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1-Fluorovitamin D_3, a Vitamin D_3 Analogue More Active on Bone-Calcium Mobilization Than on Intestinal-Calcium Transport†

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ABSTRACT: The chemical synthesis, spectral characterization, and biological activity of 1-fluorovitamin D_3 are described. The title compound was synthesized through direct fluorination of 1α-hydroxyvitamin D_3 3β-acetate and characterized by ultraviolet, nuclear magnetic resonance, and mass spectroscopy. Similar responses of bone-calcium mobilization in vitamin D deficient rats are given by 200–300 ng of 1-fluorovitamin D_3 and 25 ng of vitamin D_3. Likewise, vitamin D_3 and 1-fluorovitamin D_3 provide the same degree of epiphyseal plate calcification in rachitic rats at daily doses of 20 and 270 ng, respectively. Thus, there appeared to be an approximate 10- to 15-fold difference in their ability to cause calcium and phosphate mobilization. However, about 1260 ng of 1-fluorovitamin D_3 is necessary to produce the same degree of intestinal-calcium transport as 12.5 ng of vitamin D_3—a difference of about 100-fold. Therefore, relative to vitamin D_3, 1-fluorovitamin D_3 demonstrates at least partial selectivity for mediating calcium metabolism in bone as opposed to mediating calcium metabolism in intestine. 1-Fluorovitamin D_3 causes maximum stimulation of bone-calcium mobilization and intestinal-calcium transport 24 h after a single, intravenous dose, like vitamin D_3 but in contrast to 1α-hydroxyvitamin D_3 which shows maximum responses at about 12 h. Nephrectomized animals do not respond to the fluoro analogue at doses higher than those which would cause significant responses in intact animals. Thus, 1-fluorovitamin D_3 probably undergoes metabolism in the kidney prior to manifesting physiological activity.

The major circulating metabolite of vitamin D_3, 25-hydroxyvitamin D_3 (25-OH-D_3),[1] is further metabolized in kidney to 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), which is a hormone that governs calcium and phosphate metabolism (DeLuca, 1974; Kodicek, 1974; DeLuca & Schnoes, 1976). To date, there is no known 1-deoxyvitamin D metabolite or analogue which functions at physiological doses in the absence of kidney tissue. The renal 25-OH-D_3-1α-hydroxylase is, additionally, a highly selective enzyme. The activity of the 1α-hydroxylase is regulated and fluctuates in response to blood calcium (Boyle et al., 1971) and phosphorus (Tanaka & DeLuca, 1973) concentrations, parathyroid hormone levels (Garabedian et al., 1972), and vitamin D status of the animal (Tanaka & DeLuca, 1974; Tanaka et al., 1975). Consequently, the 25-OH-D_3-1α-hydroxylase represents a crucial control point in expression of 1,25-$(OH)_2D_3$-like activity by vitamin D metabolites and analogues.

An analogue of vitamin D_3 blocked at carbon 1 should be an important research tool and could be clinically useful. Through competition for the renal 25-OH-D_3-1α-hydroxylase, an analogue incapable of 1α-hydroxylation could be an antimetabolite of 25-OH-D_3. The research utility of such an agent rests on the realization that besides the 25-OH-D_3-1α-hydroxylase at least two other enzymes which have vitamin D metabolites as substrates exist in kidney tissue (Knutson & DeLuca, 1974; Ghazarian & DeLuca, 1977; Tanaka et al., 1978). Experimental protocols which excise kidney to demonstrate essentiality of 1α-hydroxylation also result in removal of the renal 24- and 26-hydroxylases, and possibly other vitamin D_3 metabolizing enzymes. Selective inhibition of the 1α-hydroxylase would permit other renal hydroxylases to remain unperturbed and present a broader research model in which to study possible function of the total vitamin D metabolism occurring in kidney.

Fluorine-substituted vitamin D_3 metabolites have been successfully used to elucidate metabolic events obligatory to vitamin D activity (Napoli et al., 1978a,b; J. L. Napoli, H. K. Schnoes, & H. F. DeLuca, unpublished results). This paper reports the chemical synthesis and biological activity of 1-fluorovitamin D_3. 1-Fluorovitamin D_3 was designed as a tool to probe the existence and effects of alternate metabolic modes when 1α-hydroxylation is hindered and also to test the feasibility of preventing vitamin D action in vitro by antagonizing

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[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; LC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; 1α-OH-D_3, 1α-hydroxyvitamin D_3; DAST, diethylaminosulfur trifluoride.

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25-OH-D_3 at the 1α-hydroxylase level.

Materials and Methods

General

High-resolution mass spectra were taken at 100–110 °C above ambient at 70 eV with an AEI Model MS-9 mass spectrometer coupled to a DS-50 data system. Ultraviolet spectra were obtained in ethanol with a Beckman Model 24 recording spectrophotometer. High-pressure liquid chromatography (LC) was carried out on a Waters Associates Model ALC/GPC 204 liquid chromatograph with a silica gel column (5-μm particles, 0.7 × 25 cm). Radioactivity was determined with a Packard Tri-Carb Model 3255 liquid scintillation counter. Scintillation fluid contained 2 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per L of toluene. Nuclear magnetic resonance (NMR) spectra were taken with a Bruker WH-270 spectrometer. Crystalline vitamin D_3 was purchased from the Philips-Duphar Co., Weesp, The Netherlands. Crystalline 1α-hydroxyvitamin D_3 (1α-OH-D_3) was a gift from the Hoffmann-La Roche Co., Nutley, NJ. Diethylaminosulfur trifluoride (DAST) was prepared by a published procedure (Middleton, 1975).

Synthesis

1α-Hydroxyvitamin D_3 3-Acetate. 1α-OH-D_3 (25 mg, 0.063 mmol) and acetic anhydride (50 μL) in 2 mL of pyridine/benzene (1:1) were heated at 50 °C for 4 h under argon. The reaction mixture was cooled, water and ether were added, and the phases were separated. The ether layer was washed consecutively with water, 1 N HCl (twice), dilute $NaHCO_3$, and saturated NaCl and dried (Na_2SO_4). The residue obtained upon evaporation of the ether was chromatographed on a silica gel plate (20 × 20 cm, 0.75 cm thick) developed with ethyl acetate/Skellysolve B (1:1). Four bands of R_fs 0.20, 0.38, 0.49, and 0.64 were obtained. The band with R_f 0.49 was 1α-OH-D_3 3-acetate (6.0 mg).

The bands with R_fs 0.64, 0.38, and 0.20 consist of 1α-OH-D_3 1,3-diacetate, 1α-OH-D_3 1-acetate, and starting material, respectively. The 1-acetate and 1,3-diacetate were combined and treated with 1 mL of 0.1 M KOH/MeOH in 1 mL of ether for 1.25 h at room temperature to produce 1α-OH-D_3. The process was repeated twice more with all of the recovered 1α-OH-D_3 to give a total of 12 mg (43%) of 1α-OH-D_3 3-acetate: UV λ_{max} 265, λ_{min} 228 nm; NMR ($CDCl_3$) δ 0.54 (s, 18-CH_3), 0.87 (d, J = 6.6 Hz, 26,27-$(CH_3)_2$), 0.92 (d, J = 6.1 Hz, 21-CH_3), 2.03 (s, acetate CH_3), 4.41 (m, 1β-H), 5.02, 5.34 (19-H's), 5.34 (3α-H), 6.02, 6.34 (ABq, J = 11.4 Hz, 6- and 7-H's).

1-Fluorovitamin D_3. To 2 mg of 1α-OH-D_3 3-acetate in CH_2Cl_2 (0.4 mL) at −78 °C was added DAST (12 μL) with good stirring. The cooling bath was removed and 5 min later the reaction was quenched with 5% K_2CO_3. Ether was added and the phases were separated. The organic phase was washed with water, and saturated NaCl, and concentrated to 0.5 mL, to which 0.1 M KOH/MeOH (1 mL) was added. After 1.5 h at room temperature the solvent was removed, ether and water were added, and the phases were separated. The ether phase was washed with water and brine and filtered through Na_2SO_4. The residue obtained after evaporation of the ether was purified by LC. A system of 0.5% isopropyl alcohol/hexane eluted the fluoro analogue (0.85 mg) in 78 mL: UV λ_{max} 265, λ_{min} 226 nm; NMR ($CDCl_3$) δ 0.54 (s, 18-CH_3), 0.87 (d, J = 6.8 Hz, 26,27-$(CH_3)_2$), 0.92 (d, J = 5.7 Hz, 21-CH_3), 4.00 (m, 3α-H), 5.03 (d of m, J = 50 Hz, 1-H), 5.12, 5.41 (multiplets, 19-H's), 6.03, 6.45 (ABq, J = 11.1 Hz, 6 and 7 H's); mass spectrum m/e (relative intensity) 402.3320 (M^+, 0.10, calcd for $C_{27}H_{43}FO$, 402.3298), 382 (M^+ − HF, 0.40), 364 (M^+ − HF − H_2O, 0.28), 349 (M^+ − CH_3 − HF − H_2O, 0.04), 289 (M^+ − side chain, 0.05), 269 (M^+ − side chain − HF), 251 (M^+ − side chain − HF − H_2O, 0.10), 154.0790 (ring A + C-6 and 7, 0.21, calcd for $C_9H_{11}FO$, 154.0794), 135.1171 (0.88, calcd for $C_{10}H_{15}$, 135.1174), 135.0786 (154 − F, 012, calcd for $C_9H_{11}O$, 135.0810).

An aliquot of 1-fluorovitamin D_3 was reinjected onto LC to check purity. The peak was homogeneous and remained so after recycling eight times through the column.

Biological Evaluation

Animals. Weanling male rats (Holtzman Co., Madison, WI) were housed individually in overhanging wire cages. They were fed vitamin D deficient diets containing normal calcium (0.47%) and normal phosphorus (0.3%), or low calcium (0.02%) and normal phosphorus, or high calcium (1.2%) and low phosphorus (0.1%) for several weeks prior to experimental use (Suda et al., 1970).

Intestinal-Calcium Transport. Animals were decapitated and their duodena were used to measure intestinal-calcium transport by the everted intestinal sac procedure (Martin & DeLuca, 1969). Aliquots (50 μL) of the mucosal and serosal fluid were spotted on filter paper disks, dried, and counted for ^{45}Ca in 10 mL of counting solution. The data are expressed as ^{45}Ca concentration in serosal fluid/^{45}Ca concentration in mucosal fluid.

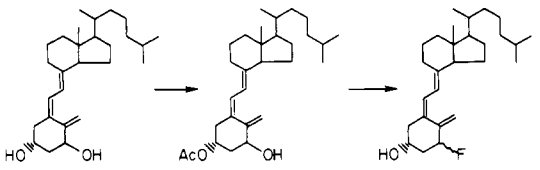
Bone-Calcium Mobilization. Blood was collected from decapitated animals and centrifuged. Serum aliquots (0.1 mL) were added to tubes containing 0.1% $LaCl_3$. Calcium concentrations of the solutions were measured with a Perkin-Elmer Model 403 atomic absorption spectrometer and corrected to reflect mg of Ca/100 mL of serum. Since the rats used in mobilization experiments were on low-calcium diets, serum calcium increases reflect mobilization of calcium from bone and not intestinal absorption.

Antirachitic Potency. Rats were rachitic after they were fed for 2 weeks with the high-calcium, low-phosphorus (rachitogenic) diet (Tanaka & DeLuca, 1971). After a further week on the diet, they were dosed daily with test compounds for 7 days and decapitated 14 h after the last dose. Radii and ulnae were collected, cleaned of adhering tissue, and soaked in distilled water for several hours. The bones were stained with 1.5% $AgNO_3$ and a calcification score was assigned for degree of endochondral calcification by the "line-test" method (*U.S. Pharmacopoeia*, 1955). This is the standardized assay by which calcification potency of vitamin D_3 like compounds is established. The activity of vitamin D_3 itself, in this assay is 40 international units (IU) per microgram as calculated from the formula

$$[(1/\text{daily dose } (\mu g)) \times 5] \times \text{line test score}$$

Results

Synthesis. To achieve fluorination of 1α-OH-D_3 exclusively at carbon 1, the 3-hydroxyl was protected by an acetate group (Figure 1). Acetylation of 1α-OH-D_3 produced the 1-acetate and the 1,3-diacetate, besides the desired 3-acetate. The mixture of unreacted 1α-OH-D_3 and the three different esters was easily resolved by preparative-layer chromatography. After hydrolysis, the 1-acetate and the 1,3-diacetate were combined with 1α-OH-D_3 obtained from chromatography and the total recovered 1α-OH-D_3 was recycled. The structure of the ester used for fluorination was established as 3-AcO-1α-OH-D_3 by UV and NMR spectroscopy. A λ_{max} of 265 nm



1-OH-D_3 1-OH-D_3 3-Acetate 1-Fluoro-D_3

FIGURE 1: Chemical synthesis of 1-fluorovitamin D_3.

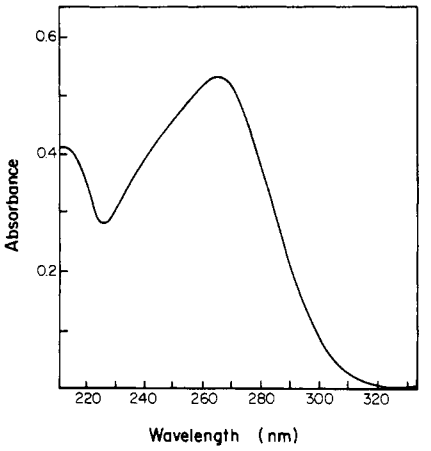


FIGURE 2: Ultraviolet absorption spectrum of 1-fluorovitamin D_3 in ethanol. An absorbance maximum at 265 nm and an absorbance minimum at 226 nm are characteristic of the triene system in vitamin D like compounds. In these compounds, the ratio $\lambda_{265}/\lambda_{226}$ further characterizes the triene system and is also a measure of purity. Values of 1.5 or better are usually sought. The ratio for 1-fluorovitamin D_3 was 1.9.

in the UV spectrum indicated the presence of a vitamin D triene chromophore. This was confirmed by appropriate proton resonances in the NMR spectrum, which further unequivocally demonstrated that the acetate was in the 3 position (acetate methyl δ 2.03) and the 1α-hydroxyl was not acetylated (1β proton δ 4.41).

Fluorination and hydrolysis of 1α-OH-D_3 3-acetate provided 1-fluorovitamin D_3. An aliquot of the analogue remained homogeneous on LC even after cycling eight times through the column. The UV spectrum of the fluoro analogue (Figure 2) is consistent with the presence of the vitamin D triene system and excludes the possibility of olefin rearrangement. The high-resolution mass spectrum (Figure 3) provides a molecular formula of $C_{27}H_{43}FO$ which corresponds to a fluoro-substituted vitamin D compound. Loss of hydrogen fluoride (*m/e* 382) and both hydrogen fluoride and water (*m/e* 364) from the molecular ion (*m/e* 402) indicates the presence of a hydroxyl and a fluoro group in the molecule. A peak at *m/e* 154, resulting from cleavage between carbons 7 and 8, is typical of vitamin D compounds and demonstrates that the fluoro substituent is in the A ring. The base peak at *m/e* 135 arises from two fragmentation processes. The minor component (relative intensity 0.12) arises from loss of fluorine from 154. The larger component (relative intensity 0.88) is a hydrocarbon fragment ($C_{10}H_{15}$) usually found in vitamin D like compounds, albeit at a much lower relative intensity. The NMR spectrum exhibited appropriate proton resonances indicative of a vitamin D–triene system and, therefore, corroborates the UV and mass spectral data. The NMR spectrum further shows that the fluoro substituent is at carbon 1 since the 3-proton at δ 4.00 appears essentially as it does in 1α-OH-D_3, whereas the large coupling constant (J = 50 Hz) of the two doublet of doublets arising from the 1-proton is indicative of geminal proton–fluorine spin–spin interaction.

Table I: Intestinal-Calcium Transport in Vitamin D Deficient Rats Administered Graded Doses of 1-Fluorovitamin D_3[a]

group	compd	dose (ng)	^{45}Ca serosal/ ^{45}Ca mucosal
1	ethanol		1.5 ± 0.1
2	vitamin D_3	25	3.1 ± 0.3[b]
3	1-fluorovitamin D_3	280	2.2 ± 0.1[b]
4		700	2.7 ± 0.2[b]
5		1260	3.0 ± 0.4[b]
6		2450	2.8 ± 0.2[b]
7		6400	3.9 ± 0.3[b]
8		12800	4.1 ± 0.4[b]
9		25600	3.5 ± 0.3[b]

[a] After 2–3 weeks on the vitamin D deficient, low-calcium diet, rats were divided into groups of 5 or 6 and each rat received a single intrajugular dose of compound in 50 μL of ethanol. Control rats (group 1) received only ethanol. Twenty-four hours later, the animals were decapitated and intestinal calcium transport was measured. Data are expressed as mean ± SEM. [b] Differ significantly from control; $p < 0.005$.

Table II: Effect of Daily 1-Fluorovitamin D_3 Doses on Intestinal-Calcium Transport in Vitamin D Deficient Rats on a Low-Calcium Diet[a]

group	compd	daily dose (ng)	^{45}Ca serosal/ ^{45}Ca mucosal
1	1,2-propanediol		2.2 ± 0.2
2	vitamin D_3	25	6.1 ± 0.5[b]
3	1-fluorovitamin D_3	30	2.7 ± 0.5
4		130	3.4 ± 1.2
5		250	3.8 ± 1.6[b]
6		620	4.1 ± 1.9[b]

[a] Rats were maintained on the vitamin D deficient diet for 2–3 weeks and then dosed intraperitoneally on each of 7 days with either compound in 50 μL of 1,2-propanediol. Controls (group 1) received only 1,2-propanediol. Twenty-four hours after the last dose, animals were killed and intestinal calcium transport was measured. Data are mean ± SEM for 5–6 rats per group. [b] Significantly different from control; $p < 0.005$.

Biological Evaluation. Graded doses of 1-fluorovitamin D_3 were assayed for ability to stimulate bone-calcium mobilization (increase serum calcium) in vitamin D deficient rats fed a low-calcium diet. Serum calcium concentration, measured 24 h after a single intrajugular dose of 1-fluorovitamin D_3, increased linearly as a function of log dose (Figure 4A). The slope was calculated from a linear regression analysis of the data as 0.76, and the correlation coefficient, *r*, was 0.96. In the same experiment, 25 ng of vitamin D_3 produced an increase of 0.6 ± 0.1 mg/100 mL (mean ± SEM of 6 animals) in serum calcium concentration. A second experiment was conducted, in which the rats were dosed (ip) each day for 7 days with 1-fluorovitamin D_3 and sacrificed 24 h after the last dose (Figure 4B). The increase in serum calcium was again a linear function of log dose. The correlation coefficient, *r*, was greater than 0.99 after linear regression analysis of the data and the slope was 0.84. In this case, 25 ng of vitamin D_3 doses daily produced an increase of 0.9 ± 0.2 mg/100 mL (mean ± SEM of 6 animals) in serum calcium. The greater responses for both compounds in the second experiment was expected in view of multiple dosing. These experiments indicate quite clearly that at least 200–300 ng of 1-fluorovitamin D_3 is necessary to produce a bone-calcium mobilization response equivalent to that produced by 25 ng of vitamin D_3.

The intestinal calcium transport responses of rats which received single doses of test compound are recorded in Table I. According to these data, about 1260 ng of 1-fluorovitamin D_3 induced transport equivalent to the 25 ng of vitamin D_3 in this assay. The intestinal calcium transport results from

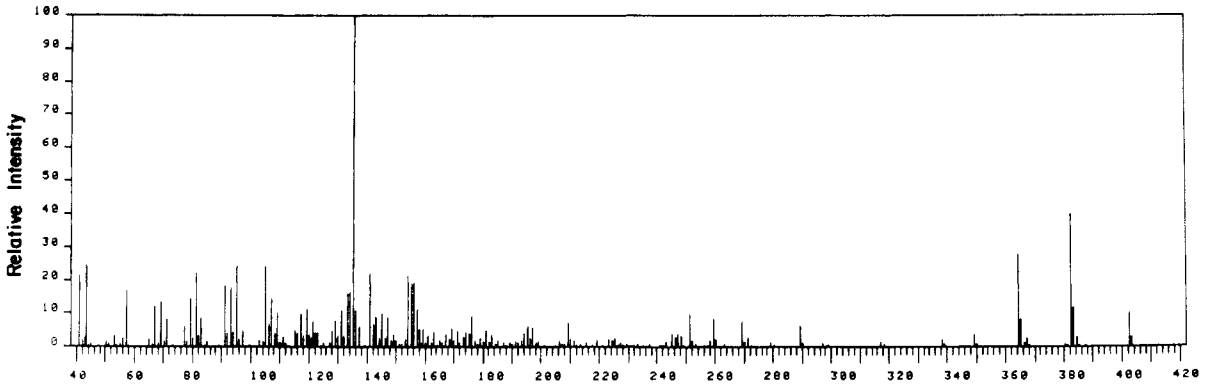


FIGURE 3: Mass spectrum of 1-fluorovitamin D_3.

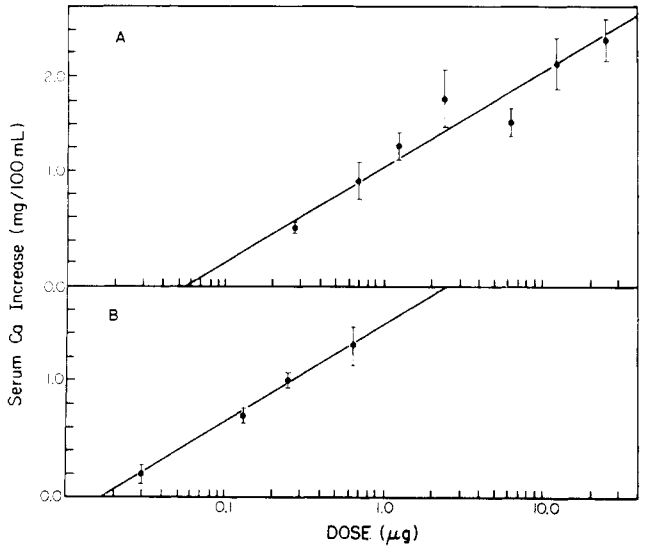


FIGURE 4: (A) Bone-calcium mobilization in vitamin D deficient rats on the low-calcium diet administered graded doses of 1-fluorovitamin D_3. The increase in serum calcium 24 h after a single intrajugular dose of the fluoro analogue in 50 μL of ethanol is plotted as a function of log dose. In the same experiment, administration of 25 ng of vitamin D_3 increased the serum calcium concentration 0.6 ± 0.1 mg/100 mL. Data are mean ± SEM from 5–6 rats per point. (B) Relationship between serum calcium concentration (mg/100 mL) increase and log daily dose (ng) in vitamin D deficient rats on a low-calcium diet administered 1-fluorovitamin D_3 intraperitoneally in 1,2-propanediol (50 μL) daily for 7 days. Dosage with 25 ng of vitamin D_3 daily produced an increase of 0.9 ± 0.2 mg/100 mL. Data are the mean ± SEM from 6 rats/point.

the animals on a daily dose regimen (Table II) corroborate the results obtained from the single dose experiment. The 620 ng/day dose of 1-fluorovitamin D_3 did not produce the intestinal-calcium transport response that was produced by 25 ng of vitamin D_3. In fact, the percent increase in the ^{45}Ca serosal/^{45}Ca mucosal value from the animals dosed with 620 ng/day 1-fluorovitamin D_3 was only half that of the vitamin D_3 dosed animals. It is well known, however, that 12.5 ng of vitamin D_3, but not less, always evokes a response equivalent to that induced by 25 ng (Holick et al., 1975; Napoli et al., unpublished results). Therefore, these two experiments demonstrate that about 100 times more 1-fluorovitamin D_3 than vitamin D_3 is necessary to produce the same intestinal calcium transport response.

The final vitamin D mediated function measured was ability to promote epiphyseal plate calcification in rachitic rats (Table III). Based on international units of vitamin D_3 activity per microgram of compound, calculated from the line-test data, about 15 times more 1-fluorovitamin D_3 than vitamin D_3 is needed to cause the same degree of cartilage calcification.

Table III: Antirachitic Potency (Line-Test) of 1-Fluorovitamin D_3 Compared with Vitamin D_3[a]

group	compd	daily dose (ng)	calcification score	IU/μg[b]
1	1,2-propanediol		0 ± 0	0 ± 0
2	vitamin D_3	20	3.6 ± 0.7	36.0 ± 7.0
3	1-fluorovitamin D_3	270	3.2 ± 0.4	2.4 ± 0.3

[a] After 3 weeks on the rachitogenic diet, rats were dosed intraperitoneally daily for 7 days with either of the test compounds in 1,2-propanediol (50 μL) or with 1,2-propanediol alone. Fourteen hours after the last dose, the animals were killed and the calcification score was determined by the line-test method as described previously (*U.S. Pharmacopoeia*, 1955). Values are mean ± SEM from 4–5 rats. [b] International units per microgram of compound.

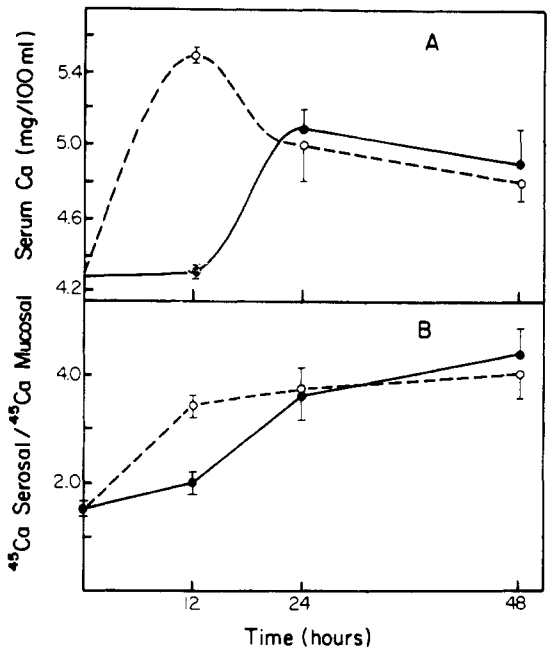


FIGURE 5: Time courses of bone-calcium mobilization (A) and intestinal-calcium transport (B) induced by either 1 μg of 1-fluorovitamin D_3 (●—●) or 25 ng of 1α-OH-D_3 (○---○). Responses were measured as described in the Materials and Methods section after a single, intrajugular dose of either compound in 50 μL of ethanol. Animals were fed the low-calcium diet for 3 weeks prior to dosing. Each point is the mean ± SEM of values from 6 rats.

Time courses of bone-calcium mobilization and intestinal-calcium transport in response to 1-fluorovitamin D_3 or 1α-OH-D_3 were compared (Figure 5). Serum calcium levels maximized about 12 h after dosage with 1α-OH-D_3 and declined somewhat rapidly from 12 to 24 h, after which the rate of decay decreased significantly. The rise in serum

Table IV: Bone-Calcium Mobilization and Intestinal-Calcium Transport in Nephrectomized Rats Administered Vitamin D_3 Analogues[a]

dose	serum calcium (mg/100 mL)	^{45}Ca serosal/ ^{45}Ca mucosal
ethanol	3.8 ± 0.2	1.2 ± 0.1
50 ng of 1α-OH-D_3	5.1 ± 0.4[b]	2.6 ± 0.2[c]
1.0 μg of 1-fluorovitamin D_3	3.7 ± 0.1	1.6 ± 0.1

[a] Animals were maintained on the normal-calcium diet for 2 weeks and then switched to the low-calcium diet. After a further week, the rats were bilaterally nephrectomized under ether anesthesia and then dosed intrajugularly with a test compound in ethanol (50 μL). Twenty-two hours later, animals were decapitated for serum calcium and intestinal-calcium transport measurements. Data are expressed as mean ± SEM of 8 rats per group. [b,c] Significantly different from control: $p < 0.01$ and $p < 0.001$, respectively.

calcium mediated by 1-fluorovitamin D_3, on the other hand, did not maximize until 24 h after dosage and did not abruptly decline, but rather remained elevated or perhaps slowly declined throughout the 48-h test period. Similarly, the intestinal-calcium transport response to 1α-OH-D_3 maximized at 12 h, but that of 1-fluorovitamin D_3 maximized only after 24 h.

1α-OH-D_3 was able to increase both serum calcium concentration and intestinal-calcium transport rate in bilaterally nephrectomized rats as expected (Holick et al., 1973; Kaneko et al., 1974). In contrast, nephrectomy eliminated the bone-calcium mobilization response to 1 μg of 1-fluorovitamin D_3 and vastly diminished the intestinal-calcium transport response (Table IV).

Discussion

The results of both bone-calcium mobilization experiments were in good agreement and established that about ten times more 1-fluorovitamin D_3 than vitamin D_3 is needed to stimulate the same degree of calcium flux from bone. Likewise, about 15 times more of the fluoroanalogue than vitamin D_3 is necessary to promote the same degree of healing of rachitic lesions. This is surprisingly good activity in view of the blocked 1 position in the new fluoro analogue. 1-Fluorovitamin D_3 is also active in stimulating intestinal-calcium transport but does not produce the same degree of stimulation as vitamin D_3 until about 100-fold more is dosed.

It has been well established that vitamin D_3 promotes intestinal-calcium transport at doses lower than those at which it stimulates bone-calcium mobilization (Holick et al., 1975; Napoli et al., unpublished results). For example, a 2.5 ng daily dose of vitamin D_3 causes a significant intestinal transport response but does not raise blood calcium concentrations. At least 12.5 ng of vitamin D_3, in a single dose or in a daily dose regimen, is needed to observe a rise in blood calcium. In other words, intestinal-calcium metabolism is more sensitive than bone-calcium metabolism to vitamin D_3. Relative to vitamin D_3, 1-fluorovitamin D_3 is more active on bone than intestine according to the data presented here, since 200–300 ng of 1-fluorovitamin D_3 approaches 25 ng of vitamin D_3 in bone-calcium mobilization response, but about 1260 ng of the fluoro compound is needed to duplicate the response that 12.5 ng of vitamin D_3 would produce in intestinal-calcium transport.

The fluorine atom in 1-fluorovitamin D_3 probably is not interacting with receptors as a hydroxyl group substitute but rather as a proton substitute since the time course of the fluoro analogue's activities more closely approximates those of vitamin D_3 and not those of 1α-OH-D_3. The relatively long delay in 1-fluorovitamin D_3 mediated responses suggests that it is metabolized prior to acting in vivo. This is confirmed by the experiment in nephrectomized rats, which also establishes kidney as the site of metabolism.

It is difficult to suggest the nature of the metabolism. Side-chain hydroxylation activates vitamin D molecules, but such compounds undergo 1α-hydroxylation before functioning at physiological doses (DeLuca & Schnoes, 1976). There is no known example of a 1-deoxy metabolite which works directly at the receptor level at low doses. This does not mean that such a circumstance could never exist. If the compound does become 1-hydroxylated, the end product is not likely to be an α-fluoro alcohol since such functionality would be unstable. Elimination of a fluorine atom, either before or after another metabolic event at carbon-1 must be considered. Nevertheless, the nature of the metabolic activation of 1-fluorovitamin D_3 is intriguing and the pursuit of it promises to broaden our knowledge of the metabolic events occurring during vitamin D_3 activation. In view of the greater activity of 1-fluorovitamin D_3 for bone-calcium mobilization than for intestinal-calcium transport, it seems reasonable to expect that the analogue is not metabolized to a known vitamin D metabolite. In order to establish the nature of the activation of 1-fluorovitamin D_3, the fate of the analogue in vivo is under investigation.

This study shows that masking the 1 position of vitamin D compounds will not necessarily produce agents potentially capable of blocking vitamin D action. It is evident that 1-fluorovitamin D_3 is far too active to be used practically as an antagonist of vitamin D_3 activity in vivo. Unexpectedly, fluorination of the 1 position resulted in an analogue which demonstrates a relative preference for stimulating bone-calcium mobilization with respect to intestinal-calcium transport. The bone receptors of vitamin D_3 metabolites are evidently structurally distinct from those in intestine. This demonstration highlights the possibility of obtaining analogues with vastly enhanced tissue specificity—compounds which would be of obvious therapeutic importance.

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Purification and Properties of the Hemagglutinin from *Wistaria floribunda* Seeds†

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ABSTRACT: Extracts of *Wistaria floribunda* seeds contain separable erythroagglutinating and lymphocyte mitogenic activities. We wish to report the purification and characterization of the erythroagglutinating lectin of these seeds. A phosphate-buffered saline (PBS) extract of the ground seeds was made to 50% ethanol and the precipitate, which contained both the agglutinin and mitogen, was dissolved in PBS. The erythroagglutinating activity was adsorbed onto insoluble polyleucyl derivatized A+H active hog gastric mucin. After desorption with 0.2 M D-galactose and removal of the sugar by dialysis, the eluate displayed three protein bands on polyacrylamide gel electrophoresis. The major component represented 85% of the mixture. Immunoelectrophoresis of the mixture demonstrated immunochemical identity among the proteins. Gel filtration through Sephadex G-200 resulted in purification of the major component. Based upon the composition and subunit molecular weight, it was concluded that the three components represent a dimer, tetramer, and octamer of a single glycopolypeptide chain of 28 000. The erythroagglutinin has a p*I* at pH 5.4 and one cystine per dimeric unit.

In order to develop sensitive carbohydrate specific reagents for the detection of saccharides of complex glycoconjugates, we have been purifying lectins which exhibit reactivity with D-galactosyl and *N*-acetyl-D-galactosaminyl residues. This report describes the purification, using affinity adsorption, and partial characterization of the hemagglutinin from *Wistaria floribunda*.

Extracts of *W. floribunda* seeds had been shown to possess both erythroagglutinating (Boyd et al., 1958) and lymphocyte mitogenic activities (Barker & Farnes, 1967). Though Boyd et al. (1958) were the first to report the hemagglutinating properties of *W. floribunda* seed extracts, Boyd & Reguera (1949) had noted, 9 years earlier, this activity in a related species, *W. chinensis*. Using aqueous extracts of *W. chinensis*, Mäkelä (1957) demonstrated that the hemagglutinating activity was inhibited best by sugars containing nonreducing terminal D-galactosyl residues. Toyoshima et al. (1971) have since shown that the protein of *W. floribunda* seeds responsible for mitogenic activity is distinct from that which causes hemagglutination. Recently, Kurokawa et al. (1976) have reported on the isolation, by classical techniques, and properties of a hemagglutinin from *W. floribunda* seeds.

Experimental Procedure

Purification of the W. floribunda Hemagglutinin. Ground *W. floribunda* seeds (100 g, F. W. Schumacher, Sandwich, MA) were extracted with 1 L of PBS for 18 h. The particulate material was removed by filtration through cheesecloth and centrifugation at 10000*g* at 4 °C for 45 min. To the resulting supernatant (fraction I) cold ethanol was slowly added at 0 °C to yield a final concentration of 50%, after which the suspension was centrifuged at 10000*g* at 4 °C for 45 min. The precipitate was resuspended in 150 mL of PBS and dialyzed against PBS (2 × 4 L). Following centrifugation of the suspension at 25000*g* for 1 h at 4 °C, the resulting supernatant (fraction II) was mixed with 1 g of polyleucyl hog gastric mucin (PLHGM)[1] (Kaplan & Kabat, 1966; Poretz, 1973) for 1 h at 4 °C. This suspension was centrifuged at 1000*g* and 4 °C for 20 min yielding a supernatant (fraction III) and the PLHGM–lectin complex. The lectin-bound adsorbant was repeatedly washed with cold PBS until the supernatant displayed an optical density at 280 nm of less than 0.05 unit. It was suspended in 100 mL of PBS containing 0.2 M D-galactose, a known inhibitor of the hemagglutinin. Following solubilization of the lectin, a supernatant was obtained by centrifugation at 1000*g* and 4 °C for 20 min and exhaustively

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[1] Abbreviations used: PLHGM, polyleucyl hog gastric mucin; PBS, phosphate-buffered saline.

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