Aryl Diazo Compounds and Diazonium Salts as Potential Irreversible Probes of the γ-Aminobutyric Acid Receptor

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The synthesis of different diazonium salts derived from homo- and heterocyclic aromatic amines bearing anionic residues is described. The chemical stabilities of these compounds were established at different pH's, and the compounds were tested accordingly in binding experiments for the rat brain γ-aminobutyric acid (GABA) receptor, for which they could ultimately be used as irreversible affinity or photoaffinity probes. The aromatic heterocyclic series studied were 2-aminoimidazole, 2-aminothiazole, and 4-aminopyridine *N*-oxide. The derived diazonium salts are unstable compounds at neutral pH unless they are able to be deprotonated to the corresponding diazo form. As such, the 2-diazoimidazole-4(5)-acetic acid (**3b**) is stable in neutral medium and recognizes the GABA receptor (IC_{50} = 70 μM). The homocyclic aromatic diazonium salts showed sufficient stability to be tested in binding experiments. The diazonium salts derived from *m*-sulfanilic acid and 8-sulfonaphthylamine were the most interesting (**10b**, IC_{50} = 10 μM; **15b**, IC_{50} < 100 μM). In this series, the compounds that deprotonate at neutral pH (hydroxybenzenediazonium derivatives **12b–14b**) showed increased chemical stability but decreased affinity for the GABA receptor. This difference between the diazoimidazole and the diazohydroxybenzene series is attributed to a different charge distribution between the two series. The ligands **3b**, **10b**, and **15b** can be used as potential irreversible probes for the GABA receptor.

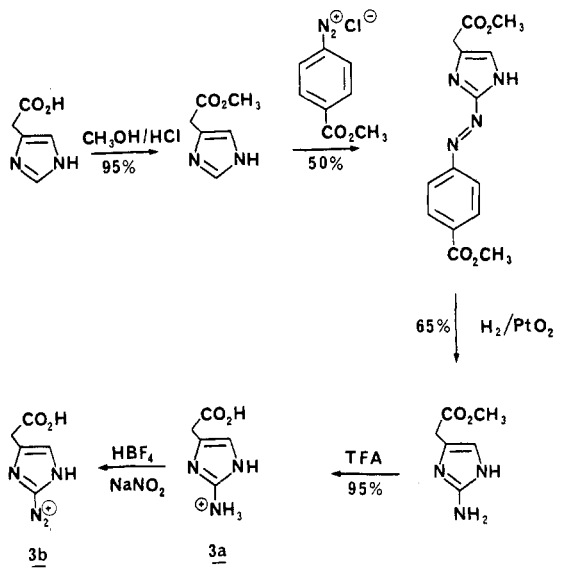
Irreversible probes (affinity and photoaffinity labels) have become useful tools for the identification of biological receptor molecules.[1] They can be used to characterize and ultimately determine the primary structure of a target receptor subunit. However, very low concentrations of neurotransmitter receptors in the central and peripheral nervous system generally make their identification difficult. As a consequence, a specific and efficient irreversible labeling is required for the characterization of these receptors.

The identification of the GABA/benzodiazepine receptor complex has progressed with the use of the photoaffinity probes flunitrazepam[2] and muscimol.[3] These reagents led to the photolabeling of the two polypeptide chains of the receptor: respectively the α (53 kD) and the β (57 kD) subunits.[4] Actually, neither of these reagents are typically photoaffinity probes in the sense that the photogenerated species are not short-lived intermediates.[5] Alternatively, a phenylazido derivative of GABA has been described as a potential photoaffinity probe of the GABA uptake system.[6]

The work presented here is related to the design of new potential irreversible probes, e.g. aromatic diazo compounds and diazonium salts, for the GABA receptor. A general property of these reagents is that they undergo an efficient photochemical activation to yield aromatic carbenes or cations, which are both highly reactive species.[7] As an example, such species are among the few that are able to react with molecular nitrogen.[8] In the absence of light, the chemical stability of aromatic diazonium salts is generally increased by electron-donating substituents on the aromatic ring, but nevertheless they are still able to react with strong nucleophiles. In that sense, they can also be used as potential affinity labels.

Different arenediazonium salts have been described as being efficient photoaffinity labels of the cholinergic enzymes and receptor.[9] For these compounds, the chemical analogy with acetylcholine relies solely on the existence of a positively charged moiety. As a consequence, it seemed reasonable to expect an arenediazonium salt to be a photosensitive substitute of a protonated primary amine such as found in the GABA molecule. Starting with this hypothesis, we synthesized several diazo compounds and

Scheme I. Syntheses of 2-Aminoimidazole-4(5)-acetic Acid (3a) and the Corresponding Diazonium Salt **3b**



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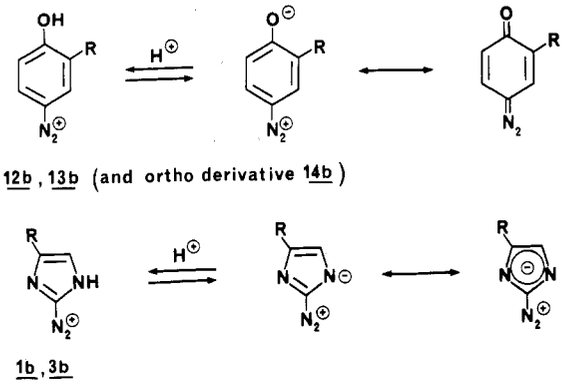
diazonium salts bearing an anionic function derived from substituted imidazole, thiazole, pyridine, and benzene rings and established their use as potential photosensitive GABA analogues by determining their affinities for the rat brain GABA receptor.

Chemistry. The compounds that were tested are listed in Table I. Most of the aromatic amine precursors were either commercially available (**1a**, **9a–12a**, **14a**, and **15a**) or known compounds (**4a**,[10] **5a**,[11] **6a**,[12] **7a**,[13] and **13a**[14]). The synthesis of 2-amino-*N*-imidazoleacetic acid was performed from **1a** by adapting a described procedure.[15] The synthesis of 2-amino-4(5)-imidazoleacetic acid (**3a**) is shown in Scheme I. A similar approach was described for the synthesis of 2-aminohistidine (**4a**),[10] but it required extensive adaptation. The amine **8a** was synthesized by *m*-chloroperbenzoic acid oxidation[16] of 3-[[*N*-[(*tert*-butyloxy)carbonyl]amino]methyl]pyridine and subsequent removal of the protective group in acidic medium.

Diazotization of the aromatic amines with sodium nitrite in acidic medium yielded the corresponding diazonium salts. For the benzene diazonium derivatives, the use of 34% aqueous tetrafluoroboric acid led in most cases to crystalline compounds. As a general rule, the diazotization reactions with the homocyclic aromatic amines afforded more stable compounds than with the heterocyclic aromatic amines. The synthesis of the diazonium salts in the 2-aminothiazole series must be carried out in the absence of halide ions, which are too nucleophilic. Fluoboric, phosphoric, or nitric acid may be used.[17] Even under these conditions, the diazonium salt **6b** decomposed as soon as it was formed. Among the different heterocyclic diazonium salts prepared, only **1b** could be obtained in crystalline form, all the others being identified by UV spectroscopy where they present intense absorption bands as shown in Table I. In addition, this table summarizes the chemical stability of the diazonium salts expressed as their half-lives in different media. As expected, with the exception of **6b**, the salts were fairly stable in acidic medium, especially in aqueous HBF_4. In neutral medium, **2b**, **5b**, and **7b** showed rapid transformation into unidentified compounds, while the half-lives of the diazonium salts **9b–11b**, **15b**, and **16b** were substantially diminished. Compounds **1b**, **3b**, and **12b–14b** showed an increased stability in neutral medium, here they change to diazo derivatives, according to their pK_a values.[18] The diazonium–diazo equilibrium is clearly evidenced by UV spectroscopy for the hydroxybenzenediazonium derivatives (Table I) for which we observed a substantial bathochromic shift going from the diazonium

Scheme II. Diazonium–Diazo Equilibrium Derived from *o*- and *p*-Aminophenols and 2-Aminoimidazoles



the 2-diazoimidazole derivatives **1b** and **3b**. According to ^{13}C NMR measurements, a dipolar ionic structure, as shown in Scheme II, was proposed for 2-diazoimidazole derivatives.[19] Such a formulation might account for the very small shifts of the λ_{max} observed with **1b** and **3b** going to the diazo structures. This shift was not observed for from the diazonium to the diazo structures. In fact, these diazo derivatives behave as typical diazonium compounds with regard to the azo coupling reactions.[20]

As a general property, all these diazonium salts or diazo derivatives are very photosensitive.[7] During our photochemical experiments, the photodecomposition was easily monitored by UV spectroscopy and by following the disappearance of the intense absorption band at the longer wavelength (results not shown).

Binding Assays. The assays were done on plasma membranes obtained from rat brain homogenates (see the Experimental Section). [^{3}H]GABA was used to determine the specific binding of the ligands to the GABA receptor. Table I summarizes the binding results as well as the chemical stabilities of the ligands in acidic and neutral media. The binding results are expressed as their IC_{50}'s or as a percentage of displacement for a given ligand concentration (ligands with weak affinity). In the heterocyclic series, among the diazotized species, only the diazoimidazole derivatives (**1b** and **3b**) showed sufficient stability in the neutral medium to be tested. The aromatic amines **9a–16a** were not tested, assuming that they are barely protonated species according to their pK_a values.

Discussion

GABA plays an important role as an inhibitory neurotransmitter. It has therefore been studied thoroughly with regard to its recognition by the GABA receptor, a postsynaptic membrane protein complex through which the inhibitory effect is mediated. A large number of chemical analogues of GABA or related drugs have been synthesized.[21] From the resulting structure–activity studies, it appeared that the charges of the zwitterionic molecule were essential for the recognition process with a postulated ideal distance of 4.8 Å between them.[22] We hypothesized that aromatic diazo compounds and diazonium salts suitably substituted with acidic functions could be considered as new GABA analogues. In fact, *p*-benzenesulfonate diazonium zwitterion **9b** has been used to label nucleophilic residues (i.e. tyrosine) of the low-affinity GABA binding site.[23] Several examples of affinity labeling of nucleophilic

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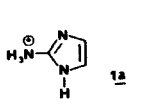
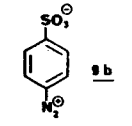
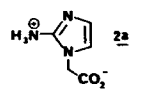
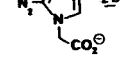
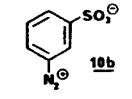
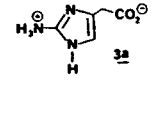
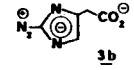
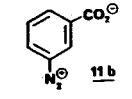
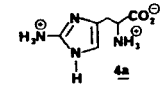
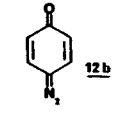
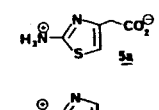
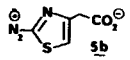
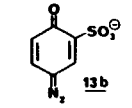
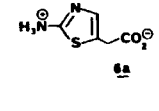
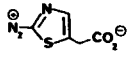
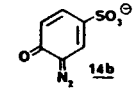
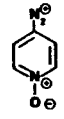
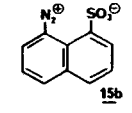
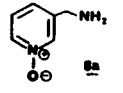
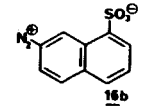
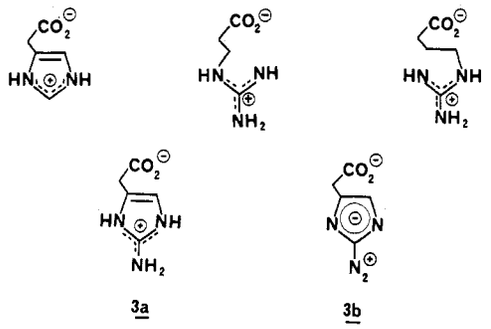
Table I. Displacement of [^{3}H]GABA: Percent or IC_{50} Value, UV λ_{max}, nm (ϵ), and Half-Lives at 20 °C of the Amines and Corresponding Diazo or Diazonium Compounds

Hétérocyclic aromatic amines		Corresponding diazotized species			Homocyclic aromatic amines	Corresponding diazotized species		
Compound (pH = 7.15)	Displacement of (^{3}H) GABA : % or IC_{50} value	Compound (pH = 7.15)	Displacement of (^{3}H) GABA : % or IC_{50} value	UV λmax_{nm} (ϵ) ; half-life time at 20°C	Compound	Compound (pH = 7.15)	Displacement of (^{3}H) GABA : % or IC_{50} value	UV λmax_{nm} (ϵ) ; half-life time at 20°C
 1a	0 % at 5.10^{-4}M	 1b	IC_{50} = 5.10^{-4}M	A) 313 (22000) ; 8h B) 306 (24000) ; 2 days C) 314 (24000) ; stable	9a	 9b	≤50 % at 5.10^{-4}M	B) 266 (16000) ; stable C) 268 (16000) ; 6h
 2a	20 % at 5.10^{-4}M	 2b	unstable	A) 313 (12000) ; 22 min [b] B) 310 (16600) ; 12h C) 299 (8000) ; 3h [c]	10a	 10b	IC_{50} = 10^{-5}M	A) 260 (11500) ; - B) 260 (11500) ; 2 days C) 260 (11500) ; 5h
 3a	30 % at 10^{-5}M	 3b	IC_{50} = 7.10^{-5}M	A) 319 (22000) ; 11h [b] B) 316 (22000) ; 2.5 days C) 323 (23000) ; stable	11a	 11b	30 % at 5.10^{-4}M	B) 258 (9800) ; stable C) 258 (8500) ; 80 min
 4a	≤ 50 % at 5.10^{-4}M	—	—	—	12a	 12b	15 % at 5.10^{-4}M	B) 313 (23000) ; stable C) 348 (44000) ; stable
 5a	25 % at 10^{-5}M [a]	 5b	unstable	A) 328 (9000) ; 25 min [b] B) 328 (12000) ; 2 days C) 321 (6500) ; 43h [c]	13a	 13b	15 % at 5.10^{-4}M	B) 306 (24000) ; stable C) 346 (44000) ; stable
 6a	≤ 50 % at 5.10^{-4}M	 6b	unstable	B) 328 unstable [b]	14a	 14b	15 % at 5.10^{-4}M	B) 260 (9600), 350 (5000) ; stable C) 390 (5900) ; stable
7a	—	 7b	unstable	B) 345 (23000) ; stable C) 304 (7800) ; unstable [c]	15a	 15b	IC_{50} = 10^{-4}M	B) 254 (15000), 341 (7800) ; 22h C) 255 (14000), 339 (7500) ; 16h
 8a	20 % at 5.10^{-4}M	—	—	—	16a	 16b	IC_{50} ≤ 10^{-4}M	A) 257 (14000), 350 (7900) ; stable C) 256 (16000), 349 (9000) ; 45 min

[a] See ref 26. [b] The diazonium salts were not isolated; the value of the extinction coefficient is an estimation, assuming that the conversion of the amine to the diazonium salt was quantitative. [c] Corresponds to a rapid transformation of the diazonium salt to unidentified compounds. (A) 1 N HCl, (B) 34% HBF_4, (C) buffer, pH 7.15.

Chart I. Chemical Structures of Imidazoleacetic Acid, 2-Aminoimidazole-4(5)-acetic Acid (**3a**), 2-Diazoimidazole-4(5)-acetic Acid (**3b**), Guanidinopropionic Acid, and Guanidinobutyric Acid



3a **3b**

residues of receptors (i.e. cysteinyl residues) with arenediazonium salts are known.[24] The use of aromatic diazonium salts as affinity reagents can be modulated by their electrophilic properties (chemical reactivity) with regard to the nucleophilic power of the target residue. In addition, the photochemical properties of these chemicals, which give rise to highly reactive species, suggest their use as potential photoaffinity probes for the GABA receptor.

Among the several diazonium salts we have synthesized, the *m*-benzenesulfonate diazonium **10b** was the most interesting compound in terms of affinity for the GABA receptor (IC_{50} = 10 μM). Comparing the four rigid sulfonate diazonium salts **9b**, **10b**, **15b**, and **16b**, there is some relationship between the distances of their two charges (5.57 Å for **9b**, 4.82 Å for **10b**, 2.42 Å for **15b**, and 5.21 Å for **16b**) and their affinities for the GABA receptor. The most potent compound **10b** has the ideal intercharge distance, although compound **15b**, which has a very small intercharge distance, shows a weak affinity for the receptor of about as much as **10b**. The weaker affinity of compound **11b** compared to **10b** confirms earlier observations demonstrating that sulfonic acid derivatives are generally better ligands for the GABA receptor than the corresponding carboxylic acids.[26]

We synthesized the two hydroxybenzenediazonium derivatives **13b** and **14b** for two reasons: (i) these compounds show a strong absorption above 300 nm, and (ii) they become very stable diazo structures in buffered neutral medium (Scheme II), both these properties being advantageous for photoaffinity reactions. These two compounds showed no affinity for the GABA receptor. In fact, according to their UV spectra, these derivatives show a typical quinonic chromophore where the charges might essentially be localized on the two nitrogen atoms. This new charge distribution can explain the loss in affinity.

The interpretation of the results in the heterocyclic series is more complex. Obviously, the use of pyridine *N*-oxide derivatives as analogues of carboxylic acid groups (**7b** and **8a**) does not seem very promising.

The azole series and especially the imidazole derivatives were developed with the expectation that 2-aminoimidazole may constitute a compromise between guanidine and imidazole. As a consequence, 2-aminoimidazole-4-(5)-acetic acid (**3a**) or the corresponding diazo compound **3b** should be an analogue of either imidazole-4(5)-acetic acid (IC_{50} = 0.5 × 10^{-6} M)[27] or guanidinopropionic acid (IC_{50} = 26 × 10^{-6} M)[27] and guanidinobutyric acid (IC_{50} = 10^{-4} M)[21] (Chart I). It was observed that the incorporation of the amino group of GABA in an amidinic or guanidinic system maintains the affinity for the GABA receptor site.[28] In fact, the binding affinities of **3a** and **3b** for the GABA receptor were similar to the affinities of the above-mentioned guanidino carboxylic acids (Table I).

The only stable photosensitive species in the heterocyclic series were the diazo structures **1b** and **3b**. The fact that a weak affinity for the GABA receptor was observed with 2-diazoimidazole **1b**, in which no carboxylic group is present, clearly differentiates these diazo structures from the hydroxybenzene series. In the 2-diazoimidazole series, the negative charge probably remains delocalized on the heterocycle, which leaves the nitrogen moiety in a diazonium form. This dipolar formulation (Scheme II) might account for the weak recognition of **1b** by the GABA receptor.

From the present structure–activity study of aromatic diazo compounds and diazonium salts as ligands of the GABA receptor, we conclude that the charged nitrogen moiety of diazonium salts can be considered as a structural substitute of the protonated primary amine of the GABA molecule. A similar analogy can be postulated for the diazo derivatives only if the nitrogen moiety remains positively charged as in the 2-diazoimidazole series. Three of the photoactivable molecules we have synthesized, 2-diazoimidazole-4(5)-acetic acid (**3b**), *m*-sulfonatobenzenediazonium tetrafluoroborate (**10b**), and 8-sulfonato-1-naphthalenediazonium chloride (**15b**) showed sufficient stability in neutral medium as well as a reasonable affinity for the GABA receptor to be candidates for irreversible binding experiments.

Experimental Section

Melting points were obtained on a calibrated Kofler hot-stage apparatus and are uncorrected. NMR spectra were recorded on Bruker WP60 (60 MHz), Bruker WP SY 200 (200 MHz) with Me_4Si as an internal reference, and Bruker AM 400 (400 MHz) spectrometers with *t*-BuOH as an internal reference; chemical shifts are reported in δ (ppm). Mass spectra were recorded on a LKB 9000S instrument with an EI source (70 eV, 100 °C) and a Thomson THW 208 instrument with a chemical-ionization source (NH_3, 100 °C). IR spectra were measured on a Beckman Acculab-4 spectrophotometer. UV spectra were obtained on a UVIKON 860 instrument. HPLC was conducted on a Waters Automated Gradient Controller instrument with a C_{18} silica gel column.

The compounds were analyzed for C, H, and N by the Service de Microanalyse du CNRS de l'Université Louis Pasteur (Strasbourg), and the results were within 0.4% of the theoretical value.

2-Aminoimidazole-1-acetic Acid (2a). The synthesis of 2-aminoimidazole-1-acetate was performed by addition of ethyl bromoacetate to the sodium salt of 2-aminoimidazole, extending

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a procedure previously described for the N-alkylation of imidazole:[15] NMR ($CDCl_3$) δ 1.3 (3 H, t), 3.8 (NH_2, br), 4.25 (2 H, q), 4.5 (2 H, s), 6.6 (2 H, q).

A 20% solution of concentrated hydrochloric acid in glacial acetic acid (4 mL) containing ethyl 2-aminoimidazole-1-acetate (0.1 g, 0.645 mmol) was refluxed for 2 h. After cooling, the solvent was evaporated under reduced pressure. The solid residue was dissolved in a minimum of hot ethanol and recrystallized after cooling by slow addition of ether: 80 mg (80%); mp 227 °C; NMR (DMSO-d_6) δ 4.7 (2 H, s), 6.92 (2 H, m), 7.8 (2 H, m). Anal. ($C_5H_8N_3O_2Cl$) C, H, N.

Methyl Imidazole-4(5)-acetate, Hydrochloride Salt. A 10% solution of gaseous HCl in MeOH (50 mL) containing imidazoleacetic acid (1 g, 6.15 mmol) was refluxed overnight. The solution was then rendered alkaline with solid Na_2CO_3, filtered, and evaporated to dryness. The residue was dissolved in THF (100 mL), and the solution was filtered. After gaseous HCl was bubbled through the solution, methyl imidazole-4(5)-acetate hydrochloride precipitated and was collected and dried: 940 mg (86%); mp 176 °C; NMR (CD_3OD) δ 3.7 (H, s), 3.9 (2 H, s), 7.5 (1 H, s), 8.9 (1 H, s).

Methyl 2-[[*p*-(Carboxymethyl)phenyl]azo]imidazole-4-(5)-acetate. A solution of $NaNO_2$ (310.5 mg, 4.5 mmol) in H_2O (5.1 mL) was cooled to 0–2 °C and added gradually to a stirred, ice-cold solution of *p*-(carboxymethyl)aniline (844.5 mg, 4.5 mmol) in HCl (2.36 N, 7.5 mL). The cold solution of the diazonium salt was stirred for 30 min and then gradually added to an ice-cold stirred solution of methyl imidazole-4(5)-acetate (795 mg, 4.5 mmol) in a 0.25 M disodium tetraborate buffer (150 mL). At the end of the addition, the pH was maintained between 9 and 9.5 with NaOH (6 N). The mixture was stirred for another 2 h. After filtration, an orange-red precipitate was obtained, which was suspended in H_2O (100 mL) in order to remove the coprecipitated salts and again collected by filtration. This procedure was repeated. The remaining residue was dried under vacuum; the powder was dissolved in acetone, from which it crystallized at −20 °C. After three recrystallizations, the pure compound was finally washed with dry ether, affording 679 mg (50%) of the dark yellow product: mp 190 °C (dec); NMR (CD_3COCD_3) δ 2.88 (3 H, s), 3.7 (2 H, s), 3.94 (3 H, s), 7.4 (1 H, s), 8 (4 H, q); mass spectrum, *m/e* (relative intensity) 302 (21, M), 223 (10). Anal. ($C_{14}H_{14}N_4O_4$) C, H, N.

Methyl 2-Aminoimidazole-4(5)-acetate, Trifluoroacetate Salt. A suspension of methyl 2-[[*p*-(carboxymethyl)phenyl]-azo]imidazole-4(5)-acetate (604 mg, 0.02 mol) in MeOH (50 mL) containing 60 mg of platinum oxide was subjected to catalytic hydrogenation (50–60 psi, Parr apparatus) at room temperature. After 24 h, an additional 60 mg of platinum oxide was added, and the reduction was continued for 24 h. At the end of the reduction, the solution was immediately acidified with 1 equiv of trifluoroacetic acid (TFA). The catalyst was removed by filtration, and the solvent was evaporated under reduced pressure. The residual material was dissolved in water and extracted with three portions of ether to remove the *p*-(carboxymethyl)aniline. The aqueous layer was evaporated under reduced pressure at room temperature to give an oily compound: 315 mg (65%); NMR (CD_3OD) δ 3.65 (2 H, s), 3.75 (3 H, s), 6.67 (1 H, s); mass spectrum, *m/e* (relative intensity) 156 (100, M + 1), 155 (20, M), 115 (10), 96 (30), 84 (12).

2-Aminoimidazole-4(5)-acetic Acid, Trifluoroacetate Salt (3a). A solution of methyl 2-aminoimidazole-4(5)-acetate, trifluoroacetate salt (250 mg, 1.1 mmol) in H_2O (60 mL) and TFA (20 mL) was stirred at room temperature for 24 h. The solution was then evaporated under reduced pressure at room temperature, and 25 mg of the residue (220 mg) was purified by HPLC (Altex-ultrasphere-ODS 5-μm column, size 10 mm × 25 cm; gradient, water/0.01% trifluoroacetic acid/acetonitrile). A 22-mg (80%) yield of pure compound was obtained: NMR (D_2O) δ 3.50 (2 H, d), 6.48 (1 H, t); mass spectrum, *m/e* (relative intensity) 142 (30, M + 1), 98 (100).

2-Aminohistidine (4a) was a gift from A. Jaganathen and L. Ehret.[29]

2-Aminothiazole-4-acetic Acid, Fluoborate Salt (5a). This compound was prepared according to the procedure previously described for the hydrochloride.[11] Ethyl 2-aminothiazole-4-acetate was hydrolyzed in a 34% aqueous solution of fluoboric acid overnight at room temperature. The product **5a** crystallized upon addition of ether: mp 180–185 °C (dec; NMR (CD_3SOCD_3) δ 3.55 (2 H, s), 6.55 (1 H, s), 7.8–8.2 (NH_2, br). Anal. ($C_5H_7N_2SO_2BF_4$) C, H, N.

2-Aminothiazole-5-acetic Acid, Fluoborate Salt (6a). The methyl ester of **6a**[12] was prepared by condensing thiourea with β-bromosuccinic semialdehyde methyl ester, which was prepared by extending a procedure described previously.[30,31] The acid hydrolysis procedure was identical with the one described for **5a**: mp 215 °C (dec; NMR (CD_3SOCD_3) δ 3.72 (2 H, s), 6.2–6.8 (NH_2, br), 7 (1 H, s). Anal. ($C_5H_7N_2SO_2BF_4$) H, N; C: calcd, 24.4; found, 25.0.

3-(Aminomethyl)pyridine *N*-Oxide (8a). 3-[[*N*-[(*tert*-butyloxy)carbonyl]amino]methyl]pyridine: to an ice-cold solution of 3-(aminomethyl)pyridine (4.32 g, 0.04 mol) in dioxane (100 mL) and H_2O (50 mL) was added solid di-*tert*-butyl dicarbonate (9.6 g, 0.044 mol). The mixture was stirred overnight at room temperature and then extracted three times with ethyl acetate. The organic layer was dried and evaporated under reduced pressure, and the residue was chromatographed on silica gel [eluent AcOEt/hexane, 1/1): 7.8 g (93%).

3-[[*N*-[(*tert*-Butyloxy)carbonyl]amino]methyl]pyridine *N*-oxide: solid *m*-chloroperbenzoic acid (3.8 mg, 2.2 mmol) was added to a solution of 3-[[*N*-[(*tert*-butyloxy)carbonyl]amino]-methyl]pyridine (4.2 mg, 2 mmol) in acetone (4 mL). The mixture was stirred overnight. The solvent was then evaporated under reduced pressure, and the residue was purified by column chromatography on silica gel (eluent AcOEt/MeOH, 90/10): 370 mg (80%); NMR ($CDCl_3$) δ 1.45 (9 H, s), 4.3 (2 H, d), 6.3 (1 H, s), 7.2 (2 H, m), 8.3 (2 H, m); IR ($CHCl_3$) 1270 cm^{-1} (*N*-oxide).

3-[[N-[(*tert*-Butyloxy)carbonyl]amino]methyl]pyridine *N*-oxide (224 mg, 10 mmol) was dissolved slowly in a mixture of concentrated HCl (1 mL) in formic acid (10 mL). The solution was stirred for 10 min at room temperature and evaporated under reduced pressure. The solid was recrystallized in 95% EtOH: 140 mg (85%); mp 210 °C; NMR (CD_3SOCD_3) δ 4 (2 H, s), 7.6 (2 H, m), 8.25 (1 H, m), 8.5 (1 H, m). Anal. (C_6H_9OCl) C, H, N.

General Procedure of Diazotization. To a well-stirred and cooled solution or suspension of the aromatic amine, 0.1–2 M in either 34% aqueous HBF_4 (−10 °C) or 1 N HCl (4 °C), was added over a 30-min period a 10% excess of either solid $NaNO_2$ or 1 M aqueous solution of $NaNO_2$. After 45 min of additional stirring at the same temperature, the diazonium salt, (i) when precipitated, was filtered off, dried under reduced pressure, and characterized (UV, Table I): **1b**,[18] **7b**,[13] **9b**,[32] **11b**,[33] **12b**,[34] **13b**,[35] **14b**,[35] **15b**,[36] **16b**,[37] or (ii) was identified in solution by UV spectroscopy (Table I): **2b**, **3b**, **5b**, and **6b**. In all cases it was checked by TLC (eluent *n*-BuOH/AcOH/H_2O, 25/4/10) that all of the starting amine had reacted.

Purification of 3b: 2-aminoimidazole-4(5)-acetic acid (**3a**) (1.75 mg, 0.0124 mmol) was diazotized in 34% HBF_4 as described above. The final solution was neutralized with Na_2CO_3 and purified through a chromatography column (Bio-Gel P-2, −400 mesh; Bio-Rad, Richmond, CA). The collected fractions were analyzed by UV spectroscopy. The pure fractions of the diazonium fluoborate **3b** of the 2-aminoimidazole-4(5)-acetic acid (**3a**) were lyophilized to yield 0.9 mg of **3b**.

The diazonium salt derived from the *m*-sulfanilic acid **10b**[38] needed special diazotization conditions: to an ice-cold 0.5 M

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aqueous solution of the sodium salt of the *m*-sulfanilic acid was added a 10% excess of a 5 M aqueous solution of $NaNO_2$. This was followed, over a 30-min period, by the addition of 2.5 equiv of 6 N HCl. The reaction mixture was then stirred for an additional 45 min, and the precipitated diazonium salt was separated by filtration, dried under reduced pressure, and characterized.

NMR spectra of the three more potent molecules **3b**, **10b**, and **15b** were recorded. **3b**: (D_2O) δ 3.72 (2 H, s), 7.48 (1 H, s). **10b**: (D_2O) δ 7.95 (1 H, t), 8.46 (1 H, d), 8.58 (1 H, d), 8.83 (1 H, s). **15b**: (D_2O) δ 8.06 (1 H, t), 8.14 (1 H, t), 8.54 (1 H, d), 8.73 (1 H, d), 9.05 (1 H, d), 9.23 (1 H, d).

Biochemical Assays. [^{3}H]GABA Binding Assays. The compounds were investigated for their ability to displace [^{3}H]-GABA from its receptor site. Experiments were performed with plasmatic membranes prepared according to Masmoudi and Rendon.[39] Bindings assays were carried out in triplicate at 4 °C for 20 min. The reaction mixture in a final volume of 0.6 mL contained 0.1 mL of plasmatic membranes suspension (about 0.08 mg of protein), 0.3 mL of [^{3}H]GABA [γ-[2,3-^{3}H(N)]aminobutyric acid, 25–40 Ci/mmol; New England Nuclear, Boston, MA] in a final concentration of 10^{-8} M, and 0.2 mL of unlabeled drug, 1 mM GABA, or 200 mM K_2HPO_4/citrate buffer, pH 7.15. At the end of the incubation, the mixture was centrifuged at 50000*g* for 30 min. The supernatant fluid was decanted, and the pellet was rinsed twice rapidly and superficially with 0.75 mL of ice-cold buffer. The pellets were dissolved in 0.250 mL of 5% SDS (sodium dodecyl sulfate). The solution (0.225 mL) was added to 6 mL of Beckman scintillation liquid "Ready-Solv HP", and bound radioactivity was evaluated by scintillation counting (Beckman LS 9800).

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Registry No. **1a**, 7720-39-0; **1a**·Na, 110295-87-9; **1b**, 50846-98-5; **2a**, 74141-18-7; **2a**·HCl, 110295-95-9; **2a** (ethyl ester), 110295-88-0; **2b**, 110295-80-2; **3a**, 73086-08-5; **3a** (methyl ester, TFA salt), 110295-91-5; **3a**·TFA, 110295-92-6; **3b**, 110295-81-3; **5a**, 110295-78-8; **5a** (ethyl ester, free base), 53266-94-7; **5b**, 110295-82-4; **6a**, 110295-79-9; **6a** (methyl ester), 110295-93-7; **6b**, 110295-83-5; **7a**, 3535-75-9; **7b**, 35332-76-4; **8a**, 106940-10-7; **9a**, 121-57-3; **9b**, 2154-66-7; **10a**·Na, 1126-34-7; **10b**, 39948-22-6; **11a**, 99-05-8; **11b**, 1743-37-9; **12a**, 123-30-8; **12b**, 932-97-8; **13a**, 2835-04-3; **13b**, 110295-84-6; **14a**, 98-37-3; **14b**, 110295-85-7; **15a**, 82-75-7; **15b**, 20653-35-4; **16a**, 86-60-2; **16b**, 110295-86-8; $BrCH_2CO_2Et$, 105-36-2; p-$HO_2CCH_2C_6H_4NH_2$, 1197-55-3; $(NH_2)_2CS$, 62-56-6; $OHCCHBrCH_2CO_2Me$, 16565-77-8; t-$BuOCO_2Bu$-t, 34619-03-9; imidazoleacetic acid, 645-65-8; methyl imidazole-4(5)-acetate hydrochloride, 51718-80-0; methyl imidazole-4(5)-acetate, 4200-46-8; methyl 2-[[(*p*-carboxymethyl)phenyl]azo]imidazole-4(5)-acetate, 110295-89-1; 3-[[*N*-[(*tert*-butyloxy)carbonyl]amino]-methyl]pyridine, 102297-41-6; 3-(aminomethyl)pyridine, 3731-52-0; 3-[[*N*-[(*tert*-butyloxy)carbonyl]amino]methyl]pyridine *N*-oxide, 110295-94-8.

N-Substituted Oxopyrimidines and Nucleosides: Structure–Activity Relationship for Hypnotic Activity as Central Nervous System Depressant

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N^3-Benzyluridine (3-(phenylmethyl)-1-β-D-ribofuranosyluracil) (**1f**) and its related compounds were synthesized and evaluated for hypnotic activity as central depressants. The primary structural modification has been carried out at the N^3 position of the pyrimidine ring in uridine. N^3-Benzyl-substituted uridine exhibited hypnotic activity as well as pentobarbital (PB) induced sleep effect on mice when administered by intracerebroventricular (icv) injection. From this result, the secondary modification was performed, namely, converting the benzyl group into a benzyl analogous group. These compounds also showed hypnotic activity, but their intensities were varied. Thirdly, changing the sugar moiety was investigated; however, it was found to be necessary for hypnotic activity. In general, introduction of benzyl analogous groups at the N^3 position of uridine increased the hypnotic activity, and modification of the sugar moiety decreased the activity. Intravenous (iv) administration failed to indicate hypnotic activity in most of the compounds tested. However, modified sugars such as 2′,3′,5′-tri-*O*-methyl or -acetyl derivatives of **1f** elicited hypnotic activity by iv injection. The majority of compounds were found to show potentiation of the PB-induced sleep, and their effects were in parallel with the hypnotic activity. The result clearly indicates that the benzyl group and β-D-ribofuranosyl, at the N^3 and N^1 positions, respectively, are necessary for hypnotic activity. The critical portion of the chemical structure for both effects appears to be the uridine moiety.

Recently, uridine has been reported to be a sleep-promoting substance that is extracted from sleep-derived rat brain stems and to have the natural sleep-promoting effect by nocturnal intracerebral infusion.[1-3] However, uridine itself does not show hypnotic activity determined by loss of righting reflex in experimental animals. In connection with this point, we found that introduction of an allyl group into the N position of barbiturates and other related compounds led to either enhancement or reduction of the sleep effect.[4-7] Furthermore, this could provide a new type of hypnotic compounds such as **1f**.[8] In the same manner, N^3-allyluridine (3-propenyl-1-β-D-ribofuranosyluracil) (**1d**) exerted central nervous system (CNS) depressant activity,

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