Exploration of Biologically Relevant Conformations of Anandamide, 2-Arachidonylglycerol, and Their Analogues Using Conformational Memories

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The endogenous cannabinoid anandamide (*N*-arachidonoylethanolamide) has been shown to possess higher affinity for the cannabinoid CB1 receptor than for the CB2 receptor. Carrier-mediated transport has been proposed to be essential for the termination of the biological effects of anandamide, while hydrolysis of anandamide is performed by a membrane-bound amidohydrolase, fatty acid amidohydrolase (FAAH). As interaction of anandamide with each of these targets occurs in different environments, the conformations of anandamide for interaction with each target may differ. To ascertain what conformations of anandamide, a highly flexible molecule, are favored in polar and nonpolar environments, the new method of Conformational Memories (CM) was used. CM has been shown to achieve complete conformational sampling of highly flexible ligands, to converge in a very practical number of steps, and to be capable of overcoming energy barriers very efficiently (Guarnieri et al. *J. Am. Chem. Soc.* **1996**, *118*, 5580). The generalized Born/surface area (GB/SA) continuum solvation models for chloroform and for water were used in the CM calculations. As a means of validation, CM was first applied to arachidonic acid because both experimental and theoretical conformational studies of arachidonic acid have been reported. CM was also applied to *sn*-2-arachidonylglycerol (2-AG), another endogenous CB ligand; to a 1,1-dimethylheptyl derivative of anandamide, an analogue with higher CB1 affinity than anandamide; and to *N*-(2-hydroxyethyl)prostaglandin-B_2-ethanolamide (PGB_2-EA), a prostanoid ligand which does not bind to CB1. Consistent with the literature, arachidonic acid was found to exist in an extended, angle-iron shape and in back-folded conformations which were U, J, or helical in shape. The angle-iron and U-shapes were both highly populated conformations with the angle-iron preferred in $CHCl_3$ and the U-shape preferred in H_2O. Results for anandamide and 2-AG paralleled those for arachidonic acid with the exception that anandamide in water does not adopt a pure extended conformation but, rather, favors a hybrid-extended/U-shape. For the dimethyl-heptyl derivative of anandamide, the U-shape was found to be predominant in both environments (42% in $CHCl_3$, 38% in H_2O), but the population of the angle-iron shape was still significant (25% in $CHCl_3$, 29% in H_2O). For all of these ligands, J-shaped conformers constituted from 7% to 17% of the conformer population, while the helical shape was less than 5%. In both $CHCl_3$ and H_2O, the presence of the five-membered ring attenuates the ability of PGB_2-EA to adopt an extended conformation. PGB_2-EA was found instead to exist predominantly in an L-shape (i.e., distorted U-shape). The low probability of PGB_2-EA adopting an extended conformation may be why PGB_2-EA shows such low affinity for the CB1 receptor. The conformational information obtained here for anandamide and 2-AG may be useful in the design of rigid analogues which mimic the preferred molecular conformations (shapes) of these ligands. Such rigid analogues may be useful in deducing the bioactive conformation of these endogenous cannabinoids, not only at the CB receptors but also at the FAAH enzyme active site and possibly at the binding site(s) on the newly proposed anandamide transporter.

Introduction

Anandamide (*N*-arachidonoylethanolamide, **1**; Chart 1), has been identified as an endogenous ligand of the G-protein-coupled cannabinoid CB1 receptor.[1] Like other cannabinoid agonists, anandamide produces a concentration-dependent inhibition of the electrically evoked twitch response of the mouse vas deferens,[1] as well as antinociception, hypothermia, hypomotility, and catalepsy in mice.[2] Anandamide inhibits forskolin-stimulated cAMP accumulation in CHO-HCR cells[3] and exhibits higher affinity for the cannabinoid CB1 receptor (K_i CB1 = 89 ± 10 nM) than for the CB2 receptor (K_i CB2 = 371 ± 102 nM).[4] Recent studies have postulated the existence of carrier-mediated anandamide transport which is essential for the termination of the biological effects of anandamide.[5,6] In brain and liver, anandamide is hydrolyzed enzymatically to yield arachidonic acid and ethanolamine.[7] The reaction is catalyzed by a membrane-bound amidohydrolase (called anandamide amidohydrolase or fatty acid amide hydrolase, FAAH) which recently was cloned.[8] Anandamide amidohydrolase appears to be located intracellularly, as suggested

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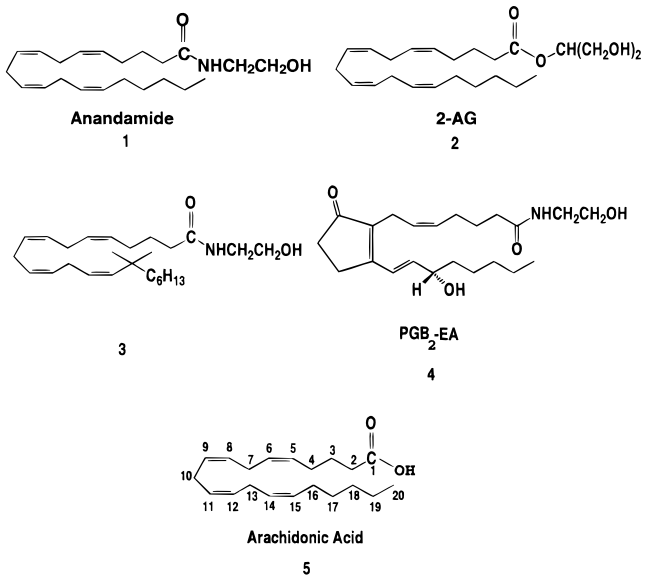
Chart 1



by both cell fractionation studies and sequence analysis of the cloned enzyme. Thus, anandamide interacts not only with the CB receptors but also possibly with a transporter and with the FAAH enzyme. Because these interactions occur in different environments, it is possible that the conformation of anandamide required for interaction with each of these targets may be different.

To explore the possible interaction of anandamide with any of its biological targets, it is first important to know what conformations are accessible to it as a function of environment and which of these conformations may be preferred. Conformational analysis of highly flexible ligands such as anandamide is a challenging problem due to the large number of conformations available to the ligand. The most commonly used method in the literature for studying such flexible ligands has been molecular dynamics.[9] However, molecular dynamics (MD) techniques reveal short timescale molecular motions but are generally incapable of a complete exploration of the conformational space of highly flexible ligands. Constrained molecular dynamics has been used effectively by Thomas et al.[10] to study a region of conformational space of anandamide in which certain functional groups of anandamide match hypothesized key CB1 pharmacophoric regions of the classical cannabinoid (−) -Δ^9 -tetrahydrocannabinol (Δ^9-THC). In this study, a low-energy looped or J-shaped conformer of anandamide was identified to be important. To date, however, a complete conformational analysis of anandamide has not been reported. In this paper, we employ a new method, Conformational Memories (CM), to explore the conformational space of anandamide in both polar and nonpolar environments. The Conformational Memories method has been shown to achieve complete sampling of the conformational space of highly flexible molecules, to converge in a very practical number of steps, and to be capable of overcoming energy barriers efficiently.[11] Rather than focus on the relative enthalpy of an individual conformer, the Conformational Memories method is based on the free energy of the system. Conformations which are identified by Conformational Memories to be highly populated are those which have low free energies.

Conformational Memories results for anandamide are compared here to those for another endogenous cannabinoid, *sn*-2-arachidonylglycerol (2-AG, **2**; Chart 1), to an analogue of anandamide with higher CB1 affinity (16,16-dimethyldocosa-*cis*-5,8,11,14-tetraenoylethanolamine, **3**; Chart 1), and to *N*-(2-hydroxyethyl)prostaglandin-B_2-ethanolamide (PGB_2-EA, **4**; Chart 1), a prostanoid ligand which does not bind to the CB_1 receptor. Because of the lack of experimental conformational data for anandamide or for any of the other compounds mentioned above, we have also applied the Conformational Memories method to the parent fatty acid of anandamide, arachidonic acid ((*all-Z*)-5,8,11,14-eicosatetraenoic acid, **5**, Chart 1), a compound for which X-ray as well as theoretical conformational analyses have been performed.

Methods

The Conformational Memories[11,12] technique employs multiple Monte Carlo[13] simulated annealing (MC/SA)[14] random walks using the MM3 force field and the generalized Born/surface area (GB/SA) continuum solvation model for chloroform or water as implemented in the Macromodel[15] molecular modeling package. All starting structures used as input to the process were minimized in vacuo or in solvent (either water or chloroform) using the MM3 force field until an energy convergence of less than or equal to 0.01 kJ/mol was achieved. The Conformational Memories technique consists of two phases, each of which is described briefly below.

1. Exploratory Phase. In the exploratory phase, repeated runs of MC/SA were carried out in order to map the entire conformational space of the flexible ligand. A starting temperature of 2070 K was used for the simulated annealing with a cooling schedule of $T_{n+1} = 0.9T_n$ for 19 discrete temperature points. At each temperature, 10 000 steps were applied to the rotatable bonds (number varies depending on ligand) with cooling to a final temperature of 310 K. Trial conformations in the MC/SA routine were generated by randomly picking 2 rotatable bonds from among the bonds being investigated, rotating each bond by a random value between ±180° and accepting or rejecting the trial conformation according to the standard Metropolis criteria[13] with a Boltzmann probability function defined at the given temperature. Three separate exploratory cycles were performed, each consisting of 50 separate runs of MC/SA. A different minimized starting structure was used for each of the exploratory cycles.

The results of the bond rotations or the step history were recorded to a "log file". To obtain structural information from this large amount of data, the log files were used as input to the Flex program which sorts, merges, and compacts the data into a collective history producing a mean field dihedral distribution function or "conformational memory" for each rotatable bond. The conformational memories for each of the ligands presented here were based on the data from 150 MC/SA runs per ligand, i.e., 2.85×10^7 individual steps per ligand. The Flex program produces a 19 × 36 spread sheet (recording 19 temperatures by 36 10° intervals with normalized populations) for each rotatable bond. The spreadsheets were imported into Deltagraph (Deltagraph TM version 4.0 (1987), Deltapoint Inc., 200 Heritage Harbor, Suite G, Monterey, CA 93940).

Figure 1 provides an example of the graphical output from the exploratory phase. On the *Y* axis are dihedral angle values from −170° to 180°. On the *X* axis are the 19 temperatures ranging from 2070 to 310 K. The population percentage is plotted on the *Z* axis. The plots yield the identification of structural motifs. For example, the graph for Figure 1, right, exhibits a classic three-state distribution seen for rotation about a bond that connects two sp^3-hybridized carbons: trans, gauche plus, and gauche minus, with trans being the most populated state. For Figure 1, left, which is for rotation about a bond that connects an sp^3-hybridized methylene carbon

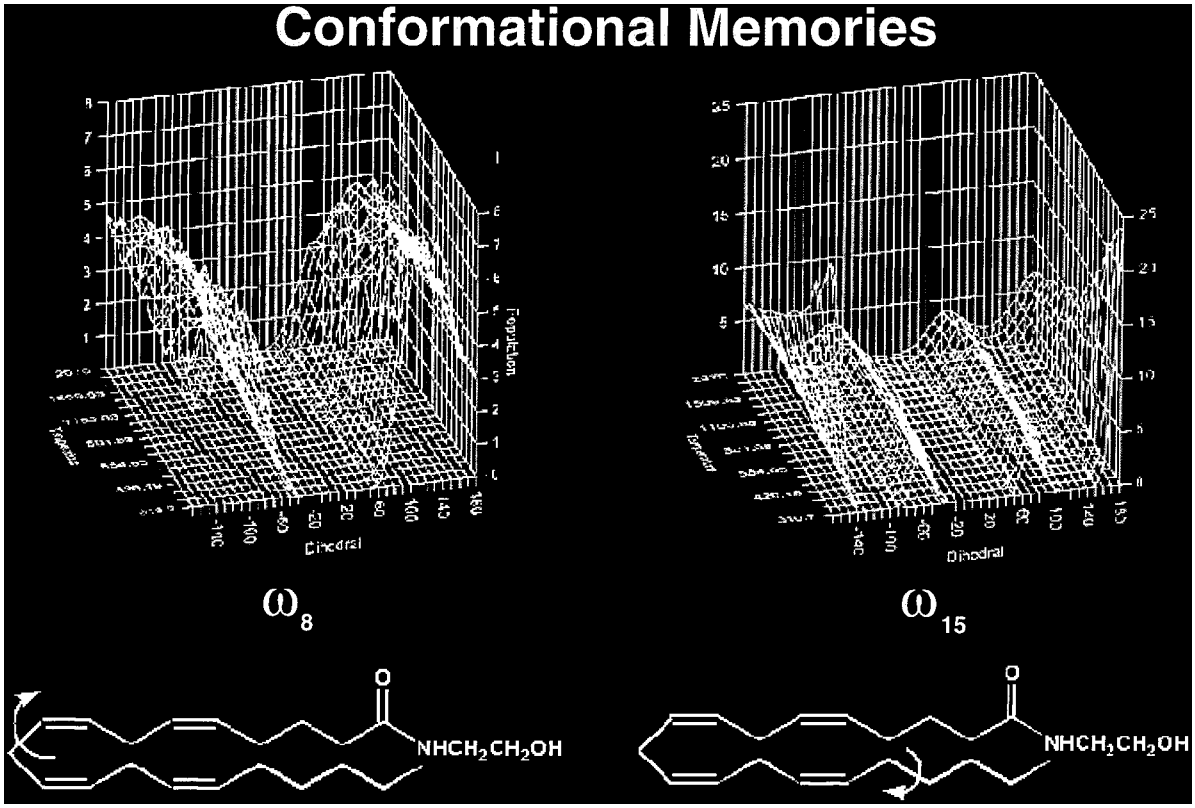


Figure 1. Conformational Memories of selected dihedral angles in anandamide: (left) dihedral 8 (ω_8, C8−C9−C10−C11); (right) dihedral 15 (ω_{15}, C15−C16−C17−C18).

to an sp^2-hybridized carbon, two peaks are evident corresponding to classic skew angles (119° and −119°) as seen in the crystal structure of arachidonic acid.[16,17]

2. Biased Sampling. The exploratory phase provides a complete mapping of the entire conformational space of the flexible ligands with no approximations. The procedure allows the elimination of conformational barriers by taking large steps (i.e., ±180°). Once the conformational memories are established, a new Monte Carlo search is performed at 310 K, sampling only the populated regions. The conformational space that is explored in the second phase has therefore been reduced by many orders of magnitude. The restriction of the sampling to the populated regions identified in the previous step is achieved by partitioning the 0−1 interval of the random number generator into 36 parts which correspond to the 36 separate 10° intervals for each rotatable dihedral angle. The partitioning of the random number generator is proportional to the population of the 10° bucket. New biased trial conformations are generated by randomly choosing two rotatable bonds, generating a new random number for each bond, determining to which of the 36 intervals each new random number for each bond belongs, and driving the dihedrals to the appropriate intervals. The exact value of the new dihedral is determined by a linear interpolation. The biased sampling runs consisted of a 500 000-step Monte Carlo random walk at 310 K with a sampling rate of 1 sample every 3333 steps. Each run resulted in 150 structures. Each group of structures was analyzed with the program Xcluster[18] that inputs the series of 150 conformations and computes the root-mean-square (rms) difference between all possible pairs of conformations. Structures 2−150 of the input sequence are then reordered on the basis of increasing rms deviation. In the new ordering, considering all 150 conformations, conformer 2 has the smallest rms deviation from conformer 1, etc. Since the conformations have been rearranged so that the rms deviation between nearest neighbors is minimized, any large jump in rms deviation between nearest neighbors is indicative of a large structural change and hence identifies a new conformational family. The clustering level for each run was chosen as the level at which the population split into two or more major families.

J-Shaped Conformer Filtration. Because a J-shape has been proposed to be low energy for arachidonic acid[16] and anandamide,[10] we screened the output files from the biased sampling runs for conformers which adopt this shape. To produce this filter, a dummy atom was created to represent the C14−C15 double bond and the distance between this dummy atom and the carbonyl carbon of the ligand was measured. Any structures with a distance of 6.5 Å or less were written to a separate file. To determine the cutoff distance, the low-energy conformers of arachidonic acid produced from the study by Rich[16] were built in Macromodel and the above distance was measured for each. The average distance for all three was 3.7 Å. In addition, a structure was built based on Thomas' model for anandamide overlayed with Δ^9-THC.[10] The distance measured as above was 4.3 Å. To allow a window of variability, 6.5 Å was chosen as the cutoff distance between the key atoms mentioned above.

Results

Torsion Angle Conventions. All torsion angles for bond rotation were designated according to conventions used by Applegate and Glomset.[19] In this convention, each torsion angle (ω) is defined in terms of the principal non-hydrogen substituent atoms attached to the atoms that form that bond. The 0° reference angle corresponds to eclipsed principal substituents as viewed along the bond axis. Positive torsion angles correspond to clockwise rotations at the far end of the bond. The following nomenclature has been adopted throughout this paper: cis, *C* ($\omega = 0°$); gauche, g^- ($\omega = 60°$) and g^+ ($\omega = -60°$); skew, s ($\omega = 120°$) and s′ ($\omega = -120°$); and trans, t ($\omega = 180°$).

Shape Conventions. Figure 2 illustrates arachidonic acid in the four principal shapes to be discussed

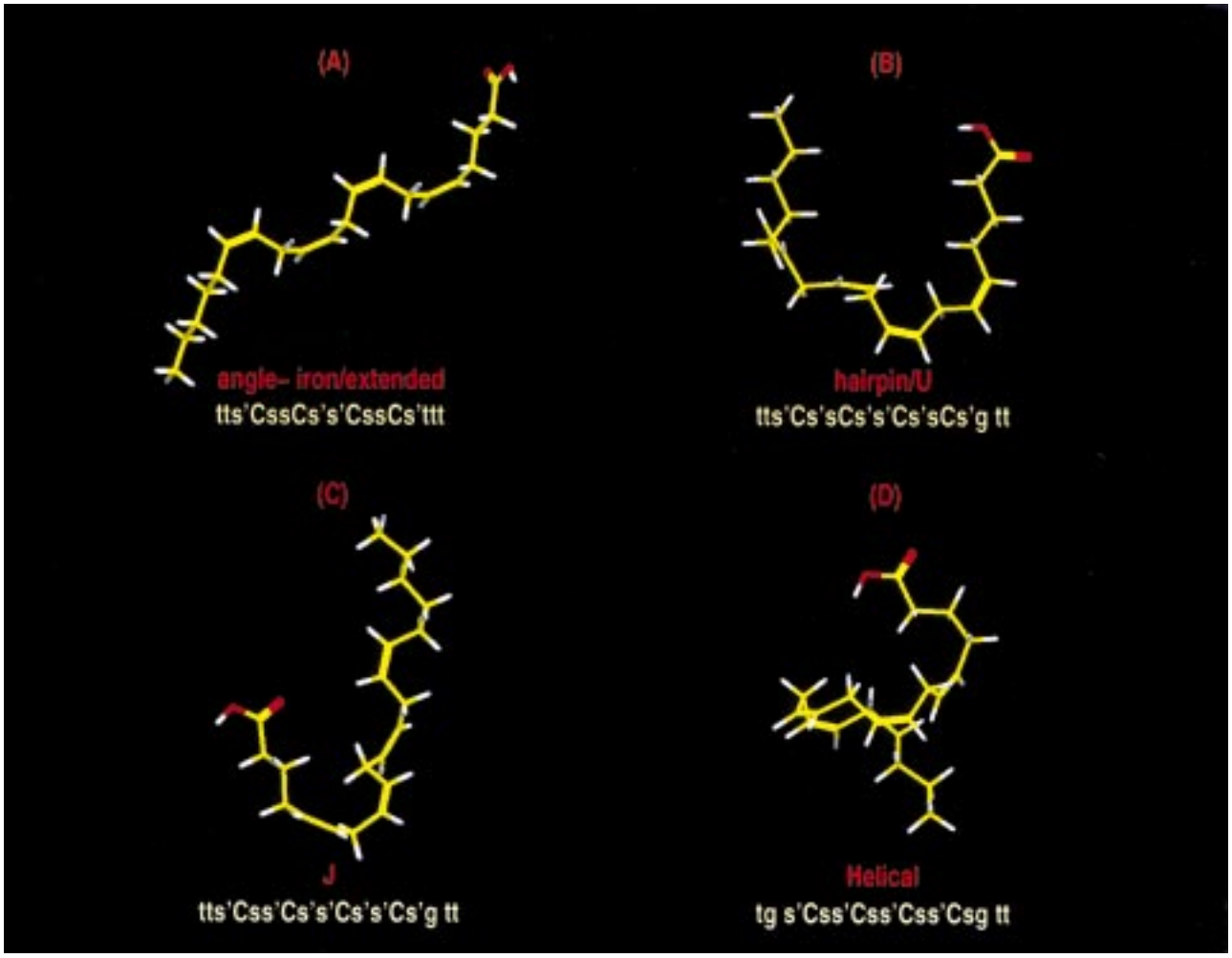


Figure 2. Illustrations of types of conformations identified for arachidonic acid: (A) the angle-iron/extended conformer; (B) the hairpin/U conformer; (C) the J-shaped conformer; (D) the helical conformer. Beneath the label for each conformer is a summary of torsion angles values (ω_1–ω_{17}) for each idealized conformer illustrated. The letter codes correspond to the following ideal torsion angles: cis, C ($\omega = 0°$); gauche, g^- ($\omega = 60°$) and g^+ ($\omega = -60°$); skew, s ($\omega = 120°$), s′ ($\omega = -120°$); and trans, t ($\omega = 180°$).

below. The ideal value of each torsion angle is described in this figure as a set of torsion angles (ω_1–ω_{17}) using the letter conventions for the torsion angles described above. The torsion angles are defined as ω_1 = C1–C2–C3–C4, ω_2 = C2–C3–C4–C5, ω_3 = C3–C4–C5–C6, ω_4 = C4–C5–C6–C7, ω_5 = C5–C6–C7–C8, ω_6 = C6–C7–C8–C9, ω_7 = C7–C8–C9–C10, ω_8 = C8–C9–C10–C11, ω_9 = C9–C10–C11–C12, ω_{10} = C10–C11–C12–C13, ω_{11} = C11–C12–C13–C14, ω_{12} = C12–C13–C14–C15, ω_{13} = C13–C14–C15–C16, ω_{14} = C14–C15–C16–C17, ω_{15} = C15–C16–C17–C18, ω_{16} = C16–C17–C18–C19, and ω_{17} = C17–C18–C19–C20. Compound **3** has two additional torsion angles: ω_{18} = C18–C19–C20–C21 and ω_{19} = C19–C20–C21–C22. The overall molecular shape of arachidonic acid and its derivatives studied here depends largely on three pairs of torsion angles along the acyl chain. These torsion angles ((ω_5, ω_6), (ω_8, ω_9), (ω_{11}, ω_{12})) govern the relative positions of the four double bonds in these compounds. In general, when each angle in a pair has the same sign, that region of the acyl chain will be extended. When the two angles in a pair have opposite signs, curvature is introduced into the acyl backbone. The extent of the curvature depends on how many torsion angle pairs have opposite signs. In the J-shape (Figure 2C), only the (ω_5, ω_6) pair has opposite signs. This introduces curvature near the carboxylic acid end of arachidonic acid in the example. In the U-shape (Figure 2B), (ω_5, ω_6) and (ω_{11}, ω_{12}) have opposite signs. This induces curvature near both ends of the molecule rendering it U-shaped. In the helical shape (Figure 2D), (ω_5, ω_6), (ω_8, ω_9), and (ω_{11}, ω_{12}) each have opposite signs. These signs alternate in the same pattern (i.e., (s,s′), (s,s′), and (s,s′)). The result of this pattern is the formation of a helical-like acyl backbone.

Structural Filtration of J-like Structures. There are two general conformations of arachidonic acid which lead to a J-like molecular shape. In one, the carboxyl end of the ligand is the long side of the J. In the other conformation, the carboxyl end is the short side of the J. This second J conformer places the carbonyl group of arachidonic acid near the C14–C15 double bond as illustrated in Figure 2C. This is the J-shape of arachidonic acid discussed by Rich[16] and Corey.[20,21]

The carboxyl group that forms the "short arm" of the J in arachidonic acid is extended by the ethanolamine portion in compounds **1**–**3**. Consequently, conformers identified by the "J-filter" (see Methods) for these three compounds do not necessarily appear to be J in shape. The discussion that follows refers to this population of conformers as one for which J-filter distances have been met. The PGB_2-EA (**4**) conformers were not filtered because the location of double bonds in the acyl chain

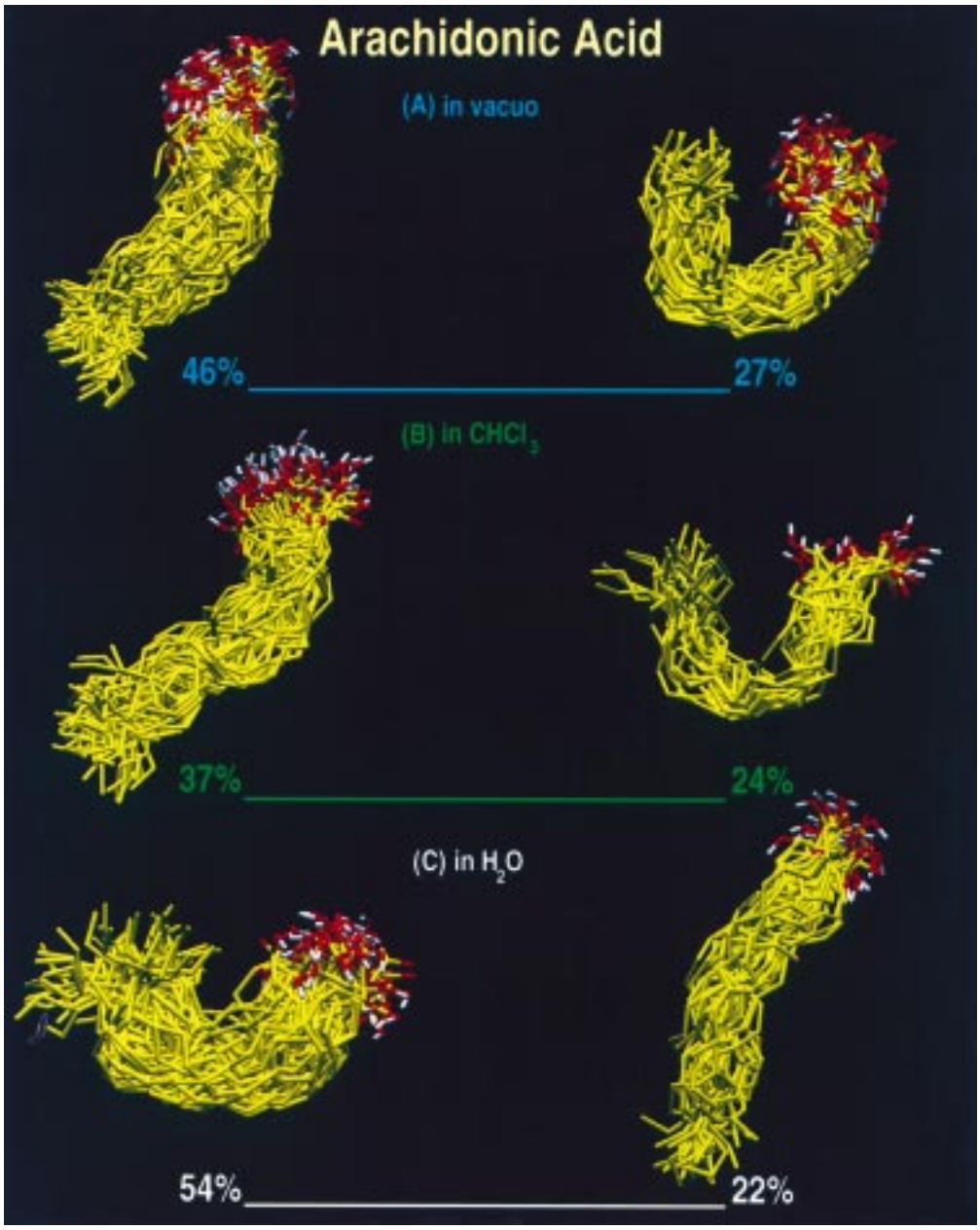


Figure 3. Conformational Memories results for the major conformational families of arachidonic acid (**5**) and their percentage populations for the following environments: (A) in vacuo; (B) in $CHCl_3$; (C) in H_2O.

of **4** differs from that in arachidonic acid and its derivatives. This change makes the PGB_2-EA and arachidonic acid acyl chains not directly comparable.

Conformational Memories. Structures from the 500 000-step biased sampling run were clustered in conformational families as described in Methods. Figures 3−7 illustrate the major clusters which emerged from the Conformational Memories analysis for each molecule studied. It is important to note that for some molecules, such as anandamide in H_2O, one shape is highly preferred (i.e., the major cluster comprised 73% of all conformers). On the other hand, for anandamide in $CHCl_3$, the population was more evenly distributed between two or more conformational families (28% extended, 23% distorted U, 11% U, etc.). In all cases, there are outlying conformations which X-cluster did not find related enough to cluster with other conformers. These represent conformations whose free energy was not sufficiently low enough to be visited frequently during the simulations. Due to all of the above, the population percentages of the clusters discussed and illustrated do not add to 100%, because only major clusters are presented and discussed here.

Conformational Memories of Arachidonic Acid (5). As summarized in Figure 3, three separate conformational analyses of **5** were performed.

A. In Vacuo. The first analysis was performed in vacuo as a basis of direct comparison with previously published theoretical studies of **5** performed in vacuo. The predominant conformational family (46%) identified for **5** was an extended structure similar to the angle-iron crystal structure of **5**,[16,17] which is illustrated in Figure 2A. U-Shaped structures (Figure 2B) formed a second conformational family that represented 27% of the sample conformers. Fifteen conformers (10%) were J-shaped (Figure 2C), analogous to those identified as minima by Rich.[16] An additional seven conformers met the J-filter distances but were part of the U-shaped

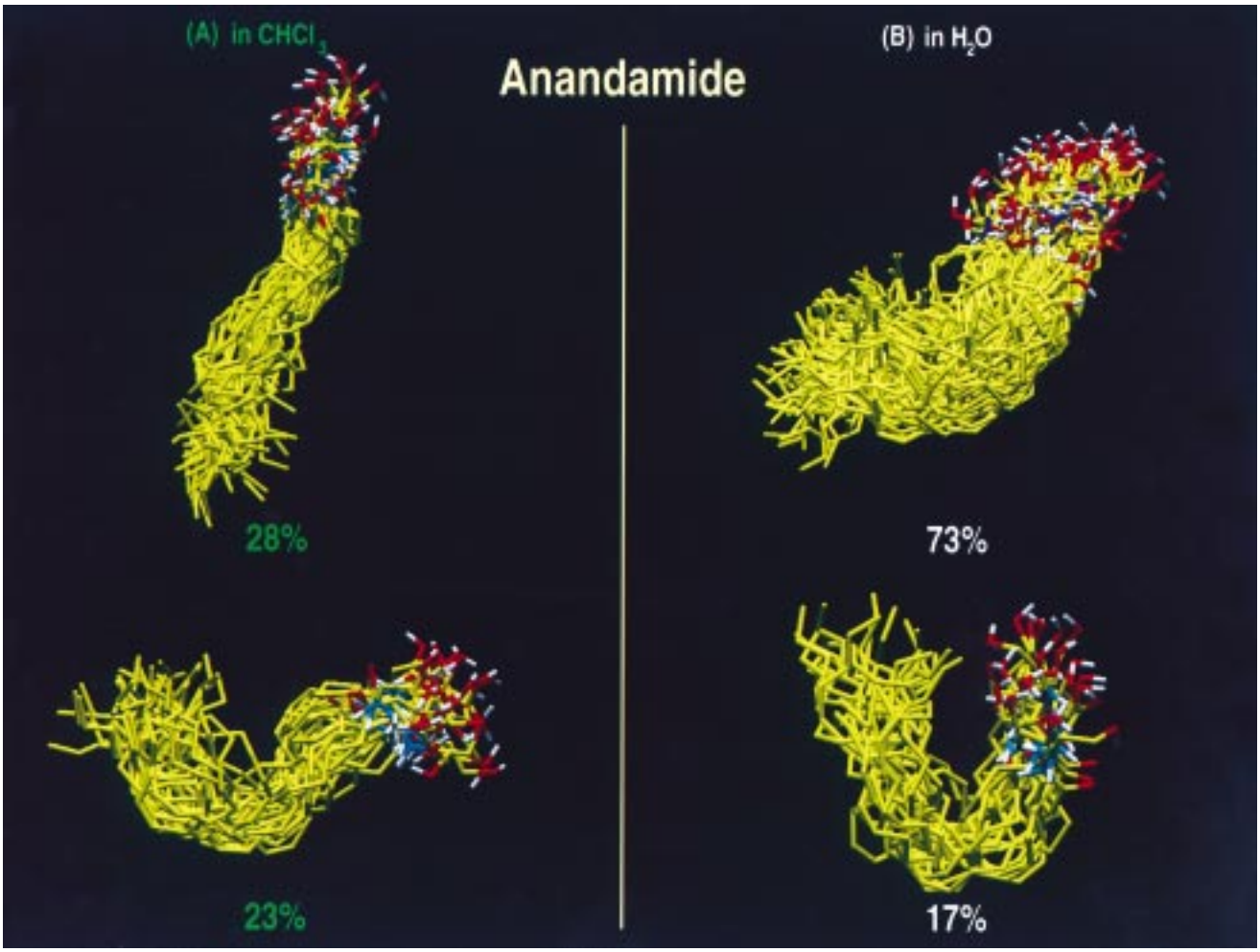


Figure 4. Conformational Memories results for the major conformational families of anandamide (**1**) and their percentage populations for the following environments: (A) in $CHCl_3$; (B) in H_2O.

cluster. Seven structures out of 150 structures were helical (Figure 2D).

B. In $CHCl_3$. Results for **5** in $CHCl_3$ showed a decrease in the number of extended conformers relative to the in vacuo results above. However, the predominant conformational family remained in the extended conformation (37%). A U-shaped conformational family comprised 24% of the population. Nine conformers (6%) were J-shaped, while two conformers in the U-shaped cluster also met the J-filter distance.

C. In H_2O. In water, the predominant conformation became the U-shape, comprising 54% of the structures. The population of the extended shape conformational family dropped to 22%. Ten conformers (7%) adopted Rich's J-shape. An additional five structures met the J-filter distances, four of which were part of the U-shape cluster.

Conformational Memories of Anandamide (1). Results for **1** are illustrated in Figure 4.

A. In $CHCl_3$. The most predominant conformational family (28%) adopts an extended angle-iron shape. The next predominant cluster (23%) has an extended U-shape. This conformational family was characterized by the (ω_8, ω_9) torsion angle pair having opposite signs (s, s′), while (ω_5, ω_6) and (ω_{11}, ω_{12}) have the same signs (i.e., (s′, s′)) and the ends of the molecule point in opposite directions. More symmetrical U-shaped structures were 11% of the population. These conformers were characterized by both the (ω_5, ω_6) and (ω_8, ω_9) pairs having opposite signs (i.e., (s, s′)), while (ω_{11}, ω_{12}) was (s′, s′) with the ends of the molecule parallel, or by the (ω_8, ω_9) pair having opposite signs (i.e., (s′,s)), while (ω_5, ω_6) and (ω_{11}, ω_{12}) have the same signs (i.e., (s, s)) with ends of the molecule in parallel. Only four conformers (3%) were J-shaped, while six others conformers met the J-filter distances (one of these was part of the extended U-shape cluster).

B. In H_2O. While the extended (angle-iron) shape was the predominant family of **1** in $CHCl_3$, none of the highly populated conformational families of **1** in water were extended conformers. Instead, the predominant conformation (73%) for **1** in water was found to be a hybrid shape which was somewhere between an extended and a U-shape, analogous to the second major cluster for **1** in $CHCl_3$. Symmetrical U-shaped structures represented 17%. Thirteen conformers (out of 150) met the J-filter distances; however, none were J-shaped.

Conformational Families of *sn*-2-Arachidonylglycerol (2). Results for **2** are summarized in Figure 5.

A. In $CHCl_3$. The major conformational family of **2** was an extended angle-iron conformation shape similar to that seen for anandamide in $CHCl_3$. This family consisted of 47% of the structures sampled. U-Shaped conformers represented 22% of the population. Sixteen conformers (out of 150) met the J-filter distances. Two

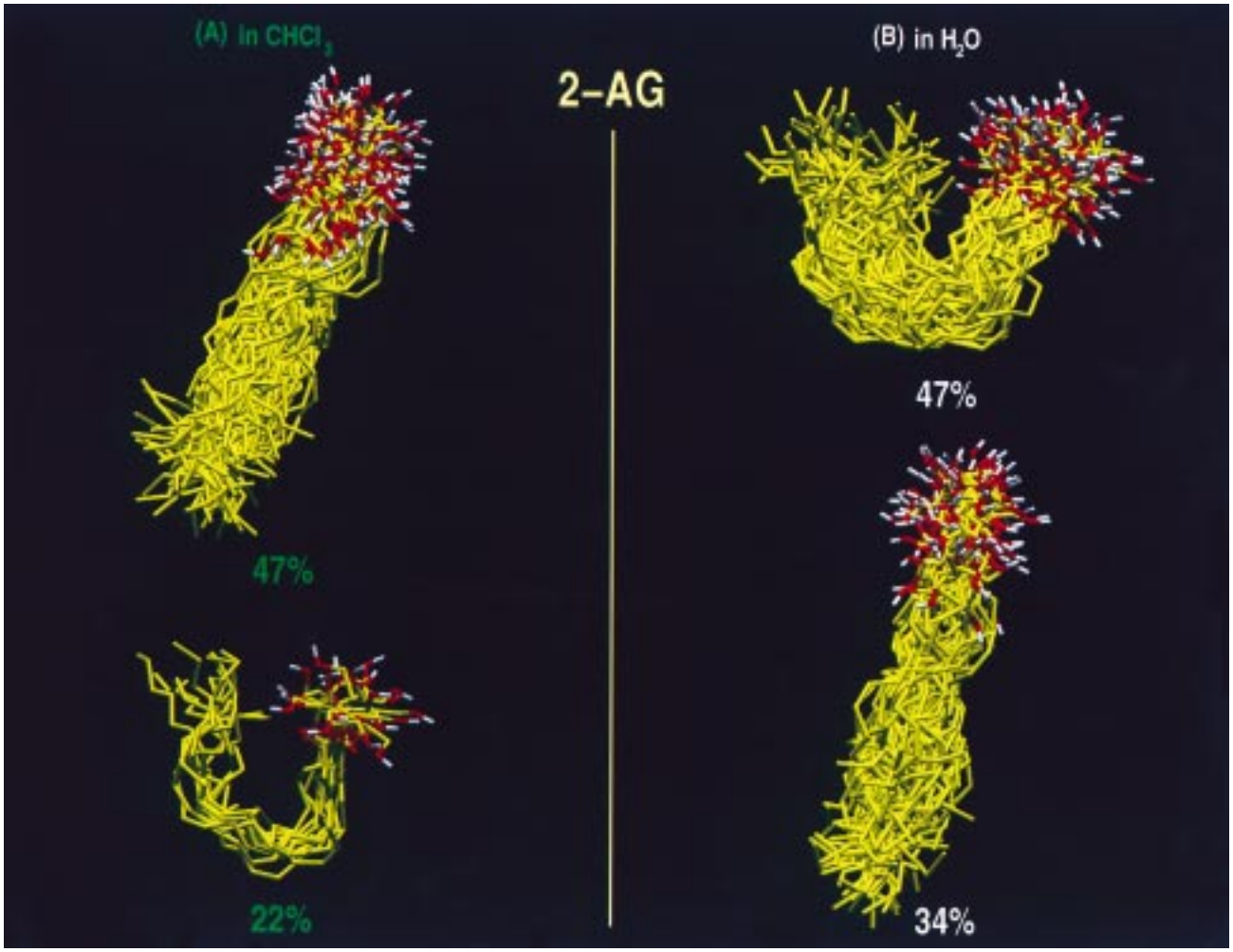


Figure 5. Conformational Memories results for the major conformational families of *sn*-2-arachidonylglycerol (2-AG, **2**) and their percentage populations for the following environments: (A) in $CHCl_3$; (B) in H_2O.

and four of these conformers were part of the major angle-iron cluster and the U-shaped cluster, respectively.

B. In H_2O. The predominant family of **2** in water was found to be the hairpin or U-shape (47%). The extended/angle-iron shape was the second major conformational family comprising 34% of the structures. Eighteen conformers met the J-filter distances. Twelve of these were part of the major U-shaped conformational family. The helical shape represented 3% of the population.

Conformational Families of Compound 3. Results for **3** are summarized in Figure 6.

A. In $CHCl_3$. Conformational Memories calculations revealed that the major conformational family of **3** in $CHCl_3$ is a distorted U-shape (42%). In this distorted conformation, the DMH side chain tends to project back toward the main chain giving the appearance of a more symmetric U than seen, for example, with anandamide. The result that the U-shape of **3** predominates in $CHCl_3$ is in contrast to results for arachidonic acid, anandamide, and 2-AG which all had more extended conformations as predominant clusters in $CHCl_3$.

In addition, what could be called an L-shape was the second major family for **3** in $CHCl_3$. The longer part of this L-shape (the C1 to C15 portion of the molecule) was in the angle-iron conformation found to predominate in $CHCl_3$ for arachidonic acid, anandamide, and 2-AG. The shorter portion of the L was formed by the 1,1-dimethylheptyl portion of **3** which begins at C16. Two torsion angles, ω_{14} and ω_{15}, were in the g^+ conformation causing this shorter portion to be nearly perpendicular to the rest of the structure. Ten structures (out of 150) met the J-shape filter criterion.

B. In H_2O. The percentage of U-shaped conformers of **3** increased in water to 38%, with 29% existing in the L-shape discussed above. Twenty-six conformers met the J-filter criterion. Thirteen of these were part of the U-shaped major family. Three structures (2%) were helical in shape.

Conformational Families of PGB_2-EA (4). Results for **4** are summarized in Figure 7.

A. In $CHCl_3$. The presence of the five-membered ring in **4** rigidifies the middle of the molecule allowing only the "ends" to move. Extended shapes (i.e., those with ends moving in opposite directions) were only 3% of the population. The two-dimensional drawings of **4** in Chart 1 might lead one to assume that a U-shape or hairpin shape would be favored for **4**, particularly since part of the molecule is constricted by the presence of the five-membered ring. In only 2% of the structures, however, were the two "ends" nearly coplanar, forming a U-shape. Instead, clustering of the structures of PGB_2-EA sampled during the biased sampling run was

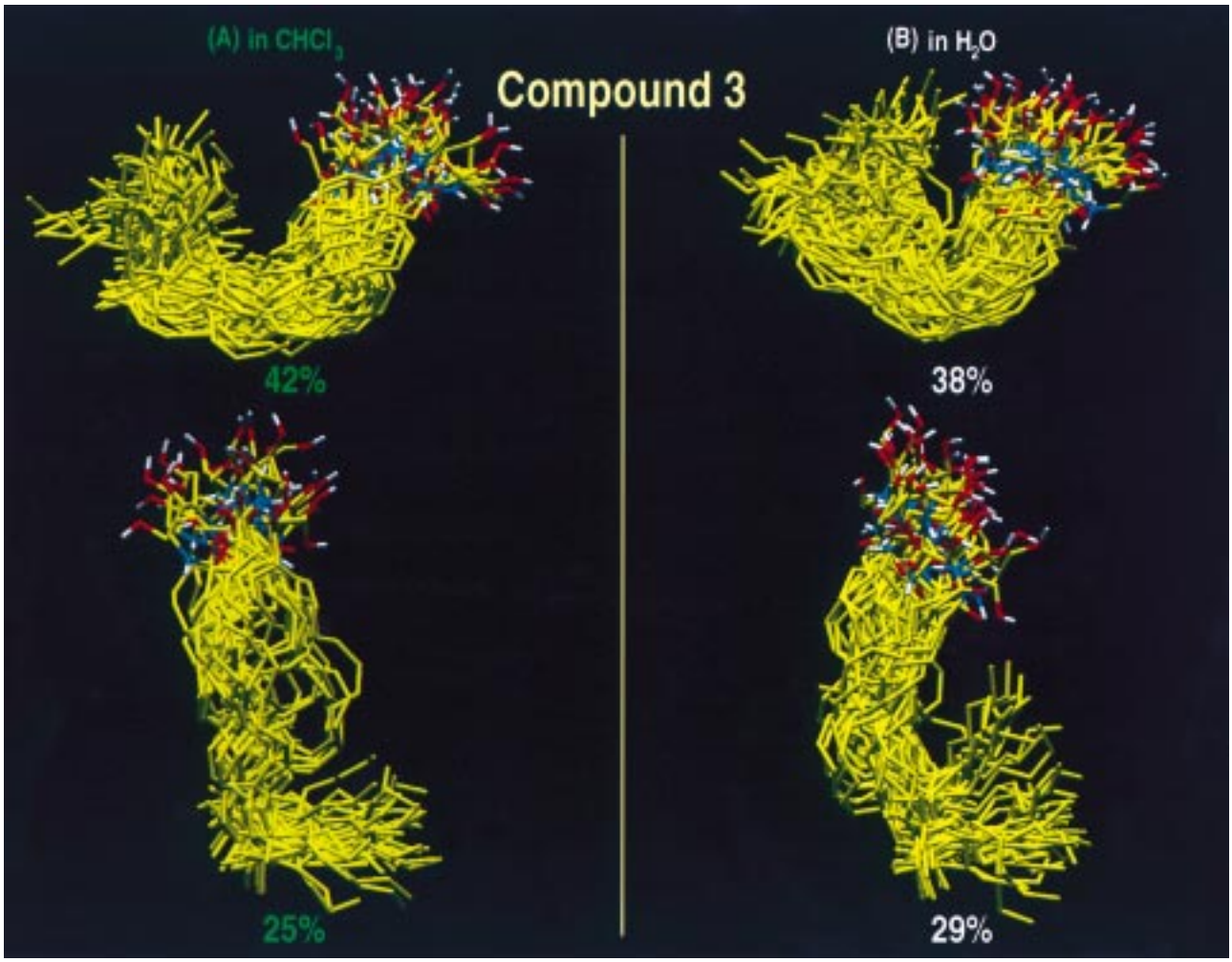


Figure 6. Conformational Memories results for the major conformational families of 16,16-dimethyldocosa-*cis*-5,8,11,14-tetraenoylethanolamine (**3**) and their percentage populations for the following environments: (A) in $CHCl_3$; (B) in H_2O.

overwhelmingly L-shaped (61%) with the two ends directed nearly normal to each other about the five-membered ring.

B. In H_2O. Symmetrical, U-shaped conformers were 29% of the conformations of **4** in water. However, the major family remained in the L-shape (47%).

Discussion

The inherent conformational flexibility of molecules such as anandamide makes the successful searching of their complete rotational space a difficult problem. In this paper, we have applied the Conformational Memories technique in the conformational analysis of anandamide and several related ligands and have explored their accessible structural space in vacuo or in solvent without restraints. By using this technique, the conformational properties of the ligands are fully characterized by the *free energy* of each of the conformations that the flexible ligand can adopt; this property includes not only the intrinsic energy of each conformational state but also the probability that the ligand will adopt each particular conformation relative to all other ones accessible in an equilibrated thermodynamic ensemble. The population or predominance of a conformational family is based on the free energy of the system. Some conformations may not fit into any one certain conformational family; however, this indicates that the free energy of each of those conformers is not favorable enough to cause a reoccurrence of that particular conformation during the Monte Carlo random walk.

In comparison to the Conformational Memories technique, single-point energy minimizations would not provide satisfactory descriptions of flexible ligands such as anandamide, while molecular dynamics simulations alone may yield only incomplete descriptions because such simulations are not always able to overcome the significant energy barriers that may separate energy wells in the conformational space of a flexible ligand. This inability to overcome barriers is highlighted below from the arachidonic acid literature.

Arachidonic Acid (5). Conformational Memories calculations identified the extended (angle-iron) shape and the more compact U (hairpin) shape as the major conformational families of **5**, the former predominating in vacuo or in $CHCl_3$ and the latter predominating in water. Experimental studies of arachidonic acid point to two general conformations of **5** as low-energy conformations. In its X-ray crystal structure, arachidonic acid has been found to exist in an extended (angle-iron) conformation in which double bonds 1 and 3 and double bonds 2 and 4 are coplanar, while the planes of adjacent double bonds are perpendicular to one another.[16,17] An X-ray crystal study of arachidonate ion complexed with adipocyte-binding protein revealed that **5** binds within

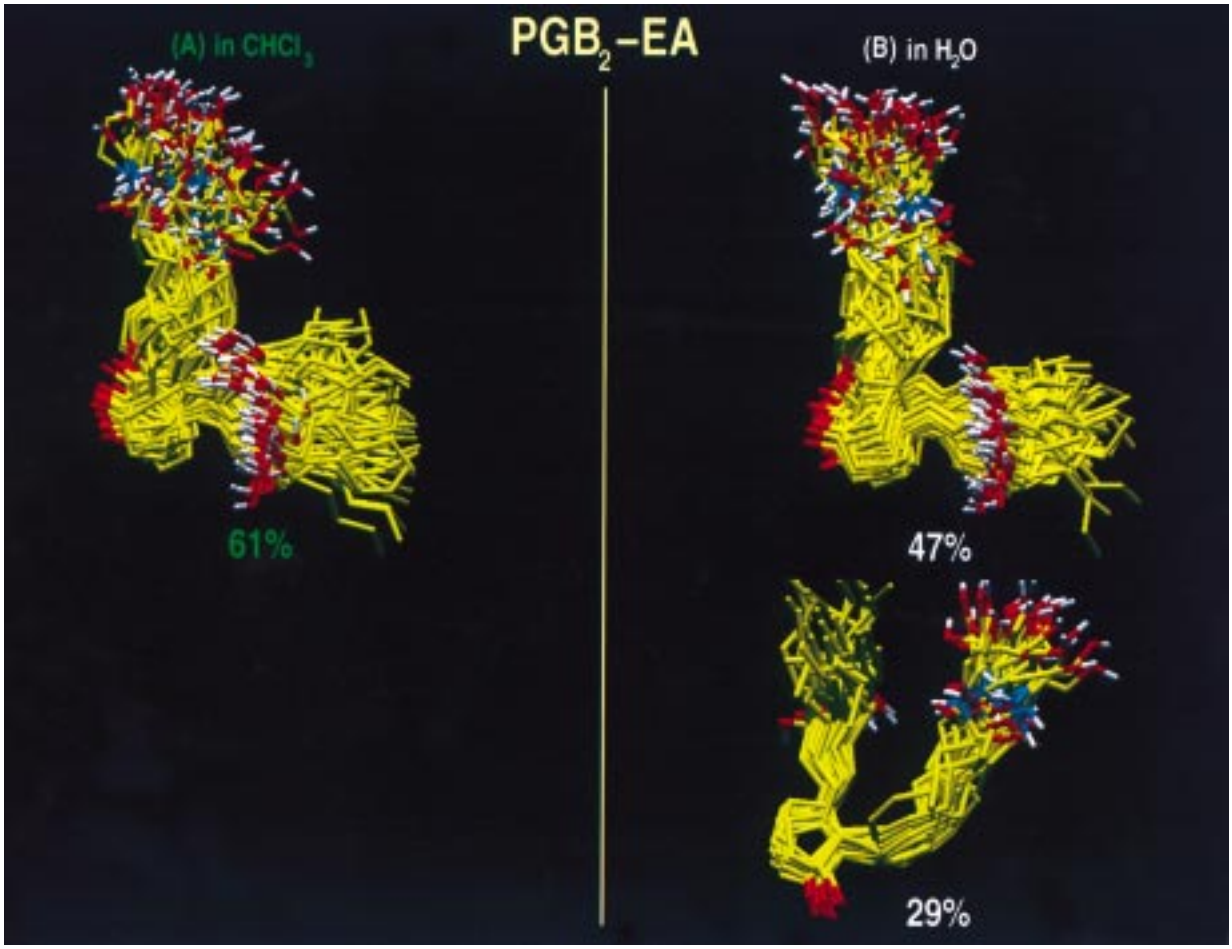


Figure 7. Conformational Memories results for the major conformational families of PGB_2-EA (**4**) and their percentage populations in the following environments: (A) in $CHCl_3$; (B) in H_2O.

the β barrel cavity of this protein. Here the carboxylate group of **5** is engaged in a strong electrostatic interaction with Arg^{126}, Tyr^{128}, and Arg^{106} through an intervening water molecule, while the arachidonate acyl chain exists in a hairpin (i.e., U-shaped) conformation for which the first double bond of arachidonic acid is distorted out of the plane formed by the other three double bonds.[22]

One important feature of the arachidonic acid unsaturated acyl chain is the great torsional mobility about the C−C bonds situated adjacent to double bonds, i.e., the two torsion angles involving each methylene carbon between adjacent pairs of double bonds in the acyl chain (ω_5, ω_6, ω_8, ω_9, ω_{11}, and ω_{12}). Rabinovich and Ripatti[23] found that polyunsaturated acyl chains in which double bonds are separated by one methylene group are characterized by the highest equilibrium flexibility compared with other unsaturated acyl chains. Rich[16] reports that a broad domain of low-energy conformational freedom exists for these C−C bonds. Results of the biased sampling phase for arachidonic acid (**5**) are consistent with Rich's and with Rabinovich and Ripatti's results. Figure 1, left, illustrates output from the exploratory phase of the CM calculation for the ω_8 torsion angle of anandamide. It is evident from Figure 1, left, that there is a relatively broad distribution of populated torsional space about the classic skew angles of 119° (s) and −119° (s′). These results are consistent with Rich's[16] report of a broad domain of conformational freedom for this type of torsion angle.

Figure 2 summarizes the key combinations of torsion angles which lead to idealized shapes of the arachidonic acid acyl chain. The descriptors of each shape in Figure 2 are largely those used by Applegate and Glomset.[19] As discussed previously in Methods, three pairs of torsion angles ((ω_5, ω_6), (ω_8, ω_9), and (ω_{11}, ω_{12})) exert the greatest influence over the shape of the acyl chain. Classic extended shapes are produced when the sign of each skew torsion angle within a pair is the same. Whenever the signs within a pair of these skew angles are opposite, curvature is introduced into the structure at that point. When the signs within each pair are opposite and follow the same sign pattern within each pair (e.g., (s, s′), (s, s′), and (s,s′)), then the acyl chain has a helical shape.

In their Monte Carlo study, Rabinovich and Ripatti[23] found that polyunsaturated fatty acids whose double bonds are separated by one methylene carbon assume an extended (angle-iron) conformation when all molecules are efficiently packed below the phase-transition temperature. Molecular dynamics (MD) or molecular dynamics/simulated annealing (MD/SA) studies (in vacuo) of arachidonic acid (**5**) and of other poly-unsaturated fatty acids related to **5** have been published by several groups.[16,19,20,23−27] These computational studies of **5** have primarily found looped or back-folded conformations to be low-energy conformers of **5**. Rich conducted a quenched molecular dynamics study of **5** in vacuo.[16] The two lowest enthalpy conformers found for

5 were J-shaped conformers in which the carboxylic acid group is in close proximity to the C14−C15 π bond. This same J-shape was reported by Corey et al.[21] as one type of low-energy minimum identified in their conformational analysis of arachidonic acid. Corey suggested that such a J-shaped conformation in solution would be energetically favorable and would be consistent with the chemistry of peroxyarachidonic acid for which an internal epoxidation leads to 14,15-epoxyarachidonic acid.[21] Using the A* algorithim followed by minimization in the "Batchmin" module of Macromodel, Leach and Prout[24] found right- and left-handed looped or "helical-like" conformations of **5** to be its lowest energy conformations. In a study of the related fatty acid 4,7,10,13,16,19-docosahexaenoic acid, Applegate and Glomset[19] found hairpin or J-shaped conformers to be the lowest-energy conformers. Other low-energy conformations of this compound were angle-iron conformers and helical structures. A simulated annealing study of **5** identified a looped conformation,[25] while a molecular dynamics study in vacuo found low-energy structures of **5** to be hairpin, helical, and crown-shaped (i.e., U-shaped conformers in which the ends of the chain come close to one another).[26] Lopez et al. also employed molecular dynamics simulations in their study of **5**, finding two low-energy conformations, one of which was U-shaped and was proposed as the bioactive conformation at its cyclooxygenase binding site.[27]

Conformational Memories results for arachidonic acid (**5**) in vacuo reported here also identified hairpin (U-shaped) and J-shaped families; however, CM also identified extended conformers similar to the angle-iron crystal structure as a significant family. It is possible that extended conformations of **5** were not identified in several of the molecular dynamics or molecular dynamics/simulated annealing calculations discussed above due to incomplete sampling of the conformational space. While the J-shape (as defined by Corey) was not among the predominant shapes found by Conformational Memories, the percentage of J-shapes was still significant (i.e., in vacuo, 22 structures met the J-filter distance criterion with 15 of these shaped as illustrated in Figure 2C). Thus, the Conformational Memories results are consistent with the chemistry of peroxyarachidonic acid.[20,21]

While no other theoretical studies of arachidonic acid (**5**) in solvent have been reported, the trends in the Conformational Memories results for **5** in $CHCl_3$ and in H_2O are consistent with the idea that in H_2O, arachidonic acid would minimize the exposure of its hydrophobic portions by forming a more compact shape (U-shape).

Anandamide (1). Thomas et al.[10] recently published a constrained molecular dynamics study of anandamide and related analogues in which a J-shaped conformation of anandamide was found to be energetically favorable. A structural correlation between this J-shaped conformer of anandamide and a classical cannabinoid template molecule was obtained by the superposition of (1) the oxygen of the carboxyamide of anandamide with the pyran oxygen in Δ^9-THC, (2) the hydroxyl group of anandamide with the phenolic hydroxyl group of Δ^9-THC, and (3) the polyolefin loop of anandamide overlaying with the cannabinoid tricyclic ring structure. Conformational Memories analysis of **1** found 10 conformers (out of 150) which met the J-filter in $CHCl_3$, 4 of which resembled a J in shape. Thirteen conformers (out of 150) in water met the J-filter, with none exhibiting the J-shape pictured in Figure 2C. These results are consistent with the idea that structures meeting the J-filter represent low-energy conformations for anandamide, as previously reported.[10]

Conformational Memories results in $CHCl_3$, however, show extended and extended U-shaped conformers as more favored for **1** than the J-shape. It is remarkable also that in H_2O, the classic angle-iron or extended conformation of **1** is no longer seen. Instead, the one predominant cluster (73%) appears to be an extended U-shaped hybrid conformation in which the ends are beginning to move toward one another.

***sn*-2-Arachidonylglycerol (2-AG, 2).** Recently, 2-AG was isolated from intestinal tissue and shown to be a second endogenous ligand present in the brain at concentrations 170 times greater than that of anandamide.[28] The FAAH enzyme which hydrolyzes anandamide to arachidonic acid and ethanolamine[8] has recently been reported to hydrolyze 2-AG at a rate 4 times faster than that for anandamide hydrolysis.[29] Conformational Memories results for 2-AG paralleled those for arachidonic acid. The extended and U-shapes were favored, with the extended shape predominating in $CHCl_3$ and the more compact U-shape predominating in water.

Compound 3. Several analogues of anandamide have been synthesized and tested for affinity at the CB1 receptor and for pharmacological activity.[30,31] Recently, two separate studies[32,33] looked at the effect of substituting a 1,1-dimethylheptyl segment for the pentyl segment of anandamide which follows the C14−C15 double bond (i.e., the C16−C20 segment). The rationale for the synthesis of these new analogues was to test if the C16−C20 segment of anandamide was analogous to the C3 pentyl side chain in classical cannabinoids. In classical cannabinoids, replacement of the C3 pentyl side chain with a 1′,1′-dimethylheptyl side chain results in as much as a 75-fold enhancement in CB1 affinity and activity.[34] In both anandamide analogue papers, substitution of the C16−C20 section of anandamide with a 1,1-dimethylheptyl side chain caused a 13-fold enhancement in CB1 affinity.[32,33] Thus, the enhancement in affinity seen for the dimethylheptyl analogues was not as great as the enhancement for analogues with the same substitution in a series of classical cannabinoids. The smaller enhancement in CB1 affinity with **3** may be due to the fact that fewer conformers exist in a CB1 appropriate conformation. Thus, although **3** may have better interactions with the receptor, presumably due to its enhanced ability for hydrophobic interaction, the lower population of conformers in the appropriate shape may modulate the affinity to be lower proportionally to that seen in dimethylheptyl analogues of classical cannabinoids.

PGB_2-EA (4). Pinto et al.[35] investigated a series of arachidonyl amides and esters in addition to a series of "rigid hairpin" conformations typified by *N*-(2-hydroxyethyl)prostaglandin amides to determine the structural requirements for binding to the CB1 receptor. As is evident in Chart 1, two-dimensional drawings of anandamide and **4** make the shapes of these two compounds look similar. The possibility existed, therefore, that **4**

may be a rigid analogue of **1**. However, all of the rigid prostaglandin analogues synthesized by Pinto et al. failed to alter [^{3}H]CP-55,940 binding to CB1 in concentrations as great as 100 μM.

Conformational Memories results reveal that while **4** can adopt a hairpin or U-shape, the angle-iron shape which predominates for **1** and **2** in $CHCl_3$ and which is the second major conformational family for **3** in $CHCl_3$ is not highly populated for **4**. (Note: The second major conformational family of **3** in $CHCl_3$ is described above as an L-shape. This family is characterized by an extended shape from which the DMH chain projects at nearly a right angle.) The very attenuated ability of **4** to adopt the more extended shapes seen in **1**−**3** may be the reason it does not bind to CB1. An alternative explanation for the lack of affinity of **4** at CB1 is that the steric bulk introduced by the five-membered ring in **4** may produce a steric clash at CB1 and prevent **4** from binding.

Conclusions

Consistent with experiment, Conformational Memories calculations for arachidonic acid identified extended conformers (such as that shown by X-ray analysis of the pure compound)[16,17] and folded or U-shaped conformers (such as that shown by X-ray analysis of arachidonic acid complexed with adipocyte-binding protein)[22] to be major conformational families of arachidonic acid. The more compact (U-shaped) structure was found to predominate in water, while the extended shape was found to predominate in $CHCl_3$ (and in vacuo). The existence of J-shaped conformers as suggested in the literature[16,20,21] was also identified by Conformational Memories. In this case, the J-shaped family was found to comprise a smaller but still significant conformational family of arachidonic acid. It appears, then, that the Conformational Memories method has done a much broader sampling of the conformational space of arachidonic acid than has heretofore been performed using molecular dynamics or molecular dynamics/simulated annealing techniques.[16,19,20,24−27] These previous studies of arachidonic acid have found primarily only compact structures (U's, J's, helical) as energy minima.

Results for **1**−**3** point to the extended (angle-iron) shape and U-shape as major conformational families in $CHCl_3$ and in water. In general, the extended shape is preferred in $CHCl_3$, while the more compact U-shape is preferred in H_2O. Due to the presence of its five-membered ring, PGB_2-EA (**4**) rarely adopts an extended conformation. This may be why **4** shows such a low affinity for the CB1 receptor. For **1**−**3** in either H_2O or $CHCl_3$, 10−26 conformers met the J-filter criterion derived from the J-shape identified by Rich[16] and Corey[20,21] in conformational studies of arachidonic acid. Compound **3** in H_2O exhibited the highest numbers of conformers which met the J-filter criterion (i.e., 26 out of 150 conformers), while anandamide and **3** in $CHCl_3$ had the fewest structures (i.e., 10 out of 150). As results reported here detected J-filtered conformations for all of the CB1 agonists studied (albeit for some in lower numbers), it is possible that the J-shaped conformation may be a biologically relevant conformation at one or more of the biological targets with which anandamide interacts. Indeed, Thomas et al.'s recent paper proposes that anandamide assumes a J-shape in its interaction with the CB1 receptor.[10]

The conformational information obtained here for anandamide and 2-AG may be useful in the design of rigid analogues which mimic the preferred molecular conformations (shapes) of these ligands. Such rigid analogues may be useful in deducing the bioactive conformation of these endogenous cannabinoids, not only at the CB receptors but also at the FAAH enzyme active site and possibly at the binding site(s) on the newly proposed anandamide transporter.

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Supporting Information Available: Tables of the torsion angles of a representative structure from each of the two highest-populated families for compounds studied here (5 pages). Ordering information is given on any current masthead page.

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