

Static images were obtained at 1-, 2-, and 24-h postinjection.

**Acute Toxicity.** Unanesthetized Swiss white mice (20–25 g) were placed in a restraining tube and injected via a tail vein with different doses of compound. Four mice were injected with each dose, and the number of survivors were determined 5 min after injection. In addition, survivors were examined 24-h postinjection.

Overt symptoms at the time of injection were noted and the LD<sub>50</sub> value for **4p**, **5m**, and **6m** were determined by using the probit-log dose method.<sup>41</sup>

**Acknowledgment.** We thank Merck-Frosst Canada Ltd. and the Natural Sciences and Engineering Research Council of Canada for their financial support.

**Registry No.** **1o**, 99540-52-0; **1o**-<sup>131</sup>I, 100839-47-2; **1m**, 100839-40-5; **1m**-HCl, 100839-66-5; **1m**-<sup>131</sup>I, 100839-48-3; **1p**, 100839-41-6; **1p**-HCl, 100839-68-7; **1p**-<sup>131</sup>I, 100839-49-4; **2o**,

76465-07-1; **2o**-HCl, 100839-64-3; **2o**-<sup>131</sup>I, 100839-50-7; **2m**, 100839-42-7; **2m**-<sup>131</sup>I, 100839-51-8; **2p**, 100839-43-8; **2p**-HCl, 100839-69-8; **2p**-<sup>131</sup>I, 100839-52-9; **3o**, 100839-44-9; **3o**-HCl, 100839-65-4; **3o**-<sup>131</sup>I, 100839-53-0; **3m**, 100839-45-0; **3m**-HCl, 100839-67-6; **3m**-<sup>131</sup>I, 100839-54-1; **3p**, 100839-46-1; **3p**-<sup>125</sup>I, 100857-94-1; **4o**-I<sup>-</sup>, 100839-55-2; **4o**-<sup>131</sup>I-I<sup>-</sup>, 100839-70-1; **4m**-I<sup>-</sup>, 100839-56-3; **4m**-<sup>131</sup>I-I<sup>-</sup>, 100839-71-2; **4p**-I<sup>-</sup>, 100839-57-4; **4p**-<sup>131</sup>I-I<sup>-</sup>, 100839-72-3; **4p**-CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, 100839-78-9; **4p**-<sup>131</sup>I-CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, 100839-84-7; **4p**-<sup>123</sup>I-CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, 100839-88-1; **5o**-I<sup>-</sup>, 100839-58-5; **5o**-<sup>131</sup>I-I<sup>-</sup>, 100839-73-4; **5m**-I<sup>-</sup>, 100839-59-6; **5m**-<sup>131</sup>I-I<sup>-</sup>, 100839-74-5; **5m**-CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, 100839-80-3; **5m**-<sup>131</sup>I-CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, 100839-86-9; **5m**-<sup>123</sup>I-CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, 100839-90-5; **5p**, 100839-60-9; **5p**-<sup>131</sup>I-I<sup>-</sup>, 100839-75-6; **6o**-I<sup>-</sup>, 100839-61-0; **6o**-<sup>131</sup>I-I<sup>-</sup>, 100839-76-7; **6m**-I<sup>-</sup>, 100839-62-1; **6m**-<sup>131</sup>I-I<sup>-</sup>, 100857-93-0; **6m** CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, 100839-82-5; **6m**-<sup>131</sup>I-CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, 100839-92-7; **6m**-<sup>123</sup>I-CH<sub>3</sub>CO<sub>2</sub><sup>-</sup>, 100839-94-9; **6p**-I<sup>-</sup>, 100839-63-2; **6p**-<sup>125</sup>I-I<sup>-</sup>, 100839-96-1; *o*-IC<sub>6</sub>H<sub>4</sub>NH<sub>2</sub>, 615-43-0; *o*-IC<sub>6</sub>H<sub>4</sub>OH, 533-58-4; *m*-IC<sub>6</sub>H<sub>4</sub>OH, 626-02-8; *p*-IC<sub>6</sub>H<sub>4</sub>OH, 540-38-5; Cl(CH<sub>2</sub>)<sub>2</sub>NMe<sub>2</sub>, 107-99-3; Cl(CH<sub>2</sub>)<sub>2</sub>NEt<sub>2</sub>, 100-35-6; *p*-NH<sub>2</sub>C<sub>6</sub>H<sub>4</sub>OH, 123-30-8; *p*-<sup>125</sup>IC<sub>6</sub>H<sub>4</sub>OH, 100839-95-0; *N*-(2-chloroethyl)morpholine, 3240-94-6.

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## 4-Amino-2-(substituted methyl)-2-butenic Acids: Substrates and Potent Inhibitors of $\gamma$ -Aminobutyric Acid Aminotransferase

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4-Amino-2-(substituted methyl)-2-butenic acids, where X (the substituted group) = F, Cl, OH, are synthesized from Cbz-protected *tert*-butyl 4-aminobutanoate. Successive substitutions at the  $\alpha$ -carbon by phenylseleno and hydroxymethyl groups, followed by elimination of the selenoxide and halide substitution at the hydroxymethyl group, afford the compounds in good yields. An unexpected degree of stereoselectivity is observed in the selenoxide elimination step, which yields the desired *E* isomer as the sole product. These compounds complement two previously reported series of compounds (Silverman, R. B.; Levy, M. A. *Biochem. Biophys. Res. Commun.* 1980, 95, 250–255; *J. Biol. Chem.* 1981, 256, 11 565–11 568) and are used in an approach to map a section of the active site of  $\gamma$ -aminobutyric acid aminotransferase (GABA-T). None of these compounds is a time-dependent inactivator of GABA-T, but all are potent competitive reversible inhibitors; the hydroxy compound has a *K<sub>i</sub>* value of 5  $\mu$ M. That these compounds are not inactivators suggests that either elimination of X does not occur or that there is no active site nucleophile in the appropriate position for reaction following elimination. With use of the fluoro analogue, enzyme-catalyzed fluoride ion release is demonstrated, indicating that elimination does occur. Unlike the previous two series of compounds (op. cit.) in which exclusive elimination occurs when the substituent is a halogen but exclusive transamination prevails for the hydroxyl-substituted analogues, in the series described here, the fluoro analogue gives a 4:1 ratio of elimination to transamination. This suggests that the 2,3-double bond stabilizes the product of azallylic isomerization of the Schiff base between the fluoro compound and pyridoxal phosphate. The results described here indicate that the design of a mechanism-based inactivator for GABA-T should not be based on electrophile generation near the 2-position of enzyme-bound GABA. Furthermore, substitution of an inhibitor with a 2-hydroxymethyl group (or other hydrogen-bonding substituent) and a 2,3-double bond may lend auspicious binding properties to the molecule for GABA-T.

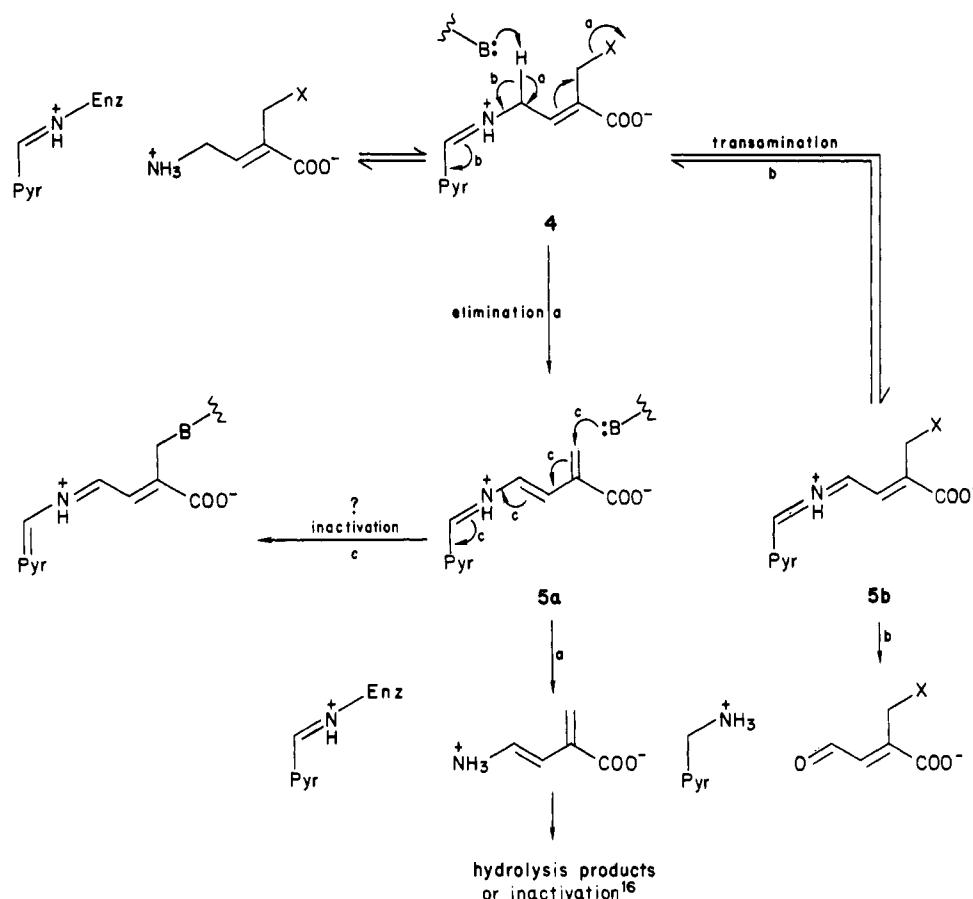
It has been shown that convulsions can occur when the level of  $\gamma$ -aminobutyric acid (GABA) in the brain diminishes below a critical amount and that direct administration of GABA into the brain terminates the seizures.<sup>1–3</sup> However, GABA does not cross the blood–brain barrier, a protective membrane that prevents xenobiotics from entering the brain. Consequently, GABA is not an effective anticonvulsant agent. Recently, efforts have been directed toward the discovery of compounds that inhibit  $\gamma$ -aminobutyric acid aminotransferase (EC 2.6.1.19; GABA-T),<sup>4–9</sup> a brain enzyme responsible for the catabolism of GABA. This would result in an increase in the con-

centration of GABA in the brain and could be an effective approach to the design of anticonvulsant agents.

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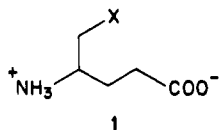
†Present address: Hercules, Inc., Research Center, Wilmington, DE 19899.

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**Scheme I.** Reactions of 4-Amino-2-(substituted methyl)-2-butenic Acids by GABA-T<sup>a</sup>

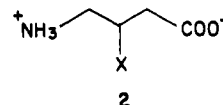
<sup>a</sup> Pyr represents the pyridine ring of PLP.

Several years ago we synthesized a series of (*S*)-5-substituted-4-aminopentanoic acids<sup>10</sup> (1) and showed that the



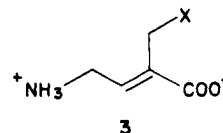
halogen-substituted analogues were potent mechanism-based irreversible inactivators<sup>11-14</sup> of GABA-T.<sup>15</sup> The mechanism proposed<sup>15</sup> for inactivation involved Schiff base formation to the pyridoxal 5'-phosphate (PLP) cofactor followed by elimination of HX and Michael addition of a nucleophile near the 4-position of the enzyme-bound inactivator. Since the time of that proposal, another mechanism for inactivation of PLP-dependent enzymes has emerged.<sup>16-18</sup> The fluoro analogue (1, X = F) was shown to be a potent in vivo inactivator of GABA-T as well,<sup>19,20</sup> indicating that it *does* cross the blood-brain

barrier. Another related series of GABA analogues, 3-substituted GABA (2), was prepared; although a mecha-



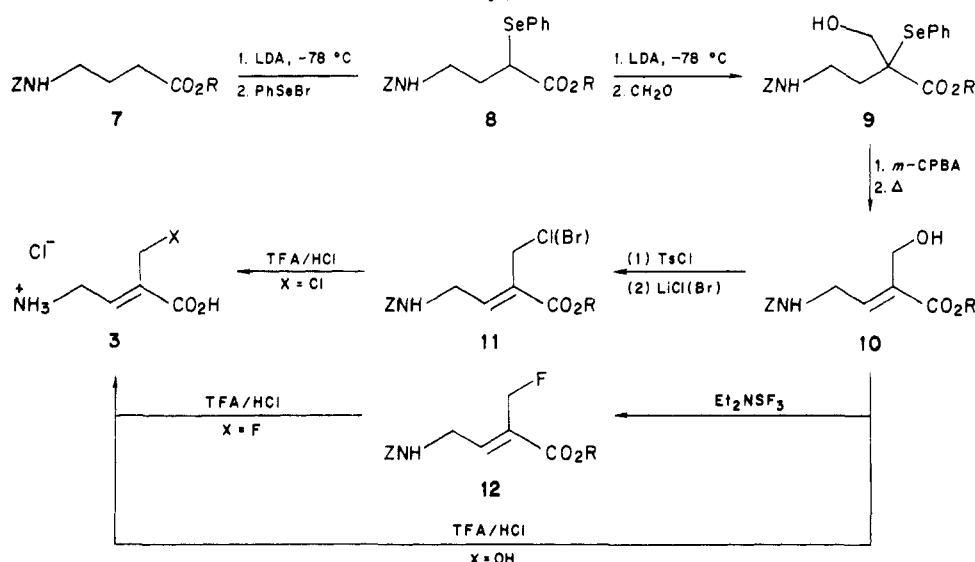
nism similar to that proposed for inactivation of GABA-T by 1 could be envisioned, these compounds were found to be substrates, but not inactivators, for the enzyme.<sup>21</sup> Following Schiff base formation with the PLP, elimination of HX from 2 (X = F or Cl) to give the proposed reactive intermediate did occur, yet no inactivation was evident. This suggested that there was no active-site nucleophile properly juxtaposed at the 3-position of these enzyme-bound intermediates. It also was found that, when X = F or Cl, virtually exclusive elimination of HX resulted, but when X = OH, only transamination occurred.<sup>21</sup>

Since inactivation was observed when an electrophilic site was generated near the 4-position of an inactivator but not when produced at the 3-position, we designed the 4-amino-2-(substituted methyl)-2-butenic acids (3), which



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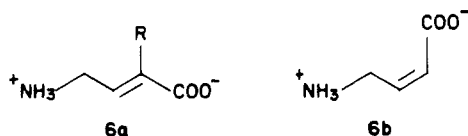
**Scheme II.** Synthetic Pathway to the 4-Amino-2-(substituted methyl)-2-butenic Acids<sup>a</sup>

<sup>a</sup> Z represents carbobenzyloxyl and R is *tert*-butyl.

could undergo an enzyme-catalyzed vinylogous elimination of HX (Scheme I); the intermediate formed (5a) would be a vinylogue of the intermediate proposed for inactivation of GABA-T by 1. If an active-site nucleophile is near the 2-position of this bound molecule, inactivation may take place (pathway c, Scheme I). This would further map the active site of GABA-T and suggest other possibilities for mechanism-based inactivators of this enzyme. The results of our study are described in this paper.

## Results and Discussion

**Syntheses.** Prior to embarking on syntheses of 3, some assurance was needed that molecules of that general structure would be capable of binding to GABA-T. Johnston et al.<sup>22,23</sup> have determined the relative ability of a series of conformationally restricted analogues of GABA to act as substrates for GABA-T. In their study, they found that *trans*-4-aminocrotonic acid (6a, R = H) is transaminated by GABA-T at 1.8 times<sup>24</sup> the rate observed for GABA, whereas the transamination rate for the *cis* isomer (6b) was negligible. This indicates that GABA



probably is bound by GABA-T in an extended conformation. Furthermore, (*E*)-4-amino-2-methyl-2-butenic acid (6a, R = CH<sub>3</sub>) undergoes catalytic transamination at a rate comparable to that for the parent compound (6a, R = H).<sup>22,23</sup> Therefore, methyl substitution at the 2-position does not interfere with binding to GABA-T. The van der Waals radius of fluorine (1.35 Å) is close in size to that of hydrogen (1.2 Å); consequently, substitution of a fluoromethyl group for methyl should not have a significant

adverse steric effect on binding to that enzyme.

The structurally related halomethylcrotonates<sup>25</sup> and 4-amino-2-alkylcrotonic acids<sup>26</sup> have been synthesized. The challenge in the present synthetic problem was to incorporate these two sensitive functional groups, the amine at C-4 and the halogen at the 2-methyl position, without encountering elimination, decarboxylation, cyclization, or polymerization. Several routes to the syntheses of 3 were attempted. The successful route to these compounds is shown in Scheme II. The protecting groups for the amino acid originally chosen were phthaloyl and benzoyl for the amino group and methyl ester for the carboxylate; however, several problems were encountered with these protecting groups and finally Cbz *tert*-butyl ester were found to be satisfactory in all of the synthetic steps. The stereochemistry of 10 was determined when the benzoyl methyl ester of 10 was being used in the synthetic sequence. The appearance of a single vinylic proton in the NMR spectrum of this compound as evidenced by a triplet at 6.73 ppm indicated that only one of the two possible isomeric allylic alcohols had been formed in the elimination step. To substantiate this conclusion, the product was photolyzed to an equilibrium mixture of the *E* and *Z* isomers. After irradiation, the NMR spectrum showed two triplets in the vinylic region, one at 6.73 ppm, which corresponded to that in starting material, and a second broader absorption at 6.30 ppm, which developed as a result of irradiation, in a ratio of 55:45. Nair and Adams<sup>27</sup> have compiled NMR data on a series of trisubstituted olefinic acids and esters. They observed that in every case the vinyl proton of the *E* isomer appeared downfield of the *Z* isomer. Furthermore, since *cis*-allylic coupling is generally slightly greater (0.3–0.6 Hz) than *trans*-allylic coupling<sup>28</sup> (although both are close to 0 Hz), it would be expected that the *cis* (*Z*) isomer would be the one that appears broader as a result of additional *cis*-allylic coupling. On the basis of these data, the sole isomer that is produced in the selenoxide elimination is

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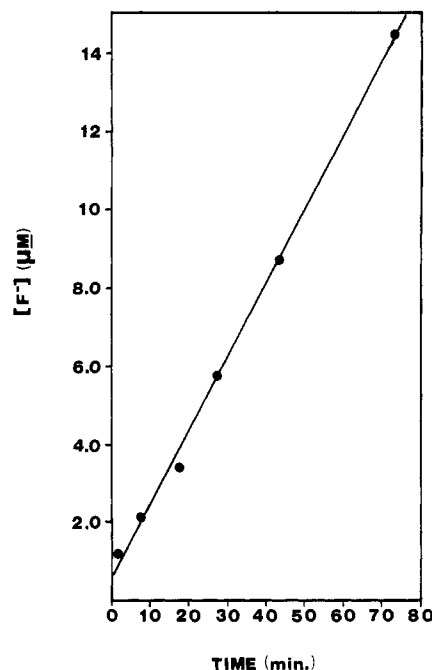
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**Table I.** Kinetic Constants for GABA and 4-Amino-2-(substituted methyl)-2-butenic Acids

compd	$K_m$ , mM	$K_i$ , <sup>b</sup> $\mu$ M	F <sup>-1</sup> release <sup>a</sup>		transamination	
			$K_m$ , $\mu$ M	$V_{max}$ , $\mu$ mol min <sup>-1</sup> mg <sup>-1</sup>	$K_m$ , $\mu$ M	$V_{max}$ , $\mu$ mol min <sup>-1</sup> mg <sup>-1</sup>
GABA	1.3 <sup>a</sup>					
	1.4 <sup>c</sup>					
3 (X = OH)		5			10	0.012
3 (X = Cl)		24				
3 (X = F)		19 <sup>a</sup>	130	0.36	140	0.088
		9 <sup>c</sup>				

<sup>a</sup> Pig brain GABA-T. <sup>b</sup> In the presence of 5 mM  $\alpha$ -ketoglutarate.<sup>c</sup> Bacterial GABA-T.assigned the *E* configuration (10).<sup>29</sup>

**Enzymology.** None of the compounds (3, X = OH, Cl, or F) showed time-dependent inhibition of GABA-T from either mammalian or bacterial sources. All were competitive reversible inhibitors; the kinetic constants are summarized in Table I. When the  $K_i$  value for 3 (X = F) was determined at varying concentrations of  $\alpha$ -ketoglutarate and was then extrapolated to zero  $\alpha$ -ketoglutarate concentration, the  $K_i$  value was 7  $\mu$ M. Since 3 (X = F) also is a substrate (vide infra) and the enzyme assay used to determine the  $K_i$  value was a coupled assay with SSADH, an experiment was carried out to show that the product of 3 (X = F) was not causing inhibition of the SSADH in the assay. The  $K_i$  value determined using a radioactive assay that measured the conversion of [<sup>14</sup>C]GABA to [<sup>14</sup>C]succinic semialdehyde was identical with that obtained with the coupled assay. Since the  $K_m$  for 4-amino-2-butenic acid was determined to be 1.3 mM, a substituent at the 2-position has a profound effect on the binding to GABA-T. Unlike the case of the 4-amino-5-halopentanoic acids (1, X = F or Cl) whose  $K_i$  values for the fluoro and chloro compounds are 395  $\mu$ M and 1.3 mM, respectively,<sup>4</sup> an increase in the size of the substituent for 3 (X = F, Cl) has a negligible effect on the  $K_i$ . This is similar to the insensitivity of the  $K_i$  of 4-amino-3-halobutanoic acids (2, X = F or Cl) on the size of the halogen substituent.<sup>21</sup> In this case, it was suggested<sup>21</sup> that the lack of a substituent effect was the result of a cavity in the active site at the 3-position of bound GABA. This also may be the case at the 2-position. The hydroxy-substituted compound, 3 (X = OH), however, differs vastly from the hydroxy compounds in the other two series. The  $K_i$  values for 4-amino-5-hydroxypentanoic acid (1, X = OH) and 4-amino-3-hydroxybutanoic acid (2, X = OH) are 70 and 67 mM, respectively,<sup>21</sup> which is  $1.4 \times 10^4$  times greater than that for 3 (X = OH). The high  $K_i$  values in the other two series was accounted for by intramolecular hydrogen bonding between the hydroxyl and amino groups (forming five-membered rings), which prevented Schiff base formation with the PLP. That the  $V_{max}$  values of these compounds were similar to that of GABA suggested that, once bound, these compounds were readily converted to product. Compound 3 (X = OH) cannot form a five- or six-membered ring by intramolecular hydrogen bonding and, therefore, its low  $K_i$  value lends further support for this intramolecular hydrogen bonding hypothesis in the other series. Since the  $K_i$  value of 3 (X = OH) is 4–5 times lower than that of 3 (X = Cl and F), there may be some stabilization via intermolecular hydrogen bonding between the hydroxyl and a group on the enzyme.

**Figure 1.** Time-dependent fluoride ion release from 3 (X = F) by GABA-T. See Experimental Section.

Since 3 (X = OH, Cl, F) were not time-dependent inactivators but were competitive inhibitors of GABA-T, it suggests that either elimination of HX does not occur (pathway a, Scheme I) or that there is no active site nucleophile properly juxtaposed for attack at the incipient electrophile (5a, Scheme I).<sup>30</sup> In order to differentiate these possibilities, enzyme-catalyzed loss of fluoride ion was monitored for 3 (X = F). A time-dependent (Figure 1) and concentration-dependent release of fluoride ion was observed. A plot of  $1/[F^-]$  (per hour) vs.  $1/[S]$  indicated saturation kinetics; a  $K_m$  of 130  $\mu$ M with a  $V_{max}$  of 0.36  $\mu$ mol min<sup>-1</sup> mg<sup>-1</sup> was determined. These experiments had to be carried out with no  $\beta$ -mercaptoethanol in the buffer at pH 8.5; otherwise, a nonenzymatic loss of fluoride ion was observed. Since the  $\beta$ -mercaptoethanol had little effect on nonenzymatic fluoride ion release at pH 7.0, it is believed that this nonenzymatic reaction is a function of thiolate concentration and may involve a Michael addition to the  $\alpha,\beta$ -unsaturated acid. In the absence of  $\beta$ -mercaptoethanol, essentially no nonenzymatic fluoride ion release was observed, even at pH 8.5. The enzyme-catalyzed loss of fluoride ion suggests that elimination is occurring and that lack of time-dependent inactivation may be the result of another void in the active site near the 2-position of bound GABA.<sup>30</sup> Elimination of fluoride ion is not surprising considering that this reaction occurs with 4-amino-5-fluoropentanoic acid (1, X = F)<sup>15</sup> and 4-amino-3-fluorobutanoic acid (2, X = F).<sup>21</sup> In both of these cases, elimination is essentially the only reaction that occurs; no transamination was observed with either. In the case of 4-amino-5-hydroxypentanoic acid (1, X = OH) and 4-amino-3-hydroxybutanoic acid (2, X = OH), only transamination occurred and no elimination was evident.<sup>21</sup> Transamination of 3 (X = OH and F) was monitored. Both undergo transamination; the  $K_m$  values are shown in Table I. The virtual identity of the  $K_m$  values for elimination (F<sup>-</sup> release) and transamination of 3 (X = F) supports the hypothesis of a common intermediate from

(29) It should be noted that, although the absolute configurations of 11 and 12 are identical with that of 10, they are assigned the *Z* configuration because of the halogens.

(30) If a mechanism related to that proposed by Metzler<sup>16</sup> were operative, it would be expected that elimination to 5a still would lead to inactivation.

which both reactions can occur (4, Scheme I, pathways a and b, respectively). Although the evidence for an elimination reaction only comes from measurement of fluoride ion release, this is a reasonable hypothesis since  $\gamma$ -proton removal could initiate both transamination and elimination reactions. A comparison of the amount of fluoride ion released and the amount of glutamate formed from  $\alpha$ -ketoglutarate (a measure of transamination) indicates that there is a ratio of 4:1 in favor of the elimination reaction (pathway a, Scheme I). This 4:1 ratio corresponds well with the  $V_{\max}$  ratio for fluoride ion release and transamination. Apparently, because of the double bond in 3 ( $X = F$ ), the extended  $\pi$ -system resulting from  $\gamma$ -proton removal stabilizes the adduct formed by azallylic isomerization (5b) and, therefore, permits transamination to occur 20% of the time. The low  $K_m$  value for 3 ( $X = OH$ ) again suggests not only lack of interference by but, possibly, a stabilization via hydrogen bonding.

In summary, the 4-amino-2-(substituted methyl)-2-butenic acid series (3,  $X = OH, Cl, F$ ) complements the previous two series.<sup>4,15,21</sup> When  $X = F$ , elimination occurs but without inactivation. Unlike the other two series of compounds,<sup>4,15,21</sup> 3 ( $X = F$ ) also undergoes transamination as well as elimination. These results may suggest that attempts to design mechanism-based inactivators should be directed toward compounds leading to electrophile generation near the 4-position of bound GABA analogues. For increased binding, incorporation of a 2-hydroxymethyl group (or other hydrogen-bonding substituent) and a 2,3-double bond into the molecule may be advantageous.

## Experimental Section

**General Analytical Methods.** Proton magnetic resonance spectra were recorded on a Varian EM 360-A spectrometer. Chemical shifts are reported as  $\delta$  values in parts per million relative to tetramethylsilane ( $\delta$  0.0) as an internal standard. For samples run in  $D_2O$ , the HOD resonance was arbitrarily set at  $\delta$  4.50. Infrared spectra were obtained on a Perkin-Elmer 283 infrared spectrophotometer. Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Elemental combustion analyses were performed by Microtech Laboratories, Skokie, IL. Enzyme activity was measured, as described under Enzymes and Assays, spectrophotometrically on either a Gilford 222 or a Perkin-Elmer Lambda 1 spectrophotometer with a constant temperature cuvette holder. Radioactivity was measured in a Beckman LS-3100 scintillation counter using 10 mL of either 3a70B (Research Products International) or Ready-Solve MP (Beckman) scintillation fluid.  $[U-^{14}C]$ -Toluene ( $4.7 \times 10^5$  dpm/mL), obtained from New England Nuclear, was used as an internal standard. Protein concentrations were determined by the method of Lowry et al.,<sup>31</sup> using bovine serum albumin as a standard. Fluoride ion concentrations were determined with an Orion Model 96-09 combination fluoride ion electrode attached to an Orion Model 601 pH meter and calibrated with sodium fluoride standard solution (Orion 94-09-06). Dowex 50  $\times$  8 ion-exchange resin was supplied by Bio-Rad Laboratories. Silica gel columns for flash chromatography utilized E. Merck silica gel 60 (230–400-mesh ASTM). Analytical thin-layer chromatography (TLC) was conducted on E. Merck silica gel 60F-254 precoated TLC plates. The chromatography of amino acids was carried out with E. Merck precoated cellulose TLC plates.

**Reagents.** Benzene and toluene were distilled under argon from sodium metal. Hexamethylphosphoramide and dimethylformamide were distilled under reduced pressure from calcium hydride. All amines were distilled under argon from barium oxide. Chloroform was distilled from phosphorus pentoxide, and methylene chloride and acetonitrile were distilled from powdered calcium hydride. Tetrahydrofuran (THF) and diethyl ether were distilled under nitrogen from sodium metal with sodium benzo-

phenone ketyl as an indicator. All other reagents and solvents were reagent grade, unless otherwise noted. All nonaqueous reactions were carried out under an argon atmosphere. The in-house distilled water was further purified by passage through a deionizer (Continental Water Conditioning Corp.) before use. Potassium pyrophosphate,  $\alpha$ -ketoglutaric acid,  $\beta$ -mercaptoethanol, NADP, and glycerol were purchased from Sigma Chemical Co. and used without further purification. GABA also was purchased from Sigma but was recrystallized from water/ethanol before use.  $[U-^{14}C]$ - $\alpha$ -Ketoglutaric acid (sp act. 240 mCi/mmol) was purchased from ICN Biomedicals, Inc., and  $[U-^{14}C]$ GABA (sp act. 224 mCi/mmol) was bought from Amersham.

**Photoisomerization of Methyl (E)-4-(Benzoylamino)-2-(hydroxymethyl)-2-butenate.** A solution of methyl (E)-4-(benzoylamino)-2-(hydroxymethyl)-2-butenate<sup>32</sup> in methanol (0.025 M) was sealed in a quartz test tube with a septum cap. The tube was placed in a merry-go-round photolysis apparatus and irradiated through a Vycor well with a 450-W Hanovia mercury arc lamp for 4 h. Evaporation of the solvent gave a yellow oil. The NMR spectrum was that of a mixture of starting material and another compound; vinyl protons appeared at  $\delta$  6.73 (t, 1 H) and 6.30 (t, 1 H) in the ratio 55:45.

**tert-Butyl 4-[(Benzoyloxycarbonyl)amino]-2-(phenylseleno)butanoate (8).** A solution of diisopropylamine (6.15 mL, 44.0 mmol) in *N,N,N',N'*-tetramethylethylenediamine (TMEDA; 5.3 mL) and THF (80 mL) was cooled to  $-78^\circ C$ , and *n*-butyllithium in hexane (21.0 mL, 2.1 M, 44.0 mmol) was added dropwise. A solution of *tert*-butyl 4-[(benzyloxycarbonyl)amino]butanoate<sup>34</sup> (4.30 g, 14.7 mmol) in THF (40 mL) was added over a 10-min period, and the resultant yellow solution was stirred at  $-78^\circ C$  for 30 min. A phenylselenenyl bromide solution was prepared by adding bromine (0.414 mL, 8.08 mmol) to a stirred solution of diphenyl diselenide (2.52 g, 8.08 mmol) in THF (45 mL). Rapid addition of this solution to the reaction mixture at  $-78^\circ C$  gave a clear yellow solution, which, after 30 min, was quenched with 2 N hydrochloric acid (35 mL). The mixture was poured into ether, and the separated ether layer was washed with 0.1 N hydrochloric acid (three times), saturated sodium bicarbonate (one time), and brine (one time). After drying ( $MgSO_4$ ), the ethereal solution was evaporated to give a bright yellow oil, which upon purification (silica gel,  $3 \times 30$  cm column, 1:49 to 3:7 ethyl acetate–hexane gradient elution) afforded 8 (5.19 g, 79%) as a yellow oil: IR (film) 3340 (br), 2970 (m), 1710 (s), 1515 (s), 1245 (s), 1130 (s)  $cm^{-1}$ ; NMR ( $CDCl_3$ )  $\delta$  1.36 (s, 9 H), 1.95 (m, 2 H), 3.25 (m, 2 H), 3.52 (m, 1 H), 5.00 (br s, 1 H), 5.03 (s, 2 H), 7.26 (s, 5 H), 7.1–7.7 (m, 5 H).

**tert-Butyl 4-[(Benzoyloxycarbonyl)amino]-2-(hydroxymethyl)-2-(phenylseleno)butanoate (9).** A solution of diisopropylamine (4.85 mL, 34.7 mmol) in TMEDA (1.95 mL) and THF (13.0 mL) was cooled to  $-78^\circ C$ , and *n*-butyllithium in hexane (16.5 mL, 2.1 M, 34.7 mmol) was added dropwise. A solution of 8 (5.19 g, 11.6 mmol) in THF (13.0 mL) was added dropwise over 10 min, and the resultant solution was stirred for 10 min. The dry ice–acetone bath was then replaced by an ethanol ice bath ( $-30^\circ C$ ), and the reaction flask was quickly fitted with a two-holed rubber stopper, which provided an inlet and an exit port. In another flask, paraformaldehyde (1.74 g,  $\sim 5$  equiv) was cracked at  $150^\circ C$ , and the formaldehyde generated was blown through the inlet, over the surface of the reaction solution, with a stream of argon. Gases were allowed to escape from the vessel via a bubbler that was attached to the exit port. The reaction solution was stirred rapidly with a large magnetic stir bar while formaldehyde was flowing through the flask; THF was added as needed to facilitate stirring. When all of the paraformaldehyde had been consumed, the reaction was quenched with 2 N hydrochloric acid (90 mL). The mixture was poured into ether and filtered, and the ether layer was washed twice with saturated ammonium chloride, dried ( $MgSO_4$ ), and evaporated to a yellow oil. Purification by silica gel column chromatography ( $3 \times 35$  cm, elution with 1:3 ethyl acetate–hexane) gave 9 (2.70 g, 49%) as a pale yellow oil: IR (5% solution in  $CHCl_3$ ) 3450 (br), 2975 (m), 1705 (s), 1507

(31) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* 1951, 193, 265–275.

(32) This compound was prepared by the same procedures to make 10, starting from methyl *N*-benzoyl-4-aminobutanoate.<sup>33</sup>

(33) Kanewskaya, S. J. *Ber.* 1936, 69, 266–273.

(34) Fosker, A. P.; Law, H. D. *J. Chem. Soc.* 1965, 7305–7312.

(s), 1230 (s), 1150 (s)  $\text{cm}^{-1}$ ; NMR ( $\text{CDCl}_3$ )  $\delta$  1.42 (s, 9 H), 1.98 (m, 2 H), 2.63 (br s, 1 H), 3.32 (m, 2 H), 3.70 (s, 2 H), 5.01 (br s, 1 H), 5.03 (s, 2 H), 7.27 (s, 5 H), 7.1–7.7 (m, 5 H).

**tert-Butyl (E)-4-[(Benzyloxycarbonyl)amino]-2-(hydroxymethyl)-2-butenate (10).** The procedure of Reich et al.<sup>35</sup> was modified. A solution of 9 (2.44 g, 5.1 mmol) in dichloromethane (13 mL) was cooled to  $-10^\circ\text{C}$ , and *m*-chloroperoxybenzoic acid (1.76 g, 10.2 mmol) was added over 15 min. The resultant mixture was stirred for 1 h at  $-10^\circ\text{C}$ . The addition of diisopropylamine (2.14 mL, 15.3 mmol) then gave a homogeneous solution, which was rapidly transferred via cannula into a flask of refluxing carbon tetrachloride. Reflux was maintained for 5 min, after which the heating bath was removed and the solution was allowed to cool. The solution was then poured into saturated aqueous sodium bicarbonate and extracted with methylene chloride. Evaporation of the extracts afforded the crude product as a yellow oil, which, upon silica gel chromatography (3  $\times$  30 cm, 1:1 ethyl acetate–*n*-hexane elution), yielded 10 (0.897 g, 55%) as a pale yellow oil: IR (5% solution in  $\text{CHCl}_3$ ) 3440 (m), 2970 (m), 1700 (s), 1500 (m), 1220 (s), 1120 (s)  $\text{cm}^{-1}$ ; NMR ( $\text{CDCl}_3$ )  $\delta$  1.47 (s, 9 H), 3.18 (br s, 1 H), 3.93 (m, 2 H), 4.27 (s, 2 H), 5.05 (s, 2 H), 5.33 (br s, 1 H), 6.60 (t, 1 H), 7.26 (s, 5 H).

**tert-Butyl (Z)-4-[(Benzyloxycarbonyl)amino]-2-(chloromethyl)-2-butenate (11).** A solution of 10 (0.274 g, 0.856 mmol) in chloroform (1.0 mL) was cooled to  $0^\circ\text{C}$ , and TMEDA (0.129 mL, 0.856 mmol) was added. Tosyl chloride (0.327 g, 1.71 mmol) was then added in four portions over a 15-min period. The tosylation of the alcohol, monitored by TLC (silica gel, 2% methanol in chloroform), was more than 90% complete after 30 min. At this point, lithium chloride (0.252 g, 6.00 mmol) was added to the reaction mixture, followed by the dropwise addition of hexamethylphosphoramide (4.0 mL), which gave a tan suspension. The suspension was stirred at  $0^\circ\text{C}$  for 1.5 h and then was poured into a separatory funnel containing ether and 1 N hydrochloric acid. The organic layer was separated and washed with 1 N hydrochloric acid (two times) and brine (one time), dried ( $\text{MgSO}_4$ ), and evaporated to give a yellow oil. Separation of the product from residual tosyl chloride was effected by silica gel column chromatography (1.4  $\times$  23 cm, 2:8 hexane–chloroform elution), giving 11 (0.203 g, 70%) as a yellow oil. Crystallization of a small sample from chloroform–hexane gave colorless crystals: mp  $93\text{--}94^\circ\text{C}$ ; IR (5% solution in  $\text{CHCl}_3$ ) 3445 (m), 2980 (m), 1705 (s), 1500 (s), 1365 (sh), 1150 (s)  $\text{cm}^{-1}$ ; NMR ( $\text{CDCl}_3$ )  $\delta$  1.48 (s, 9 H), 3.98 (m, 2 H), 4.25 (s, 2 H), 5.07 (s, 2 H), 5.10 (br s, 1 H), 6.71 (t, 1 H), 7.28 (s, 5 H).

**(Z)-4-Amino-2-(chloromethyl)-2-butenic Acid Hydrochloride (3, X = Cl).** A solution of 11 (0.195 g, 0.575 mmol) in trifluoroacetic acid (1.8 mL) was treated with concentrated hydrochloric acid (0.30 mL) and was allowed to stand for 1 h with occasional agitation. The solution was then poured into 20 mL of water and extracted twice with ethyl acetate. Evaporation of the aqueous solution under vacuum gave the crude product (0.0647 g, 60%) as a yellow solid, which upon recrystallization from ethanol–ethyl acetate afforded colorless crystals (0.0494 g, 46%): TLC (silica gel; 1-butanol–acetic acid–water, 4:1:2),  $R_f$  0.48; mp  $153^\circ\text{C}$  dec; IR (KBr) 3410 (br m), 2970 (br s), 1690 (br), 1650 (m), 1415 (m), 1270 (m), 1195 (m)  $\text{cm}^{-1}$ ; NMR ( $\text{D}_2\text{O}$ )  $\delta$  3.78 (d, 2 H), 4.17 (s, 2 H), 4.50 (s, HDO), 6.71 (t, 1 H). Anal. ( $\text{C}_5\text{H}_9\text{Cl}_2\text{NO}_2$ ) C, H, N; Cl: calcd, 38.11; found, 36.86. Further recrystallization led to increased decomposition.

**(E)-4-Amino-2-(hydroxymethyl)-2-butenic Acid Hydrochloride (3, X = OH).** The procedure described for the synthesis of 3 (X = Cl) was employed in the conversion of 10 (0.275 g, 0.856 mmol) to the amino acid hydrochloride (3, X = OH) (0.203 g, 70%): mp  $134\text{--}136^\circ\text{C}$ ; IR (KBr) 3250 (br), 3010 (br), 1710 (s), 1475 (m), 1340 (m), 1270 (m), 1160 (m), 1010 (s)  $\text{cm}^{-1}$ ; NMR ( $\text{D}_2\text{O}$ )  $\delta$  3.75 (d, 2 H), 4.15 (s, 2 H), 4.50 (s, HDO), 6.66 (t, 1 H). Anal. ( $\text{C}_5\text{H}_{10}\text{ClNO}_3$ ) C, H, Cl, N.

**tert-Butyl (Z)-4-[(benzyloxycarbonyl)amino]-2-(fluoromethyl)-2-butenate (12).** The procedure of Middleton<sup>36</sup> was adapted. A solution of 10 (1.65 g, 5.14 mmol) in methylene

chloride (6 mL) was added dropwise to a cold solution ( $-78^\circ\text{C}$ ) of (diethylamino)sulfur trifluoride (1.65 g, 10.3 mmol) in methylene chloride (9 mL) over a 15-min period. After 1 h at  $-78^\circ\text{C}$ , the dry ice–acetone bath was removed and the reaction solution was stirred for 1 h at room temperature. The solution was then poured into a separatory funnel containing methylene chloride and brine, and the organic layer was separated and washed with brine (two times), dried ( $\text{MgSO}_4$ ), and evaporated to a yellow oil. Chromatography of the crude product (silica gel, 2.95  $\times$  39 cm column, 1:4 ethyl acetate–hexane elution) afforded 12 (0.911 g, 55%) as a yellow oil: IR (5% solution in chloroform) 3445 (m), 2975 (m), 2920 (m), 1705 (s), 1500 (s), 1365 (sh), 1200 (s), 1135 (s)  $\text{cm}^{-1}$ ; NMR ( $\text{CDCl}_3$ )  $\delta$  1.46 (s, 9 H), 4.00 (m, 2 H), 5.06 (s, 2 H), 5.09 (d,  $J_{\text{HF}}$  = 47 Hz, 2 H), 5.25 (br s, 1 H), 6.82 (m, 1 H), 7.28 (s, 5 H).

**(Z)-4-Amino-2-(fluoromethyl)-2-butenic Acid Hydrochloride (3, X = F).** The procedure described for 3 (X = Cl) was employed in the conversion of 12 (0.898 g, 2.78 mmol) to 3 (X = F) (0.300 g, 64%) as a yellow solid. This solid was re-evaporated several times from dilute hydrochloric acid and recrystallized from methanol–ethyl acetate to give the hydrochloride salt as colorless crystals (0.208 g, 44%): mp  $128\text{--}131^\circ\text{C}$  dec; IR (KBr) 3400 (m), 3000 (br s), 1700 (s), 1390 (s), 1270 (m), 1170 (m)  $\text{cm}^{-1}$ ; NMR ( $\text{D}_2\text{O}$ )  $\delta$  3.82 (m, 2 H), 4.50 (s, HDO), 5.04 (d,  $J_{\text{HF}}$  = 47 Hz, 2 H), 6.81 (m, 1 H). Anal. ( $\text{C}_5\text{H}_9\text{ClFNO}_2$ ) C, H, Cl, F, N.

**Enzymes and Assays.** Pig brain GABA-T, Gabase, and succinic semialdehyde dehydrogenase (SSADH) were obtained and assayed as described previously.<sup>15</sup>

**Time-Dependent Inactivation of GABA-T by 3 (X = F).** Pig brain GABA-T (0.011 unit) was incubated at  $30^\circ\text{C}$  with 3 (X = F) (0.5 mM) in 50 mM potassium pyrophosphate buffer, pH 8.5, containing 0.8 mM  $\alpha$ -ketoglutarate. An identical sample, which contained no 3 (X = F), served as a control. At time intervals up to 100 min, 30- $\mu\text{L}$  aliquots of either sample or control were assayed in a final volume of 575  $\mu\text{L}$ . The same experiment was carried out with Gabase (0.43 unit), using 10 mM of 3 (X = F).

**Inhibition of GABA-T by 3 (X = OH, Cl, F) Using a Coupled Assay.** A 30- $\mu\text{L}$  aliquot of pig brain GABA-T (0.011 unit) was diluted to a total volume of 600  $\mu\text{L}$  to give a solution containing 50 mM potassium pyrophosphate buffer (pH 8.5), 5 mM  $\alpha$ -ketoglutarate, 1 mM NADP excess SSADH, and varying concentrations of GABA and inhibitors at  $25^\circ\text{C}$ . Initial rates, which were linear for at least 5 min, were measured by recording the increase in the 340-nm absorbance with time. These rate data were treated by the method of Lineweaver and Burk,<sup>37</sup> and kinetic constants were obtained from a linear regression analysis (correlation coefficients  $> 0.99$ ) of the best line. A similar experiment was performed with use of Gabase in the presence of 5 mM  $\beta$ -mercaptoethanol.

**Inhibition of GABA-T by 3 (X = F) Using a Radioactive Assay.** Pig brain GABA-T (0.007 unit) was incubated at  $25^\circ\text{C}$  with 5 mM  $\alpha$ -ketoglutarate and varying concentrations of [ $^{14}\text{C}$ ]GABA in the absence of 3 (X = F) (run 1) or in the presence of 11  $\mu\text{M}$  3 (X = F) (run 2) or 22  $\mu\text{M}$  3 (X = F) (run 3) in a total volume of 200  $\mu\text{L}$ . The reactions were initiated by addition of enzyme and allowed to proceed for 60 min at which time the reactions were quenched by the addition of 20  $\mu\text{L}$  of 20% (w/w) aqueous TCA. The quenched reaction mixtures were applied to Dowex 50 ( $\text{H}^+$  form) (0.5  $\times$  5.5 cm), and the columns were washed with 5 mL of water. The eluant, containing non-amines, were collected and counted for radioactivity. The data from each run were plotted as  $\text{cpm}^{-1}$  vs.  $[\text{GABA}]^{-1}$ .

**GABA-T-Catalyzed Transamination of 3 (X = OH and F).** Transamination of 3 (X = OH and F) was determined by measuring the conversion of [ $^{14}\text{C}$ ]- $\alpha$ -ketoglutarate to [ $^{14}\text{C}$ ]glutamate at various concentrations of 3 (X = OH and F) as described previously.<sup>21</sup> A sample containing no enzyme and one containing no substrate served as controls. Kinetic constants ( $K_m$  and  $V_{\text{max}}$ ) were determined from linear regression analyses (correlation coefficients  $> 0.99$ ) of Lineweaver–Burk plots.<sup>37</sup>

**GABA-T-Catalyzed Release of Fluoride Ion from 3 (X = F).** Pig brain GABA-T (0.022 unit) was incubated at  $25^\circ\text{C}$  with various concentrations of 3 (X = F) in a total volume of 600  $\mu\text{L}$

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of 50 mM potassium pyrophosphate buffer, pH 8.5, containing 5 mM  $\alpha$ -ketoglutarate. Controls for each concentration of **3** ( $X = F$ ) excluding enzyme were run concurrently. After a 60-min incubation period, a 550- $\mu$ L aliquot of each sample was removed and added to 550  $\mu$ L of a total ionic strength adjusting buffer (57 mL of glacial acetic acid, 58 g of NaCl, and 0.30 g of sodium citrate diluted to 500 mL with  $H_2O$ ; pH 5.25) in a plastic vial and the concentration of fluoride ion measured with a fluoride ion electrode. Kinetic constants ( $K_m$  and  $V_{max}$ ) were determined from linear regression analyses (correlation coefficients > 0.99) of Lineweaver-Burk plots.<sup>37</sup>

**Time-Dependent Fluoride Ion Release from **3** ( $X = F$ ).** Pig brain GABA-T (0.11 unit) was incubated at 25 °C in 5.0 mL of a solution containing 0.12 mM **3** ( $X = F$ ), 5 mM  $\alpha$ -ketoglutarate, 50 mM potassium phosphate, and 5 mM  $\beta$ -mercaptoethanol at pH 7.0. A similar sample containing no enzyme served as a control. At various time intervals, 550- $\mu$ L aliquots of the reaction mixture

were removed, quenched by addition to an equal volume of total ionic strength adjusting buffer, and analyzed for fluoride ion concentration. Virtually no fluoride ion was released in the control after 60 min. The concentration of fluoride ion in the reaction mixture was plotted as a function of time.

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**Registry No.** GABA, 56-12-2; GABA-T, 9037-67-6; (*Z*)-**3** ( $x = Cl$ ), 100702-76-9; (*Z*)-**3** ( $x = Cl$ ), free base, 100702-84-9; (*Z*)-**3** ( $x = F$ ), 100702-78-1; (*Z*)-**3** ( $x = F$ ), free base, 100702-86-1; (*E*)-**3** ( $x = OH$ ), 100702-77-0; (*E*)-**3** ( $x = OH$ ), free base, 100702-85-0; **7** ( $R = Bu-t$ ), 5105-79-3; **8** ( $R = Bu-t$ ), 100702-79-2; **9** ( $R = Bu-t$ ), 100702-80-5; (*E*)-**10** ( $R = Bu-t$ ), 100702-81-6; (*Z*)-**11** ( $R = Bu-t$ ), 100702-82-7; (*Z*)-**12** ( $R = Bu-t$ ), 100702-83-8; PhSeBr, 34837-55-3; PhCONHCH<sub>2</sub>CH=C(CH<sub>2</sub>OH)CO<sub>2</sub>Me-(*E*), 100702-87-2.

## Improved Glucose Tolerance in Rats Treated with Oxazolidinediones

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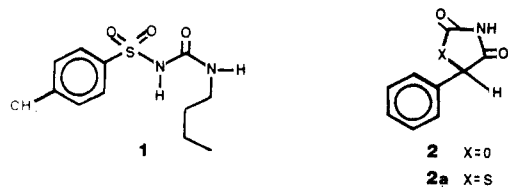
Central Research, Pfizer Inc., Groton, Connecticut 06340. Received August 9, 1985

5-(2-Chloro-6-methoxyphenyl)oxazolidine-2,4-dione (**49**) is the most potent agent selected from a series of 5-substituted oxazolidinediones that were found to cause improvements in glucose tolerance in previously fasted rats and potentiation of insulin release in response to a glucose challenge. These compounds were unique in not producing hypoglycemia below the normal fasting glycemia levels. Substituent effects at positions 2-6 of the phenyl ring were investigated. Optimal positions for substitution were found to be the 2-, 5-, and 6-positions. Variations in the oxazolidinedione ring generally lead to loss of activity. The synthesis and structure-activity relationships of this series are detailed.

The search for drugs to treat non-insulin-dependent diabetes mellitus (NIDDM), recently estimated as 2.1% diagnosed in the population of the United States,<sup>1</sup> has been widely pursued for the past four decades. The culmination of early efforts was reported in the milestone papers of Haack<sup>2a</sup> and Ruschig<sup>2b</sup> describing the sulfonylurea (SU) drugs, e.g., tolbutamide (**1**), and was more recently reviewed by Sarges<sup>1</sup> and others.<sup>3</sup> However, after nearly 30 years of research, therapy for diabetics of this type remains largely restricted to the sulfonylureas<sup>4</sup> (SUs). Although SUs are valuable therapy, they possess disadvantages, e.g., primary or secondary failure of efficacy and induction of hypoglycemia, which have stimulated scientists to seek better antidiabetic drugs. Numerous other non-sulfonylurea classes of compounds have been clinically investigated in NIDDMs, with the most promising agents being varieties of aromatic and aliphatic carboxylic acids. Unfortunately, none of these has been sufficiently efficacious or well tolerated in man to have reached market status.

Because the greatest majority of hypoglycemic drugs discovered thus far have been acidic,<sup>5</sup> our research began a number of years ago to focus on acidic substances, excluding, however, the sulfonylurea class and the carboxylic acid functionality.<sup>6</sup> Attention was focused initially on heterocyclic acids and  $\beta$ -dicarbonyl structures. Among a group of simple aromatic acidic heterocycles chosen empirically for testing in the fasted rat glucose tolerance test, compounds **2** and **2a** showed remarkable improvements in iv glucose tolerance. Unique to **2**, as compared to **1**, was the powerful control of glycemia without a subsequent hypoglycemic effect commonly found with sulfonylureas<sup>7</sup> and carboxylic acids (Figure 1). Because of the significance of this observation and the attractive toleration profile found upon further pharmacological experimentation, **2** was selected as a lead to pursue extensive structure-activity relationship (SAR) studies. Some of the results of these investigations are reported here.

**Chemistry.** Recent advances in the synthesis of oxazolidinediones,<sup>8</sup> as well as earlier synthetic procedures,<sup>9</sup> were used to prepare the target molecules.<sup>10</sup> The method shown in Figure 2, starting with the readily available aldehydes, generally was found most expedient and reliable.



- (1) Sarges, R. *Prog. Med. Chem.* **1981**, *18*, 191.
- (2) (a) Haack, E. *Arzneim.-Forsch.* **1958**, *8*, 444. (b) Ruschig, H.; Korger, G.; Aumüller, N.; Wagner, H.; Weyer, R.; Bander, A.; Scholz, J. *Arzneim.-Forsch.* **1958**, *8*, 448.
- (3) Other reviews: Keller, U.; Berger, W. *Schweiz. Med. Wochenschr.* **1983**, *113*, 645. Rasmussen, C. R. Maryanoff, B. E.; Tutwiler, G. F. *Ann. Rep. Med. Chem.* **1981**, *16*, 173 and references therein.
- (4) The extent of biguanide therapy is minor and limited to some foreign markets; see ref 1.

- (5) The  $pK_a$  of tolbutamide, **1**, is 7.14 in 1:1 dioxane- $H_2O$ ; other drugs are metabolized to acids.<sup>1</sup>
- (6) The Sarges review summarizes the outcome of many of these clinical drug candidates.<sup>1</sup> Wreber, H.; Aumüller, W.; Muth, K.; Weyer, R.; Heerdt, R.; Fauland, E.; Bander, A.; Pfaff, W.; Schmidt, F. H.; Stork, H. *Arzneim.-Forsch.* **1969**, *19*, 1326. Rufer, C.; Biere, H.; Ahrens, H.; Loge, O.; Schroder, E. *J. Med. Chem.* **1974**, *17*, 708.
- (7) Latter time points of Figure 1. The dangers of unpredictable hypoglycemic episodes involve coma and sometimes death.
- (8) Schnur, R. C.; Sarges, R.; Peterson, M. J. *J. Med. Chem.* **1982**, *25*, 1451 and references therein.
- (9) The chemistry of oxazolidinediones has been reviewed: Clark-Lewis, J. W. *Chem. Rev.* **1958**, *58*, 63.
- (10) Schnur, R. C. U.S. Patent 4 399 296, 1983; 4 407 811, 1983; 4 448 971, 1984.