Biochemistry

Volume 9, Number 8 April 14, 1970

25-Hydroxydihydrotachysterol$_3$. Synthesis and Biological Activity*

T. Suda, R. B. Hallick, H. F. DeLuca,† and H. K. Schnoes

ABSTRACT: 25-Hydroxydihydrotachysterol$_3$ has been synthesized by two methods and isolated in pure form. Its structure has been established by nuclear magnetic resonance, mass spectra, and ultraviolet absorption spectra. This compound has weak antirachitic activity but is a potent bone mobilization agent. In both capacities it is more effective than is dihydrotachysterol$_3$. It is also more effective than dihydrotachysterol$_3$ in increasing intestinal calcium transport and bone mobilization in thyroparathyroidectomized rats. Its biological activity suggests that it may be the drug of choice in the treatment of hypoparathyroidism or other similar bone diseases.

In 1939, von Werder studied the products from the reduction of tachysterol by sodium and propanol from which he isolated dihydrotachysterol. This compound which can be considered a reduction product of vitamin D possesses slight antirachitic activity but is remarkably effective in elevating serum calcium concentration in conditions of hypoparathyroidism. Westerhof and Keverling Buisman (1956, 1957) examined various reduction methods and isolated a number of isomers of which dihydrotachysterol$_2$ was the only effective compound in the elevation of serum calcium concentration. Using liquid ammonia and lithium metal a 40% yield of dihydrotachysterol$_2$ from tachysterol$_2$ could be obtained.

In this laboratory, the probable metabolically active form of vitamin D$_3$, namely, 25-HCC[1] was discovered (Lund and DeLuca, 1966) isolated, identified (Blunt *et al.*, 1968a,b), and synthesized (Blunt and DeLuca, 1969). This substance proved to be more effective than vitamin D$_3$ in intestinal calcium transport and in bone mobilization (rise in serum calcium) (Blunt *et al.*, 1968c). Other evidence using isolated bone (Trummel *et al.*, 1969) and intestinal (Olson and DeLuca, 1969) systems strongly suggest that vitamin D must be hydroxylated in the 25 position before it is effective.

Because both vitamin D and dihydrotachysterol are effective in the mobilization of bone, it seemed possible that dihydrotachysterol might also require hydroxylation before it can be effective. 25-OH-DHT$_3$ has therefore been synthesized and its biological activity was examined as compared with DHT$_3$. It has been found more effective than dihydrotachysterol$_3$ in the mobilization of bone and as an antirachitic agent. Its antirachitic activity is nevertheless low as compared with 25-hydroxycholecalciferol and vitamin D$_3$. It is the purpose of this communication to report these results.

Experimental Procedure

Crystalline dihydrotachysterol$_2$ and dihydrotachysterol$_3$ were a gift from the Phillips-Duphar Co., Weesp, the Netherlands.

Ultraviolet absorption spectra were determined in ether in a Beckman DB-G or a Cary 11 recording spectrophotometer. The molar extinction coefficients of the 25-hydroxytachysterols and the 25-hydroxydihydrotachysterols are assumed to be the same as those of the corresponding nonhydroxylated sterols. The following molar extinction coefficients are used: 25-hydroxytachysterol, $\lambda_{281\,m\mu}$ (ϵ 24,600) (Fieser and Fieser, 1959); 25-hydroxydihydrotachysterol, $\lambda_{251\,m\mu}$ (ϵ 38,900) (Westerhof and Keverling Buisman, 1956); cholesta-5,7-diene-3β-25-diol, $\lambda_{281\,m\mu}$ (ϵ 11,000); 25-hydroxycholecalcif-

* From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received August 5, 1969.* Published with the approval of the director of the Wisconsin Agricultural Experiment Station. Supported by the Harry Steenbock fund of the Wisconsin Alumni Research Foundation.

† To whom to send proofs at the Department of Biochemistry, University of Wisconsin.

[1] Abbreviations used are: 25-HCC, 25-hydroxycholecalciferol; 25-HEC, 25-hydroxyergocalciferol; 25-OH-DTH$_3$, 25-hydroxydihydrotachysterol$_3$, the subscript referring to the side-chain skeleton of vitamin D$_3$; DHT$_3$, dihydrotachysterol$_3$.

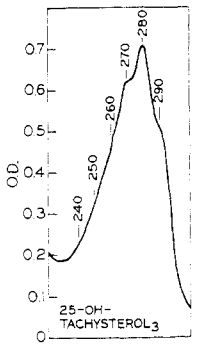

FIGURE 1: Ultraviolet absorption spectrum of 25-hydroxytachysterol$_3$.

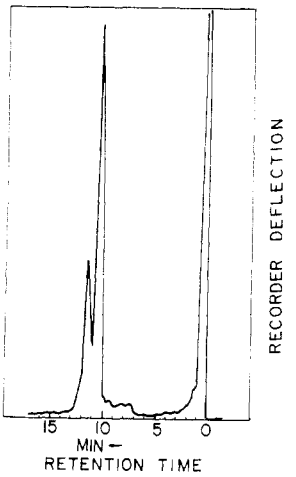

FIGURE 3: Gas–liquid partition chromatography of 25-hydroxydihydrotachysterol$_3$ obtained by sodium–*t*-amyl alcohol reduction.

erol, $\lambda_{264\,m\mu}$ (ϵ 18,000) (Blunt and DeLuca, 1969). Nuclear magnetic resonance spectra were obtained in deuteriochloroform solvent from a Varian Associates Model HA-100 spectrometer coupled to a time-averaging computer or from a Varian Associates Model T-60 spectrometer. Gas–liquid partition chromatography was performed in an F & M Model 402 gas chromatograph using a 4-ft glass column packed with 3% W-98 on 80–100 mesh Diatoport S. High-resolution mass spectra were reported with an A. E. I. MS-9 or C. E. C. 21-110B mass spectrometer coupled with photoplate recording. Infrared spectra were recorded on a Beckman IR-5 spectrometer.

3β-Acetoxy-26-norcholesten-25-one. 26-Nor-5-cholesten-3β-ol-25-one (5 g) was refluxed in 250 ml of acetic anhydride and a few drops of pyridine for 45 min. The cooled solution was poured into 800 ml of water. After 3 hr, the white solid was filtered. Recrystallization gave 5.12 g of 3β-acetoxy-26-nor-5-cholesten-25-one, mp 145–147.5° (MeOH, plates). The infrared spectrum of the product showed ν_{max} 1730 and 1715 cm^{-1}, corresponding to the carbonyl absorption of the acetate and methyl ketone, respectively. Nuclear magnetic resonance displayed δ 0.67 (C-18, H_3), 1.02 (C-19, H_3), 2.02 (acetate, H_3), 2.12 (C-26, H_3), and 5.42 (d,C-6, H_3). Concentration of the mother liquor yielded another 0.15 (MeOH, plates).

3β-Acetoxy-26-norcholesta-5,7-dien-25-one. The 5,7-diene was produced as previously described (Blunt and DeLuca, 1969). The dehydrobromination mixture from 1 g of 3β-acetoxy-26-nor-5-cholesten-25-one was evaporated with a rotary evaporator. The absorbancy at 282 mμ of the crude reaction mixture indicated a maximum yield of 465 mg of 3β-acetoxy-26-norcholesta-5,7-dien-25-one. Recrystallization from methanol gave 458 mg of white crystals, mp 109–111° (prisms). Ultraviolet spectrum showed the presence of 323 mg of the 5,7-diene. Gas–liquid partition indicated that the only impurity was unreacted starting material. The product displayed ultraviolet maxima at 272, 282, and 294 mμ.

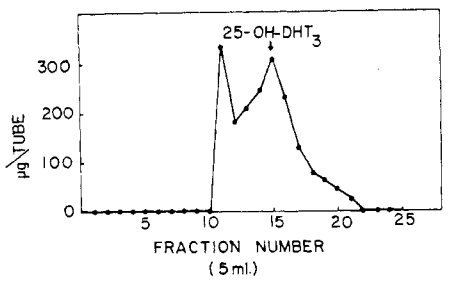

FIGURE 2: Partition column chromatography of 25-hydroxydihydrotachysterol$_3$ obtained by sodium–amyl alcohol reduction. Elution profile represents material with ultraviolet maxima at 242.5, 251, and 260.5 mμ.

Cholesta-5,7-diene-3β,25-diol. The above product (450 mg) was dissolved in a mixture of 10 ml of ether and 1 ml of benzene; 5 ml of 1 mM ethereal methylmagnesium iodide was added dropwise. After standing overnight at room temperature, ammonium chloride solution was added. The product was extracted with ether, applied to a 14-g silicic acid column, and eluted with ether. Ultraviolet spectra indicated 236 mg of cholesta-5,7-diene-3β,25-diol, with ultraviolet maxima at 272, 282, and 294 mμ. Gas–liquid chromatography indicated the presence of 5-cholestene-3β,25-diol as expected.

25-Hydroxytachysterol$_3$. The irradiation of cholesta-5,7-diene-3β,25-diol was performed as previously described (Blunt and DeLuca, 1969). All irradiations were carried out in 400 ml of ether or 400 ml of ethanol in a jacket around a double-walled, water-cooled, quartz well. An Hannovia high-pressure quartz mercury vapor lamp, Model 654A, was ignited for 3 min before placement in the immersion well. During the irradiation the solution was flushed continuously with nitrogen and stirred vigorously. Cholesta-5,7-diene-3β,25-diol (76 mg) was irradiated for 4 min. The products were applied in ether–Skelly B (a petroleum fraction, bp 67–69°, 1:1) to a 60-cm multibore column (Fischer and Kabara, 1964; Blunt and DeLuca, 1969) prepared from 14 g of heat-activated silicic acid (Mallinckrodt, 100 mesh). The column was eluted with a convex gradient obtained by running 100% ether into a 250-ml erlenmeyer constant-volume mixing chamber initially filled with ether–Skelly B (1:1). Fractions (5 ml) were collected. 25-Hydroxytachysterol$_3$ (17 mg) was obtained in fractions 30–39, which showed ultraviolet maximum at 281 mμ, and ultraviolet spectrum identical with tachysterol (Figure 1). Gas–liquid partition chromatography indicated the presence of some cholesta-5,7-diene-3β,25-diol and 5-cholestene-3β,25-diol.

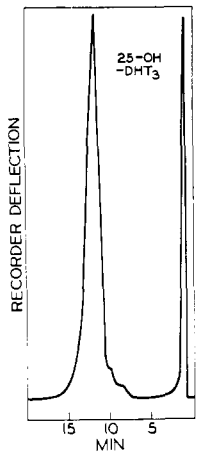

FIGURE 4: Gas–liquid partition chromatography of 25-hydroxydihydrotachysterol$_3$ obtained by lithium–ammonia reduction.

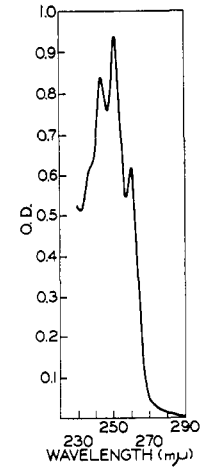

FIGURE 5: Ultraviolet absorption spectrum of 25-hydroxydihydrotachysterol$_3$.

25-Hydroxydihydrotachysterol$_3$. METHOD A. REDUCTION BY SODIUM–*t*-AMYL ALCOHOL (Westerhof and Keverling Buisman, 1956). About 1 g of sodium metal was heated with 10 ml of dry xylene at 150° under nitrogen atmosphere in a flask fitted with a stirrer and reflux condenser. 25-Hydroxytachysterol$_3$ (17 mg) was dissolved in 10 ml of dry xylene and added to the above sodium–xylene mixture with a 3-ml rinse. In the course of 15 min a mixture of 5 ml of dry *t*-amyl alcohol and 3 ml of xylene was added with vigorous stirring, and then an additional 13 ml of *t*-amyl alcohol was added. After this, stirring was continued for 75 min. The mixture was then cooled and the excess of sodium was removed with 100% ethanol. After evaporation of the xylene, the residue was extracted twice with ether. The ethereal solution was washed three times with water and dried with anhydrous Na_2SO_4. The crude reduction mixture showed ultraviolet absorption maxima at 242.5, 251, and 260.5 mμ, and in addition, ultraviolet absorption maxima at 272, 282, and 294 mμ, suggesting the presence of some cholesta-5,7-diene-3β,25-diol. This mixture was applied to a multibore silicic acid column as described above. Fractions (5 ml) were collected. Ultraviolet analysis indicated the presence of 3 mg of 25-hydroxydihydrotachysterol$_3$ in fractions 14–26, while later fractions contained cholesta-5,7-diene-3β,25-diol. Gas–liquid partition chromatography showed that the fractions containing 25-hydroxydihydrotachysterol$_3$ were contaminated by cholesta-5,7-diene-3β,25-diol and another component which is assumed to have the dihydrovitamin D-I diene structure described by Westerhof and Keverling Buisman (1956), a side product of sodium–*t*-amyl alcohol reduction of tachysterol$_2$. The 3 mg of 25-hydroxydihydrotachysterol$_3$ was applied to a partition column. Celite (20 g) was mixed with 15 ml of a stationary phase (80% methanol–20% water equilibrated at 4° with an equal volume of Skelly B) and packed in a 1-cm diameter column. The column was eluted with mobile phase (Skelly B equilibrated with an equal volume of stationary phase). Fractions (5 ml) were collected. 25-Hydroxydihydrotachysterol$_3$ was eluted in fractions 13–20, but was contaminated by cholesta-5,7-diene-3β,25-diol in fractions 17–21 and 25-hydroxydihydrovitamin D_3 in fractions 11–16. Figure 2 shows the elution profile of materials with absorption maxima at 242.5, 251, and 260.5 mμ. Figure 3 shows the gas–liquid partition chromatography record from fraction 14, demonstrating the contamination by 25-hydroxydihydrovitamin D_3-I. We were unable to isolate pure 25-hydroxydihydrotachysterol$_3$ from this mixture.

25-Hydroxydihydrotachysterol$_3$. METHOD B. REDUCTION BY LITHIUM IN AMMONIA (Westerhof and Keverling Buisman, 1956). About 3–4 ml of dry ammonia was condensed in a round-bottom flask with a stirrer and cooled to −50° with an acetone–solid carbon dioxide bath. Dry, precooled ether (1 ml) was added with stirring, followed by small amounts of lithium until a lasting blue color was achieved. 25-Hydroxytachysterol$_3$ (9 mg) was added in 1 ml of ether, and the reaction was allowed to proceed for 5 min, and then terminated with the addition of 100 mg of NH_4Cl. The reaction mixture was diluted slowly with water, and extracted three times with ether. The ether was washed three times with water and dried over anhydrous Na_2SO_4. The crude product displayed ultraviolet maxima at 242.5, 251, and 260.5 mμ; yield 3.8 mg. The material was chromatographed on a multibore silicic acid column as described before. Fractions 22–27 were pooled, containing 1.2 mg of 25-hydroxydihydrotachysterol$_3$, with ultraviolet maxima at 242.5, 251, and 260.5 mμ. Gas–liquid partition chromatography indicated the presence of 2 minor contaminants, one of them being 5-cholestene-3β,25-diol. Some material with the dihydrotachysterol ultraviolet spectrum also eluted in fractions 9–12, apparently due to elimination of the 25-hydroxy function during the lithium–ammonia reduction. The pooled 25-hydroxydihydrotachysterol$_3$ fractions were applied to a Celite partition column as described above. 25-Hydroxydihydrotachysterol$_3$ (0.8 mg) was obtained in pure form. Figure 4 shows the gas–liquid partition chromatography record of this product. The purification of the lithium in ammonia product was not hampered by other unwanted reduction products. Additional trial reductions yield a total of 5.4 mg of pure 25-hydroxydihydrotachysterol which was used for identification.

Structural Verification of 25-OH-DHT$_3$. The 25-hydroxydihydrotachysterol$_3$ obtained in pure form by method B was identified in three ways. The ultraviolet spectra shown

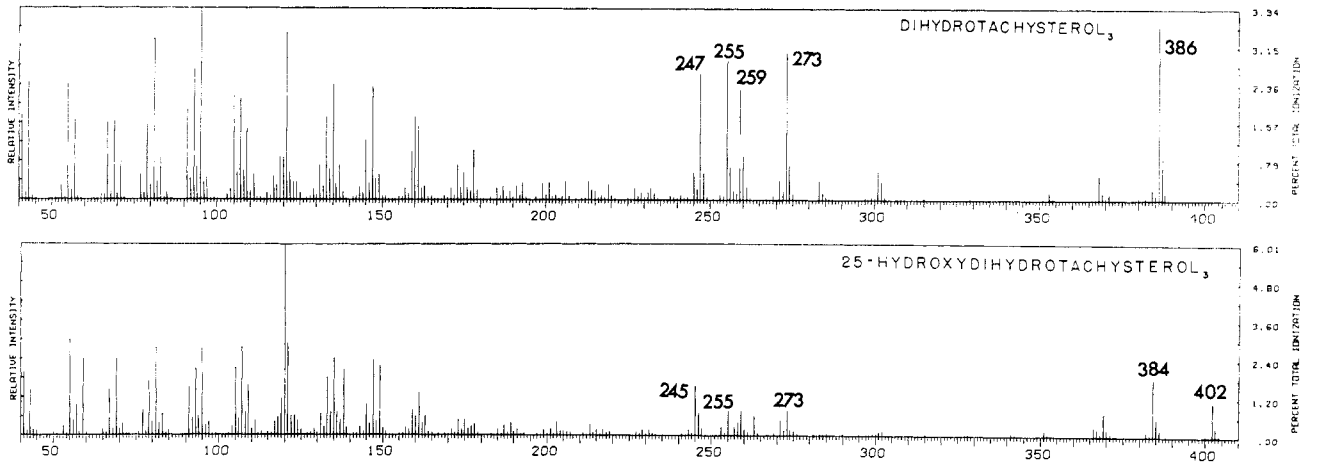

FIGURE 6: Mass spectra of 25-hydroxydihydrotachysterol$_3$ and dihydrotachysterol$_3$.

in Figure 5 is identical with that of dihydrotachysterol, *i.e.*, ultraviolet maxima at 242.5, 251, and 260.5 mμ. The high-resolution mass spectra of dihydrotachysterol$_3$ (Figure 6, Table I) and 25-hydroxydihydrotachysterol$_3$ also exhibits a very similar pattern. The molecular ion of the latter occurs at *m/e* 402.3506 ($C_{27}H_{46}O_2$, calcd 402.3498) in agreement with the structural assignment. Both spectra show the fragmentation sequence typical for the vitamin D series, namely, M-side-chain-H_2O, resulting in the ions at 273 and 255. A less common fragmentation, loss of ring A and C-6-7, is likewise observed for both compounds yielding in the case of dihydrotachysterol$_3$ the peak at *m/e* 247, and in the case of its 25-hydroxy derivative the peaks at *m/e* 263 and 245. The peak at *m/e* 259 occurring in both spectra appears to involve fragmentation of both the side chain and of ring A; a plausible explanation is given by the sequence: M $\rightarrow$ 301 $\rightarrow$ 259, *i.e.*, the elimination of a six-carbon fragment from the side chain to yield an ion of composition $C_{21}H_{33}O$ (301.2598) followed by loss of ketone involving the hydroxyl function of ring A. Differences between the two spectra concern primarily the appearance of a peak at *m/e* 59 (C_3H_7O) due to the 25-hydroxy grouping, the more pronounced loss of the elements of H_2O (*m/e* 384) from the molecular ion, and the enhancement of the peak at *m/e* 120 (C_9H_{12}, 120.0922). The fragmentation mechanism leading to this last peak is not clear, but may be connected with the much more enhanced elimination of H_2O from the molecule.

The comparison of the nuclear magnetic resonance spectra of dihydrotachysterol$_3$ and 25-hydroxydihydrotachysterol$_3$ provides final confirmation of the structural assignment. The expected side-chain structure is seen in comparison of Figures 7 and 8.

Especially evident is the 1.2-ppm singlet from the 26,27 methyl protons, the 0.90-ppm doublet (J = 8 cps) from the C_{21} protons and the 0.54-ppm singlet from the C_{18} protons. In this case of the dihydrotachysterol$_3$ note the 0.54-ppm singlet, but the 0.87 ppm (J = 6.5 cps) doublets from the $C_{26,27}$ methyl protons as well as the 0.93-ppm (J = 5 cps) doublet from the C_{21} methyl protons. (The singlet near the 0.54-ppm singlet in both spectra is a side band of tetramethylsilane.) The AB quartet of the olefinic protons of the dihydrotachysterol-conjugated diene system is shown in Figure 9. Both dihydrotachysterol$_3$ and 25-hydroxydihydrotachysterol$_3$ display 6.13 and 5.91 ppm (J = 10.5 cps) for the

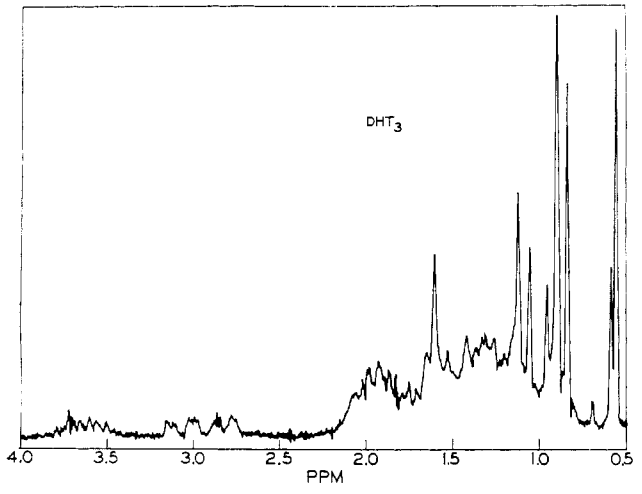

FIGURE 7: Nuclear magnetic resonance spectra of dihydrotachysterol$_3$.

TABLE I: High-Resolution Mass Spectra of 25-OH-DHT$_3$.

m/e		
Calcd, 402.34984[a]	Found, 402.35062[a]	$C_{27}H_{46}O_2$ (M)
384.33924	*384.33985*	$C_{27}H_{44}O$
273.25821	*273.25698*	$C_{19}H_{29}O$
259.24258	*259.24273*	$C_{19}H_{31}$
255.21126	*255.21066*	$C_{19}H_{27}$
245.22962	*245.22606*	$C_{18}H_{29}$
120.09389	*120.09226*	C_9H_{12}

[a] Molecular weight.

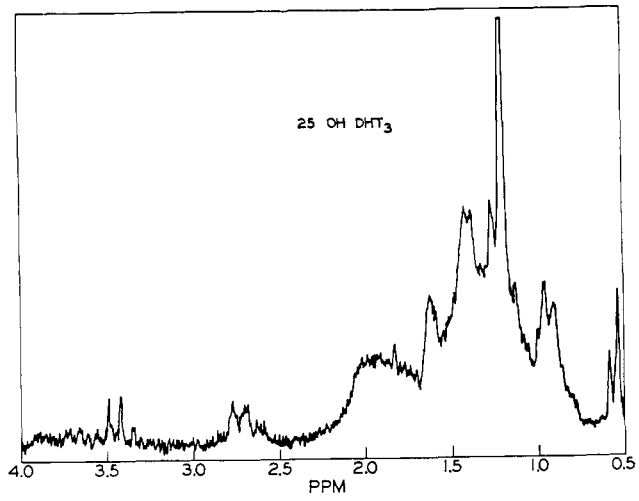

FIGURE 8: Nuclear magnetic resonance spectra of 25-hydroxydihydrotachysterol$_3$.

protons at C_6 and C_7. Thus the structure was confirmed as 25-hydroxydihydrotachysterol$_3$ (Figure 10).

Biological Activity of Synthetic 25-Hydroxydihydrotachysterol$_3$. All assays were performed as described previously (Blunt *et al.*, 1968a–c) except some assays with thyroparathyroidectomized rats, which were carried out according to Harrison and Harrison's procedure (Harrison *et al.*, 1968). For bioassays in thyroparathyroidectomized rats, 71 Holtzman male rats, 5-weeks old, weighing 120–130 g, were maintained on a low Ca diet (DeLuca *et al.*, 1961) for 7 days. They were given 75 IU of vitamin D_3 every 3 days until they were killed. After 1 week of feeding, 1% calcium gluconate in 2.5% glucose was substituted for the drinking water and 24 hr later the rats were thyroparathyroidectomized surgically. The 1% calcium gluconate solution was continued for 48 hr postoperatively, and then 0.45% sodium chloride in 2.5% glucose was substituted. Thirty-two rats died within 24 hr after surgery. Three days following operation, the rats were bled from the tail for determination of serum calcium. Animals with serum calcium concentration below 7.0 mg/100 ml were considered to be parathyroidectomized; 6 rats of 39 rats which survived were incompletely parathyroidectomized. Their serum calcium

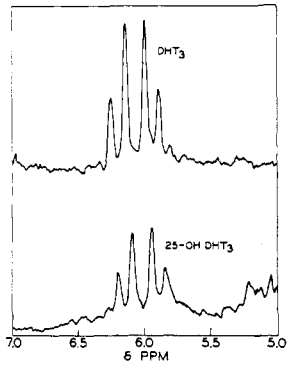

FIGURE 9: Downfield nuclear magnetic resonance spectra of 10 mg of crystalline dihydrotachysterol$_3$ and 5.4 mg of 25-hydroxydihydrotachysterol$_3$ from method B.

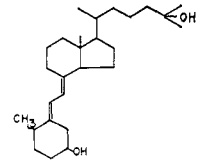

FIGURE 10: Structure of 25-hydroxydihydrotachysterol$_3$.

concentrations were 7.9 ~ 10.1 mg/100 ml. These 6 rats were considered to have functioning parathyroids which had not been removed and were used as operated thyroidectomized controls. The 33 parathyroidectomized rats were divided into 7 groups and given by stomach tube a single dose of varying amounts of dihydrotachysterol$_3$ or 25-hydroxydihydrotachysterol$_3$ dissolved in 0.1 ml of cottonseed oil, while the operated controls and a group of parathyroidectomized rats were given only oil. Twenty-four hours following dosage, the animals were bled for determination of serum calcium and used for measurement of duodenal transport of calcium. The transport efficiency of the *in vitro* preparation was measured in terms of the serosal:mucosal ratio (Harrison *et al.*, 1968).

Like dihydrotachysterol$_3$, 25-hydroxydihydrotachysterol$_3$ is the only slightly antirachitic (Table II); however, it is definitely more antirachitic than is dihydrotachysterol$_3$ (five times) and dihydrotachysterol$_2$ (ten times) by the rat line test assay (U. S. Pharmacopeia, 1955). Certainly its antirachitic activity is much less than the very potent 25-hydroxycholecalciferol (Blunt *et al.*, 1968c) and 25-hydroxyergocaliferol (Suda *et al.*, 1969) (Table II).

The greatest activity of the 25-hydroxydihydrotachysterol$_3$ is in the mobilization of bone mineral to elevate plasma calcium. A 2.5-μg dose acts more rapidly and is more effective than a dose of dihydrotachysterol$_3$ in vitamin D deficient rats (Figure 11). However, it is not as effective as 25-hydroxycholecalciferol (Blunt *et al.*, 1968c).

25-Hydroxydihydrotachysterol$_3$ is also more effective than dihydrotachysterol$_3$ in surgical hypoparathyroid rats in its ability to elevate plasma calcium. As little as 10 μg/rat elevated plasma calcium with 50 μg giving a higher elevation than 100 μg of dihydrotachysterol$_3$ (Table III). Note that an isomer (probably 25-hydroxydihydrovitamin D_3-I) isolated from the sodium and *t*-amyl alcohol reduction was found inactive even at 100 μg. The 25-hydroxydihydrotachy-

TABLE II: Antirachitic Activity of 25-OH-DHT$_3$ in Rats.[a]

	IU/μg	Ratio	
Vitamin D_2	40	1.0	
Vitamin D_3	40	1.0	
25-HEC	60	1.5	Suda *et al.* (1969)
25-HCC	56	1.4	Blunt *et al.* (1968)
DHT_2	0.09	$^1/_{450}$	von Werder (1939)
DHT_3	0.16	$^1/_{250}$	
25-OH-DHT_3	0.8	$^1/_{50}$	2 IU/2.5 μg

[a] Carried out by the U. S. Pharmacopeia method (1955). There were eight rats in each group.

TABLE III: Serum Calcium Response of Thyroparathyroidectomized Rats to 25-OH-DHT_3.

Group			Serum Calcium (mg %)	
Rats	Dose	Amount	Before Dose	24 hr after Dose
Surgical control	Oil	0.1 ml	9.3 ± 0.80 (6)	9.8 ± 0.76 (6)
TPTX	Oil	0.1 ml	5.9 ± 0.35 (4)	5.5 ± 0.76 (4)
TPTX	DHT_3	50 μg	5.3 ± 0.75 (4)	6.3 ± 0.75 (4)
TPTX	DHT_3	100 μg	5.7 ± 0.45 (6)	7.5 ± 0.36 (6)
TPTX	25-OH-DHT_3	10 μg	5.5 ± 0.33 (6)	6.0 ± 0.49 (6)
TPTX	25-OH-DHT_3	25 μg	5.5 ± 0.35 (6)	6.7 ± 0.75 (6)
TPTX	25-OH-DHT_3	50 μg	5.4 ± 0.67 (4)	7.7 ± 0.70 (4)
TPTX	25-OH Dihydrovitamin D_3-I[a]	100 μg	5.6 ± 0.17 (3)	5.2 ± 0.20 (3)

[a] Tubes 11 and 12 of Figure 2. The methods used are those of Harrison and Harrison (1968).

$sterol_3$ was also at least as effective as $dihydrotachysterol_3$ in elevating calcium transport in hypoparathyroid animals (Figure 12). Of interest is the lowered calcium transport in the thyroparathyroidectomized animals and its elevation by $dihydrotachysterol_3$ as well as 25-hydroxydihydrotachy$sterol_3$.

Discussion

A new compound, 25-hydroxydihydrotachy$sterol_3$, has been synthesized and has great potential in the treatment of hypoparathyroidism. Certainly it is more effective than the most favored of present drugs, namely, $dihydrotachysterol_3$. Although it is not as effective as 25-hydroxycholecalciferol (Blunt *et al.*, 1968c) or 25-hydroxyergocalciferol (T. Suda and H. F. DeLuca, unpublished results) in inducing the mobilization of bone in vitamin D deficiency, it does not have the calcification properties of the D vitamins and the 25-OH-D vitamins. Thus it is an ideal agent to be used specifically for bone mobilization or elevation of plasma calcium in hypoparathyroidism or other bone diseases. It is certainly more effective than either $dihydrotachysterol_2$ or $dihydrotachy$$sterol_3$ and thus should be superior to these two agents in such cases.

It is of great interest that 25-hydroxydihydrotachy$sterol_3$ is more effective than $dihydrotachysterol_3$ in calcium metabolism which might be inferred from the fact that 25-hydroxycholecalciferol is more effective than vitamin D_3. Strong evidence is now accumulating that 25-hydroxycholecalciferol represents the metabolically active form of vitamin D_3 (Trummel *et al.*, 1969; Olson and DeLuca, 1969). Thus it is inviting to suspect that $dihydrotachysterol_3$ must also be hydroxylated in the 25 position before it is effective. This possibility is currently under investigation as well as the synthesis of [^{3}H]$dihydrotachysterol_3$ to examine the possibility that $dihydrotachysterol_3$ is converted *in vitro* into 25-hydroxydihydrotachy$sterol_3$.

Our results confirm the finding (Harrison *et al.*, 1968) that $dihydrotachysterol_3$ increases intestinal transport of Ca^{2+} in the hypoparathyroid rat. It also seems clear that surgical hypoparathyroidism is followed by a decrease in Ca^{2+} transport by intestine, a question which has remained controversial for many years.

The synthesis of the 25-hydroxydihydrotachy$sterol_3$ was carried out by the two methods originally described by the chemists at Philips Duphar Co. (Westerhof and Keverling

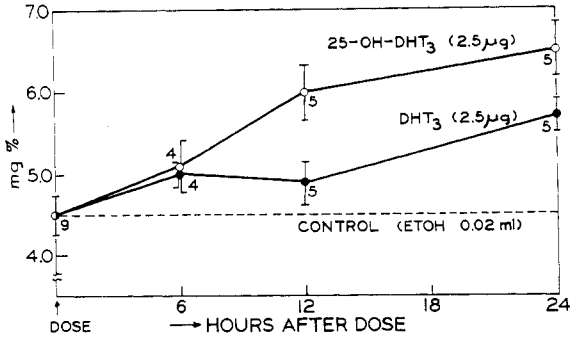

FIGURE 11: Serum calcium response of vitamin D deficient rats on a low calcium diet to $dihydrotachysterol_3$ and 25-hydroxydihydro$tachysterol_3$.

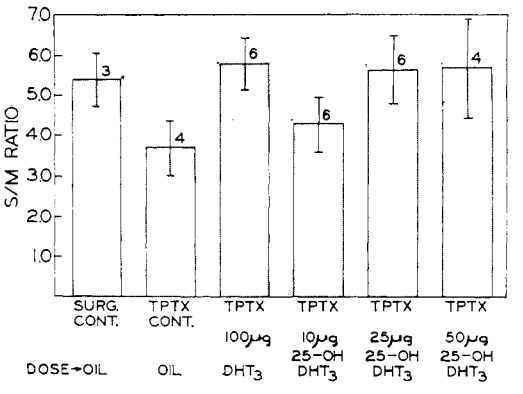

FIGURE 12: Comparison of intestinal calcium transport responses of thyroparathroidectomized rats with $dihydrotachysterol_3$ and 25-hydroxydihydro$tachysterol_3$.

Buisman, 1956) for dihydrotachysterol$_2$. 25-Hydroxydihydrotachysterol has been synthesized in overall yield of 0.6% from 26-nor-5-cholesten-3β-ol-25-one. In results not published here we have also been able to reduce both 25-hydroxyprecholecalciferol and 25-hydroxycholecalciferol to 25-hydroxydihydrotachysterol$_3$ with lithium in ammonia. The yields were comparable with those reported by Westerhof and Keverling Buisman (1956) for preergocalciferol and ergocalciferol, respectively.

Our experiments also confirm the findings of the Philips group that *t*-amyl alcohol and sodium produce unwanted reduction products which are difficult to separate from the desired dihydrotachysterol$_2$ or in our case 25-hydroxydihydrotachysterol$_3$. However methods herein described will allow separation of the 25-hydroxydihydrotachysterol$_3$ from the other reduction products. By far the best method is, nevertheless, the liquid ammonia and lithium metal reduction which is less subject to the many side-reaction products.

Acknowledgment

We are indebted to Professor Biemann (Massachusetts Institute of Technology, Cambridge, Mass.) for the high-resolution mass spectra, to Miss Martha Petrie, Mr. Roland Randall, and Professor P. Bender, Chemistry Department, University of Wisconsin for the nuclear magnetic resonance spectra. We thank Dr. M. J. Melancon for performing the thyroparathyroidectomies and Mr. Lloyd Hein for carrying out the vitamin D assays at the Wisconsin Alumni Research Foundation.

References

Blunt, J. W., and DeLuca, H. F. (1969), *Biochemistry 8*, 671.

Blunt, J. W., DeLuca, H. F., and Schnoes, H. K. (1968a), *Biochemistry 7*, 3317.

Blunt, J. W., DeLuca, H. F., and Schnoes, H. K. (1968b), *Chem. Commun., No. 14*, 801.

Blunt, J. W., Tanaka, Y., and DeLuca, H. F. (1968c), *Proc. Natl. Acad. Sci. U. S. 61*, 1503.

DeLuca, H. F., Guroff, G., Steenbock, H., Reiser, S., and Mannatt, M. R. (1961), *J. Nutrition 75*, 175.

Fieser, L. F., and Fieser, M. (1959), Steroids, New York, N. Y., Reinhold, p 90.

Fischer, G. A., and Kabara, J. J. (1964), *Anal. Biochem. 9*, 303.

Harrison, H. E., Harrison, H. C., and Lipshitz, F. (1968), *Proc. 3rd Parathyroid Conf. Excerpta Med. Intern. Congr. Ser. No. 159*, p 455.

Lund, J., and DeLuca, H. F. (1966), *J. Lipid Res. 7*, 739.

Olson, E. B., and DeLuca, H. F. (1969), *Science 165*, 405.

Suda, T., DeLuca, H. F., Schnoes, H. K., and Blunt, J. W. (1969), *Biochemistry 8*, 3515.

Trummel, C., Raisz, L. G., Blunt, J. W., and DeLuca, H. F. (1969), *Science 163*, 1450.

U. S. Pharmacopeia (1955), 14th Revision, Easton, Pa., Mack, p 889.

von Werder, F. (1939), *Hoppe-Seyler's Z. Physiol. Chem. 260*, 119.

Westerhof, P., and Keverling Buisman, J. A. (1956), *Rec. Trav. Chim. Pays-Bas 75*, 453.

Westerhof, P., and Keverling Buisman, J. A. (1957), *Rec. Trav. Chim. Pays-Bas 76*, 680.