Sequentially Labile Water-Soluble Prodrugs of Alprazolam

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A 1,4-benzodiazepine analogue alprazolam (1) undergoes ring-opening hydrolysis under acidic conditions to form a triazolobenzophenone (2). At a neutral pH, 2 rapidly and quantitatively cyclizes back to 1. This facile reversible reaction was utilized in developing water-soluble prodrugs of 1 in which the "solubility anchoring" acyl moieties are formyl, acetyl, succinyl, glycyl, leucyl, and γ-aminobutyryl groups. These compounds were prepared directly from 2 and corresponding acids through DCC coupling in a 1:1 mixture of H_2O and THF. They should be stable in aqueous media at room temperature. Promoieties used should be nontoxic at an intended dose level. In vitro human serum hydrolysis study showed that only glycyl and leucyl amides were able to regenerate 1 within a reasonable period of time. Ex vivo competitive receptor binding assay in mice with [^{3}H]flunitrazepam indicated that leucyl amide prodrug should be able to produce a rapid onset of the intrinsic central nervous system activity of 1 when parenterally administered. For all compounds studied, the final outcome of the biological response appears to be kinetically controlled, rather than by an unfavorable equilibrium, particularly by the first step in which the amide bond is hydrolyzed in vivo.

Currently 1,4-benzodiazepine analogues are one of the most commonly prescribed minor tranquilizers. However, when a rapid onset of action is required, via a parenteral route of administration, a significant problem arises from the fact that 1,4-benzodiazepines are characteristically insoluble in aqueous media. This report describes the use of the facile reversible 1,4-benzodiazepine ring-opening reaction of alprazolam (1; 1-methyl-6-phenyl-4*H*-*s*-triazolo[4,3-*a*][1,4]benzodiazepine)[1] in developing water-soluble prodrugs for parenteral dosage forms. It is specifically concerned with modification of the terminal amino function of the ring-opened compound (2) in such a way that not only the desired solubility (greater than 1 mg/mL) but also a rapid in vivo conversion to 1 can be achieved. Note that the latter condition can be met only when in vivo hydrolysis of promoiety as well as the subsequent spontaneous cyclization step is fast (Scheme I).

Among various 1,4-benzodiazepines which are known to undergo reversible ring-opening reactions,[2] 1 is unique in that its reverse cyclization is quantitative and relatively fast at a physiological pH; at pH 7.4, $t_{1/2}$ is in the order of 10 min at 25 °C[1] and 5 min at 37 °C.[3] It thus warrants a rapid onset of the intrinsic central nervous system (CNS) activity of 1, provided the first step in the two sequential events (i.e., hydrolysis of promoiety) is fast when parenterally administered. Although various peptidoaminobenzophenones have been proposed as potential prodrugs,[4] their onset of action could well be controlled by spontaneous cyclization rate.

Results and Discussion

Chemistry. At pH <2, over 90% of the total alprazolam exists as 2 whereas virtually all exists as 1 so long as pH >5.5.[1] This facile reversibility not only makes it feasible for derivatives of type 3 to serve as sequentially labile prodrugs of 1 but also provides a simple synthesis of **3a–f**.

When an acidic solution that contains 2 is rapidly neutralized, the primary amino group generated will interact with an activated carboxyl function present in a promoiety to form an amide bond. In addition, two side reactions should occur: intramolecular nucleophilic addition of the primary amino group of 2 across the benzophenone carbonyl group to form 1 and hydrolysis of the activated carboxyl function added. Our previous study established that the cyclization reaction becomes slower as pH increases.[1] The hydrolysis of activated carboxyl groups is catalyzed by OH^-. These two considerations led us to believe that an optimal pH for the synthesis of **3a–f** should be around pH 9. Also in an attempt to suppress the water reactivity during the amide bond formation, as much as 50% of THF was maintained in the reaction system. Other aspects of synthesis of **3a–f** are rather straightforward. Due to the side reactions described above, the overall yield was generally low, 20–70%.

As potential prodrugs of 1, **3a–f** appear to meet most of the important pharmaceutical property requirements. All of them, with a possible exception of **3c** in which an intramolecular catalysis can be important as in the case of other succinic acid esters of amides,[5] should be stable in aqueous solutions under a typical storage condition for

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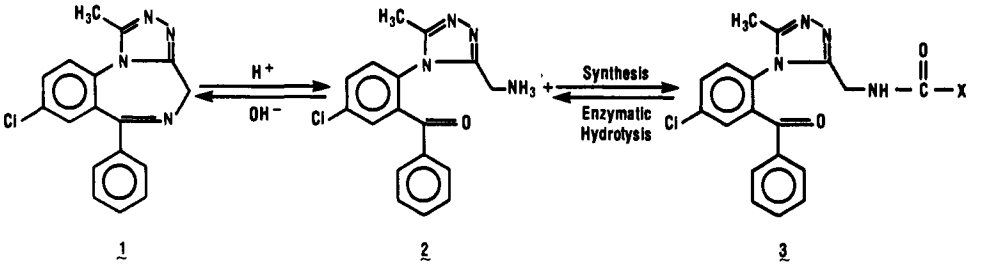
Scheme I



1 2 3

parenteral solutions. Solubility of the ionizable compounds **3c–f** exceeds the concentration desired in the development, approximately 1 mg equiv to 1/mL at room temperature. The solubility of **3a** in water and **3b** in 5% ethanol was 1.8 and 1.6 mg/mL, respectively. Finally, except for **3a**, safety of the promoieties should not be of great concern; they are either nontoxic carboxylic acids or naturally occurring L-amino acids.

Serum Hydrolysis. In developing a water-soluble prodrug for a parenteral dosage form, selection of a promoiety should take the requirement into consideration that the bond between the parent compound and the promoiety should be stable in formulations but rapidly cleaved in vivo. In this regard, it is not surprising that most successful prodrugs are those in which the conversion to the parent compound is catalyzed by a host of enzymes encountered after administration. Since most of the natural substrates for the abundant proteolytic enzymes are peptides, the present study deals largely with amino acid amides of **2**.

Unless one carries out an exhaustive animal or human pharmacokinetic study, it is impossible to prove that a given derivative is indeed rapidly and quantitatively converted in vivo to the parent compound. Results of in vitro serum hydrolysis are therefore useful only when a positive finding is observed. That is, even if a derivative fails to regenerate the parent compound in serum, there is still a good chance that it may be cleaved in other organs rich in various enzymes, such as the liver. That a derivative is rapidly hydrolyzed in serum should be thus considered as a sufficient, not a necessary, condition for the derivative to become a promising prodrug.[6]

As shown in Figure 1, amino acid amides of **2** (**3d** and **3e**) are subject to hydrolysis in the whole human serum following first-order kinetics, whereas **3a–c** are not; $t_{1/2}$ is in the order of 260 and 60 min at 37 °C for **3d** and **3e**, respectively. This finding supports that amino acids do indeed provide some substrate specificity for the enzymes present in the serum. Since the HPLC procedure adopted was able to quantitate both **1** and derivatives **3** and since the mass balance was well-maintained (over 95% recovery) throughout a kinetic run, it was possible to rule out any buildup of the intermediate **2** in the process, **3** → **2** → **1**. Finally, it is not clear in the present study whether the 4.3-fold difference in the serum hydrolysis rate oberved for **3d** and **3e** is significant in terms of the ultimate biological response, although there appears to be some difference in the dose–response competitive binding study (see below).

Receptor Binding Studies. In contrast to conventional in vitro competitive binding assay with isolated receptor site,[7] the procedure adopted in the present study more closely approximates the in vivo situation in that **3a–f** were directly administered to whole mice so that hydrolysis (**3** → **2**) as well as cyclization (**2** → **1**) can take place. With isolated receptors for 1,4-benzodiazepines, a negative finding may signify only that the derivatives do not have intrinsic binding affinity and/or that the receptor site apparently is not capable of converting a derivative to the parent compound.

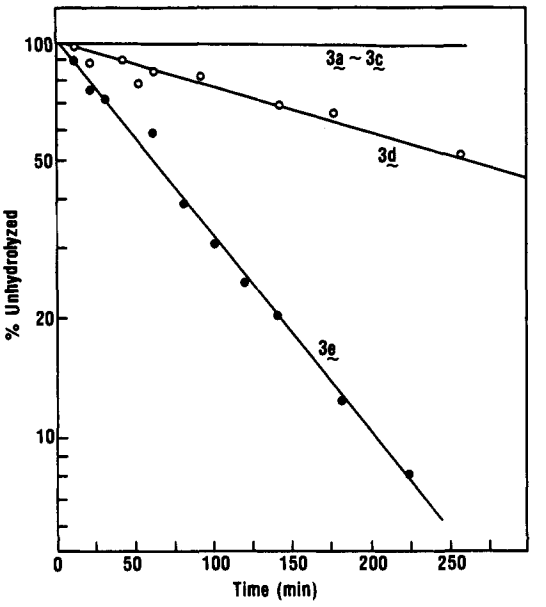


Figure 1. Normalized first-order plots for the in vitro serum hydrolysis of **3a–e** at 37 °C. Disappearance of the substrates was monitored by HPLC procedures (see text). Initial substrate concentrations were 65–75 μg/mL for **3a–c**, 89 μg/mL for **3d**, and 4.3 μg/mL for **3e**. Values of correlation coefficient are 0.956 and 0.993 for **3d** and **3e**, respectively.

A series of dose–response studies with **1** and **3d–f** (Figure 2), in which mice were killed 30 min after the iv administration of drugs, yielded the following potency ranking in terms of ED_{50}: alprazolam (**1**) > leucylamide (**3e**) > glycylamide (**3d**) > GABA amide (**3f**), with ED_{50} values (μmol/kg, iv) of 2.9, 6.0, 9.0, and 320 (not reported in Figure 3), respectively. In vitro conversion of **2** → **1** is quantitative at pH 7.4,[1] and so is assumed for **3** → **2** in vivo (i.e., amide hydrolysis). Since the **2** → **1** process is independent of the structure of starting materials, the ED_{50} values observed should reflect the magnitude of bioconversion rate of **3** → **2**. In this regard, two points are rel-

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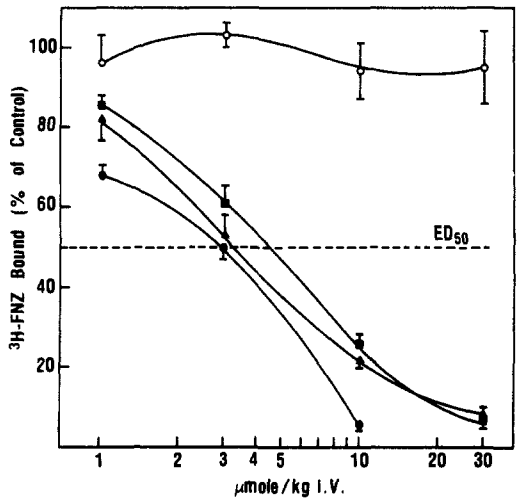

Figure 2. Dose–response curves for 1 (●), **3d** (■), **3e** (▲), and **3f** (○), in terms of competitive binding to the benzodiazepine receptor of mice with [^{3}H]flunitrazepam as the ligand. Binding assay was carried out 30 min after 1 and **3d–f** were intravenously administered. Each data point is the mean of four independent measurements and vertical bars represent standard errors.

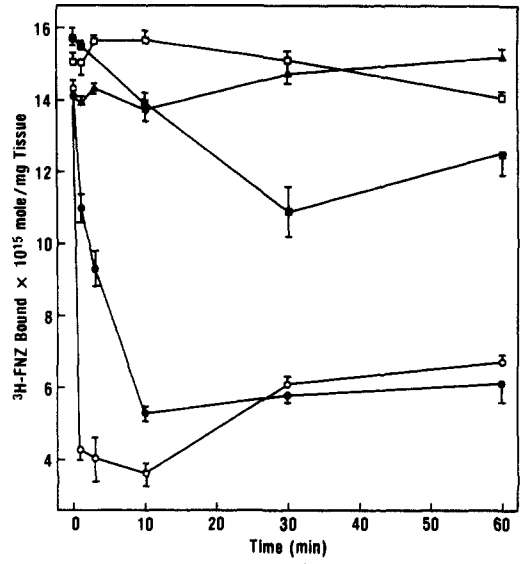

Figure 3. Time course of the CNS activity observed after iv administration of 1 (○, 3 μmol/kg), **3a** (■, 10 μmol/kg), **3b** (□, 10 μmol/kg), **3c** (△, 10 μmol/kg), and **3e** (●, 6 μmol/kg). The CNS activity is expressed in terms of competitive binding to the benzodiazepine receptor of mice with [^{3}H]flunitrazepam as the ligand. Each data point represents the mean of four independent measurements and vertical bars represent standard errors.

evant: First, the arbitrarily chosen 30-min waiting period prior to decapitation of mice must have played a critical role in determining the magnitude of ED_{50} values. It is quite conceivable that a shorter waiting period could indeed result in more spread values of ED_{50}. Second, enzyme specificity should ultimately determine the final outcome of biological response. Unfortunately, due to paucity of data, the present study is not able to identify a class of enzyme systems primarily responsible for the bioconversion **3** → **2**.

GABA amide of **2** (**3f**) failed to show any binding at all. This may be due to sluggish hydrolysis in the general circulation, poor binding of intact **3f** if it passes through the blood–brain barrier at all, or elimination of intact **3f** by other competing metabolic pathways. Although the present study does not provide any concrete evidence for any of the above possible reasons, it is most likely that **3f** is not hydrolyzed in the circulation and other organs such as liver. Note that all derivatives which showed no hydrolysis in serum showed little or no binding (see more below). It is being postulated that **3a–f** must be converted to 1 before they pass the blood–brain barrier. In the absence of any structural feature specific for active transport across the blood–brain barrier, lipophilicity is the major factor which determines the transport rate.[8] Note that the apparent oil/water partition coefficient of **3a–f** at pH 7.4 is much smaller than that of 1.

Since the present ex vivo dose–response studies appeared to yield kinetically controlled ED_{50} values for **3a–f**, i.e., ED_{50} values not controlled by equilibrium, a series of time–course studies were carried out for a few selected compounds (Figure 3). Extent of displacement of bound **3e** by [^{3}H]flunitrazepam (FNZ) became indistinguishable from that by the parent compound 1 after 30 min when twice as much **3e** was administered. Once again, the delayed binding of **3e** to the receptor site is attributed to possibly slow in vivo hydrolysis during circulation. Although the extent of displacement of **3e** by [^{3}H]FNZ observed 60 min after the administration of **3e** is comparable to that produced by the half amount of 1, **3e** does not seem to be bioequivalent to 1 on a molar basis. It is possible that intact **3e** itself may undergo its own metabolism and/or elimination while the **3e** → **2** → 1 process is taking place.

Compounds **3a–c**, which are not hydrolyzed in human serum, failed to show any significant binding to the 1,4-benzodiazepine receptor even after more than 3-fold excess over 1 was administered. In summary, the acyl moiety of **3** appears to determine the hydrolytic lability in vivo, which in turn governs the extent of binding to the receptor.

Experimental Section

Melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. NMR, IR, and UV spectra were recorded with Varian Model T-60, Perkin-Elmer Model 621 grating IR, and Zeiss Model DMR-21 spectro(photo)meters, respectively. Precoated silica gel GF TLC plates (Uniplate from Analtech) were used with the following solvent systems: A, CH_3OH/2-propanol/$CHCl_3$ (5:15:80); B, HOAc/CH_3OH/CH_2Cl_2 (5:5:90); C, CH_3OH/2-propanol/EtOAc (10:20:70); and D, 28% NH_4OH/CH_3OH/2-propanol/CH_2Cl_2 (1:4:15:80:). Some of these solvent systems were also used in preparative LC procedures. The ninhydrin test[9] was exclusively used in detecting free amino function on TLC plates. Solvents used throughout the present study are of the distilled-in-glass grade (Burdick and Jackson) and all amino acid derivatives are those of the L isomer.

5-Chloro-2-[3-(formaminomethyl)-5-methyl-4*H*-1,2,4-triazol-4-yl]benzophenone (3a). In 20 mL of dry THF, 1.13 mL of formic acid (30 mmol; Aldrich) was reacted with 4.12 g of dicyclohexylcarbodiimide (DCC; 20 mmol; Aldrich) for 5 min at room temperature. After dicyclohexylurea was filtered off, the filtrate was added to the following aqueous solution immediately after the pH of the latter was adjusted to 9.0. In 15.0 mL of 0.33 N HCl, 926 mg of 1 (3.0 mmol) was dissolved to generate **2**, and the pH was rapidly adjusted to 9.0 with 1.0 N NaOH. After 30 min at room temperature, the reaction mixture was evaporated to dryness under vacuum at 60 °C. The solid residue was the taken up into 100 mL of $CHCl_3$ and filtered. Concentrated filtrate was subject to LC separation on a silica gel column (Lobar size C column from E. Merck) using solvent system A. Elution was constantly monitored at 307 nm in a flow-through cell using a Gilford Model 250 spectrophotometer. Fractions containing **3a** were combined and evaporated to result in 473 mg of solid (yield,

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51%). The analytical sample was crystallized from EtOAc: R_f 0.23 (A); mp 178.0–180.3 °C; NMR ($CDCl_3$) δ 8.27 (s, br, 1 H, NH), 8.08 (s, 1 H, COH), 7.6 (m, 8 H, Ph), 4.3 (m, 2 H, CH_2), 2.2 (s, 3 H, triazole CH_3). Anal. Calcd for $C_{18}H_{16}O_2N_4Cl$: C, 60.76; H, 4.53; N, 15.74; Cl, 9.96. Found: C, 61.25; H, 4.26; N, 15.27; Cl, 10.03.

5-Chloro-2-[3-(acetaminomethyl)-5-methyl-4*H*-1,2,4-triazol-4-yl]benzophenone (3b) and 5-Chloro-2-[3-(hemisuccinaminomethyl)-5-methyl-4*H*-1,2,4-triazol-4-yl]benzophenone (3c). In 15 mL of 0.33 N HCl, 926 mg of 1 (3.0 mmol) was dissolved to generate **2**. Immediately after the pH of this solution was rapidly adjusted to 9.0 with 1.0 N NaOH, 8.5 mL of acetic anhydride (90 mmol; Malinckrodt) or 9.0 g of succinic anhydride (90 mmol; Eastman) in 20 mL of THF was added with stirring. After 30 min at RT, the reaction mixture was evaporated to dryness under vacuum at 60 °C.

The residue containing **3b** was taken up in 100 mL of $CHCl_3$ and filtered. Filtrate was concentrated and subject to LC separation on a silica gel column (Lobar size C column from E. Merck) using solvent system A. Elution was constantly monitored at 307 nm as described above. Fractions containing **3b** were combined and evaporated to result in 803 mg of **3b** (yield, 73%). The analytical sample was crystallized from EtOAc: R_f 0.40 (A); mp 209.1–211.5 °C; UV (H_2O) λ_{max} 260 nm (ϵ 9.2 × 10^3), nearly identical to UV spectrum of **2** in an acidic solution,[1] IR ($CHCl_3$) 3640 and 3610 cm^{-1} (w, NH), 1670 cm^{-1} (s, C=O), 1590 and 1315 cm^{-1} (m and w, amide bands II and III); NMR ($CDCl_3$) δ 8.2 (s, br, 1 H, NH), 7.7 (m, 8 H, Ph), 4.4 (m, 2 H, CH_2), 2.2 (s, 3 H, triazole CH_3), 1.9 (s, 3 H, $COCH_3$). Anal. Calcd for $C_{19}H_{17}N_4O_2Cl$: C, 61.87; H, 4.65; N, 15.19; Cl, 9.61. Found: C, 61.48; H, 4.74; N, 14.95; Cl, 9.51.

In the case of **3c**, the residue obtained from evaporation was taken up into ca. 100 mL of CH_3CN and filtered. The filtrate was evaporated, and the residue dissolved in 200 mL of EtOAc was washed with equal volumes of 0.05 M acetate buffer at pH 4.5 and H_2O. After EtOAc was evaporated, the residue was crystallized twice from $CHCl_3$ to yield 350 mg (27%): mp 185.0–186.5 °C; NMR (Me_2SO) δ 8.2 (s, br, NH), 7.7 (m, 8 H, Ph), 4.2 (m, 2 H, CH_2) 2.3 (m, 4 H, CH_2CH_2), 2.1 (s, 3 H, triazole CH_3). Anal. Calcd for $C_{21}H_{19}N_4O_4C$: C, 59.10; H, 4.49; N, 13.12. Found: C, 59.15; H, 4.89; N, 13.12.

5-Chloro-2-[3-(peptidoaminomethyl)-5-methyl-4*H*-1,2,4-triazol-4-yl]benzophenones (3d–f). In 20 mL of dry THF, 7–12 mmol of *tert*-butyloxycarbonyl (Boc)-amino acid (Sigma) was reacted with 7–8 mmol of DCC for 5–10 min at room temperature. For the synthesis of **3f**, Boc derivative of γ-aminobutyric acid (GABA; Aldrich) was prepared by using 2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile (Boc-ON; Aldrich).[10] An oil homogeneous on TLC was obtained: R_f 0.77 (B); NMR ($CDCl_3$) δ 3.18 (q, 2 H, CH_2NH), 2.35 (t, 2 H, CH_2CO), 1.8 (q, 2 H, CH_2), 1.45 (s, 9 H, *t*-Bu). After dicyclohexylurea was filtered off, the filtrate was added with stirring to the following aqueous solution at pH 9.5–10 immediately after it was prepared. For the synthesis of Boc-**3e**, 18.3 mg of *N,N*-dimethylaminopyridine (0.15 mmol; Aldrich) was also added as a nucleophilic catalyst.[11] In 15.0 mL of 0.33 N HCl, 926 mg of 1 (3.0 mmol) was dissolved to generate **2**, which usually took about 20 min at room temperature, pH was rapidly adjusted to 9.5–10 with 3 N Na_2CO_3. The DCC coupling was allowed to take place for 60 min at room temperature, concentrated under vacuum, and the solubles in 100 mL of EtOAc or $CHCl_3$ were collected through filtration. The filtrate was then washed successively with 100 mL of 0.1 M formate buffer at pH 4.1 and 0.1 M carbonate buffer at pH 9.7, both saturated with NaCl and H_2O. After drying with anhydrous Na_2SO_4, the solvents were removed under vacuum. Residues containing Boc derivatives of **3d–f** were further purified by means of the LC procedures described below.

In the case of Boc-glycyl and γ-Boc-GABA amides (**3d** and **3f**), reaction mixtures were loaded on two commercially obtained reverse-phase LC columns in series (RP-8 Lobar size B columns from E. Merck), eluted with mobile phases consisting of CH_3OH/H_2O, and elution was constantly monitored at 307 nm as described earlier. For separation of Boc-**3d**, a gradient system, starting from a 55:45 mixture of CH_3OH and H_2O to 100% CH_3OH at an increment rate of 0.25%/min, was adopted using a commercially available system (a modified DuPont Model 838 programmable gradient system). For Boc-**3f** separation, an isocratic mode was adopted using a 65:35 mixture of CH_3OH and H_2O as mobile phase. For separation of Boc-**3f**, two normal-phase silica gel columns in series (Lobar size B columns from E. Merck) were used along with solvent system C as the mobile phase. In all preparative LC procedures, flow rate was on the order of 4.5–8.0 mL/min, and provided by a commercially obtained pump (Milton Roy miniPump Model NSI-33R). Eluents containing products were combined and evaporated to yield 282 mg (20% yield) of Boc-**3d** and 436 mg (27% yield) of Boc-**3e**.

Boc derivatives of **3d–f** thus obtained were treated with 50 mL of 50% freshly distilled trifluoroacetic acid (TFA; Aldrich) in dry CH_2Cl_2 for 60 min at room temperature. After the solvents were thoroughly removed under vacuum at 60 °C, remaining residues were taken up into EtOAc, and TFA salts of **3d** and **3e** were precipitated out with ether. In the case of **3f**, the TFA salt was directly converted to chloride salt (see below). For TFA salt of **3d**: mp 85–88 °C; NMR (CD_3OD) δ 7.7 (m, 8 H, Ph), 4.4 (s, 2 H, CH_2N), 3.65 (s, 2 H, CH_2CO), 2.2 (s, 3 H, CH_3). Anal. Calcd for $C_{21}H_{19}N_5O_4F_3Cl{\cdot}H_2O$:C, 48.90; H, 4.10; N, 13.57; Cl, 6.87. Found: C, 49.23; H, 3.96; N, 12.48; Cl, 7.16. For TFA salt of **3e**: R_f 0.59 (D); mp 120–123 °C; IR (Nujol) 1670 cm^{-1} (s, C=O), 1590 and 1315 cm^{-1} (m and w, amide bands II and III); NMR (CD_3OD) δ 7.7 (m, 8 H, Ph), 4.3 (d, 2 H, CH_2N), 3.9 (t, 1 H, $C_\alpha H$), 2.15 (s, 3 H, triazole CH_3), 1.7 (m, 3 H, $C_\beta H_2C_\gamma H$), 1.0 (d, 6 H, $C_\gamma(CH_3)_2$). Anal. Calcd for $C_{25}H_{27}H_5O_4F_3Cl$: C, 54.21; H, 4.91; N, 12.64; Cl, 6.40. Found: C, 53.65; H, 4.88: N, 12.74; Cl, 6.38.

The TFA salt of **3f** was directly converted to the chloride salt without characterization. Thoroughly dried residue obtained after deprotection of Boc-group was dissolved in 10 mL of H_2O and added to 5 mL (wet vol) of Dowex 2X-8 anionic exchange resin (200–400 mesh, Cl^- form; Bio-rad). The suspension was then poured into a glass column of 10 mm i.d. × 300 mm long (Amicon), which already contained 20 mL of the same resin, and eluted with water at a flow rate 1.6 mL/min. Fractions containing the product were combined and freeze-dried overnight (Labconco Model FD-5) to yield 200 mg of a white solid (overall yield 13%): R_f 0.10 (D); mp 150–155 °C NMR (D_2O) δ 7.6 (m, 8 H, Ph), 4.3 (s, br, 2 H, CH_2NHCO), 2.9 (t, 2 H, CH_2N^+), 2.25 (s, 3 H, triazole CH_3), 1.8–2.1 (nr, 4 H, $OCCH_2CH_2$).

In Vitro Serum Hydrolysis of 3a–e. After the blood from a healthy noraml female Caucasian volunteer was allowed to coagulate at room temperature for 60 min, the whole blood was centrifuged at 1000*g* for 20 min at 4 °C. Supernatant was once subject to centrifugation at 3000*g* for 20 min at 4 °C. The supernatant was decanted into fractions of about 10 mL and kept as frozen at −10 °C until used.

Hydrolysis was initiated by adding a methanolic stock solution of **3a–c** or aqueous stock solution of **3d–f** to a given volume of serum that had been equilibrated at 37 °C. Initial substrate concentration varied from 4.3 μg (equivalent to 1)/mL for **3e** to 81.0 μg/mL for **3d**, and the final concentration of methanol was always less than 10%. With proper time intervals, an aliquot of a 0.5-mL serum sample was withdrawn, immediately diluted with 5.0 mL of H_2O, and passed through a small reverse-phase column (Sep-PAK C-18, Waters Assoc.). After being washed with 20 mL of H_2O, the column was finally eluted with 5.0 mL of methanol. This methanol-rich eluent was then concentrated under a stream of N_2 at room temperature to a volume of ca. 1 mL and diluted exactly to 2.0 mL with the mobile phase used in the following HPLC assay. By use of a constant-volume loop (Rheodyne Model 7000), 20 μL of a sample was injected into a reverse-phase column (C-18, 10 μm; Waters Assoc.) coupled with an RP-18 precolumn (Rainin) and eluted with the mobile phase; CH_3OH/H_2O (60:40) for **3a** and **3b**, $CH_3OH/H_2O/H_3PO_4$ (65:35:0.1) for **3c**, and CH_3OH/0.1 M citrate buffer at pH 6.0 (60:40) for **3d**. For the hydrolysis of **3e**, instead of 20 μL, 1.0 mL of the final sample was loaded and eluted with a mobile phase, CH_3OH/0.01 M acetate buffer at pH 6 (70:30), which contained sodium dodecyl sulfate (50 mg/L). Other conditions for the HPLC assay were as follows:

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detector, DuPont Model 837 spectrophotometer; pump, Altex Model 110A; λ = 222 nm for **3a–d** and 262 nm for **3e**; P = 2500–3500 psi at FR = ca. 2 mL/min. Values of k' under these HPLC conditions were 1.5 for **3a**, 2.2 for **3b**, 0.93 for **3c**, 0.84 for **3d**, 2.5 for **3e**, and 2.8 for **1**.

Ex Vivo Competitive Binding Studies. Male mice, weighing 25–30 g, used in the present study had been kept under constant temperature and diurnal lighting prior to their experimental use, and all were killed nearly at the same time of day to avoid any possible effects of circadian rhythm. Stock solutions of **3a–f** were prepared in 0.9% NaCl at a concentration range of 6×10^{-4} to 10^{-3} M, and that of **1** was made in 2% ethanol at 3×10^{-4} M. All drugs were administered through an iv route using a tail vein. Control mice received equal volume of the vehicle alone.

In the dose–response studies, mice were sacrificed by decapitation 30 min after varying doses of **2** and **3d–f** were administered. In the time–course studies, mice were killed 1, 3, 10, 30, and 60 min after 3 μmol/kg of **1**, 10 μmol/kg of **3a–c**, and 6 μmol/kg of **3e** were administered.

After decapitation, mice brain was quickly removed from the skull. The whole brain minus cerebellum was homogenized for 30 s in 50 vol (ca. 10–15 mL) of 0.05 M Tris buffer at pH 7.4 and 4 °C, using a Brinkman Polytron homogenizer (PCU-2-110) at no. 6 setting. Tritium-labeled flurnitrazepam ([^{3}H]FNZ; New England Nuclear; specific activity 84.8 Ci/mmol) binding was measured by incubating a 1.0-mL aliquot of the homogenate, 0.10 mL of [^{3}H]FNZ, which resulted in a final concentration of about 0.77 nM, 0.1 mL of H_2O or a flurazepam (Hoffman LaRoche) solution, and 0.8 mL of 0.05 M Tris buffer at pH 7.4. This 2.0-mL mixture was incubated for 30 min at 25 °C and then filtered through a Whatman GF/B filter disk under vacuum. The incubation tube was rinsed with 5 mL of ice-cold Tris buffer and filtered through again. The filter disk was finally washed 3 times with 5-mL aliquots of the same buffer. The filter disk was then placed in a scintillation vial containing 15 mL of Amersham-Searle ACS cocktail, and the radioactivity was measured by a liquid scintillation counter (Beckman Model LS3133T).

Specific binding is defined as the total binding minus binding in the presence of 10 μM flurazepam. Specific binding represents over 90% of the total binding.

Registry No. 1, 28981-97-7; **2**, 85966-74-1; **3a**, 102537-66-6; **3b**, 102537-67-7; **3c**, 102537-68-8; **3d**, 64194-69-0; **3d**·TFA, 102537-73-5; BOC-**3d**, 102537-71-3; **3e**, 102537-69-9; **3e**·TFA, 102537-74-6; BOC-**3e**, 102573-67-1; **3f**, 102537-70-2; **3f**·TFA, 102537-75-7; BOC-**3f**, 102537-72-4; $BOCNHCH_2CO_2H$, 4530-20-5; (L)-$BOCNHCH(i\text{-}Bu)CO_2H$, 13139-15-6; $BOCNH(CH_2)_3CO_2H$, 57294-38-9; succinic anhydride, 108-30-5.

Ribose as the Preferential Target for the Oxidized Form of Elliptinium Acetate in Ribonucleos(t)ides. Biological Activities of the Resulting Adducts

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The covalent binding of the oxidized form of elliptinium acetate, an antitumor drug, to various ribonucleos(t)ides is described. In the absence of a strong nucleophile on the bases, e.g., a sulfhydryl group, the main target of this quinone imine derivative is the sugar moiety. With unmodified regular bases, the first electrophilic addition always occurs on the 2′-oxygen of ribose (more slowly for pyrimidine than for purine); in a second step, cyclization of the reoxidized product leads to a spiro derivative: only one stereoisomer is detected with purine nucleoside; the other stereoisomer appears as a minor product (10–20%) with nucleotides and pyrimidine nucleosides. With modified bases, no change is observed except for bases exhibiting an additional strong nucleophilic center: oxidized elliptinium alkylates thioguanine and thioguanosine on the sulfur atom and in this last case not on the ribose moiety. All spiro derivatives are less cytotoxic than the parent compound even if the base is an antimetabolite (azauridine); however, thio–elliptinium adducts maintain high cytotoxicity.

Elliptinium acetate (**1**; 9-OH-NME or N^2-methyl-9-hydroxyellipticinium acetate) is an antitumor agent currently used for therapeutic treatment of osteolytic metastases of breast cancer.[1,2] In addition to its high affinity for DNA,[3] this molecule can easily be oxidized to a quinone imine derivative **2**.[4,5] The strong electrophilic properties of this oxidized form of 9-OH-NME were shown (i) in vitro with various nitrogen[6,7] (amino acids), sulfur (cysteine, glutathione), and oxygen[5,6] (H_2O_2, CH_3OH) nucleophiles and (ii) in vivo in humans and rats with the recovery of cysteinyl- and glutathionyl–elliptinium adducts as metabolites.[8–10] Previously, it was shown that elliptinium acetate binds to guanosine and adenosine in an unexpected manner, on the sugar moiety of nucleosides.[11,12] This high regioselectivity for the addition of **2** to nucleosides was interpreted in terms of a preliminary stacking between the pyridocarbazole **2** and nucleoside bases.

In the present paper, we report the synthesis and biological properties of various elliptinium adducts with purine and pyrimidine nucleosides and nucleotides and also with other nucleosides bearing modified bases. We intended to modulate the stacking effects between **2** and

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