solution (2.4 mL, 17.4 mmol) in acetonitrile (35 mL) was stirred for 4 h at room temperature and concentrated in vacuo. The residue was dissolved in $CHCl_3$ and then the $CHCl_3$ solution was washed with water and concentrated. The resulting oil (0.41 g) after chromatography was dissolved in THF, to which 10% KOH solution (40 mL) was added. The solution was allowed to reflux for 10 min, made acidic with 10% HCl solution, and extracted with $CHCl_3$. The $CHCl_3$ layer was washed with water and concentrated. Recrystallization of the crude product by AcOEt gave 13 (0.25 g, 71%) as a light yellow powder: mp 233–235 °C; IR (Nujol) 3300, 3250, 1600 cm^{-1}; NMR ($CDCl_3$) δ 1.23 (d, J = 7 Hz, 6 H, $CHMe_2$), 1.27 (d, J = 7 Hz, $CHMe_2$), 2.7–3.3 (m, 2 H, 2 $CHMe_2$), 3.02 (d, J = 5 Hz, 3 H, $NHMe$), 3.79 (s, 3 H, OMe), 6.4–7.5 (m, 11 H, CH, aromatic H, tropolone H). Anal. ($C_{29}H_{33}NO_4$) C, H, N.

Biological Assays. Assays of antitumor activity were carried out as described previously.[2b]

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Registry No. **1a**, 92832-17-2; **2a**, 92832-11-6; **3**, 108149-77-5; **4**, 108149-78-6; **5**, 108149-79-7; **6**, 108149-80-0; **9**, 108149-81-1; **10**, 108149-82-2; **11**, 108149-83-3; **12**, 108149-84-4; **13**, 108149-85-5.

Synthesis of β-Carboline–Benzodiazepine Hybrid Molecules: Use of the Known Structural Requirements for Benzodiazepine and β-Carboline Binding in Designing a Novel, High-Affinity Ligand for the Benzodiazepine Receptor

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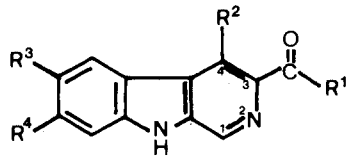
Hybrid molecules incorporating pharmacologically important structural features of both 3-carboxy-β-carbolines and 1,4-benzodiazepines were synthesized, and their affinities for the benzodiazepine receptor were determined in vitro. One of these hybrids, 8,14-dioxo-13,14-dihydro-8*H*-indolo[3′,2′:4,5]pyrido[2,1-*c*][1,4]benzodiazepine (**13**), demonstrated high affinity for the receptor, displacing both benzodiazepines (IC_{50} = 23 nM) and β-carbolines (IC_{50} = 47 nM) from their binding sites. Of the compounds synthesized, **13** also most closely satisfied the structural requirements that generally ensure a high affinity of both β-carbolines and benzodiazepines for the receptor (e.g., aromaticity of the β-carboline, presence of a carbonyl at C-3 of the β-carboline and of a π_2-region on the benzodiazepine). The hybrids not fulfilling these requirements had no affinity for the receptor. In vivo pharmacological properties of **13** could not be demonstrated because of its metabolic instability and/or its poor transport into the brain. The results are discussed in terms of a possible overlapping of β-carboline binding sites with those of benzodiazepines on the receptor.

The benzodiazepine receptor, which has been shown to mediate the sedative, anxiolytic, muscle-relaxant, and anticonvulsant effects of 1,4-benzodiazepines in the central nervous system,[1-5] is known to recognize a wide variety of apparently unrelated structures besides the benzodiazepines (represented by diazepam (**1**), Figure 1). For example, β-carbolines such as β-CCM (**2**) also display high



2 R^1 = OCH_3; R^2=R^3=R^4=H (β-CCM)
3 R^1 = OCH_2CH_3; R^2=R^3=R^4=H (β-CCE)
4 R^1 = $NHCH_3$; R^2=R^3=R^4=H (FG 7142)
7 R^1 = R^3 = R^4 = OCH_3; R^2 = CH_2CH_3 (DMCM)

affinities for the benzodiazepine receptor equivalent or superior to those of the benzodiazepines themselves.[6,7] However, though β-CCM can displace diazepam from its receptor sites (and vice versa), this compound also possesses pharmacological properties mediated by the benzodiazepine receptor that are completely opposite to those generally associated with benzodiazepines. Thus, β-CCM is a potent convulsant in various species[9-12] and demonstrates anxiogenic properties in mice.[13] Other analogues of β-CCM also display such inverse properties to various degrees. The ethyl ester, β-CCE (**3**), is proconvulsant in mice[14] and baboons[15] and anxiogenic in rats.[16] The methyl amide derivative, FG 7142 (**4**), also has attenuated convulsant properties[17] and is anxiogenic in certain para-

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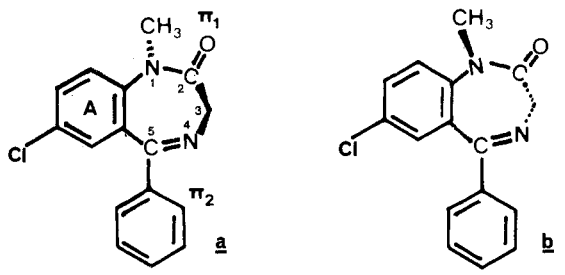
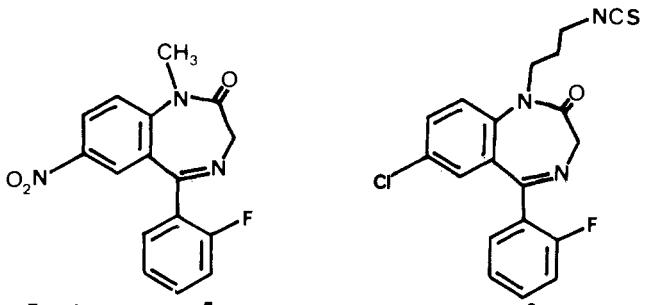


Figure 1. Two conformational possibilities for diazepam, 1. Conformer a is most likely the pharmacologically active one.

digms,[16] including subjective tests in humans.[18] The term inverse agonist has been applied to such substances demonstrating activities opposite to those of the agonist benzodiazepines.[8]

Little is known at present concerning the exact nature of the recognition sites for β-carbolines and benzodiazepines on the benzodiazepine receptor. That is, for any given benzodiazepine receptor protein, do the recognition sites for both benzodiazepines and β-carbolines coincide exactly, do they merely overlap, or are these recognition sites in fact completely separate (though probably close-by)?

Much evidence indicates that benzodiazepine receptor agonists (e.g., benzodiazepines in general) and inverse agonists (e.g., β-CCM, β-CCE) do *not* occupy identical sites on the receptor. Thus, irreversible photolabeling of the benzodiazepine receptor with flunitrazepam (**5**) greatly



Flunitrazepam **5** Irazepine **6**

diminishes the affinity of the receptor for benzodiazepines[19] without appreciably affecting the affinities of either β-CCE[20-22] or the propyl ester, β-CCP.[23] Similar results were obtained when an alkylating benzodiazepine, irazepine (**6**), was used as the irreversible ligand.[24] Moreover, the in vitro binding affinities of convulsant β-carbolines such as β-CCM, β-CCE, and DMCM (**7**) have been found to be decreased by the addition of γ-aminobutyric acid (GABA) to the incubation medium[25-27] while the affinities of benzodiazepines are substantially increased

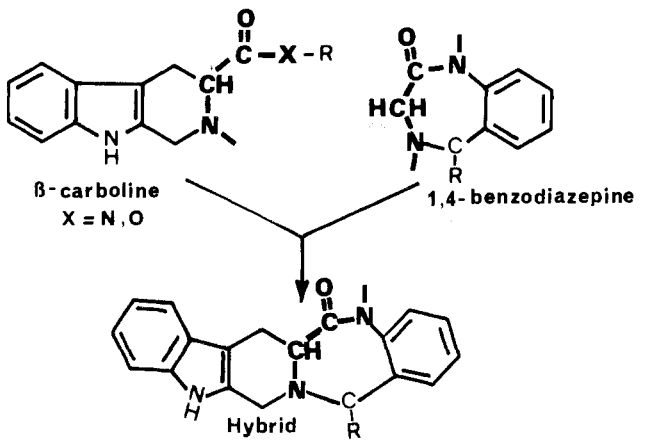


Figure 2. General structural formulas of 3-carboxy-β-carbolines (ester or amide) and 1,4-benzodiazepines, showing their common atoms. The hybrids incorporate both molecules joined by these common atoms.

by this treatment.[28-30] Finally, an octadecapeptide derived from a putative endogenous ligand of the benzodiazepine receptor called DBI (diazepam binding inhibitor) has been found to preferentially inhibit the binding of [^{3}H]-β-CCM over that of [^{3}H]flunitrazepam.[31] It is thus not unreasonable to assume that β-carbolines bind to the benzodiazepine receptor in some way different from that of benzodiazepines,[25,32] the binding sites of each class of compounds being either entirely distinct or else overlapping.

We have used this latter possibility as the starting point in designing novel benzodiazepine receptor ligands with the hope of obtaining molecules with a narrower pharmacological profile than either benzodiazepines or β-carbolines. As our working hypothesis, we have assumed, on the basis of the above observations, that benzodiazepines and β-carbolines occupy such separate but overlapping sites, or domains, on any given benzodiazepine receptor protein. The overlapping region was taken, naively, to accommodate a structural feature common, and essential, to both classes of compounds, that is, the —N—C—C(=O)—X— unit (Figure 2, X = N, O). If this is so, a hybrid molecule that incorporates both a 1,4-benzodiazepine moiety and a 3-carboxy-β-carboline moiety connected by this common structural unit should occupy, at once, the available binding sites for each separate type of molecule with high affinity. Such a hybrid could reveal important topographical features of the benzodiazepine receptor as well as perhaps exhibiting interesting pharmacological properties. In this paper, we describe the synthesis of two benzodiazepine–β-carboline hybrids (**13** and **19**) and their interactions with the benzodiazepine receptor.

Chemistry. Hybrid molecule **13** was synthesized as shown in Scheme I. Thus, reaction of tetrahydro-β-CCE (**8**)[33] with o-nitrobenzoyl chloride (**9**) proceeded smoothly under mildly basic conditions, yielding the benzoylated β-carboline **10**. No competitive benzoylation of the less

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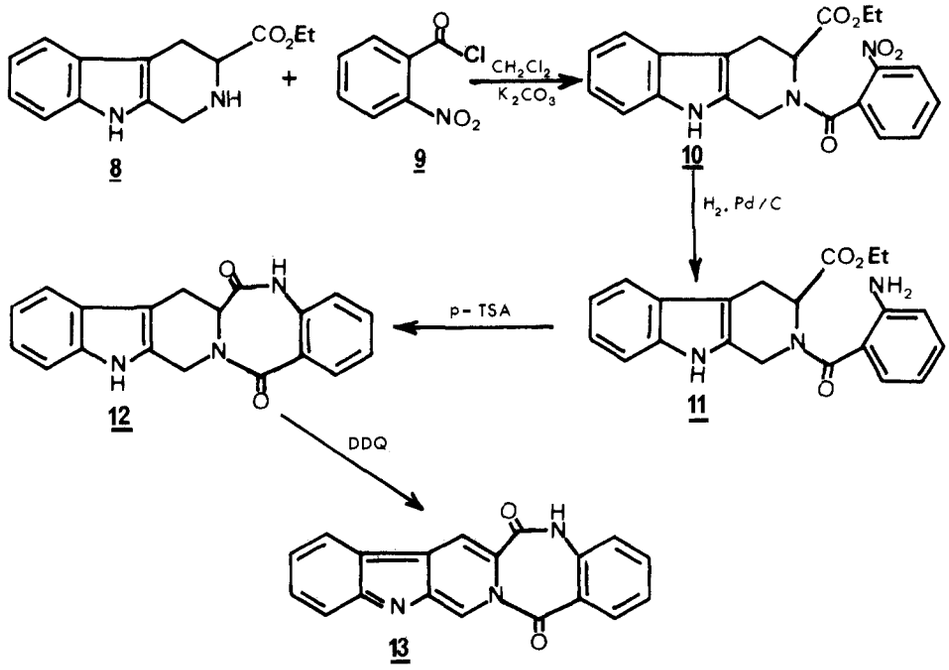
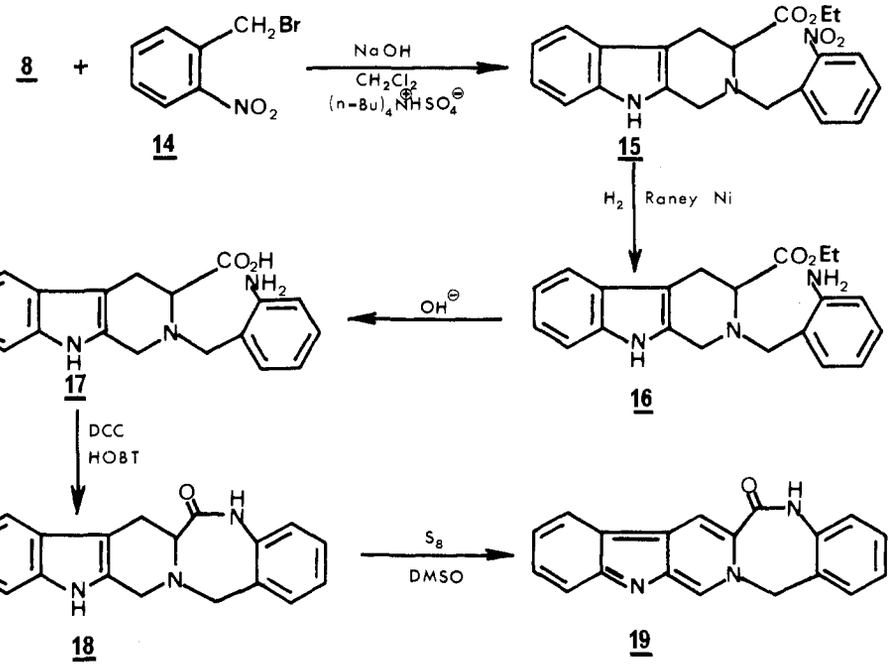
Scheme I



Scheme II



basic indolic nitrogen was observed. Catalytic hydrogenation of **10** with palladium on activated carbon gave the amine **11** quantitatively. Ring closure was effected by boiling a solution of **11** in dioxane containing a catalytic amount of *p*-toluenesulfonic acid, the 1,4-benzodiazepine derivative **12** being precipitated as it formed. Dehydrogenation of compound **12** with 2 equiv of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) under anhydrous conditions[34-36] produced the benzodiazepino-β-carboline **13**. The mass spectrum of **13** indicated loss of four hydrogen atoms while the NMR spectrum showed only aromatic protons for this compound, with only one D_2O-exchangeable proton. Treatment of **12** with only 1 equiv of DDQ led to 50% conversion to **13** rather than to partial dehydrogenation to give a dihydro derivative.

Synthesis of the second hybrid molecule, **19** (Scheme II), by an analogous route met with some difficulties, partly due to the benzylic nature of the intermediates. Thus, coupling of tetrahydro-β-CCE (**8**) with o-nitrobenzyl bromide (**14**) to give **15** was performed by using phase-transfer catalysis.[37] The addition of a small quantity of potassium carbonate to the reaction mixture prevented agglomeration of the powdered sodium hydroxide during

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Table I. Benzodiazepine Receptor Affinities of β-Carboline–Benzodiazepine Hybrids

compound	IC_{50} vs. [^{3}H]Flu, nM[a]	IC_{50} vs. [^{3}H]-β-CCE, nM[b]
flunitrazepam	3	8[c]
β-CCE	2.5	6
12	>100 000	>100 000
13	23	47
18	>100 000	>100 000
19	6000	2000

[a] Concentration of compound required to inhibit 50% of 1 nM [^{3}H]flunitrazepam binding to in vitro preparations of rat cerebral cortex membranes at 0 °C; average of duplicate determinations. [b] Concentration of compound required to inhibit 50% of 0.4 nM [^{3}H]-β-CCE binding to in vitro preparations of rat cerebral cortex membranes at 0 °C; average of duplicate determinations. [c] Ref 52.

the relatively long reaction period (22 h).[38] An attempted hydrogenation of the nitro group of **15** using palladium on carbon as catalyst led almost exclusively to debenzylation and formation of **8**. The use of active Raney nickel as catalyst alleviated this problem, and the amine derivative **16** was produced in acceptable yield. Contrary to the facile, acid-catalyzed cyclization of **11** to **12**, only decomposition products were obtained when **16** was submitted to the same reaction conditions. The ester group of **16** was thus removed by refluxing this compound in 90% ethanol containing exactly 1 equiv of sodium hydroxide. Use of excess base led to debenzylation. The carboxylic acid derivative **17** so obtained was cyclized by using dicyclohexylcarbodiimide in dimethylformamide. One equivalent of 1-hydroxybenzotriazole was added to the reaction mixture to minimize formation of N-acylated dicyclohexylurea adducts.[39] The final step, dehydrogenation of the cyclized product **18** to give **19**, was first attempted by using 2 equiv of DDQ, as described for the preparation of **13**. However, though compound **19** was indeed formed under these reaction conditions, as shown by mass spectral analysis of the reaction mixture, this product could not be efficiently separated from residual DDQ and the quinol byproduct. Secondary reaction products were also evident.[40] However, treatment of **18** in dimethyl sulfoxide with 2 equiv of elemental sulfur at 100 °C for 2 days[7,35] led to clean conversion to the desired aromatic derivative **19**.

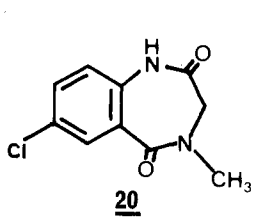
Results and Discussion

The results of the in vitro displacement studies using compounds **12**, **13**, **18**, and **19** are shown in Table I.[41] Thus, while compound **19** inhibits only weakly [^{3}H]flunitrazepam binding to rat brain membrane preparations, compound **13** demonstrates high affinity for the benzodiazepine receptor.[42] Compound **13** also inhibits [^{3}H]-β-CCE binding just as effectively. None of the intermediate compounds, including the saturated precursors of **13** and **19** (**12** and **18**, respectively) showed any significant affinity for the benzodiazepine receptor. These results can be rationalized in the light of the known structural prerequisites for β-carboline and benzodiazepine binding to the receptor.

Diazepam can exist in two different conformations as a result of flipping of the C-3 methylene group (Figure 1). On the basis of X-ray crystal data and NMR studies of benzodiazepine derivatives substituted at C-3, Blount et al.[43] have shown that the most likely conformation of diazepam recognized by the benzodiazepine receptor is that in which the C-3 methylene group adopts the position shown in Figure 1a. Dreiding models indicate that the seven-membered ring of the active molecule **13** cannot assume a conformation identical with that of the active form of diazepam since the aromatic ring fused to the 3,4-position of the benzodiazepine portion of the molecule restricts rotation about this bond. Nevertheless, the conformational differences between the seven-membered rings of the active form of diazepam and **13** are only minor. The activity of **13** in displacing [^{3}H]flunitrazepam is thus in line with the observation that small geometrical differences in the benzodiazepines are unimportant in determining their efficient binding to the benzodiazepine receptor.[44] Attempts to form crystals of **13** suitable for X-ray analysis thereby allowing direct comparison with the known X-ray structures of other benzodiazepines have so far been unsuccessful.

In the case of compound **12**, the saturated precursor of **13**, the absence of an sp^2 carbon at C-3 of the seven-membered ring allows the latter to attain a conformation that is completely superimposable with that of diazepam shown in Figure 1a.[45] Nevertheless, compound **12** is almost totally inactive. It thus appears that a more important criterion for binding of the hybrid molecules to the receptor is complete aromaticity of the β-carboline portion of the molecule. This same restriction has been observed for the β-carbolines themselves, the tetrahydro derivative of β-CCE (**8**) for instance displaying little affinity for the benzodiazepine receptor.[7,46] This, in fact, is a good indication that the receptor indeed specifically recognizes the hybrid **13** as a β-carboline.

The importance of the β-carboline moiety of **13** in ensuring its high affinity for the receptor is further suggested by the fact that both compounds **20**[47] and **21**,[2] in which this ring system is completely absent or is replaced by pyrrolidine, are practically devoid of activity.



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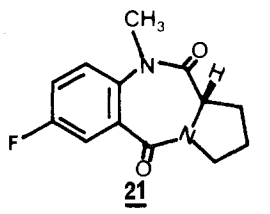

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(42) The possibility that compound **13** is hydrolyzed in the in vitro incubation medium to give potentially high affinity β-carboline-3-carboxamides was discounted by appropriate control experiments. Thus, solutions of **13** in Me_2SO, Me_2SO + Tris·HCl, or Me_2SO + Tris·HCl + high concentrations of membrane preparations showed absolutely no decomposition even after 3 h at room temperature (binding experiments being in fact conducted for 1 h at 0 °C) as shown by thin-layer chromatography on reverse-phase silica gel plates (Merck RP-8) using ethanol–water, 6:4, as developer.
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(45) Compound **12** possesses a chiral center at C-3 (β-carboline numbering), giving rise to two enantiomers, 3*R* and 3*S*. Since it has been shown in analogous anthramycin-type derivatives that the 3*S* isomer is the more active one with respect to the benzodiazepine receptor,[43] this configuration of **12** was chosen for comparison of molecular models.
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Table II. In Vivo Displacement of [^{3}H]Flunitrazepam by Hybrid 13 in Various Brain Regions of Mice

brain region	total binding[a] of [^{3}H]Flu[b] (n = 25)	binding of [^{3}H]Flu after a saturating dose of diazepam[c] (n = 21)	total binding of [^{3}H]Flu after 13[d] (n = 4)
cerebellum	71.2 ± 4.8	24.1 ± 2.0	78.8 ± 6.1
cortex	85.0 ± 5.0	26.7 ± 2.3	88.0 ± 5.6
hippocampus	95.5 ± 5.9	25.6 ± 1.6	96.8 ± 12.3

[a] In pCi/mg of wet tissue. [b] 200 μCi/kg administered 20 min before decapitation. [c] 10 mg/kg administered 40 min before [^{3}H]Flu and 60 min before decapitation. [d] 10 mg/kg administered 10 min after [^{3}H]Flu and 10 min before decapitation.[50,51]

The difference in the binding properties of **13** and **19** suggests, furthermore, that the receptor also recognizes the hybrids as benzodiazepines. On the basis of structure–activity studies on a large number of benzodiazepines, Fryer[48] has invoked the necessity, among other factors, of π-electrons (carbonyl or phenyl group) in the vicinity of C-5 (π_2-region, Figure 1a) to assure effective binding of these molecules to the receptor. Compound **19**, in which the C-5 carbonyl of **13** has been replaced by a methylene group, has thus lost this π_2-region and is correspondingly inactive. The presence of a π_2-region is, of course, a necessary but not sufficient condition for binding, as shown, again, by the poor affinities of compounds **20** and **21**.

In vivo results with the high-affinity derivative **13** have been disappointing. The substance injected in mice (20 mg/kg suspended in Tween 80, sc) produced no detectable effects by itself; the animals were not sedated or stimulated, motor coordination was unchanged, and there was no evidence of myoclonic activity. Compound **13** at doses up to 20 mg/kg did not antagonize convulsions provoked by pentylenetetrazole (PTZ) (80 mg/kg) or by β-CCM (10 mg/kg) in mice.[10] Moreover, **13** did not reverse the anti-PTZ actions of flunitrazepam (0.075 mg/kg) nor did it block the ataxic effect of diazepam (2 mg/kg) as measured by the rotarod test.

This lack of activity of **13** prompted us to investigate its benzodiazepine displacing potency in vivo.[49] Thus, mice were injected with [^{3}H]flunitrazepam followed 10 min later by injection of hybrid **13** (10 mg/kg). After 10 min, the animals were sacrificed, the brain areas known to contain high proportions of benzodiazepine receptors (cerebellum, cortex, hippocampus) were homogenized and filtered, and the [^{3}H]flunitrazepam bound was determined by scintillation counting. As shown in Table II, no significant displacement by **13** could be observed in any of these brain structures, suggesting that either the hybrid does not cross the blood–brain barrier and thus has no access to the benzodiazepine receptors or that it undergoes metabolic degradation before reaching the brain. Thus, though the mere binding of a molecule to a receptor is by no means a guarantee per se of pharmacological efficacy, the absence of demonstrable in vivo activity for **13** can be attributed, at least for the moment, to unfavorable pharmacokinetic factors.

In conclusion, the hybrid that most effectively inhibits the binding of both flunitrazepam and β-CCE in vitro, compound **13**, is in fact that which most completely incorporates the structural elements of both β-carbolines and benzodiazepines necessary for their respective activities. These elements include (1) a π_2-region with respect to the benzodiazepine moiety, (2) a completely aromatic β-carboline skeleton, and (3) a carbonyl group at C-3 of the β-carboline moiety. To further test our hypothesis that β-carboline and benzodiazepine binding sites on the benzodiazepine receptor may overlap and in the hope of obtaining a pharmacologically useful compound, we are currently synthesizing hybrids in which other structural parameters known to affect the binding of β-carbolines and benzodiazepines to the receptor are modified.

Experimental Section

Chemistry. Melting points were determined on a Büchi apparatus and are uncorrected. IR spectra of samples were obtained in Nujol or neat with a Perkin-Elmer 297 instrument. UV spectra were run on a Perkin-Elmer Lambda 5 spectrophotometer. Proton NMR spectra were determined on Varian T-60 or Bruker 200-MHz instruments. Chemical shifts are given as δ values with reference to Me_4Si as internal standard. Thin-layer chromatography was performed on Merck silica gel 60 plates with fluorescent indicator generally with use of 9:1 toluene–ethanol as developer. The plates were visualized with UV light (254 and 366 nm). Merck silica gel 60 (230–400 mesh) was used for all column chromatography. Mass spectral measurements were done on an AEI MS-9 or an AEI MS-50 spectrometer. Elemental analyses were performed at the ICSN, CNRS, Gif-sur-Yvette, France.

(3*RS*)-Ethyl 2-(*o*-Nitrobenzoyl)-1,3,4,9-tetrahydro-β-carboline-3-carboxylate (10). A mixture of ethyl 1,2,3,4-tetrahydro-β-carboline-3-carboxylate (**8**) (5.0 g, 20.5 mmol), *o*-nitrobenzoyl chloride (**9**) (3.8 g, 20.5 mmol), and anhydrous sodium hydrogen carbonate (8.6 g) in dry dichloromethane (250 mL) was stirred for 30 min at 0 °C. The reaction mixture was then filtered, the precipitate was washed with dichloromethane, and the combined filtrate and washings were extracted twice with brine (250 mL). The organic phase was dried over sodium sulfate, the solvent was removed under vacuum, and the resulting yellow foam was purified by column chromatography on silica gel with 9:1 toluene–ethanol as developer, yielding **10** as a yellow solid (8.0 g, 90%). An analytical sample was crystallized from ethanol: mp 130–132 °C; EIMS, m/z 393 (M^+), 320 ($M^+ - CO_2CH_2CH_3$), 243 ($M^+ - O_2NC_6H_4C{=}O$). Anal. ($C_{21}H_{19}N_3O_5$) C, H, N.

(3*RS*)-Ethyl 2-(*o*-Aminobenzoyl)-1,3,4,9-tetrahydro-β-carboline-3-carboxylate (11). A mixture of the nitro derivative **10** (2.84 g, 7.2 mmol) and 10% palladium on activated carbon (200 mg) in 1:1 dichloromethane–ethanol (200 mL) was hydrogenated for 4 h at 40 psi. The reaction mixture was filtered on Celite, and the catalyst was washed copiously with ethanol. The combined filtrate and washings were then concentrated under vacuum, and the residue was purified by column chromatography on silica gel with 9:1 toluene–ethanol as developer, providing the amino compound **11** as a colorless oil (2.48 g, 95%): IR (neat) 1640 (N—C=O), 1725 (O—C=O), 3370 cm^{-1} (NH_2); EIMS, m/z 363 (M^+), 243 ($M^+ - H_2NC_6H_4C{=}O$). Anal. ($C_{21}H_{21}N_3O_3$) C, H, N.

(14a*RS*)-8,14-Dioxo-13,14,14a,15-tetrahydro-5*H*,6*H*,8*H*-indolo[3′,2′:4,5]pyrido[2,1-*c*][1,4]benzodiazepine (12). A solution of the amine derivative **11** (600 mg, 1.65 mmol) in 1,4-dioxane (30 mL) containing a catalytic amount of *p*-toluenesulfonic acid (30 mg) was refluxed under a nitrogen atmosphere for 5 h. The white precipitate that formed was collected by filtration and washed with dioxane, yielding compound **12** as a fine powder (420 mg, 80%): mp 335 °C dec; EIMS, m/z 317 (M^+); NMR (Me_2SO-d_6) δ 3.05 (1 H, dd, $J_{14a,15a}$ = 7 Hz, $J_{15a,15b}$ = 16 Hz, H-15a), 3.49 (1 H, d (partially hidden by H_2O), $J_{15a,15b}$ = 16 Hz, H-15b), 4.65 (1 H, d, $J_{6a,6b}$ = 17 Hz, H-6a), 4.78 (1 H, d, $J_{14a,15a}$ = 7 Hz, H-14a), 5.07 (1 H, d, H-6b), 7.12–7.75 (7 H, m, Ar), 7.98 (1 H, d, Ar), 10.68 (1 H, s, NH indole, D_2O exchangeable), 11.16 (1 H, s,

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NH amide, slow exchange with D_2O). Anal. ($C_{19}H_{15}N_3O_2 \cdot 0.25H_2O$) C, H, N.

8,14-Dioxo-13,14-dihydro-8*H*-indolo [3′,2′:4,5]pyrido[2,1-*c*][1,4]benzodiazepine (13). A suspension of compound **12** (308 mg, 1 mmol) in dry 1,4-dioxane (50 mL) was treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (450 mg, 2 mmol) at reflux under a nitrogen atmosphere for 40 min. The reaction mixture was then cooled to room temperature, and after 1 h, the precipitated quinol was removed by filtration, the filtrate was concentrated, and the residue was chromatographed on silica gel with 9:2 toluene–ethanol as developer, yielding **13** as a white powder (140 mg, 45%). An analytical sample was crystallized from dioxane–ethanol: mp 340–342 °C dec; IR (Nujol) 1550, 1680, 3315 cm^{-1}; UV (dioxane) 291 nm (log ϵ = 5.4); EIMS, m/z 313 (M^+), 285 ($M^+ - CO$); NMR (Me_2SO-d_6) δ 7.49 (1 H, t, J = 7.0 Hz, H-2), 7.82 (3 H, m, Ar), 7.96 (1 H, d, J = 8.0 Hz, Ar), 8.16 (1 H, t, J = 7.0 Hz, Ar), 8.36 (1 H, d, J = 8.0 Hz, Ar), 8.62 (1 H, d, J = 8.0 Hz, Ar), 9.22 (1 H, s, H-15), 9.36 (1 H, s, H-6), 12.29 (1 H, s, NH, D_2O exchangeable). Anal. ($C_{19}H_{11}N_3O_2 \cdot 0.5H_2O$) C, H, N.

(3*RS*)-Ethyl 2-(*o*-Nitrobenzyl)-1,3,4,9-tetrahydro-β-carboline-3-carboxylate (15). A mixture of β-carboline **8** (5.0 g, 20.5 mmol), *o*-nitrobenzyl bromide (**14**) (5.0 g, 23 mmol), potassium carbonate (1.66 g, 12 mmol), powdered sodium hydroxide (0.9 g, 22 mmol), and tetra-*n*-butylammonium hydrogen sulfate (0.68 g, 2.2 mmol) in dry dichloromethane (150 mL) was stirred for 22 h at room temperature. The reaction mixture was then filtered, the filtrate was washed with brine (2 × 100 mL), and the organic phase was dried over sodium sulfate. After evaporation of the solvents, the residue was purified by chromatography on silica gel with 9:1 toluene–ethanol as developer. Compound **15** was obtained as an oil, which crystallized upon trituration with dichloromethane (3.2 g, 44%) and which could be recrystallized from dichloromethane–hexane: mp 117–119 °C; IR (neat) 1530, 1705, 1725, 3420 cm^{-1}; EIMS, m/z 379 (M^+), 306 ($M^+ - CO_2C_2H_5$), 243 ($M^+ - CH_2C_6H_4NO_2$). Anal. ($C_{21}H_{21}N_3O_4$) C, H, N.

(3*RS*)-Ethyl 2-(*o*-Aminobenzyl)-1,3,4,9-tetrahydro-β-carboline-3-carboxylate (16). A mixture of the nitro compound **15** (3.0 g, 7.9 mmol) and freshly activated Raney nickel (3 g) in ethanol (150 mL) was hydrogenated at atmospheric pressure for 4 h. The reaction mixture was then filtered, the catalyst was washed copiously with ethanol, and the combined filtrate and washings were evaporated under vacuum, yielding compound **16** as an oil, which was used without further purification in subsequent steps (2.5 g, 90%). A sample was crystallized from chloroform–hexane for analysis: mp 148–150 °C; IR (Nujol) 1735, 3380, 3420 cm^{-1}; EIMS, m/z 349 (M^+), 276 ($M^+ - CO_2C_2H_5$), 243 ($M^+ - H_2NC_6H_4CH_2$). Anal. ($C_{21}H_{23}N_3O_2 \cdot {}^1/_3H_2O$) C, H, N.

(3*RS*)-2-(*o*-Aminobenzyl)-1,3,4,9-tetrahydro-β-carboline-3-carboxylic Acid (17). A solution of the ester **16** (2.35 g, 6.7 mmol) in 90% ethanol (100 mL) containing 1 N sodium hydroxide (7 mL) was refluxed for 2 h. The reaction mixture was cooled and was concentrated to half-volume under vacuum. Water (30 mL) was added, and the solution was carefully neutralized with glacial acetic acid, whereupon a heavy precipitate formed. The solid **17** was collected by filtration, washed with water, and dried (1.3 g, 60%): mp 266–267 °C (CO_2 evolved); EIMS, m/z 321 (M^+), 215 ($M^+ - H_2NC_6H_4CH_2$), 171 ($M^+ - H_2NC_6H_4CH_2 - CO_2$). Anal. ($C_{19}H_{19}N_3O_2 \cdot 0.5H_2O$) C, H, N.

(14a*RS*)-6,8,13,14,14a,15-Hexahydro-14-oxo-5*H*-indolo-[3′,2′:4,5]pyrido[2,1-*c*][1,4]benzodiazepine (18). A solution of the carboxylic acid **17** (500 mg, 1.56 mmol), dicyclohexylcarbodiimide (320 mg, 1.56 mmol), and 1-hydroxybenzotriazole (210 mg, 1.56 mmol) in dry dimethylformamide (50 mL) was stirred at room temperature for 2 h. The solvent was then removed under vacuum, and the residue was taken up in ethyl acetate. The insoluble urea was removed by filtration, and the filtrate was washed successively with saturated aqueous sodium hydrogen carbonate, water, and brine. The organic phase was dried over sodium sulfate, the solvent was evaporated, and the residue was chromatographed on silica gel with 9:1 toluene–ethanol as developer, yielding **18** as a solid (200 mg, 42%). The material was crystallized from dichloromethane–hexane: mp 183–185 °C; IR (Nujol) 1680, 3400 cm^{-1}; EIMS, m/z 303 (M^+); NMR (Me_2SO-d_6) δ 2.69 (1 H, dd, $J_{14a,15a}$ = 3.0 Hz, $J_{15a,15b}$ = 15.0 Hz, H-15a), 3.02 (1 H, dd, $J_{14a,15a}$ = 3.0 Hz, $J_{14a,15b}$ = 10 Hz, collapses to a doublet with J = 10 Hz when H-15a is irradiated, H-14a), 3.08–3.28 (1 H, m, collapses to a doublet with J = 10 Hz when H-15a is irradiated, H-15b), 3.78 (1 H, d, $J_{6a,6b}$ = 14 Hz, H-6a), 3.79 (1 H, d, $J_{8a,8b}$ = 15 Hz, H-8a), 3.92 (1 H, d, $J_{6a,6b}$ = 15 Hz, collapses to a singlet when H-6b is irradiated, H-6a), 3.99 (1 H, d, H-8b), 4.25 (1 H, d, H-6b), 6.98–7.57 (8 H, m, Ar), 10.24 (1 H, s, NH, D_2O exchangeable), 10.87 (1 H, s, NH, D_2O exchangeable); HRMS, m/z calcd for $C_{19}H_{17}N_3O$ 303.1371, found 303.1335. Anal. Calcd for $C_{19}H_{17}N_3O \cdot 1/4H_2O$: C, 74.14; H, 5.69; N, 13.65. Found: C, 74.61; H, 5.83; N, 12.92.

13,14-Dihydro-14-oxo-8*H*-indolo[3′,2′:4,5]pyrido[2,1-*c*]-[1,4]benzodiazepine (19). A mixture of compound **18** (50 mg, 0.16 mmol) and sulfur (10 mg, 0.32 mmol) in dry dimethyl sulfoxide (5 mL) was kept at 100 °C for 48 h. The reaction mixture was cooled and filtered, and the filtrate was evaporated to dryness under vacuum, leaving **19** as a yellow solid (45 mg, 94%). The highly insoluble material was purified by dissolving in dimethyl sulfoxide and precipitating with water (15 mg, 31%): mp 336–338 °C dec; EIMS, m/z 299 (M^+), 166 ($M^+ - CH_2C_6H_4NHCO$); NMR (Me_2SO-d_6) δ 6.25 (2 H, s, H-8), 7.45 (2 H, br s, Ar), 7.62 (2 H, br s, Ar), 7.98 (4 H, m, Ar), 8.81 (1 H, s, H-15), 9.48 (1 H, s, H-6), 11.78 (1 H, br s, NH, D_2O exchangeable); HRMS, m/z calcd for $C_{19}H_{13}N_3O$ 299.1058, found 299.1020. Anal. Calcd for $C_{19}H_{13}H_3O \cdot 0.4S$: C, 74.27; H, 4.23; N, 13.68. Found: C, 73.79; H, 4.33; N, 13.24.

Biological Methods. In Vitro Benzodiazepine Receptor Binding Assays. Male Sprague–Dawley rats were decapitated, the brains were excised, and the cortex was dissected. Each cortex was homogenized in 5 mL of ice-cold Tris·HCl (50 mM, pH 7.4) with a Polytron. The homogenate was centrifuged a first time at 460*g* for 3 min, and the supernatant was recentrifuged at 22400*g* for 20 min. The resulting supernatant was discarded, and each pellet was resuspended in 3 mL of buffer and centrifuged again at 22400*g* for 20 min. The resulting pellet was again suspended in 3 mL of buffer, rehomogenized, divided into 1-mL fractions, and stored at −20 °C for at least 24 h before use. For the inhibition studies, the thawed membrane preparations were diluted with 20 volumes of ice-cold buffer and 900-μL aliquots containing 60 μg of protein were incubated at 0 °C for 60 min with [^{3}H]flunitrazepam (76.9 Ci/mmol, NEN, final concentration of 1 nM) or with [^{3}H]-β-CCE (80 Ci/mmol, NEN, final concentration of 0.4 nM) and varying concentrations of the test compound ranging from 10^{-5} to 5×10^{-10} M (final concentrations for a total volume of 1 mL). Nonspecific binding was measured in the presence of 1 μM nonradioactive flunitrazepam (or 1 μM nonradioactive β-CCE in the case of [^{3}H]-β-CCE) and represented 10–15% of the total binding. Incubations were terminated by adding 3 mL of cold buffer to each incubation tube, filtering through Whatman GF/B glass fiber filters, and washing each filter four times with 3 mL of ice-cold buffer. Radioactivity retained on the filters was counted in 10 mL of Aquasol scintillation solution with an LKB Wallac 1215 Rackbeta 2 counter. Each value was determined in duplicate. IC_{50} values (the concentration of ligand inhibiting 50% of flunitrazepam (or β-CCE) binding) were determined by Hofstee analysis. Results are given in Table I.

In Vivo Studies. Drugs. Flunitrazepam (Hoffmann-La Roche, Basel, Switzerland) and compound **13** were used as a suspension in Tween 80 and saline. Diazepam (Hoffmann-La Roche) was used as the commercially available preparation. Methyl β-carboline-3-carboxylate (β-CCM), synthesized by one of us (R.H.D.), was dissolved in 0.1 N HCl (1 mg in 100 μL) and diluted to volume with saline. Pentylenetetrazole (PTZ) (M. Richard Laboratories, Sauzet, France) was dissolved in saline. Unless otherwise stated, all drugs were administered subcutaneously (sc) at a dose volume of 0.05 mL/10 g of body weight.

Convulsion Studies with Compound 13. To study the anticonvulsant activity of **13**, male Swiss mice (25 g) were treated with **13** (up to 20 mg/kg) 10 min before treatment with a convulsant dose of either PTZ (80 mg/kg) or β-CCM (10 mg/kg). Latency times to convulsions were noted and compared to those of PTZ and β-CCM alone. Antagonism of the anti-PTZ activity of flunitrazepam was studied by first administering **13** (up to 20 mg/kg) 10 min after injection of an anticonvulsant dose of flunitrazepam (0.075 mg/kg) but 10 min before treatment with PTZ (80 mg/kg).

Rotarod Studies with Compound 13. The rotarod deficit was evaluated on a 2.5-cm-diameter wooden rod rotating at 4 rpm.

Mice were placed backwards on the rotating rod in such a way that they had to turn around to face the movement. Mice were pretrained for a 5-min period 30 min before the beginning of the experiment. The ability of each mouse to stay on the rod for 1 min was then pretested just before the first drug treatment. The occasional mouse not succeeding this pretest was eliminated. Mice were then injected with diazepam (2 mg/kg) followed 10 min later by compound 13 (up to 20 mg/kg) or saline and tested every 10 min during the next hour. Each experiment was repeated on 10 mice.

In Vivo Inhibition of [^{3}H]Flunitrazepam Binding by Compound 13. Tritiated flunitrazepam (200 μCi/kg diluted in saline to a dose volume of 0.1 mL/10 g of weight) was administered through the tail vein of mice (Swiss, male) 20 min before decapitation. The test molecule 13 (10 mg/kg) suspended in Tween 80 was administered 10 min after [^{3}H]flunitrazepam treatment and 10 min before decapitation.[50] Total binding values were obtained from mice treated only with [^{3}H]flunitrazepam. Nonspecific binding values were determined from mice injected with diazepam (10 mg/kg)[51] 60 min before decapitation.

After decapitation, brains were rapidly excised. The cerebellum, cortex, and hippocampus were dissected over ice and weighed and then homogenized with a Polytron in an appropriate volume of ice-cold Tris·HCl (50 mM, pH 7.4): 3 mL for the cortex and cerebellum and 1.5 mL for the hippocampus. Two 600-μL aliquots of these crude homogenates (12 mg of wet tissue) were immediately filtered through Whatman GF/B glass fiber filters and washed twice with 5 mL of ice-cold buffer. Radioactivity retained on the filters was counted in 10 mL of Aquasol scintillation liquid (NEN) by using an LKB Rackbeta 2 counter. Results are given in Table II.

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(50) In initial experiments, compound 13 was, like diazepam, injected 20 min or more before [^{3}H]flunitrazepam. However, no displacement of the radioactive ligand by 13 was observed. In view of the potential metabolic instability of 13, this compound was therefore injected 10 min *after* [^{3}H]flunitrazepam. Control experiments have shown that, when this protocol is used, standard β-carbolines (e.g., β-CCE) effectively displace tritiated benzodiazepines in vivo.

(51) The dose of diazepam that half-maximally inhibits the in vivo binding of [^{3}H]flunitrazepam in mice (ID_{50} value) is ca. 0.5 mg/kg.[49b] A much higher dose of diazepam was used here (10 mg/kg) merely to determine nonspecific binding, that is, the maximum [^{3}H]flunitrazepam binding inhibition that can be expected by the test compound. The dose of compound 13 chosen for the in vivo binding experiments (10 mg/kg) thus represents 20 times the ID_{50} of diazepam, and, despite a fivefold-lower affinity than diazepam, 13 would be expected to displace at least a fraction of the bound [^{3}H]flunitrazepam if it reached the brain.

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N^{10}-Substituted 5,8-Dideazafolate Inhibitors of Glycinamide Ribonucleotide Transformylase

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A series of 5,8-dideazafolates bearing ethyl, isopropyl, cyclopropylmethyl, propargyl, 3-cyanopropyl, carboxymethyl, 2-carboxyethyl, phenacyl, 3-fluorobenzyl, and 5-uracilylmethyl substituents at N^{10} were tested as inhibitors of purified L5178Y glycinamide ribonucleotide transformylase (GAR TFase), which requires 10-formyltetrahydrofolate as cofactor. All of these cofactor analogues exhibited competitive inhibition against N^{10}-formyl-5,8-dideazafolate, with K_i's ranging from 2 to 32 μM.

We have recently demonstrated that both 5,8-dideazafolate (1) and the N^{10}-acetyl (7) analogue could inhibit the GAR TFase catalyzed formylation of glycinamide ribonucleotide (GAR) by N^{10}-formyl-5,8-dideazafolate in a competitive manner.[1] This reaction is the first of two folate-dependent formyl group transfers in the de novo purine biosynthetic pathway. Previous studies had indicated that the N^{10}-formyl analogue was utilized very efficiently as a substrate by GAR TFase isolated from both avian[2] and mammalian sources[3,4] and that this analogue was very effective as an affinity ligand for enzyme purification.[3,5]

Recently, a series of N^{10}-substituted 5,8-dideazafolates that displayed preferential inhibition of thymidylate synthase (TS) vs. dihydrofolate reductase (DHFR) were reported.[6] Several of these analogues were also quite cytotoxic toward L1210 cells in culture. However, none of the new analogues were more effective than the N^{10}-propargyl compound (5), which is the most potent antifolate inhibitor of TS (K_i = 4.5 nM) described to date[7] and which has an ID_{50} of 5 μM for L1210 cells in culture.[6] Since other 5,8-dideazafolates have proven to be extremely useful tools for studies on GAR TFase,[3-5] we wished to examine the interaction of these new 5,8-dideazafolate analogues with L5178Y GAR TFase in order to gain insight into the

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