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Vitamin D: Structure-Function Analyses and the Design of Analogs

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Abstract There is continuing and emerging new interest in the development of vitamin D analogs resulting from the recognition that analogs of 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] may be therapeutically useful. Side chain analogs of this steroid hormone are of particular interest because a family of lead structures have recently emerged for possible use in the treatment of certain types of cancers and skin diseases. Because of the chaotic array of side chain structures which exhibit useful therapeutic indices for these purposes, a more systematic approach towards developing intelligible structure-function information needs development. Accordingly, a method has been devised to analyze analogs as to their side chain topology based on identifying specific occupancy volumes through conformational analysis. Dot maps have been constructed as an indication of the volume in space which the side chain of 1α,25-$(OH)_2$-D_3 or analogs is permitted to occupy. Volume exclusion analyses based on comparison of structural and biological data for 1α,25-$(OH)_2$-D_3 and analogs are anticipated to lead to a more cogent model for drug design. A cautionary note on the limitations of this approach is discussed.

Key words: conformational analysis, molecular mechanics computations, side chain topology, dot maps, steroid hormones

One of the continuing goals of this laboratory is to develop a detailed understanding at the molecular level of the biochemical mode of action of vitamin D (calciferol). The design, chemical synthesis, and biological evaluation of analogs could lead to compounds which may not only serve as useful biochemical research tools for obtaining mechanistic information, but also for more effectively designing drugs for chemotherapeutic applications. In order to develop the concepts related to the design of new vitamin D analogs, it is useful to consider as shown in Figure 1 the metabolic pathway [1–3] leading to the steroid hormone 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3, **6**), the physiologically active form of vitamin D_3, which is usually depicted in textbooks [4] as its s-trans conformer **4** rather than its steroidal, s-cis conformer **3.** After its photochemical production in the skin from 7-dehydrocholesterol **(1)**, previtamin D_3 **(2)** undergoes a hydrogen shift to afford vitamin D_3. The latter is hepatically converted to 25-hydroxyvitamin D_3 (25-OH-D_3, **5**), which then undergoes renal metabolism to 1α,25-$(OH)_2$-D_3 and/or 24R,25-dihydroxyvitamin D_3 **(7)** depending upon the physiological status of the animal. Although 1α,25-$(OH)_2$-D_3 is considered to be the active form of vitamin D_3, there is evidence that **7** possesses its own unique physiological role. More than 30 other metabolites and/or catabolites have been characterized to date.

After renal secretion, 1α,25-$(OH)_2$-D_3 **(6)** migrates to certain target tissues where biological responses (Fig. 1) occur by the action of specific proteins induced via a steroid hormone type genomic pathway [1–4]. In this pathway, the steroid 1α,25-$(OH)_2$-D_3 binds to an intracellular receptor, the vitamin D receptor (VDR), which triggers the induction of new proteins more directly responsible for the biological response. The most classical biological responses associated with vitamin D action are calcitropic, which includes intestinal calcium absorption (ICA) and bone calcium mobilization (BCM). This steroid induced, genomic pathway is considered to mediate these responses through calcium binding protein synthesis. VDR has been shown to be homologous to those of other steroid hormone receptors. The receptor gene for 1α,25-$(OH)_2$-D_3 belongs to the same super family of trans-activating regulators of gene-transcription which include, besides the receptors for the steroidal glucocorticoids, mineral corticoids and sex hor-

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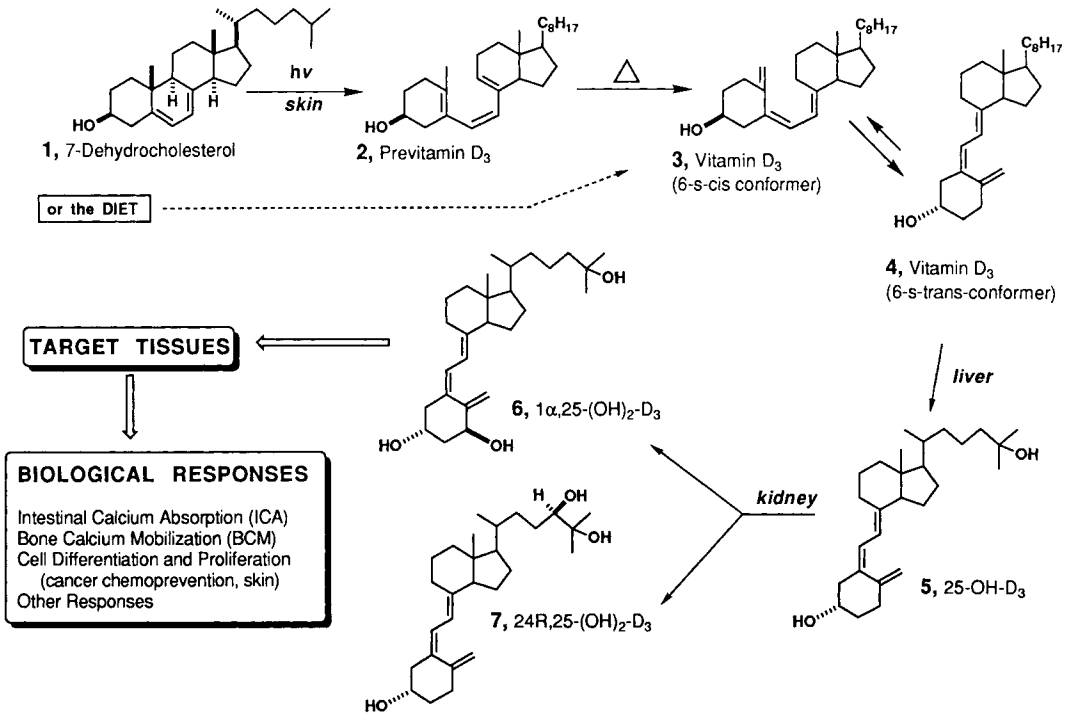

Fig. 1. Vitamin D metabolism. The principal metabolic pathway leading from 7-dehydrocholesterol (**1**) to $1\alpha,25\text{-}(OH)_2\text{-}D_3$ (**6**) is summarized. The steroid hormone **6** is considered to migrate to certain target tissues where a genomic response leads to specific biological responses mediated through protein synthesis.

mones, those for thyroid hormone and retinoic acid [5].

A reasonable hypothesis is that most if not all forms of vitamin D interact selectively with various proteins (receptors, enzymes and/or transport proteins) and other biological matrices (e.g., lipids) as they traverse the aqueous milieu of the endocrine maze [1–4]. Besides VDR, other important proteins include DBP (vitamin D-binding protein, the serum vitamin D transport protein) and the P-450 enzymes in the liver (which produces **5**) and kidney (which produces **6** and **7**). An intriguing question surrounds the role played by the putative $1\alpha,25\text{-}(OH)_2\text{-}D_3$-responsive, membrane-receptor system present in the intestine, which leads to a non-genomic, very rapid vitamin D-dependent stimulation of intestinal calcium transport known as transcaltachia [6].

Besides its traditional role as a hormone in calcium homeostasis, $1\alpha,25\text{-}(OH)_2\text{-}D_3$ induces differentiation and affects cellular proliferation, suggesting possible use in the treatment of certain cancers and skin disorders [7,8]. There has accordingly been an increased interest in the further development of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ analogs. The clinical utility of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ is limited because therapeutically effective doses induce hypercalcemia through its normal action in stimulating ICA and BCM [9,10]. This has led investigators towards the development of an analog with a more useful therapeutic index, specifically directed towards analogs with high cell differentiating ability and low calcitropic action (ICA and BCM). As shown in Figure 2, a series of remarkably diverse side chain analogs of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ exhibiting promising therapeutic indices have been reported and examples include 1α,25S,26-trihydroxy-22,23-didehydrovitamin D_3 (**8**) [11], 16,17,23,23,24,24-hexadehydro-1α,25-dihydroxyvitamin D_3 (**9**) [12], 1α,25-dihydroxy-22-oxavitamin D_3 (**10**) [13], 1α,24R-dihydroxy-22,23,26,27-tetradehydrovitamin D_3 (**11**) [14], and 24a-homo-1α,25-dihydroxyvitamin D_3 (**12**) [15]. From efforts in our own laboratories, the conformationally restricted side-chain analogs **13, 14,** and **15** (known as the arocalciferols) have also emerged as possible lead structures [10]. One facet of the mode of action of these steroids leading to cellular differentiation is also thought to entail their binding to an intracellular receptor followed by a genomic response characteristic of other steroid hormones [1–4]. The diversity of side chain structural units in some of the most promising analogs

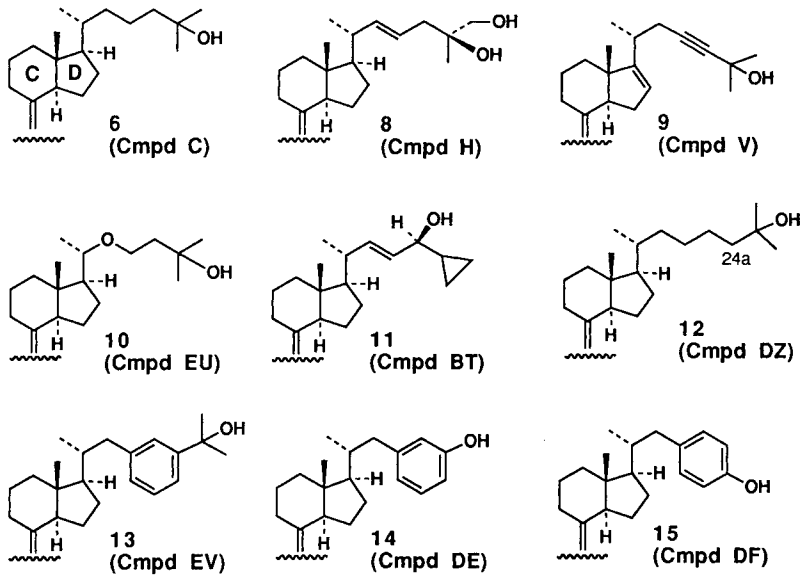

Fig. 2. Side chain analogs of 1α,25-$(OH)_2$-D_3. Only partial structures depicting the CD ring and side chain are given in this figure. Structure **6** is that of 1α,25-$(OH)_2$-D_3 whose complete structure is shown in Figure 1. Analogs **8–15**, discussed in the text, contain A-ring and triene components as in **6**. It should be noted that the parenthetic labels (Cmpd C, H, etc.) are code letters used at the University of California, Riverside, for a data base system currently being developed for structure-function analyses.

such as those shown in Figure 2 in comparison to the native hormone 1α,25-$(OH)_2$-D_3 makes difficult an assessment of intelligible structure-activity correlations needed in designing yet more effective analogs for therapeutic purposes.

Serendipity rather than well layed plans often seem to characterize the drug discovery process. Nevertheless, lead structures in the form of side-chain analogs such as those shown in Figure 2 provide at least a basis for the design of a new generation of analogs of possible use in the cancer chemoprevention area. As a step towards developing a better understanding of the side chain structural requirements of 1α,25-$(OH)_2D_3$ and its analogs, it is the purpose of this Prospect article to describe the dynamic structural nuances (i.e., the conformational characteristics) of the flexible side chain of this steroid. To date, more attention has been paid to the structural features of the triene and A-ring components of vitamin D, topics which will only be briefly reviewed towards the end of this article. With the emergence of the side chain as a key area for chemical modification in the design of new drugs, it is useful to first focus on this structural unit of vitamin D.

THE SIDE CHAIN

Unlike the other more classical, mammalian steroid hormones which bear truncated side chains (progesterone, cortisol and aldosterone) or no side chain at all (estradiol and testosterone), 1α,25-$(OH)_2$-D_3 is unique because it possesses the fully intact, conformationally flexible eight carbon side chain characteristic of cholesterol itself. The side chain of 1α,25-$(OH)_2$-D_3 may assume any number of conformational orientations and it is hardly possible at this juncture to predict how the side chain is best oriented when 1α,25-$(OH)_2$-D_3 binds to receptor.

A conformational analysis [16] of the side chain of 1α,25-$(OH)_2$-D_3 in the form of model system **16** is summarized in Figure 3. Due to the flexibility of the steroid side-chain, relatively free rotation about the six single bonds indicated by the curved arrows renders the analysis complex. There are 729 possible staggered rotamers (3^6, assuming 120° rotations) involving six single bonds of an aliphatic chain, but conformational searching of the steroid's side chain using a standard molecular mechanics program (Serena Software, Bloomington, Indiana) leads to 1,083 side-chain minimum energy conformers within 4.5 kcal/mole of the global minimum (lowest energy) conformer. Far more than the 729 conformers are found due to small variations in the dihedral angles of the normal staggered conformations. Of these, 393 side chain conformers lie within 4 kcal of this same minimum. Panel 1 seeks to depict an overlay of all

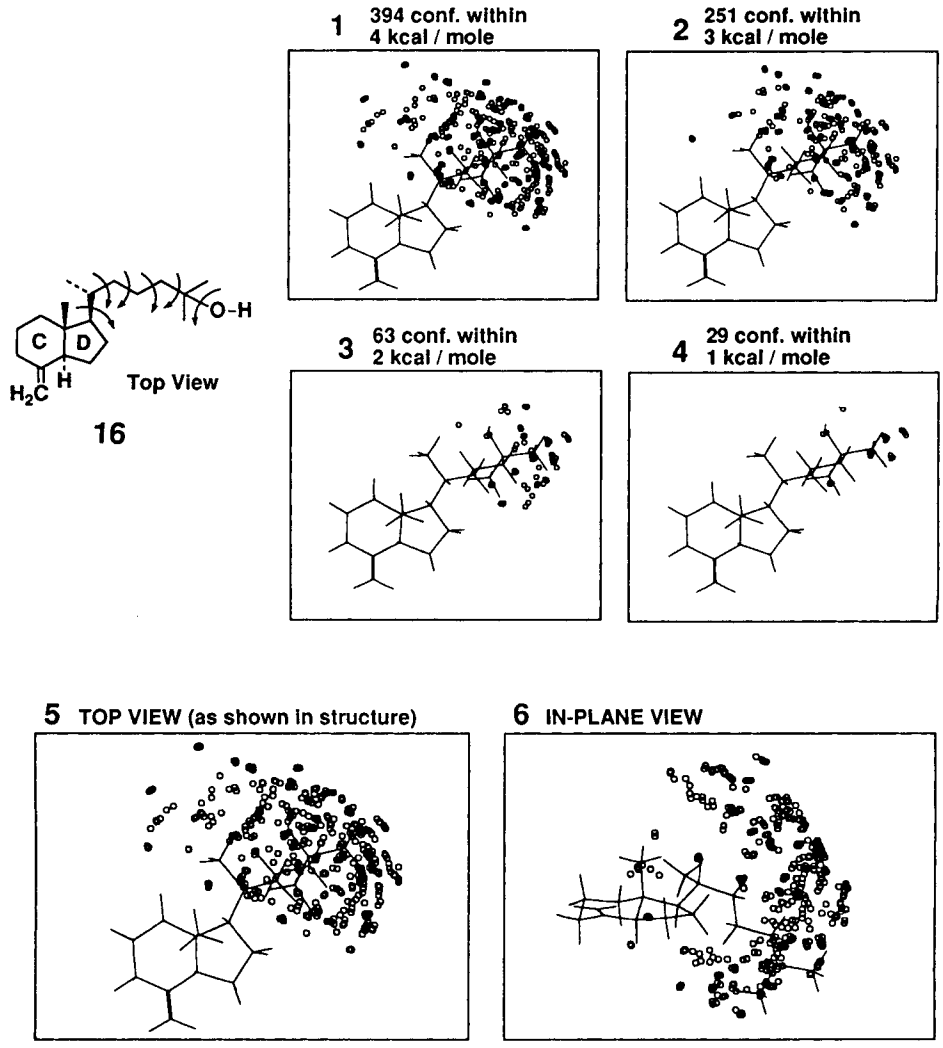

Fig. 3. Dot maps for the conformational analysis of the side chain of 1α,25-$(OH)_2$-D_3. Structure **16** is the 1α,25-$(OH)_2$-D_3 CD-side chain component utilized in this molecular mechanics evaluation. **1–4** depict the overlay comparison of the CD fragment as viewed from the top view shown approximately in the line drawing structure **16**. **5** is identical to **1** wherein **6** depicts an in-plane view of this same dot map.

394 conformations in the form of dot maps wherein the circles represent the oxygen atom of the side chain hydroxyl for each of 394 conformational minima. The superimposed line drawing represents the top view (or CD-ring face on view as depicted in structure **16**) of the lowest energy conformation (the global minimum). It should be noted that the overlays of the remaining 393 conformational minima were superimposed atop the CD hydrindane moiety to give the root mean square best fit of the relatively rigid CD-ring system wherein only the position of the oxygen atoms (indicated by the circle) with respect to the CD ring is shown for the 393 other conformers. Panels 2, 3, and 4 represent the 251, 63 and 29 conformational minima which lie within 3, 2, and 1 kcal/mole, respectively of the global minimum. In each of the four panels, 1–4, the reference line drawing is that of the same global minimum conformation wherein this reference structure is most easily seen in panel 4 (but they are identically positioned in all four of these panels). Panels 1–4 hardly do justice to the three-dimensional orientation of the various conformational minima projected into the third dimension. We have accordingly selected two nearly orthogonal views of these dot maps to reflect that "volume" in space which the side chain can occupy with respect to the rigid CD ring. This is represented by a side by side comparison of the dot maps shown in panel 5 (which is identical to that shown in panel 1) and panel 6.

In panel 6 the CD ring is viewed along the plane of the CD fragment such that the line of sight is along an axis slightly below the exocyclic methylene in the C ring. This view shows clearly that the side chain easily rotates into orientations both above and below the approximate plane defined by the CD ring. Note however that panel 5 reveals that the 394 lowest energy side chain conformations hover over the CD ring defined by a line drawn through carbons 12 and 17 (steroid numbering).

It must be strongly emphasized that these dot maps for the 1α,25-$(OH)_2$-D_3 side chain seek to define a volume in space which the side chain is "permitted" to sweep within the restrictions of covalent bonds of the side chain. The assumption here is simply that the side chain must be "parked" within this volume upon receptor binding. A further assumption is made that this type of energy minimization analysis provides a reasonable approximation of all possible orientations of the side chain irrespective of whether the side chain resides at a local minimum or not. In no way does it imply that the side chain resides at the global minimum (or at any other local minimum) at the time that it ligates to protein. It is interesting to note, however, that at its global minimum, the side chain is not fully extended as in simple aliphatic chains as implied in the simple line drawing shown in structure **16** of Figure 3. As most easily seen in panel 4 the global minimum side chain conformation of 1α,25-$(OH)_2$-D_3 consists of a nearly 90° bend at the C-22, 23 single bond (extending behind the plane of the CD-ring), presumably as a consequence of steric repulsion between C-23 of the side chain and C-16 of the D-ring if the side chain is full extended as in structure **16.**

In sum, the dot maps of Figure 3 identify an occupancy volume (or a "parking zone" [17,18]) for the location of the side chain hydroxyl group which is assumed to be most important in the binding of 1α,25-$(OH)_2$-D_3 to its receptor protein, possibly through hydrogen bonding. With the occupancy volume or parking zone so defined for 1α,25-$(OH)_2$-D_3 the important question is in which direction is the side chain really oriented upon receptor binding? It is assumed that the side chain occupies a fairly restricted topology at the binding site of receptor. Although only one global minimum energy conformation exists according to these molecular mechanics computations, the side chain can easily adopt quite different orientations as a consequence of perturbations by protein. The goal of analog design then is to carry out studies to define the precise orientation of the side chain upon receptor binding. To be sure, this might be accomplished by co-crystallization of 1α,25-$(OH)_2$-D_3 bound to its receptor and then direct x-ray structural analysis. This kind of experiment is still somewhat far into the future and, in the meantime, one is faced with the greater need to produce new analogs which may be useful in drug therapy.

In order to make more practical progress, one approach towards defining the optimal topology of the side chain is to synthesize a host of analogs of the hormone 1α,25-$(OH)_2$-D_3 to allow for a volume exclusion analysis [18] (i.e., define side chain "parking zones" and "no parking zones"). For example, as shown in Figure 4, a side by side comparison is made of the dot maps for the still unknown arocalciferol model system **17** with that of the 1α,25-$(OH)_2$-D_3 model system **16.** The presence of the aromatic ring rigidifies the side chain of 1α,25-$(OH)_2$-D_3 by bridging across the C-22 and C-24 carbon atoms of the natural hormone. The side chain of the aromatic analog can orient in only two distinct regions, one approximately above the C-ring and one extended away from the D-ring. This should be compared to the near continuum of side chain orientations as depicted for model system **16.** Thus by computer modeling, it will be possible to identify side chain analogs with limited occupancy volumes. This is to be followed by synthesis and then comparison of their biological activities versus those of the natural hormone **16.** If the analog is biologically active then the spatial orientations common to both analog and 1α,25-$(OH)_2$-D_3 can be deemed a "parking zone" or allowed occupancy volume. The spatial arrangement not common to an analog and 1α,25-$(OH)_2$-D_3 would be a forbidden occupancy volume ("a no parking zone"). What is urgently needed for this type of volume exclusion analysis is a series of analogs with restricted rotations in order to define the *minimum allowed occupancy volume which satisfies both 1α,25-$(OH)_2$-D_3 and the biologically active analog.* Thus by this analysis it is hoped that an "active topography" for inducing cell differentiation may be accessed.

CD-RING, TRIENE, AND A-RING STRUCTURE

Although the primary emphasis of this Prospect review article has focused on the heretofore

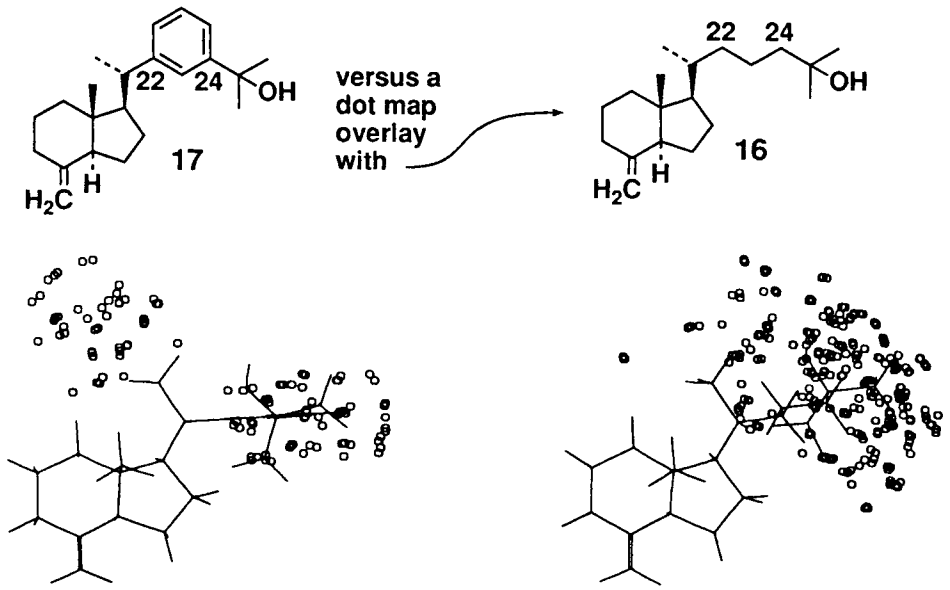

Fig. 4. Overlay of dot maps. Comparisons can be made of occupancy volumes for any analog such as the still unknown arocalciferol model system **17** versus that of the natural hormone model system **16.** Only the top views are shown. This type of comparison seeks to answer the question, What side chain orientations (topologies) are the most effective for ligand binding?

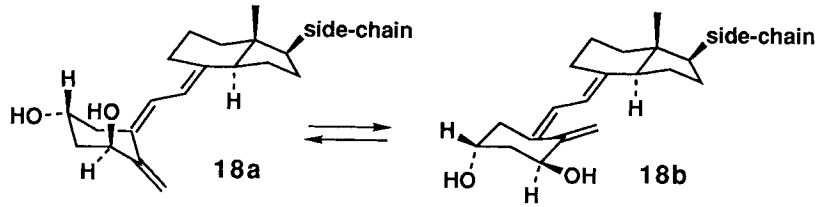

Fig. 5. A-ring conformations for 1α,25-$(OH)_2$-D_3. This schematic line drawing depicts approximately the two A-ring chair conformations **18a** and **18b** as discussed in the text.

less well analyzed vitamin D side chain, it is obvious that the complete vitamin D structure including the CD-ring, triene and A-ring components taken collectively with the side chain must be considered in developing structure-function relationships useful for understanding vitamin D action. The CD-ring of 1α,25-$(OH)_2$-D_3 is the most rigid component of the structure [16] and its conformational analysis was included in the energy minimization of the side chain in model system 16 (Fig. 3). Because of the rigidity of the CD-ring, a trans-hydrindane, we consider it useful to simplify the analysis by discussing primarily the relative relationship of the conformational features of the side chain with respect to the triene moiety and the A-ring wherein the CD fragment is treated as a rigid, insulating anchor.

Although the x-ray crystallographic structure determination of 1α,25-$(OH)_2$-D_3 has not been successfully achieved, solid state structural results of various other vitamin D derivatives and analogs have been published [19–26]. In addition, evaluation of solution structures have been carried out primarily through ^{1}H-NMR spectral studies [27–38]. The collective sum of these investigations both in the crystalline state and in solution can be summarized as shown in Figure 5. The principal conclusion as suggested from the initial, dynamic solution studies for 1α,25-$(OH)_2$-D_3 is that the A-ring exists in dynamic equilibrium between nearly equimolar populations of two chair A-ring forms **18a** and **18b** [28–29]. In one chair conformation, **18b,** the C-1 hydroxyl is equatorially oriented whereas the C-3 hydroxyl is axial. In the second conformation, **18a,** the C-1 hydroxyl is axial whereas that at C-3 is equatorial. As for the triene component, the intercyclic diene unit, the $\Delta^{5,6}$ and the $\Delta^{7,8}$ double bonds are nearly co-planar and s-trans (or transoid) in orientation. The exocyclic dou-

ble bond at the $\Delta^{10,19}$ position is oriented above or below the plane defined by the $\Delta^{5,7}$ diene unit as implied in structures **18b** and **18a,** respectively. The barrier to A-ring chair-chair inversion between **18a** and **18b** is approximately 10 kcal/mole so that in solution, both forms exist to an equal extent and the interconversion is extraordinarily rapid. The ability to rotate about the $\Delta^{6,7}$ single bond is even more facile. It was Delaroff [27] who first showed in solution that the nature of the $\Delta^{6,7}$ single bond is transoid on the basis of ^{1}H-NMR studies. There is no question however that the cisoid conformation (6-s-cis conformer) must be easily accessible for all of the D vitamins. However from a spectroscopic [27–38] and x-ray crystallographic standpoint [19–26], there is no direct evidence for detectable levels of the cisoid conformation.

Referring to the conformational equilibrium between 6-s-cis conformer **3** and its 6-s-trans form **4** shown in Figure 1, there is no question that vitamin D_3 or any of its metabolites including 1α,25-$(OH)_2$-D_3 can chemically equilibrate with the corresponding previtamin form. At equilibrium 1α,25-$(OH)_2$-D_3 exists to the extent of 10% in its previtamin structure as recently reported by this laboratory [39]. In order for 1α,25-$(OH)_2$-D_3 or vitamin D_3 to chemically equilibrate (via a [1,7]-sigmatropic hydrogen shift) with the previtamin form, they must assume the 6-s-cis conformation (i.e., **3**) in order to back rearrange to the corresponding previtamin structure. Thus, there must be kinetically competent concentrations of the cisoid conformation so that it is well possible that at the time that 1α,25-$(OH)_2$-D_3 binds to its receptor, it is fully capable of binding to receptor in its 6-s-cis steroid like conformation.

We speculated in the early 1970s [40] that it is the A-ring chair conformation **18b** which binds to receptor. However, no direct evidence for this hypothesis has thus far emerged. The important matter here is that in order to fully appreciate our understanding of structure-function relationships of 1α,25-$(OH)_2$-D_3 in drug design, we must fully understand not only the occupancy volume of the side chain, but in reality, the occupancy volume of the entire molecule including the dynamic A-ring and triene moieties which are anchored with respect to one another by the relatively rigid trans-hydrindane CD unit. Because of the complexity of the problem, and the likely possibility that conformational perturbations of the side chain would have relatively small effects on the A- and triene moieties, by keeping the latter components common to all of our analogs at least for this one area of our research (i.e., the design of side chain analogs) we can assume that whatever the topology of the lower half of the structure of vitamin D, it will be possible to develop cogent structure-function relationships through changing systematically only one small part of the molecule, namely the side chain. Future studies could be directed towards freezing out the lower half of the molecule in a conformational sense together with manipulations of the side chain, but interpretations could turn out to be rather complex. The insulatory effect of the rigid CD anchor provides a reasonable justification of this approach.

A CAUTIONARY NOTE AND SUMMARY: "RUSTING" OF THE LOCK AND KEY ANALYSIS [41]

It is important to appreciate the fact that this Prospect article simply describes facets of the dynamic topological structure of vitamin D; it is not at all suggested that any specific conformation corresponds to the active topology of the hormone 1α,25-$(OH)_2$-D_3 at the time that this ligand binds to protein. As indicated above, one can qualitatively describe the vitamin D molecule as consisting of two flexible units attached to a relatively rigid steroidal CD-fragment serving as an anchor; at one end (at C-8 of the steroid skeleton) there is a modestly flexible triene and A-ring component and, at the other end (C-17), there resides an even more flexible 25-hydroxylated cholesterol like side-chain.

As summarized recently by Jorgensen [41], it is reasonable to expect that flexible molecules distort to form optimal interactions with binding partners in this lock (protein) and key (ligand) model for designing drugs. A very important practical consequence of this expectation is that any attempt to design drugs by analogy to the structures of flexible, unbound active substances could lead to extreme frustrations. The tacit assumption that the favored conformation of a free ligand corresponds to that which resides in the actual "parking zone" in the ligand-receptor complex was criticized some time earlier by Marshall et al. [18]. Unbound ligands more generally possess shapes which are quite different from their topology in the bound state. Likewise, bound and unbound protein receptors may also assume quite different shapes in the more general case. While it may be possible to

identify examples [40] where the shape of the ligand and the shape of the receptor protein assume the same shapes in the bound state, one cannot generally expect this to be the case. Finally, it should be clearly noted that even in the event that the ligand protein structure has been fully established through physico-chemical techniques such as X-ray crystallography or through multi-dimensional NMR analyses, there remains the uncertainty that the specific three-dimensional structure so obtained may have little bearing on the actual structure of the ligand receptor complex in the in vivo situation.

The implication of this cautionary note is not meant to suggest that one should terminate any attempts to carry out structural studies on free ligand, free receptor protein and/or the ligand-protein complex. Rather, it is stated here simply to suggest the extreme caution with which limited data should be interpreted.

With regard to the vitamin D molecule, the important point is that it is a conformationally flexible molecule and as such all accessible conformations irrespective of energetic considerations should be considered in the initial analysis. The active topology of vitamin D at the time that it interacts with receptor must be within its occupancy volume simply due to the restrictions of the presence of strong covalent bonds. The idea then is to incorporate conformationally rigidifying units such as rings in the side chain to identify more precisely the essential critical volume which is associated with the active topology of the vitamin D molecule. It is anticipated that through cooperative efforts between chemists and biochemists involving design, chemical synthesis and biological evaluation, one might obtain information on the required topology of the $1\alpha,25\text{-}(OH)_2\text{-}D_3$ molecule for active agonist behavior. There is less to be learned if specific analogs are biologically inactive. This is because for inactive analogs, the lack of activity may have its origin in undue steric or electronic effects. Such factors are undoubtedly significant, but at this first level of structure-function analysis, if vitamin D cannot assume the proper shape, it is unlikely to be viable for competent protein activation for inducing biological activity. The availability of a family of conformationally restricted analogs of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ would be highly desirable for the further development of cogent structure-function concepts in this area of research.

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