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Structural Determinants of Affinity for the Phencyclidine Binding Site of the *N*-Methyl-D-aspartate Receptor Complex: Discovery of a Rigid Phencyclidine Analogue of High Binding Affinity

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SUMMARY

To learn more about the binding conformation of phencyclidine (PCP) and to arrive at analogues of higher affinity, which may serve as noncompetitive *N*-methyl-D-aspartate receptor antagonists, eight optically pure PCP analogues were designed with the aid of computer. These compounds represent conformationally constrained versions of PCP in which the motion of the phenyl ring is frozen, thus allowing a determination of the orientation of the phenyl ring relevant to binding. The analogues were synthesized by a Diels-Alder strategy and tested in a radioligand binding assay to evaluate their affinity for the PCP binding site of the *N*-methyl-D-aspartate receptor complex. One of the analogues was found to bind with nanomolar affinity (IC_{50} = 19 nM) and to be 73-fold more potent in binding than its enantiomer. These results, which further elucidate the structural determinants of high affinity binding, should aid both in the design of higher affinity molecular probes of the PCP binding site and in the discovery of potential neuroprotective agents.

PCP (phencyclidine or "angel dust"), which was first introduced in the 1950's as a dissociative anesthetic, has since become a major drug of abuse due to its ability to elicit hallucinations, excitation, and feelings of tranquility (1). This synthetic compound exhibits various dose-dependent behavioral effects in humans and mammals and many actions in the central nervous system (2–17). In particular, PCP has been shown to block the ion channel that is coupled to the NMDA subtype of L-glutamate receptors in the brain, a receptor class that has captured the interest of most neurobiologists (8). The search for highly selective and potent PCP analogues as one type of regulator (i.e., noncompetitive antagonists) of NMDA receptor function, thus, continues to attract the attention of many researchers. Much of the current interest in such antagonists stems from a considerable body of evidence garnered in the past few years that reveals that the brain damage accompanying anoxia, stroke, hypoglycemia, epilepsy, etc., may be due to overactivation of these specific receptors (18, 19).

To better pursue the search for highly selective and potent noncompetitive antagonists (or open channel blockers) of the NMDA receptor, compounds that may thus prove useful as neuroprotective agents or as probes for the isolation of the NMDA receptor, it is essential to learn more about the binding conformation of PCP. Such studies can indirectly provide information as to the nature of the binding elements that anchor PCP to its binding sites. Although numerous PCP analogues have been synthesized over the past several decades, only three structural analogues of PCP (i.e., TCP, *m*-OH-PCP, and MK-801) have been shown to possess higher binding affinities than PCP itself (20–22). These compounds have, however, not served to define the binding conformation of PCP.

In this manuscript we report the successful design and synthesis of a novel class of rigid PCP analogues, one member of which exhibits 19 nM affinity for the PCP binding sites. This analogue serves to more rigorously characterize the conformation of PCP that is relevant to molecular recognition at the PCP binding sites.

Experimental Procedures

Materials. (+)-[^{3}H]MK-801 was purchased from New England Nuclear Research Products. MK-801 was obtained as a gift from Merck, Sharp and Dohme (West Point, PA). PCP, TCP, PCA, and PCE were prepared by Dr. Werner Tueckmantel in our laboratories. The mate-

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ABBREVIATIONS: PCP, 1-phenylcyclohexylpiperidine; NMDA, *N*-methyl-D-aspartate; TCP, 1-[1-(2-thienyl)cyclohexyl]piperidine; *m*-OH-PCP, 1-[1-(3-hydroxyphenyl)cyclohexyl]piperidine; MK-801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine; PCA, 1-phencyclohexylamine; PCE, 1-phenylcyclohexylethylamine; MTPA, α-methoxy-α-(trifluoromethyl)phenylacetyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IC_{50}, concentration giving 50% inhibition.

rials employed in the synthesis of the PCP analogues **1–8** were purchased from the Aldrich Chemical Company (Milwaukee, WI). The rat brains were purchased from Pel Freez (Rogers, AR).

Synthesis. In total, four structurally different compounds were synthesized. The synthesis makes use of the Diels-Alder adduct prepared from indene-3-carboxylic acid and butadiene. The carboxylic acid group was transformed to amine via the Hofmann reaction, and the amine was resolved by use of L-(+)- and D-(−)-tartaric acid to provide **1** and **2** (scheme in Fig. 1). The optical purity of these compounds was checked by ^{1}H NMR analysis of their (*S*)-(−)-MTPA amide derivatives (23). The enantiomerically pure amines were individually hydrogenated to provide **3** and **4**. Additionally, these compounds were *N*-ethylated to provide **5** and **6**, which upon hydrogenation gave rise to **7** and **8**[1]. These optically pure compounds are also of considerable importance to further elucidating the stereochemical requirements of the PCP binding sites.

X-ray analysis. A single crystal X-ray analysis was carried out on the salt prepared from compound **1** and L-(+)-tartaric acid. A colorless plate-like crystal of $C_{17}H_{21}NO_6$, having approximate dimensions of 0.11 × 0.34 × 0.44 mm, was cut from a larger plate and mounted in a nonspecific orientation on an Enraf-Nonius CAD4 automated diffractomer. All intensity measurements were performed using Cu Kα radiation (λ = 1.5418 Å) with a graphite crystal, incident beam monochromator.

The automatic peak search and reflection-indexing programs[2] in conjunction with a cell-reduction program showed the crystal to be monoclinic and, from the systematic absence of *0k0*, *k* odd, the space group was determined to be $P2_1$ (No. 4) (24). A total of 2008 reflections were collected and these were corrected for Lorentz, polarization, and background effects. The data were corrected for absorption effects using the Gaussian integration method.[3] After averaging of equivalent forms (*R* factor for averaging is 0.006) and rejection of any systematically absent data, there were 1869 unique reflections of which 1830, having $I > 3\sigma(I)$, were used in the structure solution and refinement.

The structure was solved using the direct methods program SHELXS-86 (25), which gave the positional parameters for all non-hydrogen atoms. Refinement of atomic parameters was carried out by using full-matrix least-squares techniques on F_o, minimizing the function $\Sigma w(|F_o| - |F_c|)$ where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively, and the weighting factor w is given by $w = 4F_o/\sigma F_o$. In the final cycle, 218 parameters were refined using 1830 observations having $I > 3\sigma(I)$. Included as a variable was a secondary extinction coefficient, which refined to a value of 2.05×10^{-5}. The final agreement factors were:

$$R_1 = \Sigma\,||F_o| - |F_c||/\Sigma|F_o| = 0.040$$

$$R_2 = (\Sigma w(|F_o| - |F_c|)/\Sigma w F_o)^{1/2} = 0.066$$

Fig. 2 shows a perspective view of the cation-anion pair. Atoms are represented by thermal ellipsoids at the 20% probability level, except for the H atoms, which are drawn at an arbitrary size. This X-ray structure, coupled with the chemical correlations of the scheme in Fig. 1, establishes the absolute stereochemistry of all members of the series. Compounds **1**, **3**, **5**, and **7** possess *R*-stereochemistry at their amine-bearing carbon centers, whereas compounds **2**, **4**, **6**, and **8** possess *S*-stereochemistry. In the crystal, compound **1** is found to adopt the phenyl-equatorial conformation, which from molecular mechanics studies (see below) represents the lower energy conformation for this molecule.

[1] For complete details of the synthesis and resolution of PCP analogues **1–8**, see reference 38.

[2] The diffractometer programs are those supplied by Enraf-Nonius for operating the CAD4F diffractometer with some local modifications and additions. The X-ray structure determination was carried out by Dr. R. G. Ball of the Merck Sharp and Dohme Research Laboratories, P. O. Box 2000, Rahway, NJ 07065, and inquiries concerning the crystallographic results should be directed to the above address quoting report number SR: sprj089c.

[3] The logic for the absorption correction is that of Coppens *et al.* (37).

Molecular modeling studies. Molecular mechanics studies were performed on PCP and compounds **1–8** using the global searching method available in the multiconformer submode of the input mode of the MacroModel program (Version 2.0), operating on a MicroVax II computer equipped with an Evans & Sutherland PS 390 graphics terminal. In global searching, torsional angles were varied in 30° increments, bond angles were varied in 10° increments, a ring closure bond was chosen on the cyclohexene, cyclohexane, or piperidine ring, the minimum closure distance was set to 1 Å, and the maximum closure distance was set to 2 Å. Batch energy minimizations were performed with the Block Diagonal Newton Raphson method and the MM2 force field (For a review of the MM2 Force Field, see Ref. 26) available within the the MacroModel program. Because PCP is known to be protonated under physiological conditions, all calculations were carried out on the ammonium derviatives.

Radioligand binding assay. Radioligand binding studies were carried out in accord with a published protocol by Reynolds *et al.* (27), to evaluate the *in vitro* affinities of the newly synthesized compounds for the PCP binding site of the NMDA receptor complex. Well washed rat brain homogenate suspensions in 1.0 ml of 10 mM HEPES (pH 7.4 at room temperature) that contained 0.3–0.6 mg of protein, 0.5–1 nM (+)-[^{3}H]MK-801, 100 μM glutamate, 30 μM glycine, and various analogues as appropriate, or 30 μM (+)-MK-801 for nonspecific binding, were incubated at room temperature for 2 hr and then the reactions were terminated by filtration under vacuum, using a 24-well cell harvester (Brandel, Gaithersburg, MD) over glass fiber filters (No. 32, Schleicher & Schuell, Keene, NH). Filters were washed with two 5 ml aliquots of assay buffer. Radioactivity trapped on filters was measured by liquid scintillation counting, using a Beckman LS 1801 scintillation counter at 50% efficiency. IC_{50} values were determined by visual inspection of log concentration-response curves. The percentage of specific binding for MK-801 in the control was 91%. The K_D of MK-801 binding under the assay conditions is 1.4 nM.[4]

Results

The relative potencies of the PCP analogues in displacing bound (+)-[^{3}H]MK-801 and their IC_{50} values are shown in Table 1. A representative plot from which the data of Table 1 were obtained is depicted in Fig. 3. The following points are noteworthy regarding structure versus activity. 1) The displacement curves from which the data of Table 1 were obtained (as depicted in Fig. 3) are all monophasic. This result indicates the displacement of [^{3}H]MK-801 from an apparent single class of binding sites. 2) Compound **3** shows an IC_{50} of 19 nM and is more potent in binding than PCP. 3) All the *R*-isomers were more potent in binding than the corresponding *S*-isomers. Assigning PCP a relative potency of 100%, the *S*-isomers ranged in potency from 2 to 6%, whereas the *R*-isomers ranged in potency from 16 to 184%. Clearly, the *R*-isomers show higher affinity than the *S*-isomers, and as a consequence minor structural variations give rise to a broader spread of binding potency than found with the *S* series. 4) The 73-fold binding difference found between the enantiomeric pair of compounds **3** and **4** is larger than the difference found previously for any such pairs of PCP analogues possessing higher binding affinities than PCP itself (22, 28–31).

Discussion

Because replacement of the phenyl ring of PCP by a nonaromatic group (e.g., a nitrile group) or a more polarizable aro-

[4] The K_D of MK-801 binding under our assay conditions was obtained from Dr. I. J. Reynolds (personal communication to A. P. Kozikowski, December, 1989).

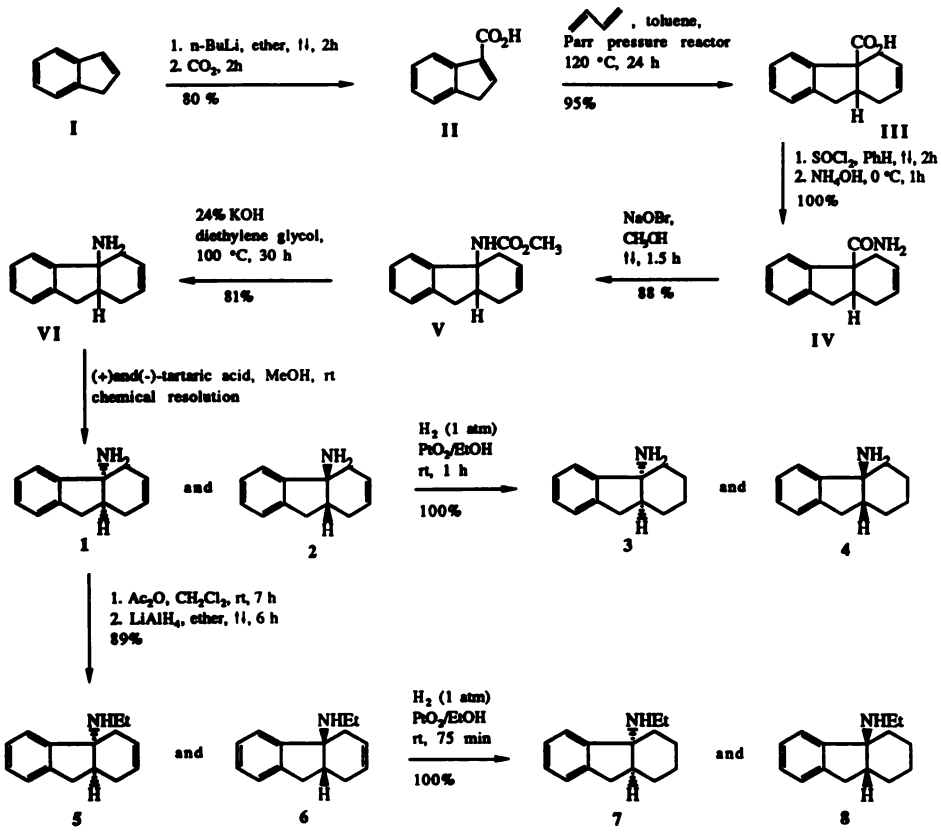

Fig. 1. Synthesis of conformationally constrained analogues of PCP. *Bu*, butyl; *Et*, ethyl; *MeOH*, methanol; Ac_2O, acetic anhydride.

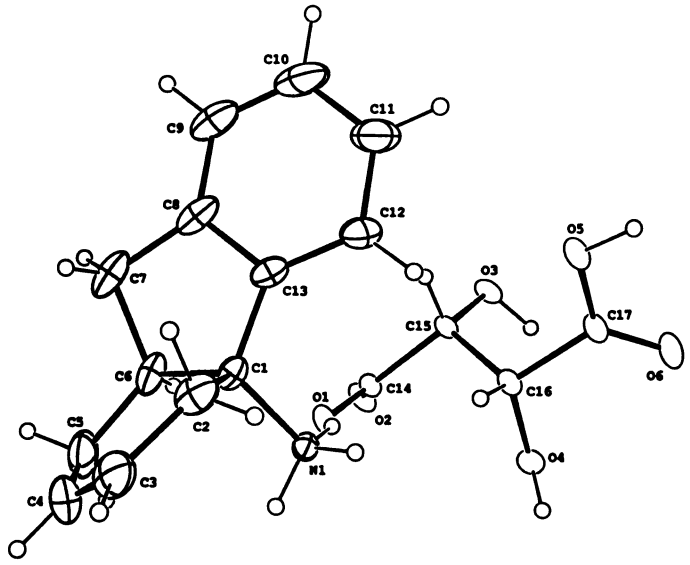

Fig. 2. Perspective view of the cation-anion pair, showing the atom-numbering scheme.

matic group (e.g., thienyl) results in a significant loss or increase in binding affinity, respectively (32), it is likely that the aromatic ring plays a critical role in binding and that, consequently, the orientation of the plane of this ring relative to the axis passing through the cyclohexyl ring carbon-nitrogen bond is critical (see ϕ in Fig. 4).

While the phenyl ring of PCP in its binding conformation has been postulated to adopt the axial position with ϕ near 90°, on the basis of variable temperature NMR experiments, X-ray crystallographic analysis, and molecular mechanics calculations (33), no direct evidence for this structural requirement has been obtained. In an attempt to examine this point in further detail so that the structural determinants of high affinity binding could be deduced, we set out to design and synthesize conformationally constrained PCP analogues, with the notion of introducing conformational constraints into these molecules that can serve to freeze certain elements of motion, thus allowing a more rigorous determination of the conformation relevant to binding. In each of these componds, the phenyl ring was constrained through the introduction of a methylene linker arm between the phenyl and cyclohexyl rings, and the piperidine ring was replaced with the simpler ethylamino or amino group

TABLE 1

Relative potencies of PCP analogues in displacing [+]-[^{3}H]MK-801 bound to rat brain homogenates

Data represent the means of three experiments, each carried out in duplicate, which varied less than 10%, and are reported ± standard error.

Agent	IC_{50}	Relative potency
	nM	%
PCP	35 ± 1	100
TCP	11 ± 1	318
1	114 ± 14	31
2	2241 ± 135	2
3	19 ± 1	184
4	1391 ± 49	3
5	224 ± 5	16
6	676 ± 16	5
7	68 ± 7	51
8	546 ± 40	6
PCA	484 ± 18	7
PCE	62 ± 12	56

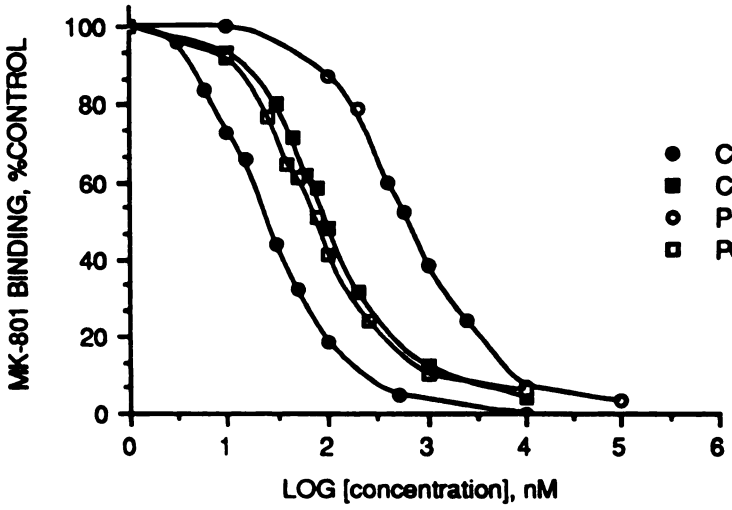

Fig. 3. Displacement of (+)-[^{3}H]MK-801 by PCP analogues. Values are the means of duplicate determinations that varied less than 10%. The experiment has been repeated three times with similar results.

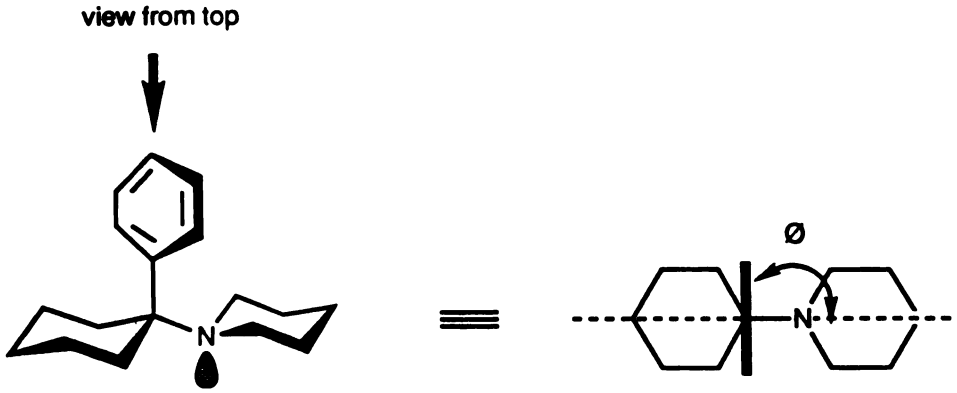

Fig. 4. View of PCP emphasizing the importance of phenyl ring orientation to binding.

(see scheme in Fig. 1). Because substitution of the ethylamino group for the piperidino group is known from structure-activity relationship studies to confer similar affinity of the ligand for the PCP binding sites,[5] this result enables us to exclude the influence of the different conformations of the piperidine ring (33). From molecular mechanics studies of these analogues, we found that axial phenyl conformations with $\phi \cong 90°$ were readily available to all these compounds (**1–8**). The global minimum energy conformations of compounds **3–8** adopt the axial phenyl conformation with $\phi \cong 90°$. In the case of compounds **1** and **2**, the equatorial phenyl isomers with $\phi \cong 137°$ (global minimum energy conformation) were 2.63 kcal/mol lower in energy than the axial phenyl isomers with $\phi \cong 90°$. Framework representations of PCP and compound **3** are shown in Fig. 5.

Because a number of regulatory sites exist on the NMDA receptor complex, these compounds could, in principle, be acting at the NMDA site, the glycine site, the zinc site, the polyamine site, or the MK-801 (PCP) site to produce the observed binding effects (34). However, because of the close structural similarities that are observed upon overlaying the phenyl ring and the two benzylic carbon atoms of rigid compound **3** with the corresponding elements of MK-801 (35) (root mean square = 0.109 Å; see Fig. 6), it is unlikely that these PCP analogues act at sites on the NMDA receptor other than the MK-801 binding site to produce the observed binding effects. Close structural similarities are also observed in the

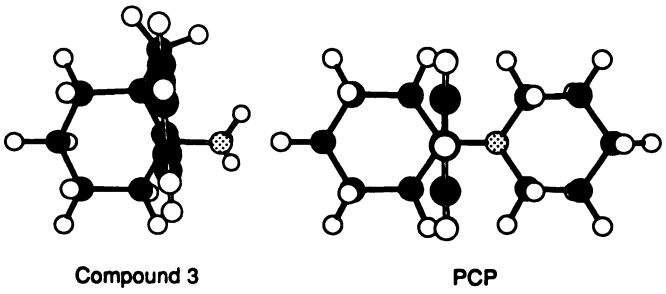

Fig. 5. Top view of energy-minimized structures of compound **3** and PCP generated by the Multic of the MacroModel Program (Version 2.0).

CPK renderings (Fig. 7) of compound **3** and MK-801. Furthermore, Anderson (36) has reported that the absence of the carbon atom in MK-801 corresponding to the methylene linker arm of compound **3** results in a reduction of binding affinity. This structural requirement for binding to the MK-801 site is consistent with our result that a large discrepancy in binding is found between compound **3**, containing the methylene linker arm, and PCA, which lacks this linker arm (see Fig. 3). The monophasic character of our MK-801 displacement curves indicates an action at a single class of binding sites and we can, therefore, exclude a simultaneous action at other sites of the NMDA receptor complex. Taken together, the foregoing considerations lead us to conclude that the *R*-isomers of this novel class of rigid PCP analogues bind to the same site as MK-801 or PCP. Consequently, our results further strengthen claims of the stereoselective nature of the PCP binding sites.

[5] K. Krueger and A. P. Kozikowski, manuscript in preparation.

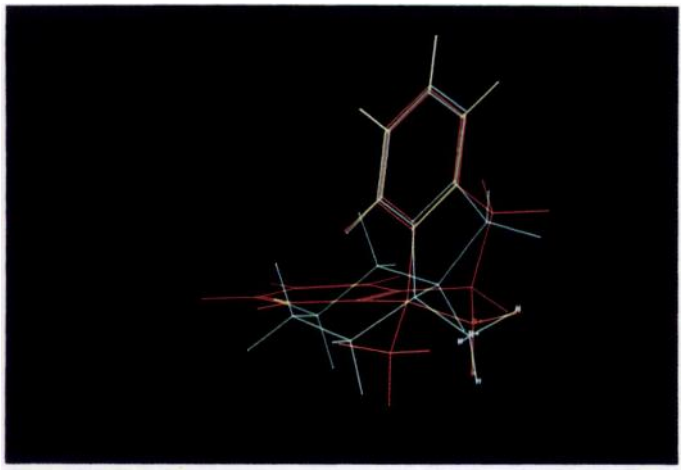

Fig. 6. Structural superposition of compound **3** (*green*) and MK-801 (*red*) generated by Analyz of the MacroModel Program (Version 2.0), emphasizing their three-dimensional correspondence.

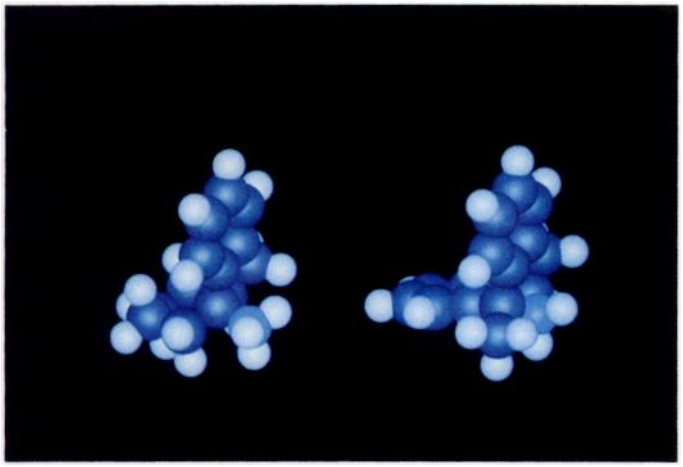

Fig. 7. CPK models of compound **3** (*left*) and MK-801 (*right*) generated by Analyz of the MacroModel Program (Version 2.0).

Compound **3** shows an affinity for the PCP binding sites very close to that of TCP, in spite of the fact that compound **3** contains the less polarizable aromatic ring. We can likely conclude that the fixation of the phenyl ring of compound **3** such that ϕ is near 90° and/or the presence of the methylene linker arm are responsible for the increased binding affinity. To further elucidate the structural requirement for the increased binding affinity, we compared the relative potencies of the freely mobile PCP analogues PCA and PCE and the rigid compounds **3** and **7** in displacing (+)-[^{3}H]MK-801 binding. As is apparent from Fig. 3, no significant difference exists in the binding potency of PCE and compound **7**; however, the binding potency of compound **3** is 25-fold higher than that of PCA. These results suggest that 1) the binding conformation of PCP adopts the axial phenyl position with ϕ near 90°, because compound **7** with a fixed phenyl ring with ϕ near 90° and PCE with a freely mobile phenyl ring show the same binding affinities; 2) the higher affinity of compound **3** stems from the presence of the methylene linker arm as opposed to a difference in the nature of the amine substituent, because a large discrepancy in binding exists between compound **3** and PCA; and 3) the binding site for compound **3** may be slightly different from that for compound **7**. These two sites may partially overlap, but they are not identical. At the binding site of compound **3**, a methylene linker arm is required for binding, whereas it is not required at the binding sites of compound **7**. When the ligands do not bear a bulky group on the nitrogen atom, they bind to the compound **3** site (e.g., compounds **1** and **3**, MK-801, and PCA). When the ligands bear a bulky group on the nitrogen atom, they bind to the compound **7** site (e.g., compounds **5** and **7**, TCP, PCP, and PCE). Thus, PCA, which has neither a methylene linker arm nor a bulky group on the nitrogen atom, binds to the compound **3** site poorly, whereas compound **3**, which has a methylene linker arm and lacks a bulky group on the nitrogen atom, binds strongly to that site. Although further experiments will be required to substantiate this two-site hypothesis, the notion does serve to rationalize the nearly equal binding potencies of PCE and compound **7** in spite of the fact that compound **7** contains the methylene linker arm and PCE does not contain such an arm.[6] Due to the presence of the bulky ethyl group on the nitrogen atoms of both PCE and compound **7**, they bind to the compound **7** site to the same degree.

In conclusion, the present work firmly establishes the structural determinants for high affinity binding to the PCP binding sites, an axial phenyl ring orientation with $\phi \cong 90°$, a methylene linker arm of absolute stereochemistry consistent with that of compound **3**, and no bulky groups on the nitrogen atom. Additional structural manipulations of compound **3** are likely to lead to further enhancements in binding affinity, and the agents so generated may prove useful as molecular probes of receptor topography, as tools for NMDA receptor isolation, or as neuroprotective drugs.

Acknowledgments

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