pig aorta strips was performed by the following modification of the method of Shepperson et al.[26] Guinea pig thoracic aortae (male, Hartley, 200–250 g) were removed and cut into rings of approximately 3 mm width and immediately bathed in Krebs–Ringer buffer bubbled with $O_2/CO_2$ (95:5%) at 37 °C. The Krebs–Ringer buffer had the following composition (mM): NaCl, 118.0; KCl, 4.7; $MgCl_2$, 1.2; $CaCl_2$, 2.6; $NaH_2PO_4$, 1.0; $NaHCO_3$, 25; glucose, 11.1; with the inclusion of 10 μM cocaine. The rings were suspended in 20-mL organ baths under a basal tension of 3 g and washed approximately 10 times over a period of 60 min. After this period, cumulative concentration–response curves were obtained to the agonists at concentrations from 3 to 200 μM in approximately 2–3-fold steps. Each agonist concentration was increased to the next step after the response to the preceding step had reached plateau. Maximum response was obtained before and after each agonist concentration–response curve by adding a supramaximal concentration of (−)-NE (30–50 μM). The tension developed by the guinea pig aortic rings was measured with Grass FTO3C force displacement transducers and recorded on Grass 79D polygraphs. Potency ($EC_{50}$) was based on the concentration of PE or FPE that afforded a contraction one-half of the maximal contraction afforded by (−)-NE.

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**Acknowledgment.** We would like to acknowledge the technical assistance of Brian Collins (Washington Center for Learning Alternatives) and the excellent clerical assistance of Colleen LePore and Judy Thomas-Younis.

**Registry No.** **2a**, 103439-04-9; **2a**·0.5oxalate, 103439-05-0; **2b**, 103439-06-1; **2c**, 103439-07-2; **2c**·0.5oxalate, 103439-08-3; **3a**, 103438-90-0; **3b**, 103438-91-1; **3c**, 103438-92-2; **4b**, 42564-51-2; **4c**, 27996-87-8; **5a**, 103438-86-4; **5b**, 103438-85-3; **5c**, 103438-84-2; **6e**, 103438-88-6; **6f**, 591-31-1; **10**, 103438-89-7; **11a**, 103438-94-4; **11b**, 103438-95-5; **11c**, 103438-93-3; **12a**, 103438-97-7; **12a**·oxalate, 103439-02-7; **12b**, 103438-98-8; **12c**, 103438-96-6; **13a**, 103439-01-6; **13a**·oxalate, 103439-03-8; **13b**, 103438-99-9; **13c**, 103439-00-5; 2-fluoro-5-aminobenzaldehyde dimethyl acetal, 103438-83-1; 3-methoxy-2-nitrobenzaldehyde, 53055-05-3; 2-amino-3-methoxybenzaldehyde dimethyl acetal, 103438-87-5; 2-fluorophenol, 367-12-4; 4-fluorophenol, 371-41-5.

# Cyclic Analogues of 2-Amino-4-phosphonobutanoic Acid (APB) and Their Inhibition of Hippocampal Excitatory Transmission and Displacement of [$^3$H]APB Binding

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Conformationally restricted analogues of 2-amino-4-phosphonobutanoic acid (APB, **2**) were prepared where the structure of APB was incorporated into cyclopentane (**3**) or cyclohexane (**4**) rings. Hydrophosphinylation of the appropriate cycloalkenones followed by Strecker amino acid syntheses provided the desired analogues. Assignments of the relative configurations for **3a** (trans), **3b** (cis), **4a** (cis), and **4b** (trans) were determined through $^{13}$C NMR studies. Compounds **3b**, **4a**, and **4b** possessed low activity as inhibitors of excitatory synaptic field potentials in the rat hippocampal perforant path. Analogues **4a** and **4b** also showed little activity in displacing [$^3$H]APB from synaptic plasma membranes. The cyclopentyl APB analogue **36**, on the other hand, was extremely potent in inhibiting the binding of [$^3$H]APB, possessing an $IC_{50}$ = 4.7 μM, thus giving further credence to the idea that the APB binding site in the rat brain synaptosomal membrane preparation is not the same as the receptor mediating APB-induced inhibition of the lateral perforant path. Of the four cyclic APB analogues, **3a** most resembled APB in its spectrum of biological activity. It showed significant potency ($IC_{50}$ = 130 μM) in inhibiting lateral entorhinal projections to hippocampal granule cells. Analogous to APB, **3a** also showed selectivity for the lateral perforant path over the medial perforant path. Its activity in the radioligand binding assay paralleled its activity in inhibiting the lateral perforant path. It thus appears that **3a** comes closest to mimicking the active conformation of APB and suggests that a folded conformation wherein the amino and phosphonate moieties are in a cis relationship to one another may approximate the active conformation of APB.

It has been proposed that L-glutamic acid (**1**) is one of the major excitatory neurotransmitters in the central nervous system.[1-3] While L-glutamate meets many of the proposed criteria for neurotransmitter status, there still exists a great need for the development of potent and specific antagonists for neuronal pathways thought to use glutamate as a neurotransmitter. The glutamic acid analogue L-2-amino-4-phosphonobutanoic acid (**2**, APB) has been shown to antagonize excitatory synapses in the lateral perforant path of the rat hippocampal slice with an apparent $K_d$ of 2.5 μM.[4] The inhibitory effect of **2** shows stereoselectivity, since the D isomer of **2** is some 40 times less potent as an antagonist. Furthermore, **2** possesses high pathway selectivity, since it is 18 times less potent in antagonizing the synaptic transmission in the medial perforant path (the lateral and medial perforant paths originate from adjacent lateral and medial areas of the entorhinal cortex and terminate distally and medially, respectively, on the dendritic field of dentate granule cells).

It also has been shown that a class of L-[$^3$H]glutamate binding sites exist that are localized in synaptic plasma membranes and are distinguished by $Ca^{2+}/Cl^-$ dependence. [$^3$H]Glutamate is displaced from these binding sites by concentrations of L-APB that are in the same range (1–10 μM) as those required for the inhibition of synaptic transmission.[5-9] This observation has led to the suggestion

[†] Department of Medicinal Chemistry.
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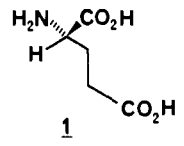
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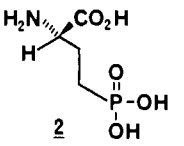
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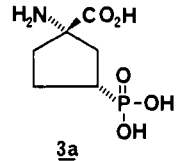
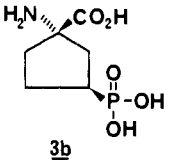
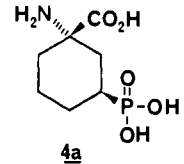
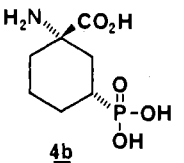

3a


3b


4a


4b

that this binding site might be the recognition site of the receptor mediating the APB-induced antagonism of synaptic transmission.[6] A DL-[$^3$H]APB binding site shows similar ligand specificity and ion dependence[10-12] as that observed for this L-[$^3$H]glutamate binding site. However, a recent investigation, by us, of the properties of this DL-[$^3$H]APB binding site suggests that this binding site is not the recognition site of the receptor mediating the APB-induced antagonism of synaptic responses.[12]

The present study was undertaken in an attempt to gain further information about the structural requirements for the antagonist activity of **2**. In particular, we wished to obtain information about the preferred conformation of **2** and thereby more clearly define the spatial arrangement of the amino, carboxyl, and phosphonate moieties at APB's recognition site(s). We chose to restrict the number of conformations available to **2** by incorporating its structure into carbocyclic rings. The 2- and 4-positions of **2** were thus linked by either two or three methylene units to give rise to the cyclopentyl APB analogues **3a** and **3b** and the cyclohexyl APB analogues **4a** and **4b**, respectively.

These APB analogues have been evaluated for their ability to inhibit excitatory inputs to granule cells and CA1 pyramidal cells in a hippocampal slice preparation and for their ability to affect [$^3$H]APB binding in a synaptic plasma membrane preparation. The results obtained have been used to compare the steric requirements of the APB receptor to those of the [$^3$H]APB binding site. A preliminary account of these results has been presented.[13,14]

## Results

**Syntheses.** The conformationally restricted APB analogues **3** and **4** were synthesized by sequential hydrophosphinylation[15] and Strecker[16] reactions on the appropriate unsaturated cyclic ketones as outlined in Schemes I and II, respectively. The cyclopentyl analogues **3a** and **3b** (Scheme I) were prepared from the known racemic diethyl 3-oxocyclopentylphosphonate **5**[15] via the Strecker synthesis using ammonium chloride and sodium cyanide to give a 81% yield of the aminonitrile **6** as a mixture of cis and trans isomers in a 1:1 ratio as suggested by $^{13}$C NMR.[17] Acetylation of this mixture produced the trans isomer **7a** and the cis isomer **7b**, which were readily separated from one another by flash chromatography. Acid hydrolysis of **7a** afforded racemic *trans*-cyclopentyl APB analogue **3a** in a 80% yield, while hydrolysis of **7b** provided racemic *cis*-cyclopentyl APB analogue **3b** in a 95% yield.

The first step toward **4** (Scheme II) was the preparation of ketophosphonate **8** in 92% yield from 2-cyclohexene-1-one. The racemic ketone was reacted with sodium cyanide and ammonium chloride to give a mixture of aminonitriles **9a** (cis) and **9b** (trans) in an approximate ratio of 1:4, respectively, as judged by the $^{13}$C NMR signals of the nitrile carbons.[17] Since previous observations had shown that on conformationally restrained cyclohexanones the Strecker reaction favored the introduction of the cyano group in an axial position,[18] the diastereoselectivity seen in the synthesis of **9** from **8** thus suggested that in this case the major isomer obtained (**9b**) was the one that possessed an axial cyano group in a trans relationship with the phosphonate moiety. This was later borne out in the $^{13}$C NMR studies that are described below. The diastereoisomeric aminonitriles **9a** and **9b** could be separated with difficulty by repeated column chromatography on silica gel. However, since some reversion to the starting phosphonoketone **8** was observed during the chromatographic separation, the mixture of aminonitriles was typically hydrolyzed without prior separation of the cis and trans isomers. Repeated cation exchange chromatography of the hydrolysis mixture provided pure samples of racemic *cis*- and *trans*-cyclohexyl APB analogues **4a** and **4b**, respectively.

**Assignment of Relative Configurations.** The assignment of relative configuration for the cyclohexyl analogues of APB, **4a** and **4b**, was carried out by use of $^{13}$C NMR spectroscopy. The $^{13}$C NMR spectra of **4a** and **4b**, although distinct, were initially difficult to interpret due to uncertainty about the effects of charged group interactions on the $^{13}$C chemical shifts. Thus, the $^{13}$C NMR spectra of the respective intermediate Strecker products **9a** and **9b** were used to assign the relative configurations through examination of their $\gamma$-gauche interactions.

Low-temperature $^{13}$C NMR studies have suggested that the phosphonate ester moiety exclusively occupies an equatorial position in cyclohexane.[19,20] Although an *A* value (i.e., the free energy difference between the equatorial and axial orientation of a substituent)[21] for the diethyl phosphonate moiety has not been reported, the $^{13}$C NMR for a set of cyclohexyl phosphorus compounds possessing either the $P(S)Me_2$ or $^+PMe_3$ tetravalent phosphorus moieties along with either a methyl group (*A* = 1.7 kcal/mol) or a *tert*-butyl group (*A* = 4–5 kcal/mol) at the 4-position have been studied. The tetravalent phosphorus moieties were found to possess a minimum *A* value of 3.0 kcal/mol.[22] Since the diethyl phosphonate

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(17) This is an approximate ratio since nuclear Overhauser effects do not allow this to be taken as a strict indication of the ratio of diastereoisomers.
(18) Edward, J. T.; Jatrangsri, C. *Can. J. Chem.* **1975**, *53*, 3339.
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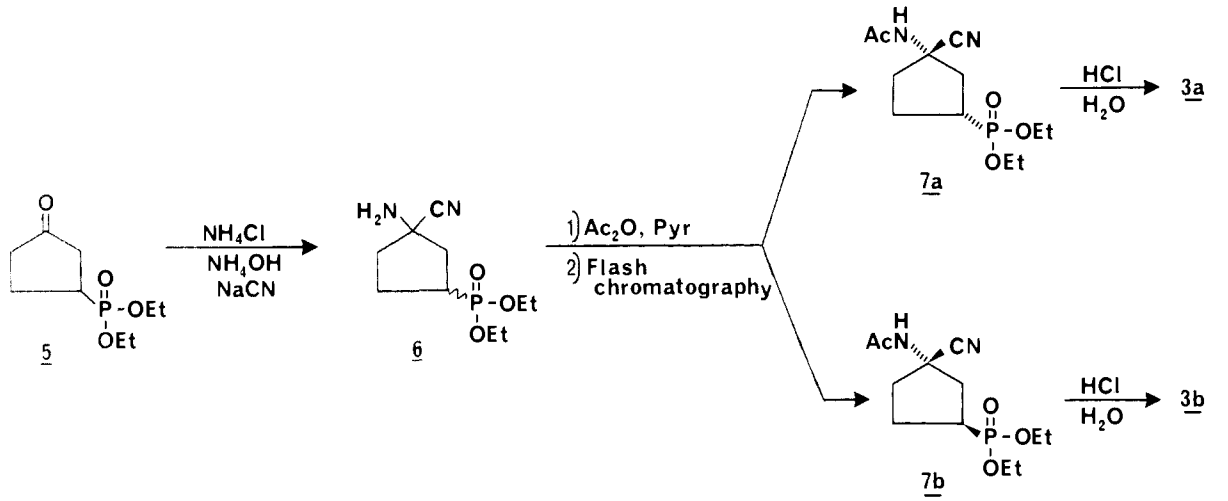
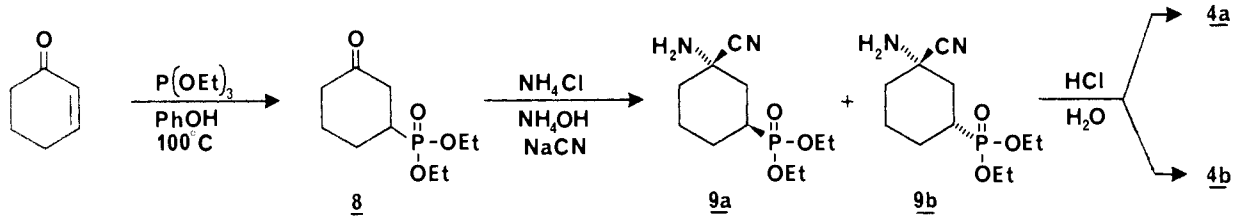
Scheme I




Scheme II




moiety is likely to have a comparable $A$ value, which is much greater than the values for the amino ($A$ = 1.2 kcal/mol) or cyano ($A$ = 0.21 kcal/mol) groups,[23] it seemed very likely, then, that the diethyl phosphonate moiety would occupy an equatorial position and possibly hold the rings in **9a** and **9b** in a chairlike conformation. This would mean that one of the diastereoisomeric aminonitriles (**9b**) would therefore have an equatorial amino group and an axial cyano moiety, while the other diastereoisomer (**9a**) would have an equatorial cyano group and an axial amino group.

A study of the $^{13}C$ NMR of 4-*tert*-butylcyclohexylamine and cyclohexylcarbonitrile has shown that an axial amino group has a larger shielding or "$\delta$-gauche" effect[24] on $C_3$ ($\Delta\delta$ = 5.60 ppm) than does the cyano group ($\Delta\delta$ = 2.54 ppm) relative to the respective equatorially substituted cyclohexanes.[23] We predicted, then, that a cyclohexane with both the amino and cyano groups on $C_1$ would show a greater shielding at $C_3$ when the amino group was axial than when the cyano moiety was axial. To test this hypothesis, we initially sought related molecules with known configurations for comparison. In particular, we felt that the corresponding aminonitriles of 4-*tert*-butylcyclohexanone would be ideal model compounds. Unfortunately, the Strecker reaction of 4-*tert*-butylcyclohexanone with ammonium chloride and sodium cyanide gives only the aminonitrile with an axial cyano group and no detectable amount of the compound with an axial amine.[25,26] As an alternative, alkyl 1-aminocyclohexanecarboxylates were examined, since procedures to make both the trans and cis isomers of ethyl 1-amino-4-*tert*-butylcyclohexanecarboxylate, **10a** and **10b**, respectively, were available.[25,26] These compounds were prepared and their $^{13}C$ spectra determined. As shown in Figure 1, the shielding effect on $C_3$ was 2.55 ppm greater in the isomer where the amino group was axial and the carboxylate moiety was equatorial, **10a**, than in the case where the carboxylate group was axial and the amino was equatorial, isomer **10b**.

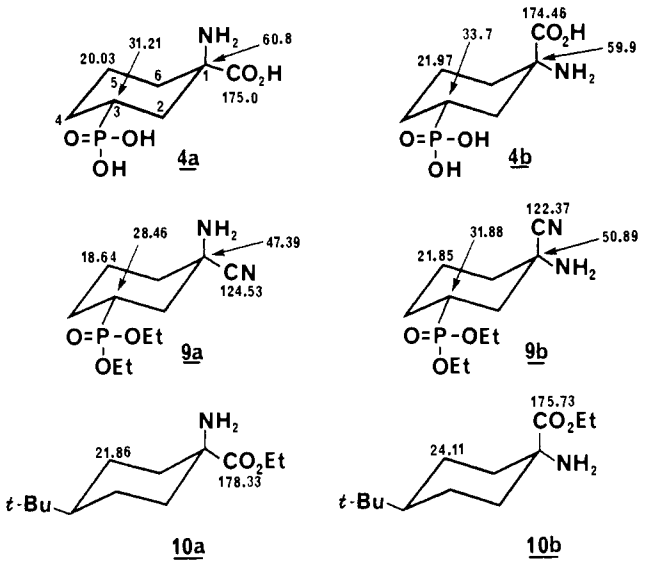



Figure 1. $^{13}C$ NMR chemical shifts and the $\gamma$-gauche effect.

Examination of the $C_3$ carbon atom resonances of the aminonitriles **9a** and **9b** (Figure 1) showed that the $C_3$ chemical shift in the minor isomer **9a** was farther upfield (3.42 ppm) than that of $C_3$ in the major isomer **9b**. This meant that in **9a** $C_3$ was more shielded. Based on the above analyses this suggested that in the minor isomer **9a** the amino group was in an axial position and therefore was in a trans relationship with the phosphonate moiety.

(22) Gordon, M. D.; Quin, L. D. *J. Org. Chem.* **1976**, *41*, 1690.
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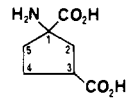
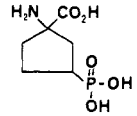
**Table I.** Correlation of the Diastereoisomers of **3** with *cis*- and *trans*-1-Amino-1,3-cyclopentanedicarboxylic Acid (**11**) by $^{13}C$ NMR[a]

| compd | | δ values, ppm | | | | | |
|---|---|---|---|---|---|---|---|
| | | $C_1$ | $C_2$ | $C_3$ | $C_4$ | $C_5$ | $CO_2^-$ |
|  | trans (**11a**) | 64.6 | 43.1 | 47.1 | 29.3 | 39.3 | 184.8 |
| | cis (**11b**) | 65.1 | 43.5 | 45.9 | 28.9 | 38.1 | 185.0 |
| | Δδ | −0.5 | −0.4 | 1.2 | 0.4 | 1.2 | −0.2 |
|  | trans (**3a**) | 66.3 | 41.9 | 38.9 | 27.7 | 40.1 | 185.0 |
| | cis (**3b**) | 66.6 | 42.1 | 37.1 | 26.7 | 38.5 | 185.0 |
| | Δδ | −0.3 | −0.2 | 1.8 | 1.0 | 1.6 | 0.0 |

[a] Samples are 0.3 M in 1 N NaOD.

Several other features of the $^{13}C$ NMR spectra of **9a** and **9b** compared favorably for the proposed assignment of relative configurations, including the shielding effects at $C_5$, $C_1$, and the nitrile carbon (Figure 1). Since $C_5$, like $C_3$, was in a γ-gauche relationship to the axial functionality on $C_1$, this carbon was expected to be most shielded when the amino group was axial. Indeed, $C_5$ in **9a** was shielded by 3.21 ppm relative to $C_5$ in **9b**. Concerning the shielding at $C_1$, Schneider and Hoppen[28] reported a greater shielding capability of $C_1$ by an axial vs. equatorial amino group (5.8 ppm) compared with the smaller difference between an axial vs. equatorial cyano group (1.3 ppm). Thus, we expected the diastereoisomer of **9** with the axial amino group and equatorial nitrile to exhibit the greater shielding at $C_1$. The lower chemical shift value for $C_1$ in **9a** compared with that for $C_1$ in **9b** was consistent with this expectation. Finally, the nitrile carbon of **9b** was shielded by 2.16 ppm relative to the nitrile carbon in the cis diastereoisomer, consistent with the notion that the axial nitrile of **9b** was shielded via γ-gauche interactions with $C_3$ and $C_5$. A similar observation was made for **10b**, where the axial ester carbonyl was shielded by 2.60 ppm relative to the equatorial ester carbonyl resonance in **10a**.

We had initially avoided basing the assignments of configuration of the amino acids **4a** and **4b** on their $^{13}C$ NMR spectra. This was primarily because we did not know what effect the charged groups would have on the conformation of the cyclohexane ring, and hence what the effect would be on γ-gauche interactions. It is of interest to note, however, that the $^{13}C$ NMR spectra of the amino acids **4a** and **4b** gave a relative pattern of shifts that was similar to that seen for the aminonitriles **9a** and **9b** (Figure 1).

In assigning the relative stereochemistry of the cyclopentyl APB analogues we also turned to $^{13}C$ NMR in the hope of producing an analysis similar to that made for the cyclohexyl analogues. The $^{13}C$ NMR spectrum of the aminonitrile intermediate **6** was of little value in this regard, since the mixture of compounds could not be separated and then hydrolyzed in order to effect a correlation with the respective amino acids. The acetylated diastereomeric intermediates **7a** and **7b** were separable; however, the $^{13}C$ NMR spectra of the acetylated intermediates gave equivocal results because the variety of conformations that could readily interconvert by pseudorotation[27] provided a complex array of possible γ-gauche interactions. The $^1H$ NMR spectra of the diastereoisomers of **7** were similar except for the amide (CON*H*) resonances, which for the higher $R_f$ diastereoisomer (**7a**) occurred 0.5 ppm farther downfield than the NH resonance for the other diastereoisomer (**7b**). The $^{31}P$ NMR spectra of the diastereoisomers of **7** also showed a downfield (2.37 ppm) shift for the diastereoisomer with the higher $R_f$, isomer **7a**, suggesting that the phosphorus atom in this isomer was less rich in electrons than the phosphorus atom in the other diastereoisomer **7b**. One possible explanation for these relative shifts is that an intramolecular hydrogen bond exists between the amide and phosphonate moieties. An oxygen atom that is doubly bonded to a phosphorus atom is known to participate in hydrogen bonding.[28,29] The phosphonate moiety would be less electron rich if it was donating electrons for a hydrogen bond. Such intramolecular hydrogen bonding would only be possible for the isomer in which the acetamido and phosphonate groups were cis to one another.

Since the cyclic amino acids **3a** and **3b** had close structural analogues in the *trans*- and *cis*-1-amino-1,3-cyclopentanedicarboxylic acids, **11a** and **11b**, respectively,[30] the relative stereochemistry of which had been determined by X-ray crystallography,[31] we thus sought to correlate the cis and trans isomers of **3** and **11** by $^{13}C$ NMR. It seemed reasonable that the conformations of these compounds in basic solutions would be similar, if unknown, due to electrostatic repulsion by the ionized acidic functionalities. The dicarboxylic amino acids were prepared as described in the literature,[30] and their $^{13}C$ NMR spectra were obtained from their 0.3 M solutions in 1 N sodium deuteroxide. The difference in chemical shifts (Δδ) was calculated for each carbon in the ring of **11**, and these Δδ values were compared with those obtained from the diastereoisomers of **3** (Table I). The high similarity in the pattern of Δδ values for $C_1$–$C_5$ suggested that the diastereoisomer of **3**, **3b**, which was shielded at $C_3$–$C_5$, possessed the cis configuration. Isomer **3a** would thus have the configuration in which the phosphonate and carboxyl moieties are trans to one another.

**Electrophysiological Studies.** The cyclic APB analogues were tested for their ability to block evoked synaptic field potentials in regions of the rat hippocampal slice as measured by extracellular recording.[4] These data for the inhibition of hippocampal excitatory pathways are summarized in Table II. For comparison, data for inhibition of these pathways by L-APB are also presented. L-APB has been shown to block synaptic transmission in a con-

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**Table II.** Inhibition of Hippocampal Excitatory Neurotransmission and Displacement of DL-[$^3$H]APB Binding by Cyclic Analogues of 2-Amino-4-phosponobutanoic Acid (APB)

| | electrophysiology | | | | [$^3$H] binding | | |
|---|---|---|---|---|---|---|---|
| compd | $IC_{50}$ (LPP[a]), μM | $IC_{50}$ (MPP[b]), μM | $IC_{50}$ (CA1[c]), μM | $IC_{50}$ (MPP)/$IC_{50}$ (LPP) | $IC_{50}$, μM | $n_{Hill}$ | $IC_{50}$ (LPP)/$IC_{50}$ (bind) |
| **2** (L-APB) | 2.5[d] | 45 | 2,500[e] | 18 | 1.5[f] | 0.8[f] | 1.7 |
| **3a**[g] | 130 | 1850 | >10000[h] | 31 | 80 | 1.0 | 1.6 |
| **3b**[g] | 960 | 2400 | 3800 | 2.5 | 4.7 | 1.0 | 200 |
| **4a**[g] | 3100 | 7300 | 8100 | 2.4 | 840 | ND[i] | 3.7 |
| **4b**[g] | 5800 | 10000 | 9600 | 1.7 | 750 | ND | 7.7 |

[a] LPP, lateral perforant path. [b] MPP, medial perforant path. [c] CA1, Schaffer collateral-CA1 pyramidal cells. [d] Data from ref 4. [e] Data from ref 32. [f] Data from ref 12. [g] Tested as racemic mixtures. [h] This value was estimated from the 18% inhibition that was observed at a concentration of 8 mM. [i] ND, not determined.

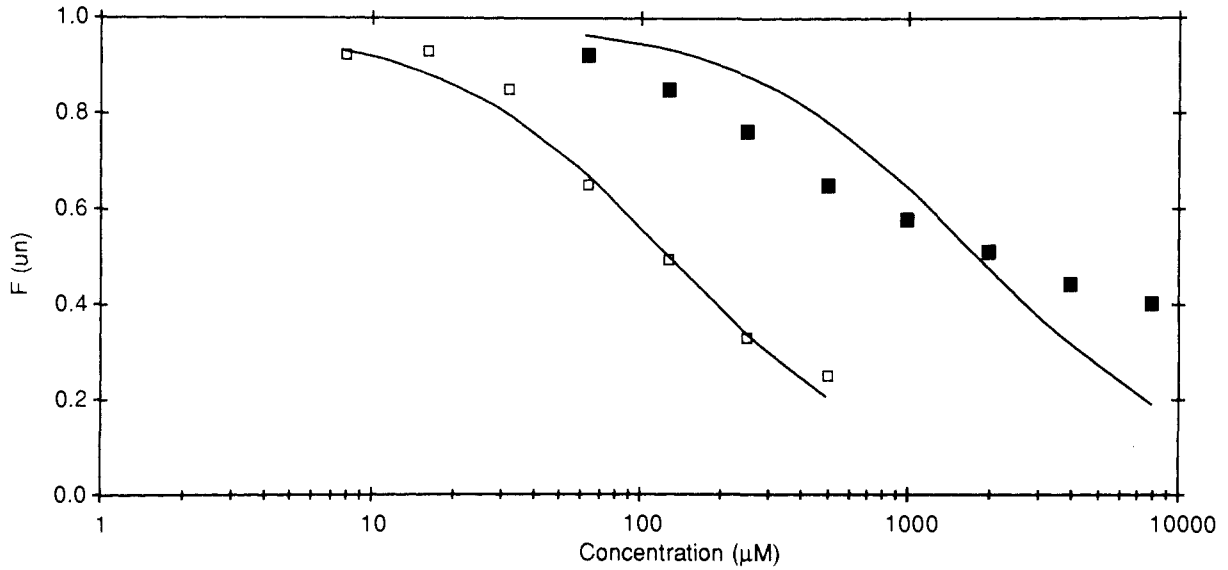


**Figure 2.** Concentration–response curves for cyclopentyl APB analogue **3a** induced inhibition of perforant path responses. Amplitudes of the synaptic field potential were recorded in the absence and the presence of increasing drug concentrations and plotted as a fraction of the original uninhibited amplitude (F(un)). The mean of data for medial perforant path responses (■, $n$ = 5) are presented. These data were derived from responses that were contaminated with no more than 9% of lateral responses. To illustrate that these data do not conform to those of a homogeneous response, a theoretical curve with a $K_d$ = 1.84 mM and a Hill coefficient of 1 was drawn through these data. The mean data obtained from four lateral perforant path responses are presented (□). Two of these sets of data were empirically corrected for no more than 30% contamination with medial responses. A theoretical curve with a $K_d$ = 130 μM and a Hill coefficient of 1 was drawn through these data.

centration-dependent and stereospecific manner. Furthermore, L-APB is pathway specific, since lateral and medial perforant paths (projections from the lateral and medial entorhinal cortex, respectively) have different sensitivities to this agent. Studies describing these properties of L-APB have been reported elsewhere.[4,35]

The cyclopentyl APB analogue **3b** and the two cyclohexyl APB analogues **4a** and **4b** all had low activity in inhibiting both lateral (LPP) and medial (MPP) perforant path neurotransmission. The fact that none of these compounds induced population spiking when stimulus intensity was just subthreshold for this response plus the fact that the data for inhibition of these responses parallel theoretical binding curves with a Hill coefficient of 1 (data not shown) suggests that these compounds may be acting as antagonists. Although **3b**, **4a**, and **4b** exhibited a small degree of pathway selectivity, being approximately 2-fold more potent for inhibition of lateral entorhinal inputs, they were substantially less selective than L-APB (**2**), which possesses an $IC_{50}$ (MPP) to $IC_{50}$ (LPP) ratio of 18. This lack of strong pathway specificity precludes definitive identification of these three compounds as antagonists. In the Schaffer collateral-CA1 region, where L-APB has been classified as the weak agonist ($IC_{50}$ = 2.5 mM),[32] the cyclohexyl derivatives of APB, **4a** and **4b**, and cyclopentyl APB analogue **3b** gave no signs of agonist activity and were found to exhibit low potency for inhibition of evoked field potentials.

The cyclopentyl APB analogue **3a**, on the other hand, showed appreciable activity in inhibiting synaptic activity in the lateral perforant path. However, with an $IC_{50}$ (LPP) value of 130 μM racemic **3a** was still about 50 times weaker than L-APB. Nevertheless, **3a** displayed pathway selectivity for inhibition of lateral vs. medial entorhinal projections to hippocampal granule cells. The $IC_{50}$ (MPP) to $IC_{50}$ (LPP) ratio was equal to 31, which is experimentally comparable to the selectivity ratio of 18 for L-APB. The concentration–response curves for **3a** are presented in Figure 2. The data for the inhibition of lateral perforant path responses paralleled a theoretical binding curve with a Hill coefficient of 1, while the concentration–response curve for the inhibition of medial responses was shallower than would be expected for a simple antagonist interacting with a single population of receptors. In the Schaffer collateral-CA1 region **3a** was found to resemble APB in that it caused population spikes indicative of agonist activity.

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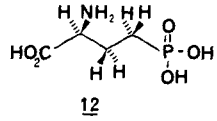
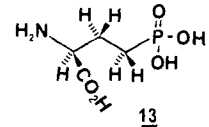
**Binding Studies.** The cyclic APB analogues were also tested for their ability to displace [$^3$H]APB from binding sites in a rat brain synaptic plasma membrane preparation.[12] The $IC_{50}$ values and Hill coefficients for the displacement of DL-[$^3$H]APB binding by **3a**, **3b**, **4a**, and **4b**, as well as L-APB (**2**), are summarized in Table II. In this preparation, DL-[$^3$H]APB had a $K_d$ = 6.0 $\mu$M and a $B_{max}$ = 380 pmol/mg of protein.[12] L-APB (**2**) possessed an $IC_{50}$ value of 1.5 $\mu$M and an $IC_{50}$ (LPP) to $IC_{50}$ (binding) ratio of 1.7. As was observed in the electrophysiological experiments, the two racemic cyclohexyl derivatives of APB, **4a** and **4b**, showed low potency for inhibition of binding. These compounds displaced approximately 50% of the binding at 1 mM. The cyclopentyl analogue **3a** was found to have an $IC_{50}$ value of 80 $\mu$M and an $IC_{50}$ (LPP) to $IC_{50}$ (binding) ratio comparable to that of APB. The cyclopentyl APB analogue **3b**, on the other hand, inhibited 50% of the binding of radioligand at 4.7 $\mu$M. The very potent activity of **3b** in the binding assay was in stark contrast to its low activity in the electrophysiological assay.

## Discussion

In the present investigation we have tried to more clearly define the biologically active conformation of 2-amino-4-phosphonobutanoic acid (**2**) by incorporating the structure of **2** into either a cyclopentane or cyclohexane ring system. These constraints would still allow some degree of flexibility in the molecules, and this would be particularly true for the cyclopentane system since conformations in this system are known to interconvert readily by pseudorotation.[27] However, we felt that these partial contraints would still provide us with some valuable information. We thus synthesized the APB analogues **3a**,**b** and **4a**,**b**. We envisioned that in the case of the two cyclohexyl APB analogues **4a** and **4b**, the phosphonate moiety, because of its size, would occupy an equatorial position and hold the rings in a chairlike conformation. In such conformations **4a** and **4b** would be capable of resembling structures **12** and **13**, respectively, two of the several possible highly extended forms of APB. The cyclopentyl APB analogues **3a** and **3b**, on the other hand, would not be capable of mimicking such fully extended conformations, but rather would resemble partially folded forms of APB, like **14** and **15**, respectively.




12

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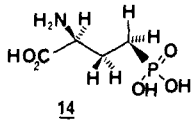


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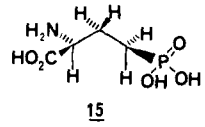


15

The analogues **3b**, **4a**, and **4b**, showed very little activity in the electrophysiological assays and little if any pathway selectivity. While one possible explanation for the inactivity of these compounds is that they are not representative of APB's biologically active conformation, it may also be that the conformational constraint that has been built into these analogues has resulted in unfavorable steric interactions. Such steric effects may be playing a role here, since we have previously shown that substitution of the $\alpha$-carbon of **2** is very sensitive to substitution. When a methyl group is placed on the $\alpha$-carbon a dramatic decrease in biological activity ($IC_{50}$(LPP) and $IC_{50}$(MPP) > 10000 $\mu$M] is observed.[33] It is interesting to note, however, that the level of electrophysiological activity seen with the cyclic APB analogues is higher than that seen previously with $\alpha$-methyl APB. Thus, it appears that by placing a bridging unit between the $\alpha$ and $\gamma$ carbons of APB we are able to overcome to some extent the loss of activity seen when there is just a methyl group on the $\alpha$-carbon of APB.

In contrast to **3b**, **4a**, and **4b**, the cyclic APB analogue **3a**, in which the carboxyl and phosphonate moieties are trans to one another, showed appreciable activity in inhibiting synaptic activity in the lateral perforant path. Although only 1/50 as potent as **2**, **3a** showed the high selectivity for the lateral vs. the medial perforant path characteristic of **2**. The inhibition data for this compound (Figure 2) also suggest heterogeneity in medial responses. This supports previous observations that suggest medial responses are comprised of two populations of responses.[4,34,35] Both baclofen[34] and L-APB[4,35] exhibit concentration–response data that cannot be fitted to a curve that assumes a single population of receptors.

Analogous to their activity in the electrophysiology studies, the cyclohexyl APB analogues **4a** and **4b** showed little activity in the radioligand binding assay. The activity of the cyclopentyl analogue **3a** in the radioligand binding assay paralleled its activity in inhibiting synaptic transmission in the lateral perforant path. Surprisingly, the cyclopentyl APB analogue **3b**, in which the phosphonate and carboxyl moieties are cis to one another, was extremely potent in inhibiting the binding of [$^3$H]APB. With an $IC_{50}$ of 4.7 $\mu$M racemic **3b** was only slightly less active than L-APB. This high potency in the binding assay is in stark contrast to its very low activity in the antagonism of synaptic activity in the lateral perforant path. This result when taken together with our previous studies with various methylated APB analogues[12] and the more recent studies of Fagg and Lanthorn[36] strongly suggests that contrary to previous suggestions[6] the APB binding site in the rat brain synaptosomal membrane preparation is not the same as the receptor mediating APB-induced inhibition of synaptic transmission in the lateral perforant path.

Of all four cyclic APB analogues, **3a** most resembles APB in its spectrum of biological activity. Similar to L-APB (**2**), **3a** showed significant potency and selectivity for inhibition of lateral entorhinal projections to hippocampal granule cells. It shows low activity in the inhibition of medial entorhinal projections to hippocampal granule cells as has been observed for L-APB.[4,35] The cyclopentyl analogue **3a** shows low potency for the inhibition of CA1 responses, and like APB, this inhibition was accompanied by the transient appearance of population spikes. Finally, like APB,[12] **3a** shows approximately equal potency for the displacement of DL-[$^3$H]APB binding and inhibition of lateral perforant path responses. It thus appears that of the four conformationally constrained analogues studied in this investigation **3a** might come closest to mimicking the active conformation of APB. Since the phosphonate and amino groups of **3a** are cis to one another, an ionic interaction between these two groups is possible, and this would certainly give rise to a highly folded conformation. Whether or not this is the conformation that **2** needs to be in to serve as an antagonist cannot be said with certainty with the current information, but it does, we feel, serve as a useful working hypothesis.

## Experimental Section

Thin-layer chromatography (TLC) was performed on Analtech 250-$\mu$m silica gel GF plates or on Analtech 250-$\mu$m $C_{18}$ RPS-F reverse-phase plates. TLC visualization was by UV light, iodine,

(36) Fagg, G. E.; Lanthorn, T. H. *Br. J. Pharmacol.* **1985**, *86*, 743.

and ninhydrin spray. Chromatographic purification was accomplished by flash chromatography[37] using Silica Gel 60 (40–63 μm) from EM Reagents. Ion-exchange chromatography was carried out on AG 50WX8 cation exchange resin (200–400 mesh) obtained from Bio Rad Laboratories. IR spectra were obtained on a Perkin-Elmer 281 IR spectrometer. $^1$H NMR spectroscopy was performed on either a JEOL FX-90-MHz or Nicolet 300-MHz spectrometer. $^{13}$C NMR spectroscopy was performed on the JEOL FX-90 instrument at 22.5 MHz, and $^{31}$P NMR spectroscopy was performed on the Nicolet instrument at 121 MHz. Melting points were obtained on a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ, and were all within ±0.4% of the theoretical values. DL-2-Amino-4-phosphono[$^3$H]butanoic acid was obtained from New England Nuclear, Boston MA.

**Diethyl (*RS*)-3-Oxocyclopentylphosphonate (5).** The procedure of Harvey[15] was followed. A solution of 2-cyclopenten-1-one (5.0 g, 61 mmol) in warm (ca. 50 °C) phenol (16 g, 170 mmol) was stirred under argon as triethyl phosphite (13 mL, 76 mmol) was added over 10 min via syringe. The temperature rose to 100 °C during the addition and was maintained at 100 °C for 24 h by external heating. The product was distilled to give 9.82 g (73%) of a clear oil: bp 102–110 °C (0.25 mmHg) [lit[15] bp 104 °C (0.15 mmHg)]; TLC, $R_f$ = 0.32 (EtOAc); $^{13}$C NMR ($CDCl_3$) δ 214.7 (d, $^3J_{CP}$ = 15 Hz, $C_3$), 61.01 (d, $^2J_{CP}$ = 7 Hz, POC), 37.71 (s, $C_4$), 36.60 (d, $^2J_{CP}$ = 7 Hz, $C_2$), 31.94 (d, $^1J_{CP}$ = 153 Hz, $C_1$), 22.67 (d, $^3J_{CP}$ = 4 Hz, $C_5$), 15.66 (s, POC*C*). Flash chromatography (EtOAc) gave an analytical sample. Anal. ($C_9H_{17}O_4P$) C, H, P.

**1(*RS*)-Amino-3(*RS*)-(diethoxyphosphinyl)cyclopentanecarbonitrile (6).** Diethyl (*RS*)-3-oxocyclopentylphosphonate (**5**; 0.76 g, 3.5 mmol), ammonium chloride (0.37 g, 7.0 mmol), and sodium cyanide (0.34 g, 7.0 mmol) were added to concentrated $NH_4OH$ (5 mL), and the mixture was stirred for 4 h in the dark at room temperature. The clear solution was extracted with $CH_2Cl_2$ (4 × 20 mL), and the combined extracts were dried ($MgSO_4$). Removal of the solvent in vacuo gave a crude oil, which was purified by flash chromatography (5% MeOH/$CH_2Cl_2$): yield, 0.77 g (89%) of a clear oil; TLC, $R_f$ = 0.36 (5% MeOH/$CH_2Cl_2$); IR (neat) 2215 (nitrile) cm$^{-1}$; $^{13}$C NMR ($CDCl_3$) δ 123.18, 123.07 (diastereomeric nitriles, equal intensity), 61.12, 61.01, 60.82, 60.71 (pair d, 2 nonequivalent POC), 54.92 (d, $^3J_{CP}$ = 13 Hz, $C_1$), 53.18 (d, $^3J_{CP}$ = 15 Hz, $C_1$), 40.39 (s, $C_2$), 40.13 (d, $^3J_{CP}$ = 12 Hz, $C_5$), 39.00 (d, $^3J_{CP}$ = 9 Hz, $C_5$), 32.82 (d, $^1J_{CP}$ = 150 Hz, $C_3$), 32.29 (d, $^1J_{CP}$ = 149 Hz, $C_3$), 23.78 (s, $C_4$), 15.54 (d, $^3J_{CP}$, POC*C*).

**1(*RS*)-Acetamido-3(*RS*)-(diethoxyphosphinyl)cyclopentanecarbonitrile (7).** A solution of 1(*RS*)-amino-3(*RS*)-(diethoxyphosphinyl)cyclopentanecarbonitrile (**6**; 0.74 g, 3.0 mmol) and acetic anhydride (4 mL, 42 mmol) in pyridine (7 mL) was stirred at room temperature with protection against moisture ($CaSO_4$ tube) for 12 h. The solvent was evaporated in vacuo to leave a brown oil containing a mixture of the cis and trans isomers of **7**. These isomers were separated by flash chromatography (5% MeOH/$CH_2Cl_2$).

**Trans Isomer (7a).** Yield, 0.17 g (24%) of a solid with mp 106–108 °C; TLC, $R_f$ = 0.45 (5% MeOH/$CH_2Cl_2$); IR (neat) 2240 (weak, CN) cm$^{-1}$; $^1$H NMR ($CDCl_3$) δ 7.64 (s, 1 H, N*H*); $^{13}$C NMR ($CDCl_3$) δ 170.13 (amide C=O), 119.63 (nitrile), 62.23, 62.17, 61.93, 61.82 (2 doublets, 2 nonequivalent POC), 54.67 (d, $^3J_{CP}$ = 11 Hz, $C_1$), 38.58 (s, $C_2$), 37.67 (d, $^3J_{CP}$ = 9 Hz, $C_5$), 31.78 (d, $^1J_{CP}$ = 149 Hz, $C_3$), 23.49 (d, $^2J_{CP}$ = 2 Hz, $C_4$), 22.27 (s, *C*C=O), 15.98 (d, $^3J_{CP}$ = 6 Hz, POC*C*); $^{31}$P NMR ($CDCl_3$) δ 33.78. Anal. ($C_{12}H_{21}N_2O_4P$) C, H, N, P.

**Cis Isomer (7b).** Yield, 0.40 g (46%) of an oil; TLC, $R_f$ = 0.34 (5% MeOH/$CH_2Cl_2$); IR (neat) 2240 (strong, nitrile) cm$^{-1}$; $^1$H NMR ($CDCl_3$) δ 7.14 (s, amide N*H*); $^{13}$C NMR ($CDCl_3$) δ 170.64 (amide C=O), 120.04 (nitrile), 62.10 (d, $^2J_{CP}$ = 7 Hz, POC), 54.45 (d, $^3J_{CP}$ = 16 Hz, $C_1$), 38.98 (s, $C_2$), 38.47 (d, $^3J_{CP}$ = 12 Hz, $C_5$), 33.08 (d, $^1J_{CP}$ = 151 Hz, $C_3$), 24.41 (s, $C_4$), 16.29 (d, $^3J_{CP}$ = 6 Hz POC*C*); $^{31}$P NMR ($CDCl_3$) δ 31.41. Anal. ($C_{12}H_{21}N_2O_4P$) C, H, N, P.

***trans*-1(*RS*)-Amino-3(*RS*)-phosphonocyclopentanecarboxylic Acid (3a).** A solution of **7a** (0.75 g, 2.6 mmol) in refluxing 6 N HCl (10 mL) was stirred for 12 h. The solvent was evaporated, and the residue was treated with $H_2O$ (2 × 10 mL), the solvent being removed by evaporation in vacuo each time. The residual white solid was dissolved in warm $H_2O$ (1 mL) and was applied to a 1 × 36 cm column of AG 50WX8 cation exchange resin ($H^+$ form, 50–100 mesh). The column was eluted with $H_2O$, and the fractions containing the desired material (monitored at 210 nm) were pooled and evaporated to give a white solid, which was recrystallized from $H_2O$/EtOH/$Et_2O$ and yielded 0.43 g (80%) of **3a**: mp 267–270 °C dec; $^{13}$C NMR (1 N NaOD) δ 185.0 (s, $CO_2$), 66.3 (d, $^3J_{CP}$ = 12 Hz, $C_1$), 41.9 (s, $C_2$), 40.1 (d, $^3J_{CP}$ = 13 Hz, $C_5$), 38.9 (d, $^1J_{CP}$ = 136 Hz, $C_3$), 27.7 (s, $C_4$); $^{31}$P NMR (1 N NaOD) δ 24.58. Anal. ($C_6H_{12}NO_5P$·0.5$H_2O$) C, H, N, P.

***cis*-1(*RS*)-Amino-3(*RS*)-phosphonocyclopentanecarboxylic Acid (3b).** A sample of **7b** (0.30 g, 1.0 mmol) was hydrolyzed in refluxing 6 N HCl (10 mL) for 11 h. The solvent was removed in vacuo and was then treated with $H_2O$ (2 × 10 mL), the solvent being removed by evaporation in vacuo each time. The residual white solid dissolved readily in $H_2O$ (1 mL) at room temperature, and this solution was applied to a 1 × 20 cm column of AG 50WX8 cation exchange resin ($H^+$ form, 50–100 mesh). The column was eluted with $H_2O$, and the fractions containing the product (monitored at 210 nm) were pooled and evaporated to give a glass that crystallized upon standing at room temperature. Drying of this material at 56 °C in vacuo over $CaSO_4$ gave 0.21 g (95%) of a white solid: mp 252–255 °C (sintered with browning); $^{13}$C NMR (1 N NaOD) δ 185.0 (s, $CO_2$), 66.6 (d, $^3J_{CP}$ = 12 Hz, $C_1$), 42.1 (s, $C_2$), 38.5 (d, $^3J_{CP}$ = 10 Hz, $C_5$), 37.1 (d, $^1J_{CP}$ = 137 Hz, $C_3$), 26.7 (s, $C_4$); $^{31}$P NMR (1 N NaOD) δ 25.40. Anal. ($C_6H_{12}NO_5P$·0.25$H_2O$) C, H, N, P.

**Diethyl (*RS*)-3-Oxocyclohexylphosphonate (8).** A solution of 2-cyclohexen-1-one (38.7 mL, 0.40 mole) in warm (ca. 50 °C) phenol (100 g, 1.10 mole) was stirred under argon as triethyl phosphite (88 mL, 0.50 mole) was added over 10 min via syringe. The temperature rose to 100 °C during the addition and then was maintained at 100 °C for 24 h by external heating (oil bath). The mixture was vacuum distilled to give 86 g (92%) of an oil: bp 106–110 °C (0.25 mmHg); TLC, $R_f$ = 0.36 (EtOAc); $^{13}$C NMR ($CDCl_3$) δ 207.78 (d, $^3J_{CP}$ = 16 Hz, $C_3$), 61.28 (d, $^2J_{CP}$ = 7 Hz, POC), 40.36 (s, $C_6$), 39.86 (d, $^2J_{CP}$ = 4 Hz, $C_2$), 35.22 (d, $^1J_{CP}$ = 145 Hz, $C_1$), 25.30 (d, $^3J_{CP}$ = 18 Hz, $C_5$), 23.81 (d, $^2J_{CP}$ = 4 Hz, $C_4$), 15.87 (s, POC*C*). Flash chromatography (EtOAc) gave an analytical sample. Anal. ($C_{10}H_{19}O_4P$) C, H, P.

**1(*RS*)-Amino-3(*RS*)-(diethoxyphosphinyl)cyclohexanecarbonitrile (9).** Diethyl (*RS*)-3-oxocyclohexylphosphonate (**8**; 3.0 g, 13 mmol), sodium cyanide (1.26 g, 26 mmol), and ammonium chloride (1.37 g, 26 mmol) were stirred in concentrated $NH_4OH$ (15 mL) for 16 h. The mixture was extracted with $CH_2Cl_2$ (4 × 35 mL), and the combined extracts were dried ($MgSO_4$). The solvent was removed in vacuo to give a faintly brown oil (2.93 g, 88%) that consisted primarily of the diastereomeric cis (**9a**) and trans (**9b**) aminonitriles in a 1:4 ratio as judged by $^{13}$C NMR. Although the diastereoisomers could be separated into their racemates by repeated flash chromatography (5% MeOH/$CH_2Cl_2$), the mixture of diastereoisomers was typically hydrolyzed without further purification.

**Cis Isomer (9a).** TLC, $R_f$ = 0.36 (5% MeOH/$CH_2Cl_2$); $^{13}$C NMR ($CDCl_3$) δ 124.53 (s, CN), 61.26 (d, $^2J_{CP}$ = 7 Hz, POC), 47.39 (d, $^3J_{CP}$ = 18 Hz, $C_1$), 35.06 (s, $C_2$ and $C_6$), 28.46 (d, $^1J_{CP}$ = 146 Hz, $C_3$), 24.11 (s, $C_4$), 18.64 (d, $^3J_{CP}$ = 17 Hz, $C_5$), 15.98 (d, $^3J_{CP}$ = 4 Hz, POC*C*).

**Trans Isomer (9b).** TLC, $R_f$ = 0.30 (5% MeOH/$CH_2Cl_2$); $^{13}$C NMR ($CDCl_3$) δ 122.37 (s, CN), 60.88 (d, $^2J_{CP}$ = 7 Hz, POC), 50.89 (d, $^3J_{CP}$ = 19 Hz, $C_1$), 36.54 (br s, $C_2$ and $C_6$), 31.88 (d, $^1J_{CP}$ = 146 Hz, $C_3$), 23.41 (s, $C_4$), 21.85 (d, $^3J_{CP}$ = 18 Hz, $C_5$), 15.55 (s, POC*C*).

**1(*RS*)-Amino-3(*RS*)-phosphonocyclohexanecarboxylic Acid (4).** The mixture of diastereomeric aminonitriles **9** (0.47 g, 1.8 mmol) was hydrolyzed in refluxing 6 N HCl (5 mL) overnight. The solvent was evaporated in vacuo, and the residue was treated with $H_2O$ (2 × 5 mL) with removal of the $H_2O$ in vacuo each time. The residual glass was dissolved in $H_2O$ and treated with activated charcoal on a steam bath. The hot mixture was filtrated through a pad of Celite followed by concentration of the filtrate to about 5 mL. This solution was applied to a 1 × 20 cm column of AG 50WX8 cation exchange resin ($H^+$ form, 200–400 mesh), and the column was eluted with $H_2O$. The column effluent was monitored at 210 nm, and, after the initial peak at the void

(37) Still, W. C.; Kahn, M.; Mitra, W. *J. Org. Chem.* **1978**, *43*, 2923.

volume, two overlapping peaks of material were obtained, a minor product (**4a**) eluting first, followed by the major product (**4b**).

**Cis Isomer (4a).** Recycling the early peaks of **4a** through the same column and combining the pure fractions gave pure **4a** as a white solid: mp >260 °C dec; TLC ($C_{18}$), $R_f$ = 0.82 (50% MeOH/$H_2O$), 0.32 (70% $CH_3CN/H_2O$); $^{13}C$ NMR ($D_2O$) δ 175.00 (s, $CO_2$), 60.8 (d, $^3J_{CP}$ = 15 Hz, $C_1$), 31.53 (s, $C_6$ or $C_2$), 31.21 (d, $^1J_{CP}$ = 140 Hz, $C_3$), 31.07 (s, $C_2$ or $C_6$), 24.75 (d, $^2J_{CP}$ = 6 Hz, $C_4$), 20.03 (d, $^3J_{CP}$ = 16 Hz, $C_5$); $^{31}P$ NMR (1 N NaOD) δ 23.93. Anal. ($C_7H_{14}NO_5P\cdot{}^1/_3H_2O$) C, H, N, P.

**Trans Isomer (4b).** In a similar fashion the late fractions of **4b** were combined and recycled through the same column. The pure fractions were combined to give pure **4b** as a white solid: mp >260 °C dec; TLC ($C_{18}$), $R_f$ = 0.80 (50% MeOH/$H_2O$), 0.26 (70% $CH_3CN/H_2O$); $^{13}C$ NMR ($D_2O$) δ 174.46 (s, $CO_2$), 59.88 (d, $^3J_{CP}$ = 15 Hz, $C_1$), 35.70 (d, $^1J_{CP}$ = 138 Hz, $C_3$), 32.78 (s, $C_2$ and $C_6$), 25.25 (s, $C_4$), 21.97 (d, $^3J_{CP}$ = 15 Hz, $C_5$); $^{31}P$ NMR (1 N NaOD) δ 24.21. Anal. ($C_7H_{14}NO_5P\cdot0.5H_2O$) C, H, N, P.

**Electrophysiological Experiments.** Experiments were performed on transverse slices of the hippocampal formation of 30–90-day-old male Holtzman rats prepared as previously described.[32,35] Slices 450 μm thick were placed in an incubation chamber at 30 °C[38] containing a medium comprised of 10 mM D-glucose, 124 mM NaCl, 3.3 mM KCl, 2.4 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, and 26 mM $NaHCO_3$ maintained under warm humid 95:5 $O_2/CO_2$ (pH 7.4). These slices were maintained with their upper surfaces exposed until stable electrical responses were obtained. Bipolar stimulation (0.1-ms duration, 10–40 V) was delivered by a pair of twisted 0.003-in. Teflon-insulated stainless-steel wires. The recording electrodes were glass micropipettes, filled with 2 M NaCl, and of 2–15 MΩ impedence. The magnitudes of the extracellular synaptic field potentials were monitored while stimulus intensity was adjusted to subthreshold for evoking a population spike.

The entorhinal inputs to granule cells were recorded in the middle or outer one-third of the molecular layer of the dentate gyrus (the medial and lateral perforant paths, respectively). Because the responses recorded in the perforant path often represent mixed medial and lateral responses,[4,35] electrode placement was confirmed by applying test concentrations of L-APB, a selective lateral perforant path antagonist.[4] The level of inhibition was used to calculate the relative contributions of each pathway to the perforant path field potential.[35] By this criterion, lateral responses were contaminated 0–40% with medial responses and medial responses were contaminated with lateral responses no more than 30%. The Schaffer collateral-CA1 pyramidal cell synaptic field potentials were measured by placing stimulating and recording electrodes in stratum radiatum of regio superior (see ref 39 for a schematic illustration of electrode placement).

After electrode placement, the slice was submerged in oxygenated and rapidly stirred medium.[38] When the response was stabilized the medium was equilibrated with drug at a threshold concentration for inhibition of field potentials. The drug concentration was doubled every 4 min, which is sufficient time for the response to restabilize.[35] The fraction of the uninhibited response was plotted as a function of the log concentration of the drug. When the inhibition data paralleled theoretical binding curves with a Hill coefficient of 1, as is observed for antagonists, the $IC_{50}$'s were weighted means of $IC_{50}$'s calculated for each experimental point.[39] When the slope of the concentration–response curve was steeper than that expected for an antagonist exhibiting a Hill coefficient of 1, as is usually observed for agonists,[32,35] the $IC_{50}$'s were read directly from the log concentration–response curve.

**Binding Experiments.** Synaptic plasma membranes were prepared as previously described.[12] Holtzman rats, 30–90 days old, were sacrificed by cervical dislocation; the forebrain was removed and homogenized in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer containing sucrose and $CaCl_2$. Intact cells were removed by differential centrifugation. The resulting synaptosomal suspension was centrifuged. This synaptosomal pellet was lysed in the hypoosmotic HEPES buffer and incubated in potassium phosphate buffer containing *p*-iodonitrotetrazolium violet and sodium succinate. This procedure increases the density of mitochondria.[40] This solution was centrifuged and the resulting pellet suspended in sucrose–HEPES buffer, which was then layered on a discontinuous sucrose density gradient. The synaptic plasma membranes were isolated from the 1.0–1.3 M interface and washed by centrifugation and resuspension in HEPES–$CaCl_2$ buffer a total of 4 times. The final pellet was suspended in HEPES–$CaCl_2$ buffer to a final concentration of 3–5 mg of protein/mL as determined by the Lowry protein assay using bovine serum albumin as a standard.[41]

Binding of DL-[$^3$H]APB was measured with a centrifugation binding assay in a total volume of 1 mL, as was also previously described.[12] Membranes (no more than 200 μg/mL) were preincubated for 5 min at 33 °C in 62.5 mM HEPES–KOH buffer (pH 7.4) and 12.5 mM $CaCl_2$. Assays were initiated by the addition of ligand and displacer in a volume of 200 μL. The final concentrations were 50 mM HEPES buffer, 10 mM $CaCl_2$, 100 nM DL-[$^3$H]APB, and up to 1 mM displacer. The assays were incubated for 1 h at 33 °C in a shaker water bath. The assays were terminated by centrifugation at 12500*g* in a microfuge for 3 min. The supernatant was aspirated and the top of the pellet rapidly washed by briefly layering cold buffer over the pellet. The pellets were solubilized overnight in sodium dodecyl sulfate and the bound radioactivity determined by liquid scintillation spectrometry. Assays were performed in triplicate, and the nonspecific contribution to binding was determined from parallel assays containing excess unlabeled ligand (1 mM).

Data for concentration–percent inhibition curves were obtained from triplicate assays on at least three different membrane preparations. The reported $IC_{50}$'s are weighted means of $IC_{50}$'s calculated for each experimental point as was done for concentration–response curves for inhibition of synaptic responses.

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