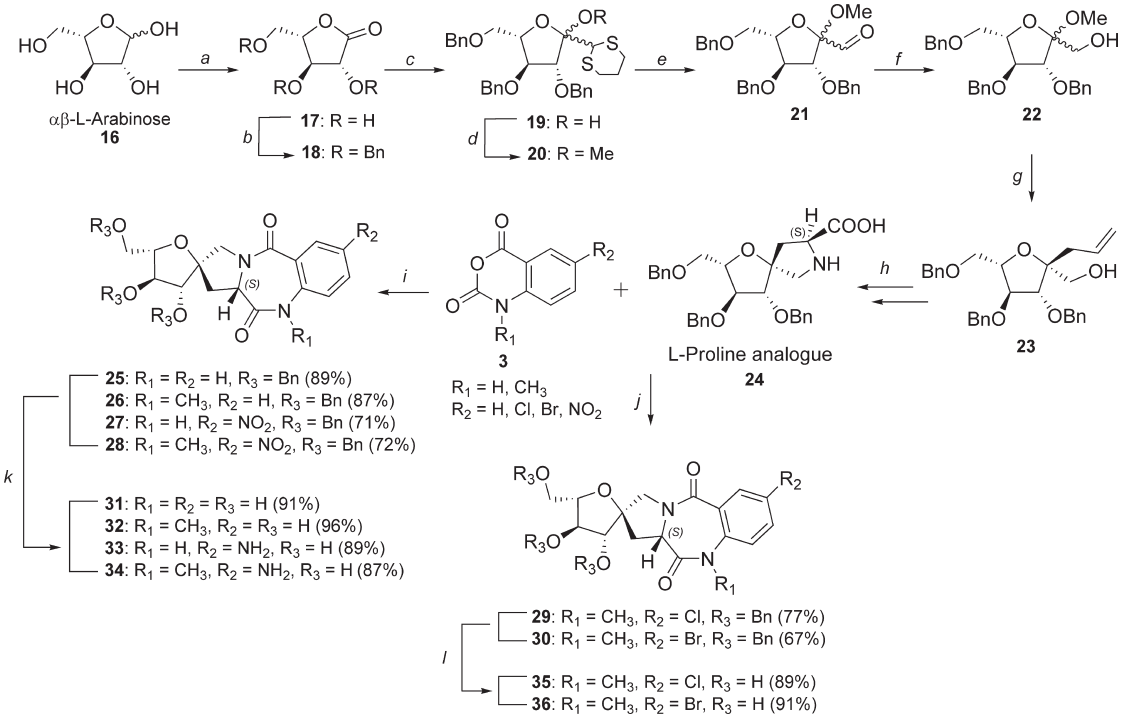
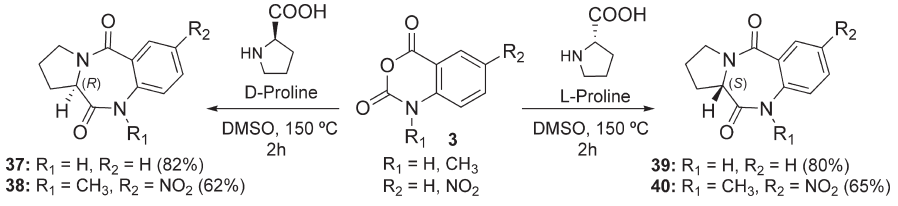
Scheme 2. Synthetic Steps toward Sugar-Based L-Proline Starting from L-Arabinose and the Following Synthesis toward L-Fructose-Based Pyrrolobenzodiazepines[a]



[a] Reagents and conditions: (a) Br_2, K_2CO_3, H_2O, rt, 2 h, 98% yield; (b) $CCl_3C(=NH)OBn$, TfOH, dioxane, 0 °C then rt, 3 h, 65% yield; (c) 1,3 dithiane, *n*-BuLi (1.6 M hexane), THF, −78 °C, 2 h, 64% yield as a mixture of anomers (1:4 α:*β* ratio); (d) CH_3OH, H_2SO_4, ms 3 Å, THF, reflux 40 °C, 12 h, 67% yield as a mixture of anomers (2:1 α:*β* ratio); (e) DBDMH, acetone, −20 °C, 15 min; (f) $NaBH_4$, EtOH, 0 °C, 30 min, 65% yield as a mixture of anomers (over two steps); (g) [i] BTSFA, CH_3CN, reflux 50 °C, 6 h, [ii] $AllSi(CH_3)_3$, TMSOTf, 0 °C then rt, 2 h, 76% yield for α anomer (8:2 α:*β*); (h) detailed synthesis can be found in the Supporting Information; (i) dry DMF, reflux, 2 h; (j) [i] dry DMF, reflux, 2 h, [ii] CH_3I, Cs_2CO_3, dry DMF, rt, 2 h; (k) $Pd(OH)_2/C$ (10% w/w), CH_3OH/EtOAc, 24 h; (l) BCl_3 (1.0 M in CH_2Cl_2), rt, 1 h.

Scheme 3. Synthetic Steps toward Pyrrolo[2,1-*c*][1,4]benzodiazepines Derived from the Free Amino Acid D- and L-Proline



2-lithium-1,3-dithiane followed by methylation of the free hydroxyl group in compound **19** using catalytic amounts of concd sulfuric acid in refluxing THF. The dithiated intermediate **20** was hydrolyzed by 1,3-dibromo-5,5-dimethylhydantoin (DBDMH), giving aldehyde **21** according to a procedure by Davis et al.[30] The α/*β*-mixture of the crude aldehyde **21** was finally reduced to the primary alcohol **22** by treatment with sodium borohydride in ethanol. Elongation at the anomeric position of compound **22** was accomplished through in situ silylation of the primary hydroxyl group, followed by Lewis acid mediated allylation by allyltrimethylsilane, affording the α-anomer **23** as the major compound, isolated in 76% yield. Synthesis of the L-proline analogue **24** was then performed as previously described for its enantiomer **2**. The sugar-based free amino acid **24** was isolated in a total yield of 3.3% over 18 steps.

Synthesis of the novel sugar-based pyrrolo[2,1-*c*][1,4]benzodiazepine-2,5-diones with (11a*S*)-configuration (compounds **25**−**30**, Scheme 2) was accomplished by heating suitably functionalized isatoic anhydrides of type **3** in dry DMF with L-proline analogue **24**. As mentioned previously for the corresponding enantiomers, *N*-methylation was performed to obtain benzodiazepine derivatives **29** and **30** and debenzylation was performed accordingly, affording compounds **31**−**36**. The pyrrolo[2,1-*c*][1,4]benzodiazepines **37**−**40**, derived from D- or L-proline, were also produced following the general procedure described for compound **39**[19] (Scheme 3) in order to be used as reference compounds for the biological assays.

2. Conformational Analysis. Conformational analysis of the water-soluble sugar-based pyrrolo[2,1-*c*][1,4]benzodiazepine-2,5-diones **10**, **11**, and **31** was performed by molecular modeling calculations and experimental NMR studies at variable temperature (dynamic NMR, DNMR). Previous data collected on hybrid pyrrolobenzodiazepines derived from D-fructose, compounds **10** ($R_1 = R_2 = R_3 = H$) and **11** ($R_1 = CH_3$, $R_2 = R_3 = H$), indicated

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Table 1. Computational Calculation of the Dihedral Angle C5a−C9a−N,10−C11 by Molecular Mechanics and Dynamics Computations

compd	conformer (oxygen-substituent)	Total energy[a] (kcal/mol)	Dihedral Angle[a,b] C5a−C9a−N, 10−C11 (deg)
(11a*R*)-**10**	pseudoaxial	25.028	+16.91
	pseudoequatorial	22.319	+17.32
(11a*R*)-**11**	pseudoaxial	30.438	+22.73
	pseudoequatorial	28.235	+24.57
(11a*S*)-**31**	pseudoaxial	25.680	−16.58
	pseudoequatorial	22.838	−17.85

[a] Calculated by ChemBio3D Ultra by applying an MM2 force field and molecular dynamics computation (step interval = 2 fs; frame interval = 1 fs; heating/cooling rate = 1.000 kcal/atoms/ps; target temperature = 300 K). Total energy and dihedral angle C5a−C9a−N,10−C11 values calculated with the two methods are consistent. [b] The dihedral angle average between the two conformers resulted +17° and +24° for compound **10** and **11**, respectively, corresponding to the helical chirality (*P*) and −17° for compound **31**, corresponding to the helical chirality (*M*).

that they adopt a rigid conformation around the benzodiazepine ring, where H-11a has a pseudoaxial position.[22] A positive dihedral angle C5a−C9a−N,10−C11 confirmed the helical chirality (*P*) in both compounds (Table 1). Additional NMR experiments run at variable temperature, to a maximum value of 363 K, showed that no conformational equilibrium of the benzodiazepine ring is present (for more details, see the Supporting Information). In all reported cases, the coalescence temperature for the pseudoequatorial/-axial equilibrium is always around 333 K.[31,32]

The conformational analysis study of the novel hybrid L-fructose-based pyrrolobenzodiazepine **31** ($R_1 = R_2 = R_3 = H$) showed a unique preferential conformation of the benzodiazepine ring. As expected, this pyrrolobenzodiazepine adopts the (*M*)-helical conformation, which is the opposite to that assumed by its enantiomer, pyrrolobenzodiazepine **10**, because this conformation places the pyrrolidine substituent in a pseudoequatorial orientation on the benzodiazepine ring. The dihedral angle C5a−C9a−N,10−C11 was also determined and found to be −17°, corresponding to the helical chirality (*M*) (Table 1 and Figure 2). NMR experiments run at variable temperature (Figure 3) showed no conformational equilibrium of the benzodiazepine ring, i.e. no protons presented coalescence between 298 and 363 K, in contrast to quaternary 1,4-benzodiazepin-2-ones, which exists as mixtures of the (*M*)- and (*P*)-conformers.[11,12]

The sugar-based pyrrolobenzodiazepines **10**, **11**, and **31** appeared always as single conformers regarding the diazepine ring, and the sterically demanding substituent present on this ring, the sugar-linked pyrrolidine, always assumes a pseudoequatorial orientation. The conformational homogeneity of the diazepine ring in the synthesized proline-derived benzodiazepines can be attributed to an amide resonance-imposed requirement for the proline moiety to occupy a pseudoequatorial orientation on the benzodiazepine ring, as reported in other cases.[11,12]

3. $GABA_A$ Receptor Binding Assay. A preliminary biological evaluation of the synthesized sugar-based pyrrolobenzodiazepines as $GABA_A$ receptor ligands has been performed. In particular, their ability to displace [^{3}H]flunitrazepam from the receptor was tested by a classical competition binding assay, using rat cortical membranes.[33] The ability of the pyrrolobenzodiazepine scaffolds to bind to $GABA_A$ receptor, at a concentration of 100 μM, was determined using the radioligand [^{3}H]flunitrazepam at a concentration of 1 nM. As control, [^{3}H]flunitrazepam without pyrrolobenzodiazepines was used to determine a comparative baseline.

Initial binding assays were performed on D-fructose-based pyrrolobenzodiazepines **4**−**7** and **10**−**13**, which presented a (*P*)-helical chirality (Scheme 1). The effect of the presence of the *N*-methyl and benzene ring substituents, such as $-NO_2$ and $-NH_2$, were evaluated. Despite having the "incorrect" conformation, some of the synthesized compounds showed affinity for $GABA_A$ receptor. Data showed that compounds **7**, **10**, and **13** possess affinity for $GABA_A$ receptor since they significantly inhibited the binding of [^{3}H]flunitrazepam in the μM range of concentration (Table 2). Results indicate that a methyl group at position 10 together with a substituent on the benzene ring is important for binding affinity. Subsequently, binding assays were performed with compounds **8**−**9** and **14**−**15** in order to evaluate the effect of a halogen (−Cl and −Br) substituent on the benzene ring. Data showed that a halogen substituent in combination with free hydroxyl groups have significant effect on binding activity (**14**−**15**). Of all the active compounds (**7**, **10**, **13**−**15**) only compound **7** is *O*-benzylated; hence almost all the sugar-based pyrrolobenzodiazepines that presented the highest binding affinities were water-soluble.

Binding assays were also performed on L-fructose-based pyrrolobenzodiazepines **25**−**36**. Data illustrated in Table 3 reinforced the previous results obtained for the corresponding enantiomers. Hence, *N*-methylation and a substituent on position 7 in the benzene ring are fundamental factors for $GABA_A$ receptor binding affinity, where the most important factor is the substituent on the benzene ring. The biological data demonstrate that $-NO_2$ and $-NH_2$ substituents are powerful enough to produce effect even in the absence of the *N*-methyl and/or *O*-benzyl groups. L-Fructose-based pyrrolobenzodiazepines with a −Cl substituent (**29** and **35**) presented higher biological activity compared to those with a −Br substituent (**30** and **36**).

Pyrrolobenzodiazepines without the sugar moiety were also characterized in receptor binding studies with [^{3}H]flunitrazepam (**37**−**40**, Table 4). Compound **40** presenting (*M*)-helical conformation shows higher binding activity than pyrrolobenzodiazepine **38** with (*P*)-helical conformation indicating that the type of conformation is important. However, for the sugar-based pyrrolobenzodiazepines, compound **10** with a (*P*)-helical conformation shows higher binding activity compared to the corresponding (*M*)-helical compound **31**. Contrary to unconstrained benzodiazepines, the effect of the helical conformation seems more complex.

The steric hindrance effect of the sugar moiety was evaluated by comparison of the pyrrolobenzodiazepines with the sugar-based pyrrolobenzodiazepines with the corresponding substituents. The binding correlated well with two exceptions. First, pyrrolobenzodiazepine **28** showed 17% reduction of the radioligand specific binding, while the corresponding pyrrolobenzodiazepine derivative without sugar moiety (compound **40**) showed an increased reduction (37%), allowing for the possibility of steric hindrance of the sugar moiety. Second, comparison of pyrrolobenzodiazepine **37** with the corresponding sugar-based

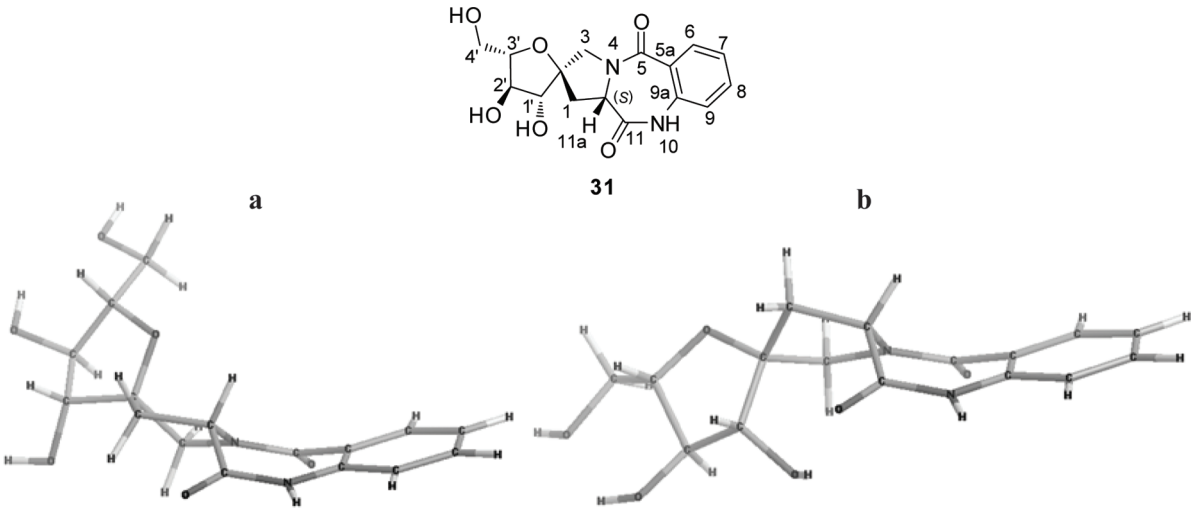


Figure 2. Oxygen-substituent pseudoaxial (a) and pseudoequatorial (b) conformers of the pyrrolo ring of compound **31**.

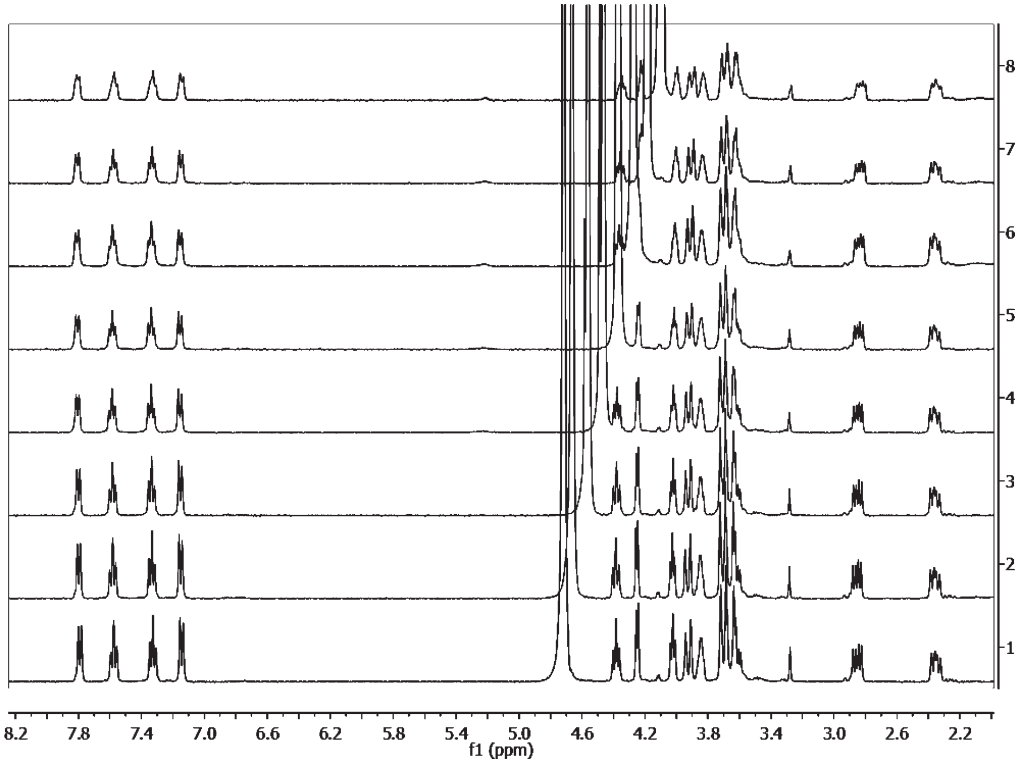


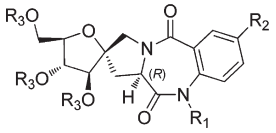
Figure 3. DNMR spectra (298K−363K) of molecule **31** dissolved in D_2O, 32 scans. Spectrum **1**, 298 K; spectrum **2**, 303 K; spectrum **3**, 313 K; spectrum **4**, 323 K; spectrum **5**, 333 K; spectrum **6**, 343 K; spectrum **7**, 353 K; spectrum **8**, 363 K.

pyrrolobenzodiazepine (compound **10**) shows that the pyrrolobenzodiazepine is not active, whereas the corresponding sugar analogue shows activity. Also, comparison between pyrrolobenzodiazepine **38** and the corresponding sugar analogue (compound **7**) shows slightly increased binding for the sugar analogue, indicating no steric hindrance of the sugar moiety. Consequently, the sugar moiety in the *S*-compounds can have a potential steric effect, whereas in the *R*-compounds the sugar moiety does not seem to inflict significant steric hindrance on the binding to the receptor and can even increase the binding affinity, indicating a possible positive participation.

CONCLUSIONS

A library of sugar-based pyrrolobenzodiazepines was synthesized with constrained enantiomeric (*M*)- and (*P*)-conformation. The locked enantiomeric conformations were achieved by controlling the absolute stereochemistry of carbon C-11a by connecting the sugar derivatized pyrrolo moiety (proline derivative) to the benzodiazepine structure. The library consisted of sugar moieties with free hydroxyl groups or *O*-benzyl groups (polar and apolar inhibitors), with or without *N*-methylation and a variety of substituents on position 7 of the benzodiazepine.

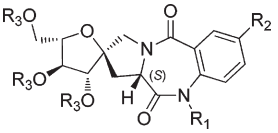
Table 2. Binding Competition Studies of D-Fructose-Based Pyrrolobenzodiazepines with [^{3}H]Flunitrazepam on GABA$_A$ Receptor, Performed on Rat Cortical Membranes



compd	R_1	R_2	R_3	%[^{3}H]Flunitrazepam specific binding[a]	significance vs control[b]
control				100.00 ± 2.00	
4	H	H	Bn	111.81 ± 2.33	ns
5	CH_3	H	Bn	96.44 ± 8.85	ns
6	H	NO_2	Bn	101.01 ± 2.10	ns
7	CH_3	NO_2	Bn	74.89 ± 1.50	$P < 0.05$
8	CH_3	Cl	Bn	92.94 ± 4.13	ns
9	CH_3	Br	Bn	111.2 ± 12.32	ns
10	H	H	H	78.22 ± 7.43	$P < 0.05$
11	CH_3	H	H	93.04 ± 12.77	ns
12	H	NH_2	H	80.17 ± 9.43	ns
13	CH_3	NH_2	H	70.63 ± 2.36	$P < 0.01$
14	CH_3	Cl	H	73.74 ± 7.31	$P < 0.05$
15	CH_3	Br	H	84.21 ± 2.84	$P < 0.05$

[a] Values are means ± SEM determined from at least three independent experiments. [b] Statistical analysis is performed with Kruskal−Wallis ANOVA for non parametric values followed by Dunns test; ns means not statistically significant and *P* means probability.

Table 3. Binding Competition Studies of L-Fructose-Based Pyrrolobenzodiazepines with [^{3}H]Flunitrazepam on GABA$_A$ Receptor, Performed on Rat Cortical Membranes



compd	R_1	R_2	R_3	%[^{3}H]Flunitrazepam specific binding[a]	significance vs control[b]
control				100.00 ± 2.40	
25	H	H	Bn	96.32 ± 3.56	ns
26	CH_3	H	Bn	105.90 ± 4.62	ns
27	H	NO_2	Bn	69.60 ± 1.50	$P < 0.001$
28	CH_3	NO_2	Bn	82.82 ± 1.24	$P < 0.001$
29	CH_3	Cl	Bn	82.33 ± 2.19	$P < 0.01$
30	CH_3	Br	Bn	93.20 ± 4.88	ns
31	H	H	H	109.70 ± 7.71	ns
32	CH_3	H	H	112.20 ± 8.17	ns
33	H	NH_2	H	73.86 ± 4.44	$P < 0.01$
34	CH_3	NH_2	H	83.55 ± 2.52	$P < 0.01$
35	CH_3	Cl	H	77.00 ± 2.27	$P < 0.001$
36	CH_3	Br	H	82.17 ± 1.69	$P < 0.01$

[a] Values are means ± SEM determined from at least three independent experiments. [b] Statistical analysis is performed with Kruskal−Wallis ANOVA for nonparametric values followed by Dunns test. ns means not statistically significant and *P* means probability.

Preliminary GABA$_A$ receptor competition binding assays using [^{3}H]flunitrazepam indicated that the conformation of the diazepam ring is not the key factor for influencing the binding affinities. The D-fructose-based pyrrolobenzodiazepines displaying a (*P*)-helical conformation presented binding affinities similar to those of the L-fructose-based pyrrolobenzodiazepines with a (*M*)-helical conformation. The D-fructose-based pyrrolobenzodiazepines, which showed the highest binding activity was *N*-methylated and carried free hydroxyl groups on the sugar. For the L-fructose-based pyrrolobenzodiazepines, *N*-methylation decreased binding activity and *O*-benzyl groups on the sugar slightly increased the binding. The effect of the benzodiazepine substituent also varied between the D- and L-fructose-based pyrrolobenzodiazepines where the binding activity increased in the following order NH_2 > Cl > NO_2 > Br for the D-series and NO_2 > NH_2 > Cl > Br for the L-series. The results further showed that the sugar moiety may or may not inflict significant steric hindrance on the binding and in some cases can increase the binding activity, possibly by participating in the binding event. However, the biological activity of the library is actually far from that of classical GABA$_A$ modulators, which falls in the nM range; nevertheless, these results indicate that sugar-based pyrrolobenzodiazepines can be considered as lead compounds and have increased the understanding of the differential recognition of (*M*)-/(*P*)-helical benzodiazepines on GABA$_A$ receptor.

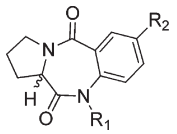
EXPERIMENTAL SECTION

1. Chemistry. *General.* All solvents were dried over molecular sieves (Fluka) for at least 24 h prior to use. When dry conditions were required, the reactions were performed under argon atmosphere. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F_{254} plates (Merck) with detection with UV light when possible, or charring with a solution containing concd H_2SO_4/EtOH/H_2O in a ratio of 5:45:45 followed by heating at 180 °C. Flash column chromatography was performed on silica gel 230−400 mesh (Merck). NMR spectra were recorded at 400 MHz (^{1}H) and at 100.57 MHz (^{13}C) on a Varian MERCURY instrument at 300 K or on a Bruker Avance 400 spectrometer at 300 K unless otherwise stated. Chemical shifts values (δ) are reported in ppm downfield from TMS as an internal standard; *J* values are given in Hz. Mass spectra were recorded on a MALDI2 Kompact Kratos instrument, with gentisic acid (DHB) as the matrix. Elemental analyses (C, H, N) were performed on a Perkin-Elmer series II 2400 analyzer, and all synthesized compounds showed a purity of more than 95%.

General Procedure for the Synthesis of Sugar-Based Pyrrolobenzodiazepines. To a solution of the proline analogue (**2** or **24**) in dry DMF, suitably functionalized isatoic anhydride type **3** was added under argon atmosphere, and the resulting mixture was stirred under reflux until complete consume of the starting material. The solvent was then removed under reduced pressure and the residue dissolved in CH_2Cl_2, filtered, and evaporated. The crude halogenated benzodiazepines (R_2 = Cl, Br) were used in the next step (methylation), whereas the crude products derived from R_2 = H, NO_2 were purified by silica gel flash chromatography to give the desired product (compounds **4**−**7** and **25**−**28**).

*L-Arabinono-1,4-lactone (**17**).* To a solution of α,β L-arabinose **16** (5 g, 0.033 mol) in water (50 mL) potassium carbonate (6.84 g, 0.050 mol) was added and the resulting mixture was cooled to 0 °C. Then bromine (3.4 mL, 0.067 mol) was added dropwise under continues stirring. The reaction was left stirring in the dark for about 1 h at rt. The solvent was then removed under reduced pressure. The crude residue was purified by silica gel flash chromatography (EtOAc/EtOH 8:2) to give the desired product **17** as a yellow oil (4.79 g, 98% yield). MS

Table 4. Binding Competition Studies of Pyrrolobenzodiazepines Derived from D- and L-Proline with [^{3}H]Flunitrazepam on GABA$_A$ Receptor, Performed on Rat Cortical Membranes



compd	R_1	R_2	%[^{3}H]Flunitrazepam specific binding[a]	significance vs control[b]
control			100.00 ± 1.49	
(11a*R*)-**37**	H	H	97.00 ± 0.91	ns
(11a*R*)-**38**	CH_3	NO_2	80.25 ± 3.68	$P < 0.05$
(11a*S*)-**39**	H	H	95.50 ± 2.47	ns
(11a*S*)-**40**	CH_3	NO_2	63.40 ± 1.57	$P < 0.001$

[a] Values are means ± SEM determined from at least three independent experiments. [b] Statistical analysis is performed with Kruskal−Wallis ANOVA for nonparametric values followed by Dunns test. ns means not statistically significant and *P* means probability.

(MALDI-TOF): $C_5H_8O_5$ calculated 148.11, observed 149.1 $[M + H]^+$, 171.1 $[M + Na]^+$. Elemental analysis calcd (%): C, 40.55; H, 5.44. Found: C, 40.61; H, 5.40. ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) 4.33 (d, 1H, J = 8.5 Hz, H-2), 4.14−4.09 (m, 2H, H-3, H-4), 3.87 (dd, 1H, J = 13.0, 1.9 Hz, H-5a), 3.65 (dd, 1H, J = 13.0, 4.0 Hz, H-5b). ^{13}C NMR (100.57 MHz, CD_3OD): δ (ppm) 176.41 (CO), 82.82, 75.66, 74.18 (C-2, C-3, C-4), 60.89 (C-5).

*2,3,5-Tri-O-benzyl-L-arabinono-1,4-lactone (**18**).* Benzyl trichloroacetimidate (28 mL, 0.153 mol) and trifluoromethanesulfonic acid (0.301 mL, 0.003 mmol) were added at 0 °C, under argon atmosphere, to a solution of **17** (5 g, 0.034 mol) in dry dioxane (100 mL). The solution was stirred for 3 h, and then quenched with a satd solution of $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was dried on sodium sulfate, filtered, and evaporated. Flash column chromatography of the residue (petroleum ether/EtOAc 9:1) afforded **18** as a yellow oil (9.25 g, 65% yield). MS (MALDI-TOF): $C_{26}H_{26}O_5$ calculated 418.48, observed 419.5 $[M + H]^+$, 457.5 $[M + K]^+$. Elemental analysis calcd (%): C, 74.62; H, 6.26. Found: C, 74.74; H, 6.22. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.33−7.06 (m, 15H, OCH_2Ph), 5.00, 4.97, 4.71, 4.68 (AB, 2H, J = 11.6 Hz, PhCH_2O), 4.55 (d, 1H, J = 11.6 Hz, PhCH_2O), 4.49−4.41 (m, 2H, PhCH_2O) 4.28−4.24 (m, 3H, H-2, H-3, H-4), 3.63 (dd, 1H, J = 11.3, 1.8 Hz, H-5a), 3.50 (dd, 1H, J = 11.4, 3.3 Hz, H-5b). ^{13}C NMR (100.57 MHz, $CDCl_3$): δ (ppm) 172.57 (CO), 137.41, 137.02, 136.72 (Cq Ph), 128.53−127.72 (OCH_2Ph), 79.20, 79.09, 78.81 (C-2, C-3, C-4), 72.48, 72.68, 72.47 (OCH_2Ph), 67.89 (C-5).

*2,3,5-Tri-O-benzyl-1-C-(1,3-dithiane-2-yl)-L-arabinose (**19**).* In a 100 mL, two-necked, round-bottomed flask equipped with a magnetic stirring bar, a rubber septum, and an argon filled balloon was placed 1,3-dithiane (0.432 g, 3.6 mmol) in dry THF (24 mL). The solution was cooled to −20 °C and *n*-BuLi (3 mL, 4.8 mmol, 1.6 M in hexane) was added slowly. After 1.5 h, the resulting mixture was cooled to −78 °C and added via cannula to a −78 °C solution of **18** (1.0 g, 2.4 mmol) in dry THF (28 mL). The reaction was stirred for 20 min and quenched at −78 °C by addition of satd solution of NH_4Cl (12 mL). To the reaction mixture was added EtOAc (80 mL) and then extracted. The organic layer was dried on sodium sulfate, filtered, and evaporated. Flash column chromatography of the residue (petroleum ether/EtOAc 8:2) afforded **19** (0.827 g, 64% yield) as a yellow oil mixture of α and β anomers (1:4 α:β ratio, as determined from the ratio integrals of the ^{1}H NMR signals). The isomers were not completely separable by chromatography. The following signal attributions were assigned from a small amount of pure β-anomer. MS (MALDI-TOF): $C_{30}H_{10}O_5S_2$ calculated 538.72, observed 539.7 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.38−7.18 (m, 15H, OCH_2Ph), 4.70 (s, 1H, H-1), 4.62−4.41 (m, 7H, OCH_2Ph, H-4), 4.14 (bm, 2H, H-3, H-5), 3.60 (bm, 1H, H-6), 3.17 (bm, 2H, H-2′), 2.53 (bm, 2H, H-4′), 2.02 (bm, 2H, H-3′). ^{13}C NMR (100.57 MHz, $CDCl_3$): δ (ppm) 137.86, 137.80, 137.50 (Cq Ph), 128.45−127.68 (OCH_2Ph), 110.10 (C-2), 84.11, 83.12, 80.23 (C-3, C-4, C-5), 73.32, 72.94, 72.30 (OCH_2Ph), 71.44 (C-6), 53.41 (C-1), 27.48, 27.34 (C-2′, C-4′), 25.16 (C-3′).

*Methyl 2,3,5-Tri-O-benzyl-1-C-(1,3-dithiane-2-yl)-L-arabinoside (**20**).* To a solution of **19** (1.0 g, 1.86 mmol) in dry THF (8 mL), ms 3 Å (1.0 g), MeOH (20 mL), and H_2SO_4 (40 μL) were added under argon atmosphere, and the resulting mixture was stirred under reflux (bath temperature 40 °C) for 12 h. After this time, a solution of 1 M NaOH was added to the reaction to neutralize the acid, then the solvent was partially evaporated, and the residue was diluted with CH_2Cl_2. The organic layer was dried on sodium sulfate, filtered, and evaporated. Purification by flash chromatography (petroleum ether/EtOAc 8:2) afforded anomers **20** (0.688 g, 67% yield, 1.5:1 α:β ratio) as a yellow oil. The following signal attributions were obtained from the separated α,β mixture. A 2D NOESY experiment for the α-anomer **20** showed a cross peak between OCH_3 and H-3, and between OCH_3 and H-5 allowing the assignment of the corresponding stereochemistry. α-Anomer: MS (MALDI-TOF) $C_{31}H_{36}O_5S_2$ calculated 552.74, observed 553.7 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.32−7.26 (m, 15H, OCH_2Ph), 4.79 (s, 1H, H-1), 4.59−4.45 (m, 6H, OCH_2Ph), 4.15 (dd, 1H, J = 10.3, 4.5 Hz, H-5), 3.97 (d, 1H, J = 1.5 Hz, H-3), 3.86 (d, 1H, J = 4.2 Hz, H-4), 3.68 (dd, 1H, J = 10.4, 5.4 Hz, H-6a), 3.54 (bm, 1H, H-6b), 3.52 (s, 3H, OCH_3), 2.91−2.75 (m, 2H, H-2′, H-4′), 2.06 (bm, 1H, H-3′a), 1.90 (bm, 2H, H-3′b). ^{13}C NMR (100.57 MHz, $CDCl_3$): δ (ppm) 138.17, 137.88, 137.66 (Cq Ph), 128.87−127.57 (OCH_2Ph), 108.59 (C-2), 87.18, 84.33, 82.90 (C-3, C-4, C-5), 73.33, 72.61, 71.67 (OCH_2Ph), 70.11 (C-6), 51.46 (C-1), 50.61 (OCH_3), 30.78, 29.68 (C-2′, C-4′), 26.41 (C-3′). β-Anomer: MS (MALDI-TOF) $C_{31}H_{36}O_5S_2$ calculated 552.74, observed 553.7 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.34−7.26 (m, 15H, OCH_2Ph), 5.30 (s, 1H, H-1), 4.58−4.47 (m, 6H, OCH_2Ph), 4.17 (dd, 1H, J = 13.5, 6.7 Hz, H-5), 4.12 (d, 1H, J = 7.1 Hz, H-3), 4.07 (d, 1H, J = 6.6 Hz, H-4), 3.59 (dd, J = 10.3, 4.8 Hz, H-6a), 3.54 (dd, J = 10.3, 5.4 Hz, H-6b), 3.41 (s, 3H, OCH_3), 2.93−2.76 (m, 2H, H-2′, H-4′), 2.05 (bm, 1H, H-3′a), 1.89 (bm, 2H, H-3′b). ^{13}C NMR (100.57 MHz, $CDCl_3$): δ (ppm) 138.24, 137.97, 137.75 (Cq Ph), 128.87−127.60 (OCH_2Ph), 105.25 (C-2), 85.45, 83.94, 79.25 (C-3, C-4, C-5), 73.68, 72.23, 72.60 (OCH_2Ph), 70.39 (C-6), 52.15 (C-1), 49.77 (OCH_3), 30.36, 29.69 (C-2′, C-4′), 25.80 (C-3′).

*Methyl 3,4,6-Tri-O-benzyl-L-arabino-hexo-2-ulo-2,5-furanoside (**21**).* To a solution of **20** (0.500 g, 0.904 mmol) in acetone (12 mL) at 25 °C was added a solution of 1.3-dibromo-5.5-dimethylhydantion (DBDMH) (0.517 g, 1.81 mmol) in acetone (8 mL) at −20 °C. The solution quickly turned red but soon faded to yellow−orange, and was stirred for 15 min. At this time, the solution was shaken with a mixture of satd aq solution of sodium sulfite and CH_2Cl_2. The organic phase was washed with sodium bicarbonate, water, and brine, dried ($NaSO_4$), and concentrated to give compound **21** as yellow oil, which was used in the next step without further purification.

*Methyl 3,4,6-Tri-O-benzyl-L-arabino-hex-2-ulo-2,5-furanoside (**22**).* To a 0 °C cooled solution of **21** (≈0.904 mmol) in dry EtOH (25 mL) was added $NaBH_4$ (0.410 g, 10.85 mmol) under argon atmosphere. The reaction mixture was left stirring for 30 min and then quenched by addition of satd aq NH_4Cl solution at 0 °C. The resulting mixture was extracted with EtOAc, and the organic layer was dried on sodium sulfate, filtered, and evaporated. Purification by flash chroma-

tography (petroleum ether/EtOAc 7:3) afforded anomers **22** (0.273 g, 65% yield over two steps, 3:1 α:β ratio) as a yellow oil. The following signal attributions were assigned from the separated α,β mixture. α-Anomer: MS (MALDI-TOF) $C_{28}H_{32}O_6$ calculated 464.55, observed 465.5 $[M + H]^+$, 503.5 $[M + K]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.33−7.22 (m, 15H, OCH_2Ph), 4.67−4.46 (m, 7H, OCH_2Ph, H-4), 4.16 (t, 2H, H-3), 4.09 (bm, 1H, H-5), 3.78 (dd, 1H, *J* = 11.9, 6.7 Hz, H-6a), 3.69 (dd, 1H, *J* = 11.9, 5.2 Hz, H-6b), 3.65 (dd, 1H, *J* = 10.7, 3.4 Hz, H-1a), 3.54 (dd, 1H, *J* = 10.7, 3.9 Hz, H-1b), 3.32 (s, 1H, OCH_3), 1.78 (bs, 1H, O*H*). ^{13}C NMR (100.57, $CDCl_3$): δ (ppm) 137.86, 137.63, 137.42 (Cq Ph), 128.49−127.78 (OCH_2Ph), 107.69 (C-2), 86.33, 82.37, 80.21 (C-3, C-4, C-5), 73.82, 73.40, 72.42 (OCH_2Ph), 69.13 (C-6), 61.49 (C-1), 48.92 (OCH_3). β-Anomer: MS (MALDI-TOF) $C_{28}H_{32}O_6$ calculated 464.55, observed 465.5 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.37−7.21 (m, 15H, OCH_2Ph), 4.62−4.46 (m, 7H, OCH_2Ph, H-4), 4.27 (d, 1H, *J* = 7.0 Hz, H-3), 4.20 (bm, 1H, H-5), 4.10 (dd, 1H, *J* = 13.0, 6.9 Hz, H-6a), 4.08 (dd, 1H, *J* = 12.0, 6.1 Hz, H-6b), 3.72 (d, 1H, *J* = 11.9 Hz, H-1a), 3.54 (d, 1H, *J* = 11.8 Hz, H-1b), 3.30 (s, 1H, OCH_3) 2.05 (bs, 1H, O*H*). ^{13}C NMR (100.57 MHz, $CDCl_3$): δ (ppm) 137.86, 137.80, 137.74 (Cq Ph), 128.38−127.76 (OCH_2Ph), 104.35 (C-2), 84.22, 83.67, 79.70 (C-3, C-4, C-5), 73.41, 72.97, 72.54 (OCH_2Ph), 71.80 (C-6), 62.53 (C-1), 49.57 (OCH_3).

*3-C-(3,4,6-Tri-O-benzyl-α-L-fructofuranos-2-yl)propene (**23**).* To a stirred solution of **22** (1 g, 2.15 mmol) in dry CH_3CN (8 mL) was added bis(trimethylsilyl) trifluoroacetamide (BTSFA) (0.568 mL, 2.15 mmol) at 100 °C under reflux and argon atmosphere. After 3 h, analysis by thin-layer chromatography indicated that starting material **22** had been consumed. The reaction mixture was clear and uniform and was allowed to cool to rt, and $AllSi(CH_3)_3$ (0.514 mL, 3.22 mmol) and TMSOTf (188 μL, 1.07 mmol) were sequentially added at 0 °C and reaction was left stirring at rt for 1 h. Water (12 mL) was added slowly to hydrolyze TMS ethers, and then the mixture was neutralized with a 1 M aq solution NaOH and concentrated in vacuo. The residue was extracted with CH_2Cl_2, the organic layer was dried on sodium sulfate, filtered, and evaporated. Purification by flash chromatography (petroleum ether/EtOAc 8:2) afforded anomer **23** (0.775 g, 76% yield) as a yellow oil. The 8:2 α:β ratio was determined from integration of the crude ^{1}H NMR spectra. MS (MALDI-TOF): $C_{30}H_{34}O_5$ calculated 474.59, observed 475.6 $[M + H]^+$. Elemental analysis calcd (%): C, 75.92; H, 7.22. Found: C, 76.10; H, 7.35. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.37−7.24 (m, 15H, OCH_2Ph), 5.78 (bm, 1H, H-2′), 5.09 (d, 1H, *J* = 10.2 Hz, H-3′a), 4.97 (d, 1H, *J* = 17.3 Hz, H-3′b), 4.75−4.45 (m, 7H, OCH_2Ph, H-4), 4.13 (d, 1H, *J* = 7.7 Hz, H-3), 3.92 (bm, 1H, H-5), 3.70 (bm, 2H, H-1a, H-6a), 3.50 (m, 2H, H-1b, H-6b), 2.30 (dd, 1H, *J* = 14.0, 6.5 Hz, H-1′a), 2.17 (dd, 1H, *J* = 14.2, 7.6 Hz, H-1′b), 1.83 (bs, 1H, O*H*). ^{13}C NMR (100.57 MHz, $CDCl_3$): δ (ppm) 138.32, 138.20, 137.73 (Cq Ph), 133.07 (C-2′), 128.71−127.07 (OCH_2Ph), 119.17 (C-3′), 84.29 (C-2), 86.65, 83.50, 79.34 (C-3, C-4, C-5), 73.65, 73.19, 72.15, 69.61, 64.41 (OCH_2Ph, C-6, C-1), 40.29 (C-1′).

Molecular Modeling. Conformational analysis of compounds **10**, **11**, and **31** was performed applying an MM2 force field in vacuum. Each model was built with ChemDraw, energy was minimized (minimum rms gradient = 0.010), and the dihedral angle C5a−C9a−N10−C11 value calculated using Chem 3D. A molecular dynamics computation was performed (step interval = 2 fs; frame interval = 1 fs; heating/cooling rate = 1.000 kcal/atom/ps; target temperature = 300 K) monitoring the same dihedral angle. The average value was calculated; in all cases, it corresponded to that found by MM calculations.

DNMR Experiments. 0.5 mg of compound **10**, **11**, and **31** was dissolved in 0.6 mL of D_2O (293−363 K). An additional DNMR experiment was performed in CD_3CD_2OD (173−293 K) for compound **10**. ^{1}H and selective 1D-NOESY (data not shown) spectra were recorded at different temperatures and then processed with MestreNova (MestreLab Research Srl). For spectra recorded in D_2O, the HDO chemical shift was calculated according to the equation $\delta = 5.060 - 0.0122T + (2.11 \times 10^{-5})T^2$.[34]

2. Biological Assays. *General.* [^{3}H]Flunitrazepam (MW unlabeled at this specific activity 313.28, $C_{16}H_{12}FN_3O_3$), flunitrazepam-[methyl-^{3}H], has a specific activity of 80.0 Ci/mmol and was purchased from International PBI SPA (Milan, Italy), American Radiolabeled Chemicals, Inc. Flumazenil (MW 303.39, $C_{15}H_{14}FN_3O_3$), 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester, was purchased from TOCRIS Bioscience, Tocris Cooksoon Ltd. (Bristol, U.K.) Centrifugation assays were performed on a centrifuge Biofuge Primo R-Heraeus and on an ultracentrifuge Beckman XL-90. Filtration assays were performed using Whatman GF/B glass fiber filters (∅ = 1−2 cm). Liquid scintillation counting was performed on a Spectrophotometer Beckman LS65000 with 40% of efficiency for tritium and was used the scintillation fluid UltimaGold F from PerkinElmer (Boston, USA). The investigations using experimental animals were conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in for example the European Community guidelines (EEC Directive of 1986; 86/609/EEC). Results are expressed in terms of percentage of control [^{3}H]Flunitrazepam specific binding and analyzed by GraphPad Prism using the Kruskal−Wallis ANOVA for nonparametric data followed by Dunn's test for specific comparisons.

Membrane Preparation. Membranes were prepared from rat cortex as described by Ahboucha et al.[33] The rat frontal cortex was homogenized, through a glass/Teflon potter at 1000 rpm, in ice-cold 0.32 M sucrose, pH 7.4 (20 mL/gr of tissue), and the homogenate was centrifuged at 2000*g* for 10 min at 4 °C. The supernatant was then ultracentrifuged at 140000*g* for 30 min at 4 °C, and the pellet was resuspended in Tris-HCl 50 mM pH 7.4 (2 mL/g wet weight) and again centrifuged at 140000*g* for 30 min at 4 °C. After the final centrifugation, the pellet was suspended in 1 mL of ice-cold Tris-HCl buffer (50 mM pH 7.4) and was manually homogenized through a glass/Teflon potter and stored frozen at −80 °C. On the day of the assay, the tissue was thawed, and the pellet, resuspended in Tris-HCl in order to obtain a final protein concentration of 2 mg/mL, which was used for displacement binding studies.

Radioligand Binding Assay. [^{3}H]Flunitrazepam (1 nM) served as radioligand, and nonspecific binding was determined in the presence of flumazenil (100 μM) and represented about 1−2% of the total binding. The water-soluble benzodiazepine derivatives synthesized were diluted in TrisHCl buffer 50 mM pH 7.4 to obtain the final concentration of 100 μM, whereas stock solutions of the benzodiazepine derivatives with the benzyl group in the sugar were prepared in ethanol and then diluted in TrisHCl buffer to obtain the final concentration of 100 μM. The binding reaction consisted of 0.3 mg of membranes incubated with the radioligand in the presence or absence of the test compounds for 90 min at 4 °C. During this incubation period, the appropriate number of filters were soaked in ice cold buffer. The binding reaction was then stopped by rapid filtration under vacuum through a glass-fiber filter. The filters were mounted in the filtration manifold, vacuum was applied, and an aliquot of the first sample was applied to the first filter. Once the sample compartment drains, the filter was washed immediately by the rapid addition of 2 mL portions of ice cold Tris-HCl buffer (two times). After being washed twice, the filters were dried under a heat lamp for 10 min and then transferred to scintillation vials and dissolved in 8 mL of scintillation fluid. Filter bound radioactivity was determined by scintillation counter, 20 min for each filter. The radioactivity values obtained from the spectrophotometer in dpm were converted into percentages, considering the value of specific binding 100%, given by the samples only with the [^{3}H]flunitrazepam. For all compounds, three independent experiments were performed in triplicate.

■ ASSOCIATED CONTENT

Supporting Information. Detailed experimental procedures and analytical and spectral data for all intermediate and final compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

BTSFA, bis(trimethylsilyl)trifluoroacetamide; DBDMH, 1,3-dibromo-5,5-dimethylhydantoin; DMF, *N*,*N*-dimethylformamide; DNMR, dynamic nuclear magnetic resonance; GABA, γ-aminobutyric acid; MM2, molecular mechanics force field; MS, mass spectrometry; SEM, standard error of the mean; rms, root-mean-square; TfOH, trifluoromethanesulfonic acid; THF, tetrahydrofuran; TMSOTf, trimethylsilyl trifluoromethanesulfonate; TLC, thin-layer chromatography.

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