Overview of general physiologic features and functions of vitamin D1–4

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ABSTRACT
Vitamin D3 is a prohormone produced in skin through ultraviolet irradiation of 7-dehydrocholesterol. It is biologically inert and must be metabolized to 25-hydroxyvitamin D3 in the liver and then to 1α,25-dihydroxyvitamin D3 in the kidney before function. The hormonal form of vitamin D3, ie, 1α,25-dihydroxyvitamin D3, acts through a nuclear receptor to carry out its many functions, including calcium absorption, phosphate absorption in the intestine, calcium mobilization in bone, and calcium reabsorption in the kidney. It also has several nongenomic functions in the body. This overview provides a brief description of the physiologic, endocrinologic, and molecular biologic characteristics of vitamin D. It also provides information on new selective analogs of 1α,25-dihydroxyvitamin D3 for therapy. Am J Clin Nutr 2004;80(suppl):1689S–96S.

KEY WORDS Vitamin D metabolism, bone, calcium homeostasis, tetany, vitamin D endocrine system, autoimmune diseases

INTRODUCTION
The discovery of vitamin D and the elimination of rickets as a major medical problem ranks as one of medicine’s great achievements (1). From the early studies of McCollum and Davis (2) in 1913, when the first vitamin was discovered, until 1940, the work leading to the identification of vitamin D and its role in bone formation and prevention of hypocalcemic tetany included many outstanding contributions. Most noteworthy was the work by Sir Edward Mellanby, who demonstrated that he could produce rickets in dogs by feeding them the diet characteristic of Scotland, ie, oatmeal; unknown to Sir Edward Mellanby was the fact that he deprived those dogs of sunlight. Because of the work of McCollum and Davis in discovering fat-soluble vitamin A, Mellanby attributed the ability of cod liver oil to cure the rachitic condition in dogs as being another property of vitamin A (3). McCollum very cleverly destroyed the vitamin A activity of cod liver oil by bubbling oxygen through the solution and heating it, but the ability to cure rickets remained in the preparation. McCollum correctly concluded that this represented a new vitamin, called vitamin D (4). Huldshinsky (5) and Chick et al (6) independently demonstrated that rachitic children could be cured with exposure to sunlight or artificially produced ultraviolet light. The puzzle was ultimately solved when Steenbock and Black (7) discovered that irradiation not only of the skin of animals but also of the food they consumed imparted antirachitic activity to either the animals or their food. Furthermore, Goldblatt and Soames (8) showed that livers taken from irradiated rats could heal rickets in rats. Therefore, 2 important discoveries occurred. First, Steenbock and Black (7) conceived that foods could be irradiated to impart vitamin D and rickets as a major medical problem would disappear. Second, the irradiation of fat-soluble substances extracted from tissues could be used to generate large amounts of vitamin D for later characterization. The structure of vitamin D2 was deduced in 1931 by Askew et al (9), and the structure of vitamin D3 was determined through synthetic means by Windaus et al (10). Vitamin D was discovered with many other vitamins and is classified as a vitamin even now. However, findings from the second half of the 20th century showed that vitamin D is truly a prohormone and not a vitamin. Vitamin D is virtually absent from the food supply. It is not found in plant materials (eg, vegetables, fruits, or grains) and is present in low abundance in meats and other animal food sources, except in rare cases such as fish liver oils and plants such as waxy-leaf nightshade (Solanum glaucophyllum).

PRODUCTION AND METABOLISM OF VITAMIN D
Vitamin D is normally produced in skin through a robust photolytic process acting on a derivative of cholesterol (ie, 7-dehydrocholesterol) to produce previtamin D, which is then slowly isomerized to vitamin D3 (11). Vitamin D3 is the natural form of vitamin D produced in skin, and vitamin D2 is derived from irradiation of ergosterol, which occurs to some degree in plankton under natural conditions and is used to produce vitamin D2 from the mold ergot (which contains as much as 2% ergosterol). We must move away from the concept that vitamin D is a vitamin.

Another important fact is that vitamin D is required throughout life. It not only is needed for the formation of bone but also likely plays an important role in several other physiologic systems. Its use may well prevent several degenerative diseases, and it may also play a role as an anticancer agent.

The structure of vitamin D3 and its numbering system are indicated in Figure 1. We now know that vitamin D3 itself is biologically inert, as clearly indicated by genetic defects that result in the disease rickets despite normal intakes of vitamin D (12). By 1967, the concept that vitamin D is converted to an active form had appeared (13, 14). By 1969, the circulating form

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of vitamin D had been isolated, chemically identified, and synthesized (15, 16). This compound, 25-hydroxyvitamin D₃ [25(OH)D₃], is now currently monitored in serum to indicate the vitamin D status of patients, as discussed below. However, 25(OH)D₃ itself is metabolically inactive and must be modified before function. The final active hormone derived from vitamin D was isolated and identified in 1971, and its structure was deduced as 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] (17) and confirmed by synthesis (18). The pathway that vitamin D must follow is illustrated in Figure 2 and forms the basis of the vitamin D endocrine system. For ~2 decades, there was consistent re-visititation of the concept that more than one hormone was derived from vitamin D, and ~33 metabolites of vitamin D were identified (19). However, it soon became clear that all metabolites were either less active or rapidly cleared and were thus intermediates in the degradation of this important molecule. The most important of these metabolites are 24,25-dihydroxyvitamin D₃ and 1α,24(R),25-trihydroxyvitamin D₃ produced by the enzyme CYP24, which is induced by the vitamin D hormone itself (20).

Much is known about the enzymes that produce 1,25(OH)₂D₃ and their regulation, but a great deal remains to be learned (20). Two enzymes are thought to function in the 25-hydroxylation step. They are not exclusively hepatic but are largely functionally active in the liver. The mitochondrial enzyme, which is not specific for vitamin D, has been cloned and a knockout mouse strain has been prepared, without any apparent effect on vitamin D metabolism, which suggests that there is an alternate 25-hydroxylase (21). A microsomal hydroxylase was recently cloned and could represent the missing enzyme (22). The 25(OH)D₃ 1α-hydroxylase was cloned by 3 different laboratories (reviewed in ref 20), and the sites of vitamin D-dependent rickets type I were identified in several studies (20). Very important was the generation of 1α-hydroxylase knockout mice, which exhibit a phenotype virtually identical to the human vitamin D-dependent rickets type I phenotype. Therefore, the enzymes that activate vitamin D have been identified.

Of major metabolic importance is the mode of disposal of vitamin D and its hormonal forms. The cytochrome P-450 enzyme now called CYP24 was isolated in pure form by Ohyama and Okuda (23) and the complementary DNA and gene were cloned, which yielded a 24-hydroxylase-null mutant (reviewed in 20). No significant phenotype resulted except for a large accumulation of 1,25(OH)₂D₃ in the circulation, which produced secondary effects on cartilaginous growth (20, 24). CYP24 is an extremely active enzyme, but the gene remains silent in vitamin D deficiency; it is induced by the hormonal form of vitamin D itself. Therefore, pulses of the vitamin D hormone program its own death through induction of the 24-hydroxylase. The 24-hydroxylase is able to metabolize vitamin D to its excretion product calcitroic acid (20). 25(OH)D₃ can also be degraded through this pathway. 24-Hydroxylase and its regulation are important factors in the determination of the circulating concentrations of the hormonal form of vitamin D.

**PHYSIOLOGIC FUNCTIONS OF VITAMIN D**

A diagrammatic explanation of the role of the vitamin D hormone in mineralizing the skeleton and preventing hypocalcemic tetany is presented in Figure 3 (20). Plasma calcium concentrations are maintained at a very constant level, and this level is supersaturating with respect to bone mineral. If the plasma becomes less than saturated with respect to calcium and phosphate, then mineralization fails, which results in rickets among children and osteomalacia among adults (24). The vitamin D hormone functions to increase serum calcium concentrations through 3 separate activities. First, it is the only hormone known to induce the proteins involved in active intestinal calcium absorption. Furthermore, it stimulates active intestinal absorption of phosphate. Second, blood calcium concentrations remain in the normal range even when an animal is placed on a no-calcium diet. Therefore, an animal must possess the ability to mobilize calcium in the absence of calcium coming from the environment, ie,
through enterocytes. Two mechanisms play a role in increasing blood calcium concentrations, especially in the absence of intestinal calcium absorption. Vitamin D hormone stimulates osteoblasts to produce receptor activator nuclear factor-κB ligand (RANKL) (25). RANKL then stimulates osteoclastogenesis and activates resting osteoclasts for bone resorption (25). Therefore, the vitamin D hormone plays an important role in allowing individuals to mobilize calcium from bone when it is absent from the diet. It is very important to note, however, that in vivo both vitamin D and parathyroid hormone are required for this mobilization event (26, 27). Therefore, 2 keys are required, similar to a safety deposit box. Third, the distal renal tubule is responsible for reabsorption of the last 1% of the filtered load of calcium, and the 2 hormones interact to stimulate the reabsorption of this last 1% of the filtered load (28). Because 7 g of calcium are filtered every day among humans, this represents a major contribution to the calcium pool. Again, both parathyroid hormone and the vitamin D hormone are required. Calcium physiologic processes are such that a single low concentration of the vitamin D hormone stimulates enterocytes to absorb calcium and phosphate. If the plasma calcium concentration fails to respond, then the parathyroid glands continue to secrete parathyroid hormone, which in- creases production of the vitamin D hormone to mobilize bone calcium from the skeleton and can ultimately lead to osteoporosis. Another important aspect is that, except for stimulating mineralization of the skeleton, the vitamin D hormone has not been found to be anabolic on bone by itself.

**VITAMIN D ENDOCRINE SYSTEM**

A diagrammatic representation of the endocrine regulation of calcium concentrations in the plasma and the vitamin D endocrine system is presented in Figure 4. Calcium-sensing proteins that sense plasma calcium concentrations are found in the parathyroid gland (29, 30). When calcium concentrations decrease below normal, even slightly, then these transmembrane proteins, coupled to a G protein system, stimulate the secretion of parathyroid hormone. Parathyroid hormone then proceeds to the osteoblasts and to the proximal convoluted tubule cells within seconds. Most importantly, in the convoluted tubule cells that serve as the endocrine gland for the vitamin D hormone, 1α-hydroxylase concentrations are markedly elevated (30, 31). This signals the vitamin D hormone, which by itself stimulates intestinal absorption of calcium or together with parathyroid hormone, at higher concentrations, stimulates mobilization of bone calcium and renal reabsorption of calcium. The increase in serum calcium concentrations exceeds the set point of the calcium-sensing system, shutting down the parathyroid gland-induced cascade of events. If the plasma calcium concentrations overshoot, then the C-cells of the thyroid gland secrete the 32-amino acid peptide calcitonin, which blocks bone calcium mobilization (32). Calcitonin also stimulates the renal 1α-hydroxylase to provide the vitamin D hormone for noncalcemic needs under normocalcemic conditions (33). The molecular mechanisms have not been entirely determined, except for the vitamin D hormone induction of 24-hydroxylase (CYP24).

An important aspect of the vitamin D endocrine system is that dietary calcium is favored to support serum calcium concentrations under normal conditions but, when this fails, the system mediates calcium mobilization from bone and reabsorption in the kidney to satisfy the needs of the organism. This results in loss of calcium from the skeleton and can ultimately lead to osteoporosis. Another important aspect is that, except for stimulating mineralization of the skeleton, the vitamin D hormone has not been found to be anabolic on bone by itself.

**MOLECULAR MECHANISMS OF VITAMIN D ACTIONS**

The vitamin D hormone functions through a single vitamin D receptor (VDR), which has been cloned for several species including humans, rats, and chickens. It is a member of the class II steroid hormones, being closely related to the retinoic acid receptor and the thyroid hormone receptor (reviewed in ref 20). It, like other receptors, has a DNA-binding domain called the C-domain, a ligand-binding domain called the E-domain, and an F-domain, which is one of the activating domains. Despite many statements to the contrary in the literature, a single receptor appears to mediate all of the functions of vitamin D, which complicates the preparation of analogs for one specific function rather than another. The human receptor is a 427-amino acid peptide, whereas the rat receptor contains 423 amino acids and the chicken receptor contains 451 amino acids. This receptor acts through vitamin D-responsive elements (VDREs), which are usually found within 1 kilobase of the start site of the target gene. The VDREs, which are shown in Figure 5, are repeat sequences...
of 6 nucleotides separated by 3 nonspecified bases. It is now clear that the 5' arm of this sequence binds the retinoic acid X receptor and the 3' arm binds the VDR. Of all of the genes identified to date, the most powerfully regulated is the CYP24 or 24-hydroxylase enzyme, which is responsible for the degradation of vitamin D (20). The programming of its own destruction is thus an important aspect of this endocrine system, which uses one of the most potent ligands known.

A diagram that describes how the VDR with its ligand affects the transcription of target genes is presented in Figure 6. Although there is little evidence for a co-repressor, we think that co-repressors will eventually be found for the VDR. When the VDR interacts with the ligand, the repressor is no longer able to bind to the receptor, and the receptor changes conformation. Together with the required RXR, the VDR forms a heterodimer at the VDREs (20). At the same time, it binds several other proteins required in the transcription complex and, most importantly, acquires an activator (20). To date, at least 3 coactivators have been identified, ie, SARC1, -2, and -3 (34) and DRIP205 (35). There may be additional coactivators, and there may be selectivity among the coactivators with respect to which gene is being expressed. Much attention is being focused on this aspect for selective regulation of target genes. Once the complex is formed, the DNA bends (36), phosphorylation on serine-205 occurs (37), and transcription is either initiated or suppressed, depending on the gene. To date, it is unclear whether the phosphorylation plays a functional role in transcription.

FUNCTIONS OF VITAMIN D UNRELATED TO CALCIUM

One of the most important findings after discovery of the receptor was that the receptor appeared not only in the target cells of enterocytes, osteoblasts, and distal renal tubule cells but also in parathyroid gland cells, skin keratinocytes, promyelocytes, lymphocytes, colon cells, pituitary gland cells, and ovarian cells (20). The expression of VDRs in these cells and not in skeletal muscle, heart muscle, and liver suggests that they must serve a function there (20). Although VDRs have been reported in liver, heart, and skeletal muscle (38–42), we and other groups failed to confirm those reports, with the use of specific monoclonal antibodies and other methods (43, 44). This led to the investigation and discovery of functions of vitamin D not previously appreciated, which takes the vitamin D system beyond bone.

An important discovery was made by Suda et al (25), who demonstrated that the vitamin D hormone plays an important role in the terminal differentiation of promyelocytes to monocytes, which are precursors of the giant osteoclasts. Those authors also found that, when the cells differentiated into a functional cell line, growth ceased. This function did not involve calcium and phosphorus and was later shown to be fundamental to vitamin D-induced production of osteoclasts through the RANKL system (for review, see reference 25).

Of great importance is the finding of the VDR in the parathyroid glands. We now know, through the treatment of renal osteodystrophy with the vitamin D hormone and its analogs, that an essential site for this therapy is the VDR in the parathyroid glands (20). An important function of the vitamin D hormone is to keep the production of the preproparathyroid gene under control and reasonably suppressed (20, 45). Furthermore, the vitamin D hormone, through its receptor, in some way functions to prevent proliferation of parathyroid gland cells. Therefore, an important function of the vitamin D hormone among normal subjects is to maintain normal parathyroid status. Among patients with renal failure, the site of production of the vitamin D hormone is destroyed and the parathyroid gland becomes vitamin D deficient; in the presence of adequate amounts of calcium in the circulation,
the parathyroid gland hyperproliferates and hypersecretes parathyroid hormone, resulting in secondary hyperparathyroidism (46). An important available treatment is the use of the vitamin D hormone and its analogs to manage the parathyroid glands of patients undergoing dialysis.

Another important area of investigation has been the immune system. Clearly, vitamin D deficiency affects the immune system, especially T cell-mediated immunity, whereas vitamin D in excess actually suppresses certain aspects of the immune system (47, 48). This has led to investigation of the use of vitamin D compounds to suppress certain autoimmune disorders. The first autoimmune disorder to come under scrutiny is multiple sclerosis, and experimental autoimmune encephalomyelitis has been used as an animal model. This disease can be suppressed or eliminated at any stage of development with adequate amounts of the vitamin D hormone administered orally each day (49). However, hypercalcemia occurs with this therapy. We now know that the increase in serum calcium concentrations plays some role in this therapeutic response. A clearer example of an autoimmune disease that is regulated by the vitamin D hormone is type 1 diabetes mellitus (50). Among nonobese diabetic rats, vitamin D deficiency caused a marked increase in incidence and a marked decrease in the lag time required for the initiation of diabetes (Figure 7). Very important is the fact that large doses of the vitamin D hormone could suppress type 1 diabetes mellitus completely (50), preventing the destruction of islet cells. Similar results were obtained with models of systemic lupus (51), inflammatory bowel disease (52), and rheumatoid arthritis (53). It is likely that the suppression of these autoimmune diseases involves the vitamin D hormone interacting with T helper lymphocytes, which in turn suppress the inflammatory responses of T helper type 1 lymphocytes. Alternative ideas have also been put forth, such as suppression of the dendritic cells that present antigens to the T cells (54). Although the mechanisms of this regulation of autoimmune diseases are not understood, the results are sufficient to warrant investigation of vitamin D analogs for the treatment of such diseases. As an extension of this function of vitamin D, the utility of 1,25(OH)2D3 in helping prevent transplant rejection has been demonstrated with both vascular and nonvascular transplants (55).

**ANALOGS OF 1,25(OH)2D3**

We now come to the area of the design of 1,25(OH)2D3 analogs with selective activities for use against specific diseases. The major problem is that the primary role of 1,25(OH)2D3 is to adjust serum calcium and phosphorus concentrations. This is its dominant role, and the design of any analog to treat a disease other than osteoporosis or osteomalacia must include the elimination or marked suppression of the plasma calcium-increasing activity. Most analog development in this field has been performed with that in mind. Years of experience with modifications of 1,25(OH)2D3 and assessments of the consequent physiologic effects have yielded some information that is very useful for designing analogs for particular uses. A recent development has been the understanding that the carbon-2 position of vitamin D not only is tolerated but actually produces a much more stable transcription complex, compared with vitamin D analogs without carbon-2 modifications (56, 57). Our group has developed analogs that are selective for actions on osteoblasts, particularly the anabolic or bone-forming actions of that cell type. The most promising of the compounds studied is 2-methylene-19-nor-20S-1α,25-dihydroxyvitamin D3 (2MD) (Figure 8). This compound is very selective for its action on bone, being 80–100 times more effective than the native hormone in stimulating bone calcium mobilization while being equally effective in the intestine. Demonstrating that osteoblastic activity is favored by this analog, incubation of 2MD with human osteoblasts caused formation of bone nodules within 2 wk (Figure 9) (58). However, incubation of the same cells with even high concentrations of 1,25(OH)2D3 produced little or no change. These results suggest strong bone-forming activity of 2MD. To test whether this analog could cause bone synthesis in vivo, 2MD was given to aged female rats that had been ovarioctomized to ensure a loss of bone mass (osteoporosis). 2MD caused a marked increase in the synthesis of new bone, yielding a high bone mass value; samples tested for breaking strength proved to be extremely strong. In the
same model, 1,25(OH)\textsubscript{2}D\textsubscript{3} administered at much higher doses was unable to induce the same levels of bone synthesis and bone mass. 2MD is now in phase 2 of development for osteoporosis and appears extremely promising as an anabolic agent for bone growth.

Two other analogs modified at carbon-2 are shown in Figure 10 (59). These compounds bind very well to the receptor and are active in transcription but, even when given orally to animals at doses as high as 70 μg/kg, are unable to increase serum calcium concentrations. However, much lower doses of the same compounds are able to suppress plasma parathyroid hormone concentrations, which shows that they are systemically active. Therefore, there is great promise that new analogs with very selective activities can be prepared. One group of analogs is very selective in stimulating bone synthesis. Another pair of analogs are devoid of calcium activity while retaining systemic activity for the parathyroid glands. The latter group might be used to treat autoimmune diseases and cancer and to suppress secondary hyperparathyroidism among patients undergoing dialysis.

**ASSESSMENT OF VITAMIN D STATUS**

One of the most important facets of vitamin D is its many physiologic activities. The importance of vitamin D being continually available is obvious. Vitamin D deficiencies compromise not only bone mineralization but also many other biological activities. It is well known that vitamin D deficiency rickets is appearing even in highly developed countries. In Europe, vitamin D fortification of foods is largely absent, and little vitamin D is made in the skin of individuals in the northern and southern regions of the planet during the winter months. To protect against bone diseases and other kinds of degenerative diseases and autoimmune diseases, adequate concentrations of vitamin D are extremely important. In the view of many scientists in the vitamin D field, the recommended dietary allowance is too low. Supplementation with vitamin D\textsubscript{3} at 2000 IU/d should be considered and should be perfectly safe. To determine safety, an assessment of vitamin D status is required. Generally, 25(OH)D\textsubscript{3} concentrations are considered the best measure of vitamin D status. Unfortunately, commercially available assays for 25(OH)D\textsubscript{3} yielded widely differing results (Figure 11) (60). To determine which of these values truly represents the 25(OH)D\textsubscript{3} concentration, we measured 25(OH)D\textsubscript{3} concentrations in the same serum samples with a chemical method and we found that only 2 values agreed with the value determined chemically. Therefore, there is

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**FIGURE 9.** Incubation of human osteoblasts with 2MD, resulting in the appearance of marked mineralized bone nodules. Osteoblasts and osteoblast precursors harvested from pediatric bone samples discarded during surgery were incubated at 10\textsuperscript{5} cells per plate with 10\textsuperscript{-8} mol/L 1,25(OH)\textsubscript{2}D\textsubscript{3}, 10\textsuperscript{-10} or 10\textsuperscript{-12} mol/L 2MD, or no vitamin D for a period of 7 d. Fresh medium was presented to the cells every 3 d. After 1 wk of incubation, the medium was replaced with ascorbic acid/β-glycerol phosphate-containing medium (without vitamin D compounds). After a 2-wk period, the cells were stained with silver nitrate to reveal bone nodules (arrows).

**FIGURE 10.** Structures of noncalcemic, carbon-2–modified analogs of 1,25(OH)\textsubscript{2}D\textsubscript{3}.

**FIGURE 11.** 25(OH)D\textsubscript{3} concentrations in 20 serum samples, as measured with assay kits from 5 different commercial laboratories and with HPLC in our own laboratory (Lab 6). The results indicate very clearly that the laboratory values differ according to the laboratory performing the tests, some of which are antibody-based tests. Only 2 methods (Lab 1 and Lab 3) yielded values approaching the values obtained with the accurate chemical or HPLC method. These results demonstrate a need for assay certification before public health parameters can be set on the basis of 25(OH)D\textsubscript{3} concentrations in plasma.
a great need to develop a standard and highly reliable 25(OH)D3 assay before supplementation levels can be set on the basis of serum 25(OH)D3 concentrations. It is well known that, when large amounts of vitamin D are given to a patient, much of the vitamin D is stored in adipose tissues. Once these sites have been saturated, the vitamin D remains in serum and is converted to 25(OH)D3, which is toxic as an analog of 1,25(OH)2D3 (61). When the dietary levels of vitamin D3 needed to achieve normal concentrations of 25(OH)D3 in the plasma are being determined, vitamin D3 itself should be measured, to confirm that vitamin D3 is not being accumulated to an extent that would result in vitamin D intoxication. These measurements with well-established, precise methods, together with a careful, clinical, dose-escalation study, should allow setting of a supplementation level that is safe and can help prevent degenerative diseases, as well as preventing or reducing the risk of autoimmune diseases.

CONCLUSIONS

Vitamin D has yielded a class of compounds that can be used for the treatment of a variety of diseases. Vitamin D3 itself is converted, in a 2-step process, to 1,25(OH)2D3. This hormone reacts with a single nuclear type 2 receptor to facilitate the activation or suppression of target genes. The proteins produced in response to the hormone then carry out classic and nonclassic functions of vitamin D. In addition to causing mineralization of the skeleton and increasing serum calcium and phosphorus concentrations, vitamin D is known to regulate parathyroid gland growth and parathyroid hormone production; it plays a role in the islet cells of the pancreas, has a significant effect on the immune system, and can help in suppression of certain autoimmune diseases and certain cancers. To obtain maximal benefits of dietary vitamin D and to reduce the risks of these diseases, intakes of vitamin D higher than currently recommended are in order. Furthermore, a standardized 25(OH)D3 assay that provides true values must be developed; findings could provide a basis for understanding what levels of supplementation must be used to yield adequate amounts of 25(OH)D3.

HFD is president and chief executive officer of Deltanoid Pharmaceuticals (Madison, WI) and serves as a consultant for Pfizer. Deltanoid is developing some of the compounds described.

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