these incubations, the rate of acetaldehyde disappearance represents a combination of the activities of the high- and low-K_m AlDH isozymes. AlDH activity is expressed in units of nanomoles of AcH oxidized per minute per milligram of protein. The protein concentration was determined by the method of Lowry et al.[21]

Effect of CP Analogues on Yeast AlDH Activity (Table I). Commercial yeast AlDH was preincubated for 10 min at 37 °C in a primary reaction mix containing 1.0 mM of the analogue (prepared in DMSO and added to mix in 5 μL), 0.074 IU of yeast AlDH, and 100 mM potassium phosphate, pH 7.4, in a total volume of 0.1 mL. At 10 min, a 20-μL aliquot of the primary mix was added to a cuvette containing 0.5 mM NAD^+, 1.0 mM EDTA, 30% glycerol, and 80 mM potassium phosphate (pH 8.0) in a final volume of 1.0 mL. This secondary reaction was initiated by the addition of benzaldehyde (0.6 μmol). The yeast AlDH activity was determined spectrophotometrically by following the increase in absorbance at 340 nm with time.

Blood Glucose Determination (Figure 3). Fasting blood glucose levels were determined 4 h after administration of the CP analogues essentially as described previously.[5]

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Metabolism of 3-(*p*-Chlorophenyl)pyrrolidine. Structural Effects in Conversion of a Prototype γ-Aminobutyric Acid Prodrug to Lactam and γ-Aminobutyric Acid Type Metabolites

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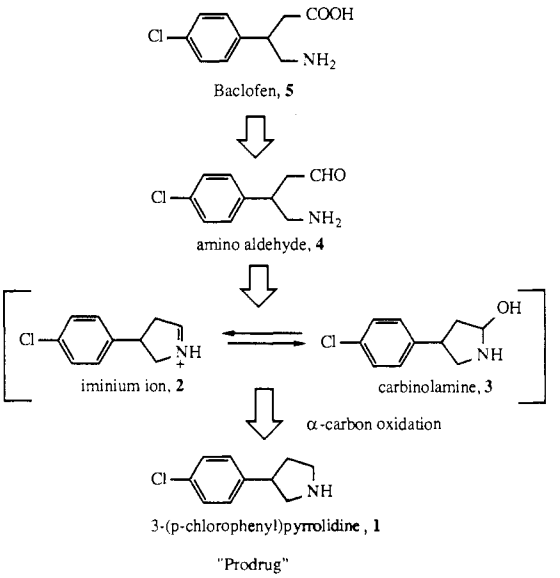
By use of rat liver or brain homogenate supernatants containing microsomes and/or mitochondria, it was found that the prototype GABAergic prodrug [3-(*p*-chlorophenyl)pyrrolidine (1)] underwent a series of α-oxidation transformations to a pair of amino acid metabolites and a pair of lactam metabolites [4-amino-3-(*p*-chlorophenyl)butanoic acid, baclofen (**5**); 4-amino-2-(*p*-chlorophenyl)butanoic acid (**10**); 4-(chlorophenyl)pyrrolidin-2-one (**6**); and 3-(*p*-chlorophenyl)pyrrolidine-2-one (11)]. With the liver homogenates, the formation of the lactam metabolites was approximately 2 orders of magnitude greater than that of the amino acid metabolites, while with the brain homogenates, the amino acid and lactam pathways were of similar magnitude. For either tissue, for both the lactam and the amino acid series, attack at the less sterically hindered 5-position of the pyrrolidine ring was greater than the attack at the 2-position (**5** > **10** and **6** > 11) with the exception of the liver homogenate mitochondrial fraction (**6** < 11). The parenteral administration of the prodrug 1 was found to give detectable brain levels of **5** as well as activity in an isoniazid-induced (GABA-inhibited) convulsion model.

γ-Aminobutyric acid (GABA), first discovered about 40 years ago in the central nervous system (CNS) of a variety of animals,[1] has been shown to be an important CNS neurotransmitter.[2] GABAergic mechanisms have been implicated in analgesia,[3] Alzheimer's disease,[4] cardiovascular function,[5,6] epilepsy,[7,8] Huntington's chorea,[9] Parkinson's disease,[10] and schizophrenia.[11] The greatest amount of research into GABA mechanisms, agonists, and antagonists has been in the area of epilepsy. Studies have shown persons who suffer from a variety of epilepsies to be deficient in brain GABA levels as compared to nonepileptics.[12,13] Also, a number of drugs currently used as anticonvulsants, including barbiturates,[8,14] benzodiazepines,[8,15] hydantions,[8] and valproic acid,[8] have been purported to act through the GABAergic pathway. While GABA itself has not been shown to be useful,[16,17] many

Scheme I. Retrometabolic Pathway to Baclofen



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attempts have been made over the last decade to produce GABAergic drugs and prodrugs. Krogsgaard-Larsen et al.[18-20] have performed an extensive series of investigations on GABAergic agonists, prodrugs, and antagonists, mostly analogues and derivatives of GABA, muscimol, and the nipecotic acid families of compounds.

A variety of simple GABA prodrugs (usually esters, amides, or Schiff bases) linked to lipophilic carriers have been developed.[21-24] Two of the most promising GABAergic agents developed during the last decade were γ-vinyl-GABA[25,26] and progabide,[27,28] both currently in clinical trials for epilepsy. An additional attempt at producing a lipophilic GABAergic agent resulted in the drug baclofen [4-amino-3-(*p*-chlorophenyl)butanoic acid, Lioresal],[29] which was shown to be efficacious against spasticity.[30] Despite the additional lipophilicity compared to GABA, less than 1% of an oral dose of baclofen was shown to cross the blood–brain barrier into the CNS,[31] where it was hypothesized to act on GABA receptors in the spinal cord.[32] However, initial attempts to establish baclofen as an agonist at the classical GABA receptor failed.[33] In the early 1980s, a new GABA receptor was discovered in the CNS[34,35] for which most of the previously known GABA agonists had little or no affinity.[36] The classical GABA receptor has been designated the $GABA_A$ receptor, while the newer receptor, for which baclofen is the only known selective agonist,[33,36] has been designated the $GABA_B$ receptor.[35]

In the present study, a "retrometabolic" approach was taken to the design of a prodrug that would mask the polarity of the prototype $GABA_B$ agent baclofen (Scheme I). In view of the low CNS absorption of baclofen, it was thought that a more lipophilic prodrug, such as **1**, would be more suited to crossing the blood–brain barrier where conversion to a baclofen-type compound (GABAergic agonist) could take place. The production of baclofen required the oxidation of an amino aldehyde, **4**, arising from the α-carbon oxidation of the prodrug, 3-(*p*-chlorophenyl)pyrrolidine (**1**). The initial α-carbon oxidation step has been hypothesized to proceed through either a iminium ion, **2**,[37,38] or carbinolamine, **3**,[39-42] intermediate, or both.[43]

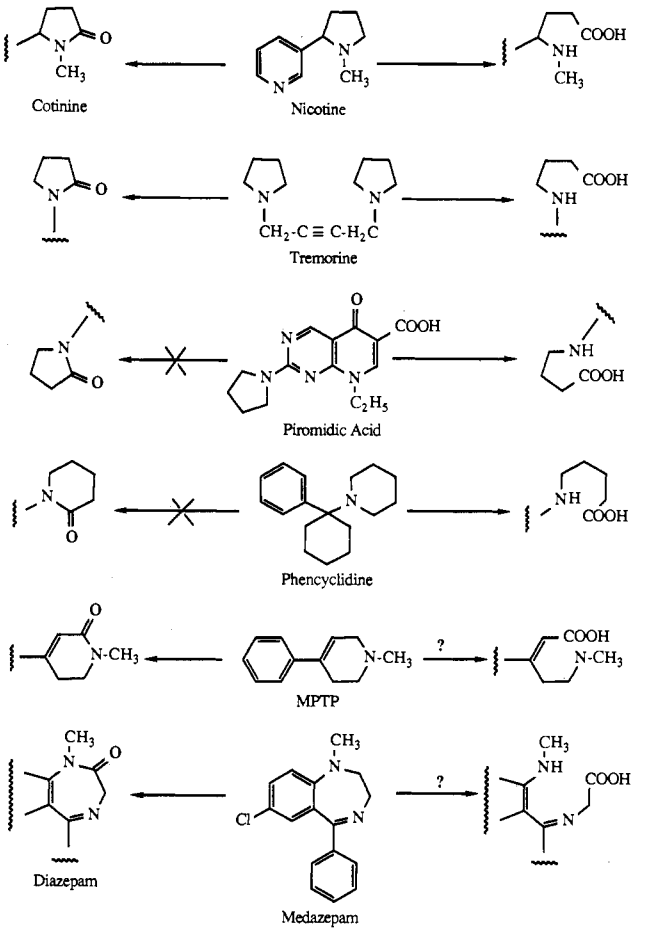
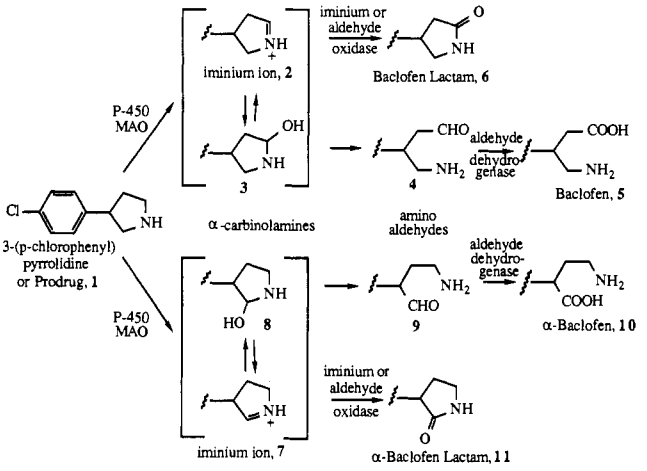


Figure 1. α-Oxidative metabolic pathways for some heterocyclic amines.

Scheme II. Proposed α-Carbon Oxidative Metabolic Pathway for Prodrug 1



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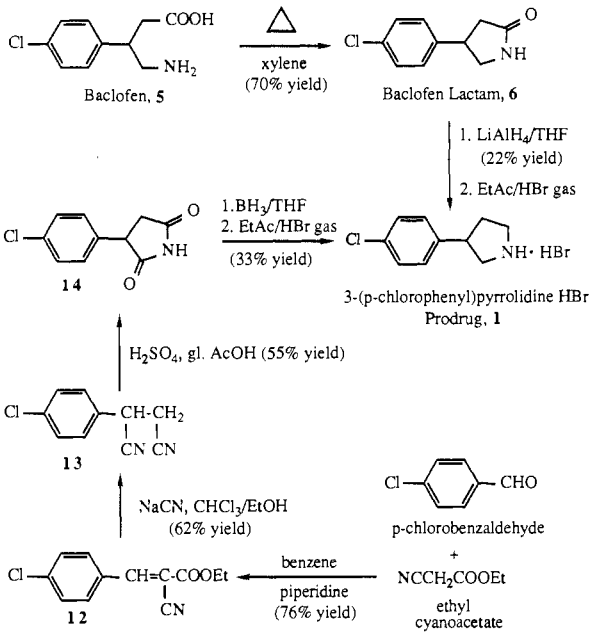
Scheme III. Synthesis of Prodrug 1 and Baclofen Lactam (6)



These hypotheses have been supported by the trapping of some iminium species[38,44] and the isolation of some carbinolamines.[40-42] The amino aldehyde **4** was hypothesized to be converted to the amino acid baclofen (**5**) in accordance with similar reports.[39,40,42]

In addition to the investigation of the fundamental suitability of pyrrolidines as prodrugs of GABAergic agents, a second major objective of the study was to determine the selectivity of the enzymatic attack at either of the two carbons adjacent to the nitrogen atom and to determine what factor might favor the formation of lactam metabolites opposed to amino acid metabolites. As shown in Figure 1, some pyrrolidines, such as nicotine[38,40] and tremorine,[45,46] give both type of metabolites, while some drugs such as piromidic acid[42] and phencyclidine[39,47] selectively produce the amino acid metabolite. Medazepam[41] and MPTP[44] have been shown to produce lactam metabolites; however, formation of the corresponding amino acid metabolites has not been investigated.

As were seen with the above series of compounds, the prototype prodrug 1 could be anticipated to lead to the formation of two lactam and two amino acid metabolites (Scheme II). From steric considerations, one could expect some selectivity with regard to position of the enzymatic attack (**5** > **10** and **6** > **11**); however, it would be difficult to anticipate the selectivity of the second oxidative step leading to the formation of the lactams or amino acids. Hopefully, the present study might shed some light on the selectivity of each of the oxidative steps and determine if there were significant differences in the selectivity in brain and liver.

Results and Discussion

Chemistry. The prodrug **1**, baclofen lactam (**6**), α-baclofen lactam (**11**), and the amino acid metabolite **10** were prepared synthetically; the amino acid metabolite baclofen (**5**) was obtained as a gift. Prodrug **1** was prepared by two methods (Scheme III): on a smaller scale from baclofen and on a larger scale from *p*-chlorobenzaldehyde. In the smaller scale preparation, baclofen (**5**) was converted to baclofen lactam (**6**) by refluxing in xylene overnight. The resulting lactam was reduced with lithium aluminum hydride in tetrahydrofuran to afford a 22% yield of the prodrug **1**. The hydrobromide salt of **1** was prepared by bubbling gaseous hydrobromic acid into a solution of the amine in ethyl acetate: this salt was found to be more satisfactory than the hydrochloride or sulfate salts. The larger scale preparation began with the condensation of *p*-chlorobenzaldehyde with ethyl cyanoacetate.[48] The resulting ethyl *p*-chloro-α-cyanocinnamate (**12**) was dissolved in chloroform/ethanol (4:3) and treated with sodium cyanide in a small amount of water. After the reaction mixture was refluxed overnight, excess cyanide was decomposed with hydrochloric acid and the succinonitrile **13** was isolated. Conversion to the succinimide **14** was accomplished by refluxing in a mixture of sulfuric acid and glacial acetic acid for 1 h. Pure succinimide **14** was obtained and reduced to the prodrug, **1**, with borane–tetrahydrofuran complex ($LiAlH_4$ reduction produced a large amount of lithium salts upon workup; borane–THF gave less salts and 33% yield).

The syntheses of α-baclofen (**10**) and α-baclofen lactam (**11**) (Scheme IV) were begun with the esterification of *p*-chlorophenylacetic acid. The resulting ethyl *p*-chlorophenylacetate (**15**) was converted to the atropic ester derivative **16** via an oxalate addition.[49] The intermediate sodium salt produced from the treatment of ethyl *p*-chlorophenylacetate (**15**) with diethyl oxalate in sodium ethoxide/diethyl ether solution was not isolated in pure form. Instead, the yellow semisolid was transferred to a flask containing a mixture of formaldehyde solution (38%) and potassium carbonate and stirred at room temperature for 2 h. Ethyl *p*-chloroatropate (**16**) was recovered from this mixture by extraction with methylene chloride, which was removed under reduced pressure on a rotoevaporator. The atropate product **16** was easily identified in the ^1H NMR spectrum of the crude reaction mixture by the presence of a broadly spaced pair of singlets at 5.9 and 6.4 ppm, representing the terminal methylene group. The atropate **16** was purified by flash chromatography using silica and a mobile phase of methylene chloride.

Treatment of the atropate **16** with nitromethane in the presence of the base Triton B produced the nitroester **17** in low yield. The nitroester was partially purified by flash chromatography using silica and a mobile phase of diethyl ether/petroleum ether. Reduction of the nitroester **17** required high pressure (H_2, 1800 psi), high temperature (70 °C), and Raney nickel catalyst (W-2 grade) for several hours. Reduction did not occur at pressures less than 1000 psi nor with the catalysts 10% palladium on activated carbon or platinum oxide. The reduction gave a mixture

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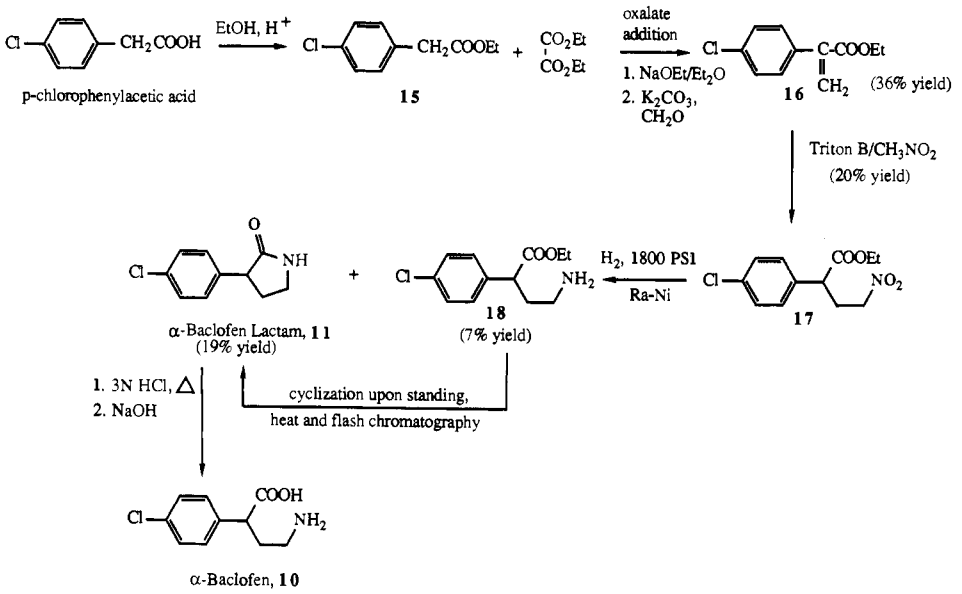
Scheme IV. Synthesis of α-Baclofen (**10**) and α-Baclofen Lactam (**11**)



of the α-baclofen ester (7%) **18** and α-baclofen lactam (18%) (**11**). The amino ester **18** was easily converted to α-baclofen lactam (**11**).

Hydrolysis of α-baclofen lactam (**11**), to the amino acid, α-baclofen (**10**), was accomplished by refluxing the lactam in 3 N hydrochloric acid for several hours. The progress of the reaction was followed by RP-HPLC/UV (50% MeOH/0.1 M phosphate buffer), by observing the disappearance of the lactam **11** (retention time 5 min) and the appearance of the amino acid **10** (retention time 3 min). After hydrolysis, the acidic solution containing α-baclofen was neutralized by careful addition of 0.1 N sodium hydroxide to a pH of 6.5–7, at which precipitation of the free amino acid **10** began to occur. Precipitation was allowed to proceed for several hours, after which the free amino acid crystals were filtered and washed with cold water.

Analytical Procedures. The amino acid metabolites were assayed by a HPLC/EC procedure, and the lactam metabolites were assayed by a procedure utilizing GC/NPD. The amino acid metabolites (and the internal standard bromobaclofen) were recovered from biological matrices by solid-phase extraction and were derivatized prior to HPLC/EC analysis. The derivatization method employed *o*-phthaldialdehyde and *tert*-butyl mercaptan reagent to convert the amino acids to products that were both fluorescent and electrochemically active. Though most literature reports of *o*-phthaldialdehyde derivatization employed mercaptoethanol,[50] *tert*-butyl mercaptan was chosen because of greater stability and greater electrochemical activity of the fluorescent products obtained.[51] Derivatization gave electrochemically active species with half-lives of 2.7 and 5.1 h for derivatives of **10** and **5**, respectively. The HPLC/EC procedure afforded detection of baclofen (**5**) and α-baclofen (**10**) to levels of 10 ng/mL in the original biological sample (brain or liver homogenate, plasma, or urine). The solid-phase extraction method gave mean recoveries of 80–90%. A typical HPLC/EC chromatogram showing the detection of baclofen in a brain homogenate sample is shown in Figure 2.

The lactams **6** and **11** were assayed by a procedure involving liquid–liquid extraction followed by GC/NPD analysis. Baclofen lactam (**6**) and α-baclofen lactam (**11**) were recovered from 5 mL of biological matrix (brain or liver homogenate or urine) by extraction with a solution of hexane/ethyl acetate (1:1). An internal standard, naphthalenamine acetate, was employed prior to extrac-

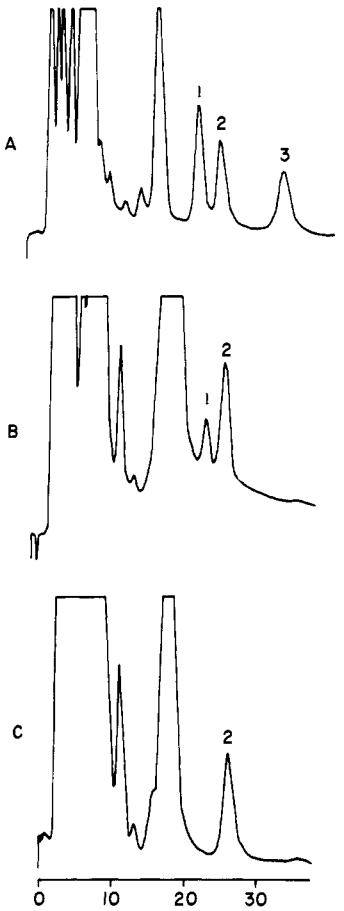


Figure 2. HPLC/EC assay for amino acid metabolites using a mobile phase of 58% MeOH/0.1 M phosphate buffer: (A) retention times of derivatives of 250 ng/mL (1) baclofen, (2) internal standard, and (3) α-baclofen in distilled water; (B) 12000*g* brain homogenate incubation mixture extract; (1) baclofen metabolite (105 ng/mL found) and (2) 250 ng/mL internal standard; (C) heat-inactivated negative-control brain homogenate containing prodrug; (2) 250 ng/mL internal standard.

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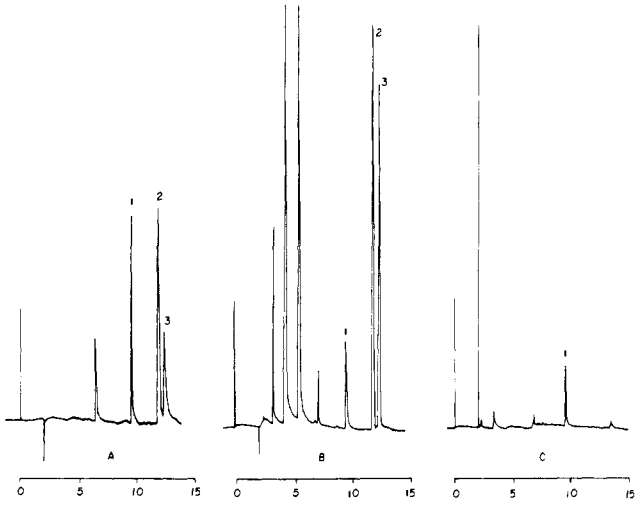

Figure 3. GC/NPD assay for lactam metabolites: (A) retention times of (1) internal standard, (2) baclofen lactam, and (3) α-baclofen lactam in methanol; (B) 700*g* liver homogenate incubation mixture extract; (1) 4 μg/mL internal standard, (2) baclofen lactam (8.4 μg/mL found), and (3) α-baclofen lactam (20.4 μg/mL found); (C) heat-inactivated negative-control liver homogenate containing prodrug; (1) 4 μg/mL internal standard.

Table I. Anticonvulsant Activity of Prodrug and Baclofen Lactam

treatment	time of first convulsion (min)	no. convulsed/ no. tested	no. deaths/ no. tested
isoniazid (control)[a]	45 ± 8	6/6[b]	5/6[b]
isoniazid + prodrug 1[c]	63 ± 25	6/6[d]	0/6[d]
isoniazid + baclofen lactam (**6**)[c]	49 ± 5	2/8[d]	1/8[d]

[a] Isoniazid 425 mg/kg, s.c. [b] Very strong convulsions continuing until death. [c] Pretreatment with 55 mg/kg, ip, 30 min prior to isoniazid. [d] Animals displayed only one brief convulsion, with most surviving.

tion. The organic layer was removed, filtered, and evaporated with a stream of nitrogen with warming from a lukewarm water bath. (The amino acids did not cyclize to the corresponding lactams under these conditions.) Recoveries of baclofen lactam (**6**) and α-baclofen lactam (**11**) were 71 and 76%, respectively. The residue was redissolved in MeOH subjected to GC/NPD (isothermal 190 °C). Detection limits for baclofen lactam (**6**) and α-baclofen lactam (**11**) were 100 ng/mL (in the original sample). A typical chromatogram showing the detection of baclofen lactam and α-baclofen lactam in a liver homogenate sample appears in Figure 3.

Anticonvulsant Screening. Prodrug 1 and baclofen lactam (**6**) were screened for anticonvulsant acitivity. Convulsions were induced in rats with isoniazid, an agent reported to produce convulsions specifically by lowering cerebellar GABA levels by about 40% in rats.[52] Subcutaneous injections of isoniazid (425 mg/kg) caused convulsions in rats after a period of about 45 min. The animals typically died with violent convulsions. Pretreatment of rats with prodrug 1 delayed the time of onset of the first convulsion by roughly 30% and prevented lethality with all animals surviving (Table I). Baclofen lactam, (**6**) pretreatment completely abolished convulsions in many animals and also reduced lethality (Table I). In further testing, baclofen lactam was also effective in preventing seizures in mice induced by maximal electroshock, pentylenetetrazole, and bicuculline (Table II), the latter of which has been shown to produce convulsions by specific $GABA_A$ receptor antagonism.[53] It was also noted that baclofen lactam did show some degree of neurotoxicity in mice as indicated in the rotorod toxicity test (Table II); indeed, it was observed that the lactam was a good sedative in rats.

Table II. Anticonvulsant Screening[a] of the Prodrug Metabolites in Mice

drug	MES ED_{50}[b] (mg/kg)	scMet ED_{50}[c] (mg/kg)	rotorod tox TD_{50}[d] (mg/kg)	Bic ED_{50}[e] (mg/kg)
baclofen lactam (**6**)[f]	68	41	117	143
baclofen (**5**)	>600	*j*	47	N/A[i]
phenytoin	9.5[g]	inactive[g]	66[g]	inactive[g]
phenobarbital	22[g]	13[g]	69[g]	38
valproic acid	272[g]	149[g]	426[g]	360[g]
progabide	75[h]	30[h]	N/A[i]	20[h]

[a] Anticonvulsant screening performed by the Anticonvulsant Screening Project, National Institutes of Health, Bethesda, MD. [b] Maximal electroshock seizure test. [c] Subcutaneous pentylenetetrazol (Metrazol) seizure theshold test. [d] Rotorod toxicity test. [e] Bicuculline seizure test. [f] This study. [g] Ref. 64. [h] Reference 65. [i] Not available. [j] Baclofen shows activity in the 50 mg/kg range, but it does produce a dose response curve that allows and ED_{50} to be calculated.

Inhibition of convulsions produced by isoniazid, an agent that lowers GABA levels, suggests that prodrug 1 has some GABAergic activity. Moreover, inhibition of convulsions induced by both isoniazid and bicuculline indicates that baclofen lactam (**6**), a metabolite of prodrug 1, also has some GABAergic activity.

In Vitro Metabolism Studies. Levels of baclofen (**5**) in the brain and liver homogenates were surprisingly consistent regardless of the fraction (Table III). Both mitochondrial/microsomal (700*g*) and microsomal (12000*g*) fractions of both brain and liver tissue were used: the 700*g* fraction has been reported to be rich in monoamine oxidase (MAO), cytochrome P-450, and soluble enzymes whereas the 12000*g* fraction contained mainly just cytochrome P-450 and soluble enzymes and little, if any, MAO.[54] About a 67% greater baclofen production was observed for the mitochondrial/microsomal fraction compared to the microsomal fraction of the brain homogenate, suggesting that monoamine oxidase (MAO) is involved in at least some of the conversion.

With the liver homogenates (Table III), the formation of the lactam metabolites was about 2 orders of magnitude higher than formation of the amino acid metabolites, while in brain, the lactam and amino acid metabolic pathways appeared to be roughly equivalent. This would suggest that the pyrrolidines might serve as prodrugs targeted for the CNS, but a significant portion of the dose would be lost to nonproductive formation of lactams in the liver.

In general, it was found that the enzymatic attack on the prodrug favored the less sterically crowded side of the pyrrolidine [leading to baclofen (**5**) and baclofen lactam (**6**)] rather than the attack at the 2-position [leading to α-baclofen (**10**) and α-baclofen lactam (**11**)]. The single exception to this generalization was for the lactam in liver where the concentration of **11** was greater than for **6**. Perhaps this was the result of the greater stability of intermediate **7** as compared to **2** (**7** can tautomerize to form a resonance-stabilized enamine while the enamine from **2** would not be resonance stabilized) and because of the greater abundance of iminium oxidase in liver tissue.

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Table III. Metabolites of Prodrug Produced in Brain and Liver Homogenates[a]

metabolite produced	amount produced[b] in liver fractions (μg/mL)		amount produced[b] in brain fractions (μg/mL)	
	700g	12000g	700g	12000g
baclofen	0.197 ± 0.004[c]	0.130 ± 0.013	0.162 ± 0.015	0.097 ± 0.009
baclofen lactam	26.4 ± 2.3	14.6 ± 0.33	2.11 ± 0.39	0.28 ± 0.02
α-baclofen	ND[d]	ND	ND	ND
α-baclofen lactam	46.7 ± 2.5	24.5 ± 0.77	0.5[e]	0.21 ± 0.10

[a] Homogenates were prepared from rats from the same lot. [b] Each liver and brain value represents mean ± standard deviation obtained from three individual incubation samples except where noted. [c] Results from two samples. [d] None detected. [e] Only observed in one sample.

A number of additional experiments were conducted to determine if the production of metabolites **5**, **6**, **10**, and **11** from the prodrug might have been artifactual in nature. At the same time the above liver and brain homogenate incubation studies were conducted, duplicate samples of the prodrug incubation mixtures were prepared by use of heat-inactivated homogenates. At the end of the incubations these heat-inactivated control samples were assayed by the GC and HPLC methods along with the test samples, and it was found that the control samples did not contain **5**, **6**, **10**, or **11**. Since the amino acids can be fairly easily converted to their corresponding lactams by chemical means, there was also some concern that the lactams seen in the incubations might have arisen from the enzymatically produced amino acid following sample workup. As a control experiment, liver microsome incubation mixtures were spiked with **5** and **10** (10 μg/mL), which were then incubated, treated and assayed in the same manner as the test samples using the prodrug. It was found that the concentrations of the amino acids (**5** and **10**) remained unchanged, and GC analysis failed to show any formation of the corresponding lactams (**6** and **11**).

In Vivo Metabolism Studies. Baclofen was also detected in brain, urine, and plasma of rats after administration of the prodrug: at low nanogram per milliliter levels in the brain 3 h postadministration of the prodrug, 55 mg/kg, ip; at low microgram per milliliter levels in the urine at 3 and 6 h postadministration of the prodrug, 55 mg/kg, ip; and at high nanogram per milliliter levels in plasma postadministration of the prodrug, 5.5 mg/kg, iv (Table IV). While α-baclofen (**10**) had not been found in the in vitro studies, α-baclofen was found in the in vivo (urine) study, but the concentration was still lower than for baclofen.

Conclusions

With regard to the selectivity of the formation of the amino acid metabolites in brain and in liver homogenates and to the selectivity of the formation of the two lactam metabolites in brain homogenates, it was observed that enzymatic attack at the 5-position of the pyrrolidine was favored over the 2-position. This would suggest that steric crowding would be a primary consideration in the future design of similar prodrugs with regard to directing the selectivity of the enzymatic attack. A glaring exception to this generalization was found for the formation of the lactam metabolites in liver homogenates. Since the liver is rich in iminium oxidase (also known as aldehyde oxidase, EC 1.2.3.1),[37] it would appear that the 2- vs 5-position selectivity of this enzyme is opposite to that of the first oxidation step. If this were the case, the 2- vs 5-position selectivity of the transformation of **1** to **6** and **1** to **11** (Scheme II) would be the product of the selectivities of the first oxidation step (favoring the 5-position) and the second oxidation step (favoring the 2-position). Thus, tissues with a high iminium oxidase/MAO ratio would favor the formation of **11** while tissues with a low iminium oxidase/MAO would favor the formation of **6**. Clearly, additional work is needed to substantiate this hypothesis using purified iminium oxidase and the respective iminium ion substrates.

Table IV. In Vivo Formation of Amino Acid Metabolites following Administration of the Prodrug[a]

metabolite	rat no.	time (h)	fluid or tissue	concn (μg/mL)[b]	cumulative amount (μg)
baclofen	1	0–3	urine	2	10
baclofen	1	3–6	urine	16	42
baclofen	2	0–3	urine	0	0
baclofen	2	3–6	urine	9.4	13
α-baclofen	1	0–3	urine	0.5	5
α-baclofen	1	3–6	urine	2.6	10.2
α-baclofen	2	0–3	urine	0	0
α-baclofen	2	3–6	urine	2.3	3.2
baclofen	3	3	brain	24.3[e]	N/A[c]
baclofen[d]	4	2	plasma	0.04	N/A
baclofen[d]	4	4	plasma	0.12	N/A

[a] Prodrug 1 administered 55 mg/kg, ip, except for plasma study. [b] Units expressed in μg/mL with the exception of brain tissue. [c] Not applicable. [d] Prodrug 1 administered 5.5 mg/kg, iv. [e] In units of ng/g.

With regard to the selectivity of the formation of lactam vs amino acid metabolites from nitrogen heterocycles, a tentative hypothesis applicable to studies using liver would be that bulky groups in close proximity to the nitrogen favor the formation of the amino acid metabolites while substrates with a more "open" nitrogen would favor formation of the lactams. Phencyclidine and piromidic acid (Figure 1), where both α-carbons were very hindered, would be predicted to give mostly amino acid metabolites. Prodrug **1**, where there is little crowding, would be anticipated to yield mostly lactams in the liver homogenate model, while nicotine and tremorine would be expected to give both the amino acid and lactam metabolites. Because of the very limited number of substrates that have been studied in the brain homogenate model, broad generalizations would not be appropriate, but at least for the substrate at hand, **1**, the brain homogenate model system gave 2 orders of magnitude lower yield of the lactam metabolites as compared to the liver homogenate model. By contrast, the yield of the amino acid metabolite was essentially the same for the two tissues.

The preliminary pharmacological evaluation of the prototype prodrug **1** demonstrated GABAergic activity in that the prodrug could inhibit the convulsions following the fall in γ-aminobutyric acid elicited by isoniazid. Since it was found that the prodrug was metabolized both in vitro and in vivo in brain to an agent with well documented $GABA_B$ activity (**5**),[55] one might expect the prodrug to be useful as a $GABA_B$ agent. However, it was also found that the prodrug was metabolized in brain homogenates to a lactam metabolite, **6**, that was subsequently found to have $GABA_A$ activity (Table II, bicuculline test). Thus, it would

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be difficult to classify the prodrug as a type A or type B GABAergic agent at this time.

Experimental Section

Chemistry. ^{1}H NMR spectra were obtained on a Varian EM-390 spectrometer (90 MHz) or a Varian VXR300 (300 MHz). ^{13}C NMR spectra were obtained on either a JEOL FX-60 spectrometer (15 MHz) or a Varian VXR-300 (25 MHz). Two-dimensional homonuclear correlated (proton–proton) (2D-COSY), attached proton test (APT), and heteronuclear correlated (proton–carbon) (HCTCOR) spectra were obtained on a Varian VXR 300 spectrometer. "Nonequivalence" in NMR signals was denoted by "noneq." Infrared spectra were obtained on a Perkin-Elmer Model 281B spectrometer, and electron-impact (70eV) mass spectra were obtained on a Finnigan 3200 GC/MS and DS. Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Elemental analyses were performed by either Galbraith Laboratories, Knoxville, TN, or Atlantic Microlab, Atlanta, GA. All flash chromatography[56] incorporated silica gel 60, 0.040–0.063 mm (230–400 mesh). Baclofen [4-amino-3-(*p*-chlorophenyl)butanoic acid, Lioresal] was obtained as a gift from CIBA Pharmaceutical Co., Summit, NJ. The term in vacuo refers to water aspirator vacuum. Extraction volumes of methylene chloride were always passed through a plug of cotton prior to evaporation in order to remove any water droplets.[57] The following abbreviations were used: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; THF, tetrahydrofuran.

4-(*p*-Chlorophenyl)pyrrolidin-2-one (6) (Baclofen Lactam). The amino acid baclofen (10 g, 0.47 mol) (CIBA Pharmaceutical Co., Summit, NJ) was placed in a flask containing 500 mL of xylene (the powdery, white, amino acid did not dissolve immediately in the xylene). The mixture was stirred at reflux for 7 h, until no observable undissolved material remained. The xylene was evaporated, yielding 7.0 g (77%) of powdery lactam, which was recrystallized from isopropyl ether/EtOH: mp 115–116.5 °C (lit.[58] mp 117–118 °C); IR (KBr) λ_{max} (cm^{-1}) 3420, 3200 (br), 2900, 1660, 1490, 1090, 830; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.5 and 2.7 (each 1 H, dd; noneq CH_2CO)*, 3.4 and 3.7 (1 H, dd and 1 H, quintet, respectively; noneq CH_2NH)*, 3.8 (1 H, t; benzyl *CH*), 7.1 (N*H*, br s), 7.2–7.3 (4 H, dd; phenyl) (* = ambiguous assignments); mass spectrum (70eV), *m/z* (relative intensity, %) 195 (M^+, 20), 138 (100), 103 (30), 77 (20).

Ethyl *p*-Chloro-α-cyanocinnamate (12). A mixture of *p*-chlorobenzaldehyde (140 g, 1 mol) and ethyl cyanoacetate (124 g, 1.1 mol) in benzene (275 mL) and piperidine (3 mL) was refluxed overnight, the water being removed with a Dean–Stark trap.[48] The solution was cooled and the solvent removed in vacuo to yield 178 g (76%) of a light yellow solid which was recrystallized from 95% EtOH: mp 91–92 °C (lit.[66] mp 91.0–92.5 °C); ^{1}H NMR (90 MHz, $CDCl_3$) δ 1.4 (3 H, t; CH_3), 4.4 (2 H, q; CH_2), 7.4 and 7.9 (4 H, dd; phenyl), 8.2 (1 H, s; benzal *CH*); ^{13}C NMR (15 MHz, $CDCl_3$) δ 14.2 (q, CH_3), 62.8 (t, CH_2), 103.7 (s, *C*–CN), 115.2 (s, *C*N), 129.7 (d), 130.0 (s), 132.2 (d), 139.5 (s), 153.2 (d, benzal *C*H), 162.2 (s, *C*O).

(*p*-Chlorophenyl)succinonitrile (13). The ester **12** (15 g, 64 mmol) was dissolved in a mixture of $CHCl_3$ (200 mL) and EtOH (150 mL) to which was added NaCN (3.4 g, 70 mmol) dissolved in 10 mL of H_2O, dropwise.[48] The solution was stirred at reflux for 18 h, cooled, acidified with 10 mL of concentrated HCl (**Caution**: cyanide gas evolved should be trapped or well ventilated!), and evaporated in vacuo, and the residue was partitioned between H_2O (150 mL, resulting pH 3) and $CHCl_3$ (150 mL). The organic layer was removed, washed with 1 N NaOH (2 × 50 mL), dried (Na_2SO_4), and evaporated to yield a brown solid, which was recrystallized from EtOH to yield 7.5 g (62%) of a pale pink solid: mp 87–90 °C (lit.[67] mp 88–89 °C); IR (KBr) λ_{max} (cm^{-1}) 2960, 2920, 2230, 2220, 1490, 1420, 1090, 830; ^{1}H NMR (90 MHz, DMSO-d_6) δ 3.30 (2 H, d; CH_2), 4.80 (1 H, t; $CHCH_2$), 7.6 (4 H, s; phenyl); ^{13}C NMR (15 MHz, DMSO-d_6) δ 22.8 (t, CH_2), 32.2 (d, $CHCH_2$), 117.1 (s, CN), 118.9 (s, *C*N), 129.2 (d), 129.4 (d), 132.6 (ns), 133.9 (s).

(*p*-Chlorophenyl)succinimide (14). Succinonitrile **13** (10 g, 53 mmol) was dissolved in a mixture of glacial AcOH (75 mL) and 98% H_2SO_4 (12 mL) and stirred under reflux and N_2 for 1 h, cooled, and evaporated in vacuo.[48] The residue was partitioned between 100 mL of H_2O and 100 mL of $MeCl_2$, and the organic layer was removed, filtered through a plug of cotton, and evaporated in vacuo. The residue was recrystallized from EtOH to yield 6.0 g of a white solid (55%): mp 122–126 °C (lit.[68] mp 130–132 °C); IR (KBr) λ_{max} (cm^{-1}) 3175, 1700, 1780, 1500; ^{1}H NMR (90 MHz, DMSO-d_6) δ 2.6–2.9 and 3.0–3.4 (each a 1 H, dd; noneq $CHCH_2$), 3.5 (1 H, br s; N*H*, D_2O exch), 4.1–4.3 (1 H, dd; $CHCH_2$), 7.4 (4 H, m; phenyl); ^{13}C NMR (25 MHz, DMSO-d_6) 37.6 (t, $CHCH_2$), 40.2 (d, $CHCH_2$), 128.3 (d), 129.5 (d), 131.7 (s), 136.6 (s), 177.1 (s, *C*O), 178.7 (s, *C*O); mass spectrum (70eV), *m/z* (relative intensity, %) 209 (M^+, 15), 140 (45), 137 (14), 138 (100), 103 (44), 102 (12), 77 (15).

3-(*p*-Chlorophenyl)pyrrolidine Hydrobromide (1) (Prodrug). Smaller Scale (Scheme III). Baclofen lactam (1 g) (**6**) was added to a solution of $LiAlH_4$ (1 g) in THF (150 mL) and stirred at reflux for 2 h. Solution was cooled externally with an ice bath and treated successively with 1 mL of H_2O, 1 mL of NaOH (15%), and 3 mL of water. Lithium salts were filtered off, and the clear, light violet colored filtrate was concentrated in vacuo and the residue partitioned between EtOAc and 0.1 N NaOH (100 mL of each). Organic solvent was removed, dried over $MgSO_4$, and removed in vacuo to yield 0.2 g (22%) of the prodrug base **1**.

Larger Scale (Scheme III). Succinimide **14** (9.2 g, 44 mmol) in dry THF (10 mL) was added, dropwise, to a solution of 176 mL (4 equiv) of borane/THF complex (1.0 M in THF), which was stirred under reflux and N_2 for 4 h. The solution was cooled, quenched with H_2O, filtered (filter cake washed with THF), and evaporated to yield 2.6 g (33%) of prodrug base **1** as a clear, colorless oil. The HBr salt of **1** was prepared by bubbling HBr gas into a solution of **1** (1 g) in EtOAc (10 mL) until the solution turned cloudy. The white precipitate was filtered off and recrystallized from isopropyl ether/EtOH: mp 142–144 °C; IR (free base, neat) λ_{max} (cm^{-1}) 3300 (br), 2860, 2960, 1490, 1090, 1010, 830; IR (HBr salt, KBr) λ_{max} (cm^{-1}) 2990, 1550, 1500, 1450, 1380, 1090, 1030, 1010, 845, 825; ^{1}H NMR (300 MHz, free base in $CDCl_3$) δ 1.8 and 2.2 (each a 1 H, m; noneq $CHCH_2CH_2$), 2.8 and 3.2 (each a 1 H, m; noneq $CHCH_2NH$), 3.1 (3 H, m; $CHCH_2CH_2NH$), 7.1 (4 H, m; phenyl), 7.4 (1 H, s; N*H*); 2D-COSY established couplings between the pairs $CHCH_2CH_2$, $CHCH_2CH_2$, and $CHCH_2NH$; ^{13}C NMR (25 MHz, free base in $CDCl_3$) δ 34.4 (t, $CHCH_2$), 44.8 (d, $CHCH_2$), 47.2 (t, CH_2CH_2NH), 54.8 (t, $CHCH_2NH$), 128.2 (d), 128.4 (d), 131.6 (s), 142.5 (s); HETCOR established couplings within the groups $CHCH_2CH_2NH$, $CHCH_2CH_2NH$, and $CHCH_2CH_2NH$; mass spectrum (HBr salt) (70eV), *m/z* (relative intensity, %) 181 (M^+, 100), 152 (12), 138 (16), 117 (51), 116 (27), 115 (69), 103 (24), 89 (26). Anal. ($C_{10}H_{13}NBrCl$) C, H, N.

Ethyl *p*-Chloroatropate (16). *p*-Chlorophenylacetic acid (48 g, 0.28 mol) (Fluka Chemical Corp., Ronkonkoma, NY) was dissolved in 200 mL of EtOH, treated with 1.5 mL of concentrated HCl, refluxed 8 h, cooled, concentrated in vacuo, and partitioned between $MeCl_2$ and 1 N KOH (250 mL of each). The $MeCl_2$ layer was removed, filtered through a cotton plug, and evaporated in vacuo to afford 50 g (90%) of ethyl *p*-chlorophenylacetate (**15**):

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^{1}H NMR (90 MHz, $CDCl_3$) δ 1.2 (3 H, t; ester CH_3), 3.6 (2 H, s; CH_2CO), 4.1 (2 H, q; ester CH_2), 7.3 (4 H, br s; phenyl); mass spectrum (70eV), m/z (relative intensity, %) 198 (M^+, 29), 126 (100), 99 (13), 89 (36). The ester **15** (100 g, 0.51 mol) was dissolved in 300 mL of Et_2O and added to NaOEt in Et_2O, prepared by dissolving 11.6 g of sodium in absolute EtOH, evaporating the EtOH in vacuo, and redissolving the NaOEt residue in 300 mL of Et_2O. To this mixture was added ethyl oxalate (74.5 g, 0.51 mol) in Et_2O (100 mL). After the mixture was stirred overnight at room temperature, the solvent was decanted and the yellow, semisolid residue was washed with Et_2O (2 × 100 mL) and dissolved in a mixture of 90 mL of formaldehyde (38%) solution and 250 mL of H_2O. The solution was cooled with an external ice bath, treated with a solution of 81 g of K_2CO_3 in 150 mL of H_2O, and then stirred for 2 h at room temperature. Extraction with $MeCl_2$ (4 × 100 mL) followed by evaporation in vacuo yielded 25 g (24%) of crude liquid product. An analytical sample was purified by flash chromatography using a mobile phase of $MeCl_2$: IR (neat) λ_{max} (cm^{-1}) 2990, 1710, 1600, 1490, 1200; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.3 (3 H, t; ester CH_3), 4.3 (2 H, q; ester CH_2), 5.9 and 6.4 (each a 1 H, s; $=CH_2$), 7.4 (4 H, m; phenyl).

Ethyl 2-(*p*-Chlorophenyl)-4-nitrobutanoate (17). Crude ethyl *p*-chloroatropate (**16**) (25 g, 0.12 mol), dissolved in 25 g Triton B (*N*-benzyltrimethylammonium hydroxide, 40% in MeOH) and cooled to 0 °C, was treated with nitromethane (31 g, 0.51 mol), dropwise. The mixture was stirred for 2 h at room temperature, the volume reduced in vacuo and the mixture extracted with $MeCl_2$ (100 mL), which was washed successively with H_2O (100 mL), 3 N HCl (100 mL), H_2O (100 mL), 1 N NaOH (100 mL), H_2O (100 mL), and saturated NaCl solution (100 mL). The $MeCl_2$ layer was then filtered through a cotton plug and evaporated to yield 25 g of crude nitroester. Product was further purified by flash chromatography using a mobile phase of $MeCl_2$: IR (neat oil) λ_{max} (cm^{-1}) 2980, 1730, 1540, 1485, 1360, 1170, 1090, 1010, 830, 760; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.2 (3 H, t; ester CH_3), 2.4 and 2.7 (each a 1 H, m; noneq $CHCH_2$), 3.7 (1 H, t; $CHCH_2$), 4.1 (2 H, q; ester CH_2), 4.1 and 4.4 (each a 1 H, m; noneq CH_2NO_2), 7.2–7.3 (4 H, m; phenyl); ^{13}C NMR (25 MHz and APT, $CDCl_3$) δ 14.0 (q, ester CH_3), 30.4 (t, $CHCH_2$), 47.5 (d, CHCO), 61.5 (t, ester CH_2), 73.0 (t, CH_2NO_2), 129.2 (d), 129.3 (d), 134.0 (s), 135.6 (s), 172.1 (s, CO); HETCOR spectra established correlations within the groups ester CH_2, ester CH_3, $CHCH_2$, CHCO, CH_2NO_2.

3-(*p*-Chlorophenyl)pyrrolidin-2-one (11) (α-Baclofen Lactam). Crude nitroester **17** (14.0 g, 52 mmol) was subjected to hydrogenation in EtOH (200 mL) with 10 g of Raney nickel, active catalyst (analogous to Raney 28 or W-2, Aldrich Chemical Co., Milwaukee, WI) [a portion of a 50% slurry in H_2O was pipetted out (**Caution**—fire hazard!) and washed (×3) with EtOH, taking care to keep catalyst wetted], at 1800 psi (H_2; **Caution**–high pressure!) in a high-pressure Parr pressure reaction apparatus (6000 psi max) at 70 °C overnight. The ethanolic solution was freed of the catalyst by slowly filtering over Celite (**Caution**—fire hazard!), taking care to keep catalyst wetted with EtOH. The ethanolic filtrate was evaporated in vacuo, and the resulting viscous oil was partitioned between $MeCl_2$ and 10% HCl (100 mL of each) (amino ester **18** was retrieved from the acidic layer). The $MeCl_2$ layer was washed with 0.5 N NaOH (2 × 50 mL), filtered through a plug of cotton, and evaporated to yield a crude solid. Flash chromatography (using a mobile phase of 2% EtOH/$MeCl_2$) followed by recrystallization from isopropyl ether/EtOH yielded 1.9 g (19%) of white crystalline α-baclofen lactam (**11**): mp 124.5–125.5 °C; IR (KBr) λ_{max} (cm^{-1}) 3100, 1700, 1490, 1285, 1090, 1010, 830, 800; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.2 and 2.6 (each a 1 H, m; noneq $CHCH_2$), 3.5 (2 H, t; CH_2NH), 3.6 (1 H, t; $CHCH_2$), 6.6 (1 H, br s; NH), 7.3 (4 H, dd; phenyl); 2D-COSY established couplings between the pairs $CHCH_2$ and CH_2CH_2NH; ^{13}C NMR (25 MHz, $CDCl_3$) δ 30.7 (t, $CHCH_2$), 40.6 (t, CH_2NH), 47.0 (d, $CHCH_2$), 128.8 (d), 129.3 (d), 133.0 (s), 137.7 (s), 178.3 (s, CO); mass spectrum (70eV), m/z (relative intensity, %) 195 (M^+, 37), 161 (34), 152 (31), 117 (100), 115 (40), 103 (17), 91 (17). Anal. ($C_{10}H_{10}NOCl$) C, H, N.

Ethyl 4-Amino-2-(*p*-chlorophenyl)butanoate (18). Amino ester **18** was obtained in 7% yield as a byproduct in the reduction of the nitroester **17** to α-baclofen lactam (**11**). The 10% HCl solution (100 mL) obtained in the workup described for α-baclofen lactam was made alkaline with concentrated NH_4OH and extracted with $MeCl_2$ (4 × 100 mL). The $MeCl_2$ was filtered through a plug of cotton and evaporated in vacuo to yield the amino ester **18** as an oil which later solidified. The product was easily converted to α-baclofen lactam (**11**) upon reflux in xylene for 1 h, upon flash chromatography (using a mobile phase of 2% EtOH/$MeCl_2$), or even upon standing for a few days at room temperature: IR (neat oil) δ_{max} (cm^{-1}) 3600–3000, 2900, 1690, 1490, 1450, 1280, 1050, 700; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.2 (3 H, t; ester CH_3), 1.3 (2 H, br s; NH_2), 1.9 and 2.2 (each a 1 H, m; noneq $CHCH_2$), 2.7 (1 H, t; CHCO), 3.7 and 4.2 (each a 1 H, m; CH_2NH_2), 4.2 (2 H, m; ester CH_2), 7.3 (4 H, m; phenyl).

4-Amino-2-(*p*-chlorophenyl)butanoic Acid (10) (α-Baclofen). α-Baclofen lactam (**11**) (670 mg, 3.4 mmol) was dissolved in 3 N HCl (3.5 mL) and stirred at reflux for 11 h. The progress of the hydrolysis was followed by RP-HPLC/UV (50% MeOH/0.1 M phosphate buffer, pH 7). The solution was cooled and washed with EtOAc (1 × 10 mL), and the aqueous layer (observed pH 1) was carefully titrated (according to a procedure used for a similar amino acid[59]) with 1 N NaOH to a pH of 6.5–7, at which the product began to precipitate as white crystals. Precipitation was allowed to occur overnight, after which the crystals were filtered and washed with ice-cold, distilled water, yielding a total of 397 mg (54%) of crystalline amino acid: IR (KBr) λ_{max} (cm^{-1}) 3500, 3000–2500, 2200, 1620, 1550, 1490, 1370, 1090, 1010, 830; ^{1}H NMR (300 MHz, D_2O, DCl, DSS) δ 2.2 and 2.4 (each a 1 H, m; noneq $CHCH_2$), 2.9 and 3.0 (each a 1 H, m; noneq CH_2NH_2), 3.9 (1 H, t; benzyl CH), 7.4 (4 H, dd; phenyl); mass spectrum (70eV), m/z (relative intensity) 213 (M^+, 0.45), 195 (56), 151.8 (44), 124.9 (21), 116.8 (100), 103 (30), 76.7 (47). Anal. ($C_{10}H_{12}NO_2Cl$) C (calcd 56.21, found 55.67), H (calcd 5.67, found 5.74), N (calcd 6.56, found 6.29).

Naphthalenamine Acetate (Internal Standard Used in the GC/NPD Method for the Lactams). 1-Aminonaphthalene (Aldrich Chemical Co., Milwaukee, WI) (1 g, 7 mmol) was placed in a 5-mL vial and treated with 1 mL of acetic anhydride. The purple crystals dissolved in a vigorous exothermic reaction, after which a precipitate formed within 1 min. The precipitate was filtered off and washed with isopropyl ether and recrystallized (X2) from isopropyl ether/EtOH to yield 0.5 g (39%) of product as white crystals; mp 157–158 °C (lit.[60] 160° C).

Metabolism Studies. Male Wistar rats weighing 180–300 g were used in all animal experiments and were obtained from Charles River Breeding Labs. Tissue homogenates were prepared from rats from the same lot. The "Tris" buffer use to homogenize tissue samples was prepared by using tris(hydroxymethyl)-aminomethane (Sigma Chemical Co, St. Louis, MO). Centrifugation of tissue homogenates was performed on a Beckman Model J2-21 centrifuge, and incubation was carried out in a Precision Scientific Model 25 shaker bath. HPLC/EC analyses were performed by using a Waters Associates Model 6000A pump, a Whatman Partisil 10 ODS-3 column, a Bioanalytical Systems, Inc., LC-3A amperometric detector, and a Hewlett-Packard 3390A integrating recorder. The internal standard used for the HPLC/EC method was bromobaclofen[4-amino-3-(*p*-chlorophenyl)butanoic acid, obtained as a gift from Drs. Riess and Dorhofer of CIBA-GEIGY, Ltd., Basle, Switzerland]. BOND-ELUT brand solid-phase extraction columns and VAC-ELUT elution chamber were obtained from Analytichem International. GC/NPD analyses were performed on a Hewlett-Packard 5890A capillary GC equipped with a 30 M × 0.25 mm J&W DB-5 fused silica capillary column and a nitrogen–phosphorus selective detector (NPD).

HPLC/EC Method for Amino Acids. Details of the HPLC/EC procedure for the analysis of the amino acid metabolites have been reported elsewhere.[61] Baclofen (**5**), α-baclofen (**10**), and internal standard (bromobaclofen) were extracted from liver homogenate supernatant or urine by using solid-phase extraction columns in a modification of the procedure of Harrison et al.[62] Solid-phase extraction columns (BOND-ELUT) packed with 1 cm of octadecylsilane were employed. Aqueous solutions were passed through these columns into 2-mL centrifuge tubes by suction from a vacuum chamber. The columns were activated just prior to use by washing with 2 × 1 mL of AcCN followed by 2 × 1 mL of 0.1% orthophosphoric acid (85%) solution. Then, 1 mL of sample (brain or liver homogenate supernatant, plasma,

or urine) spiked with internal standard was passed through the column, followed by 1 mL of 0.1%; orthophosphoric acid (85%) solution. After discarding the previous wash, the column was eluted with 1.5 mL of 0.05 M potassium dihydrogen phosphate–AcCN (75:25). A 100-μL aliquot of this eluate was derivatized with 25 μL of *o*-phthalidialdehyde/*tert*-butyl mercaptan reagent (75 mg of *o*-phthalidialdehyde, 5.0 mL of MeOH, 50 μL *tert*-butyl mercaptan, and 5.0 mL of 0.18 M sodium tetraborate decahydrate solution). A 50-μL aliquot of the resulting solution was injected onto the HPLC/EC system within 1–2 min. Detection limits were 10 ng/mL for **5** and **10** when expressed as concentrations in the original, unextracted sample.

GC/NPD Method for Lactams. Five milliliters of a biological sample (brain or liver homogenate or urine) spiked with internal standard (naphthalenamine acetate, preparation described herein) was extracted (2 × 5 mL) with hexane/EtAc (1:1) in 20-mL screw-top test tubes (1.5 × 15 cm) and centrifuged (3000 rpm × 5 min). The organic layer was removed, filtered through 3 g of sodium sulfate, and dried with a stream of nitrogen with warming in a lukewarm water bath. The residue was redissolved in 25 μL of MeOH and 1 μL injected onto the capillary GC/NPD system under isothermal conditions (190 °C, helium carrier gas flow rate of 1 mL/min, injector and detector, 220 °C). Retention times were as follows: internal standard, 9.5 min; baclofen lactam (**6**), 12 min; and α-baclofen lactam (**10**), 12.5 min. Detection limits for baclofen lactam (**6**) and α-baclofen lactam (**10**) were 50 ng/mL (in the original sample).

Brain and Liver Homogenates. Rats were sacrificed by using a guillotine, and the brain (about 1.5–2 g per rat) and liver (about 12–15 g per rat) were removed and placed on aluminum foil chilled on crushed ice. The tissue samples were briefly washed with distilled water to remove hair and blood and then immediately returned to the crushed ice. While the liver sample was maintained at ice temperatures, scissors were used to mince the liver lobes into pieces less than 5 mm^2; brain required no mincing and was homogenized directly. The tissue sample was quickly weighed and then transferred to an ice-chilled Potter–Elvehjem tube with a volume of ice-cold 0.05 M, pH 7.4, Tris buffer/0.15 M KCl solution equal to 3 times the weight of the tissue (i.e., 3 mL of buffer/g of tissue). The tissue was homogenized with seven passes of the Teflon pestle while maintaining ice temperatures. Prior to centrifugation, liver homogenates were pooled and brain homogenates were pooled to ensure uniformity.

Two different fractions of tissue homogenate were obtained by centrigutation at 0 °C for 10 min. Centrifugation at 700*g* gave a fraction containing mitochondria, microsomes, and soluble enzymes, while centrifugation at 12000*g* gave a fraction containing only the microsomal and soluble enzymes. A glass pipette was used to transfer the homogenate supernatant to an ice-cooled polycarbonate storage tube, taking care not to such up any of the very soft pellet at the bottom of the centrifuge tube. Homogenate that was not immediately used was preserved by quick-freezing in a dry ice–2-propanol bath and storing at −90 °C for future use.

Incubation Conditions.[63] Typically, three test samples, one heat-inactivated control, and one blank were simultaneously prepared for incubation. Each incubation sample contained (1) 1.0 mL of stock solution of prodrug 1 (5.0 mM in distilled water) or blank stock solution, (2) 1.0 mL of tissue (brain or liver) homogenate, and (3) 3.0 mL of cofactor solution (0.75 mM NADP with 4.5 waters of hydration or 15.2 mg; 8.33 mM glucose 6-phosphate or 54.2 mg; 8.33 mM anhydrous magnesium sulfate or 25.1 mg; all dissolved in 25 mL of Tris–KCl buffer). The "blank" sample was prepared by substituting 1.0 mL of water for the 1.0 mL of prodrug stock solution. Also, one "heat-inactivated control" was prepared in which the 1.0 mL of tissue homogenate was heated on a steam bath for 10 min before the substrate stock solution and cofactors were added. Each of the 5.0-mL incubation mixtures were placed in a 50-mL glass beaker with one glass marble. The beakers were fastened into a shaker bath and agitated at a temperature of 37 °C and at a rate of 120 oscillations per minute while maintaining a 100% oxygen atmosphere (O_2, 200 mL/min) before and during the experiment.

In Vivo Tests. Plasma Samples. One rat (217 g) was anesthetized with xylazine (Rhompun, 6 mg/kg, im) and ketamine (80 mg/kg, im) and surgically implanted with Tygon catheters (0.15-in. i.d., 0.30-in. o.d.) into the carotid artery and into the left superior vena cava. Catheters were exteriorized at the back of the neck, filled with heparinized saline, and plugged with stainless steel pins. Surgical incisions were closed with cyanoacrylite glue. Anesthesia was partially reversed with yohimbine (0.2 cm^3 of a 2 mg/mL solution) shortly after completion of the surgery. Animals were allowed a recovery period of 48 h with water and food provided ad libitum. Prodrug 1 (10 mg/mL in saline) was administered intravenously (5.5 mg/kg) with a slow infusion over a period of 5 min. Blood samples (2 mL) were collected at 2 and 4 h and centrifuged to obtain plasma. Immediately after collection of this large amount of blood, a blood transfusion (2 mL) from a donor rat was performed to maintain adequate blood pressure. Plasma samples were assayed by HPLC/EC.

Urine and Brain Samples. Rats housed in metabolism cages were administered the prodrug (55 mg/kg, ip). Urine samples were collected at 3 and 6 h and assayed by HPLC/EC. One rat was decapitated 3 h postadministration the brain removed, homogenized with Tris–KCl buffer, and assayed by HPLC/EC.

Anticonvulsant Screening. Anticonvulsant studies were performed by using rats from the same lot. A positive control group of four rats were administered 425 mg/kg isoniazid (180 mg/mL in saline), subcutaneously, and placed in cages and observed for the next several hours for convulsions. Another group of eight rats were pretreated with prodrug 1 (55 mg/kg, ip, using a 200 mg/mL solution in saline), prior to administration of isoniazid. A third group of rats were pretreated with baclofen lactam (**6**) (55 mg/kg, ip, using a 200 mg/mL solution in dimethyl sulfoxide), prior to administration of isoniazid.

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