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SYNTHESIS AND BIOLOGIC ACTIVITY OF 3β-THIOVITAMIN D_3

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Summary—We synthesized 3β-thiovitamin D_3 from 7-dehydrocholesterol and tested its biological activity and protein binding properties. The thiovitamin was found to be a weak vitamin D agonist at high doses *in vivo*. It was poorly bound by both vitamin D-binding protein as well as by the intestinal cytosol receptor for 1,25-dihydroxyvitamin D. It did not increase the synthesis of calcium binding protein in the chick embryonic duodenum and did not block the activity of 1,25-dihydroxyvitamin D_3 in this system. We conclude that 3β-thiovitamin D_3 is a weak vitamin D agonist *in vivo* with no agonist activity or antagonist activity to 1,25-dihydroxyvitamin D_3 in the chick embryonic duodenum.

INTRODUCTION

In order to assess the influence of thiol groups on the biological activity and protein binding characteristics of vitamin D_3, we synthesized 3β-thiovitamin D_3‡ **5** and tested its biological activity *in vivo* and *in vitro*. Previous reports showed that 3β-thio-7-dehydrocholesterol could be synthesized from cholesterol and 7-dehydrocholesterol [1–3]. The same investigators also irradiated the 3β-thio-7-dehydrocholesterol to obtain the vitamin in the form of a mixture of photochemical products [2, 4]; however, the photolysed mixture was not purified further before biological testing. Consequently, biological activity experiments were not conclusive as it was uncertain what the exact constituents of the administered doses were. We now report the synthesis of the pure thiovitamin and the results of bioactivity and binding potency studies. In addition, we report on studies of the biological activity of the 3β-thiovitamin in a chick embryonic duodenum organ culture system in which vitamin D analogs induce the synthesis of vitamin D dependent calcium binding protein.

EXPERIMENTAL

General

Ultraviolet (u.v.) spectra were taken in ethanol with a Beckman Model 35 recording spectrophotometer (Beckman Instruments, Palo Alto, CA). Mass spectra were obtained on a Kratos MS50/DS-55 mass spectrometer–computer system (Kratos Instruments, U.K.). High performance liquid chromatography (HPLC) was performed on a Waters liquid chromatograph equipped with a Model M-6000 A pump, a Model 450 variable wavelength detector set at 265 nm (all from Waters Associates, Milford, MA), and a Model 3380 A Hewlett–Packard integrator (Hewlett–Packard, Avondale, PA). HPLC conditions for purification and final spectroscopic characterization were: Varian Micropak MCH-10, 50 cm × 8 mm, C-18 reverse phase column, ethanol eluant, 3 ml/min flow rate. Nuclear magnetic resonance spectra (NMR) were obtained in deuterated chloroform with 0.03% tetramethylsilane on an IBM NR-80 Fourier transform nuclear magnetic resonance spectrometer (IBM Instruments, Danbury, CT). Radioactivity was determined with a Beckman LS-9000 β-scintillation counter (Beckman Instruments, Palo Alto, CA). $^{45}CaCl_2$ was obtained from New England Nuclear (New England Nuclear, Boston, MA). Photolysis was carried out with a Rayonet photochemical reactor (Southern New England Ultraviolet Company, Hamden, CT).

Biological measurements

Serum calcium was measured in 0.1% lanthanum chloride as diluent with a Perkin–Elmer 2380 atomic absorption spectrometer (Perkin–Elmer Instruments, Norwalk, CT). Calcium transport in the duodenum was determined by the everted gut sac method of Martin and DeLuca[5]. Binding assays using vitamin D binding protein and chick intestinal cytosol receptor were performed as described before [6]. The induction of the vitamin D-dependent calcium binding protein was studied in organ-cultured duodena as described in detail elsewhere [7]. The rats were administered the appropriate dose of the vitamin D_3 compound dissolved in 50 μl of ethanol, 24 h before the experiment. Control rats received vehicle alone.

Animals

Male, albino, weanling rats (50–60 g) were obtained from the Holtzman Co. (Madison, WI). They

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‡Abbreviation: 3β-thiovitamin D_3, 5(Z),7(E),3β-thio-9,10-secocholesta-5,7,10(19)-triene.

were maintained in individual overhanging wire cages and fed a 0.02% calcium, 0.3% phosphorus, vitamin D-deficient diet *ad libitum* for 3 weeks prior to use [8].

Synthesis (Fig. 1)

3β-p-Toluenesulphonoxycholesta-5,7-diene **2**. Cholesta-5,7-dien-3β-ol, **1** (10 g, 26 mmol), which had been previously dried *in vacuo* over phosphorus pentoxide, was added to *p*-toluenesulphonyl chloride (10 g, 52 mmol) in dry pyridine (20 ml). This reaction was allowed to stir at room temperature in the dark, under nitrogen for 24 h. The mixture was poured into cold saturated sodium bicarbonate solution (300 ml). After 1 h, the precipitate was filtered and thoroughly washed with cold water and then methanol, until colorless. Recrystallization from acetone (500 ml) gave **2** (10.8 g, 20.0 mmol, 77% yield) as fluffy white needles: m.p. 101–102°C; NMR δ 4.46 (m, 1H, 3α-H), 5.08–5.47 (m, 2H, 6,7-H), 7.80, 7.34 (ABq, 4H, J = 9.8 Hz, aromatic); mass spectrum *m/z* (assignment, relative intensity) 538 (M^+ ($C_{34}H_{50}O_3S$: calcd 538.4347, found 538.3403), 4), 366 (M^+ – TsOH, 100), 351 (366 – CH_3, 62), 253 (366 – side chain, 40), 172 (TsOH, 28).

3β-Isothiouronium-p-toluenesulphonoxycholesta-5,7-diene **3**. 3β-*p*-Toluenesulphonoxycholesta-5,7-diene **2** (9.3 g, 17 mmol), was refluxed under nitrogen with thiourea (9.5 g, 125 mmol) in anhydrous pyridine (9.3 ml) and absolute ethanol (93 ml) for 5 h. The reaction vessel was cooled to room temperature and cold ether (100 ml) was added. The reaction mixture was stirred for 30 min and then refrigerated overnight. The precipitate was collected by filtration and washed with cold ether, water and acetone. The crystals were washed with boiling ethanol, yielding 2.9 g of **3** (4.7 mmol, 28%): m.p. 230–231°C (decomposes); mass spectrum *m/z* (assignment, relative intensity) 614 (M^+ ($C_{35}H_{54}O_3S_2N_2$), O (not seen)), 366 ($M^+ - C_8H_{12}O_3S_2N_2$, 68), 253 (366 – side chain, 100).

3β-Thio-cholesta-5,7-diene **4**. 3β-Isothiouronium-*p*-toluenesulphonoxycholesta-5,7-diene **3** (2.6 g, 4.2 mmol) was refluxed under nitrogen with sodium hydroxide (0.72 g, 18.0 mmol) in absolute ethanol (55 ml). After disssolution, water (3.2 ml) was added and the mixture refluxed for $2\frac{1}{2}$ h. The mixture was cooled and poured into ice water (500 ml) and glacial acetic acid (1.5 ml) was added. After stirring for 30 min, the mixture was extracted with ether. The ether extract was dried with anhydrous sodium sulphate, concentrated to about 40 ml, and mixed with acetone (200 ml). Thiol **4** was precipitated as a lead salt by adding lead acetate (1.6 g, 4.9 mmol) dissolved in boiling 70% alcohol to the ether–acetone solution. The yellow precipitate was filtered, washed with cold acetone, and dissolved in benzene. A small insoluble white residue was removed by filtration. Hydrogen sulphide was passed through the solution, and the lead sulphide precipitate removed by filtration. The filtrate was evaporated to dryness and kept *in vacuo* overnight to remove the excess H_2S, yielding white crystals of **4** (1.34 g, 3.3 mmol, 78%): m.p. 127–129°C; u.v. λ_{max} 294 nm, 282, 272, 262; NMR δ 2.73 (m, 1H, 3α-H), 5.55, 5.35 (ABq, 2H, J = 6.5 Hz, 6,7-H); mass spectrum *m/z* (assignment, relative intensity) 400 (M^+ ($C_{27}H_{44}S$: calcd 400.4032, found 400.3221), 100), 385 ($M^+ - CH_3$, 8), 366 ($M^+ - H_2S$, 18), 351 (366 – CH_3, 65), 253 (366 – side chain, 24).

Photolysis of 3β-thio-cholesta-5,7-diene **4**. A solution of **4** (27 mg, 0.067 mmol) in 75 ml anhydrous ether at 0°C was irradiated at λ = 300 nm for 3 min. The solution was flushed thoroughly with nitrogen prior to and during irradiation. The ether solution was refluxed under nitrogen for 72 h. The solvent was concentrated to 1 ml under reduced temperature and pressure, and purified by preparative HPLC (see Experimental, General; 10.8 min elution time) to give 3β-thiovitamin D_3 **5** (5.8 mg,* 0.014 mmol, 21%): u.v. λ_{max} 264 nm, λ_{min} 228 nm ($\lambda_{max}/\lambda_{min}$ = 1.76); mass spectrum (Fig. 2) *m/z* (assignment, relative intensity) 400 (M^+ ($C_{27}H_{44}S$: calcd 400.4032, found 400.3419), 27), 385 ($M^+ - CH_3$, 6), 366 ($M^+ - H_2S$, 12), 351 (366 – CH_3, 25), 253 (366 – side chain, 26), 152 ($C_9H_{12}S$, 66), 143 ($C_{11}H_{11}$, 65), 118 (152 – H_2S, 100).

RESULTS

We employed a four-step synthesis of 3β-thiovitamin D_3 **5** from cholesta-5,7-dien-3β-ol **1** (Fig. 1) [3]. Esterification of **1** with *p*-toluenesulphonyl chloride in pyridine gives *p*-toluenesulphonate **2** in 77% yield. Thiourea reacts with tosylate **2** to give isothiouronium-*p*-toluenesulphonate **3** in 28% yield. Alkaline hydrolysis of **3** gives thiol **4** in 78% yield. Photolysis of **4** with λ = 300 nm followed by thermal rearrangement in ether affords 3β-thiovitamin D_3 **5** in 21% yield.

As shown in Fig. 3, a dose of at least 500,000 pmol/rat of **5** is necessary to elicit an increase in intestinal calcium transport comparable to 50 pmol/rat of vitamin D_3. With respect to bone calcium mobilization, a dose of at least 500,000 pmol/rat of **5** is necessary to elicit a response comparable to 50 pmol/rat of vitamin D_3 (Fig. 4). Thiovitamin **5** binds very poorly to vitamin D binding protein and the intestinal cytosol receptor. The B-50† value of each vitamin D metabolite tested (Fig. 5) is listed in order of decreasing ability to displace radiolabeled 25-hydroxyvitamin D_3 from rat plasma vitamin D binding protein: 25-hydroxyvitamin D_3 (3.9×10^{-9} M), 24(R),25-dihydroxyvitamin D_3 (4.7×10^{-9} M), 25(S),26-dihydroxyvitamin D_3 (5.4×10^{-9} M), 1α,25-dihydroxyvitamin D_3 (7.1×10^{-8} M), vitamin D_3 (4.0×10^{-7} M), 1α-hydroxyvitamin D_3 (1.3×10^{-6} M), and 3β-thiovitamin D_3 (2.1×10^{-5} M). The B-50 value of each

*Weight of purified vitamin was determined from u.v. measurements by assuming the ε of **5** to be 18,200.

†B-50 value is defined as the concentration of material necessary to cause 50% displacement of the radiolabel.

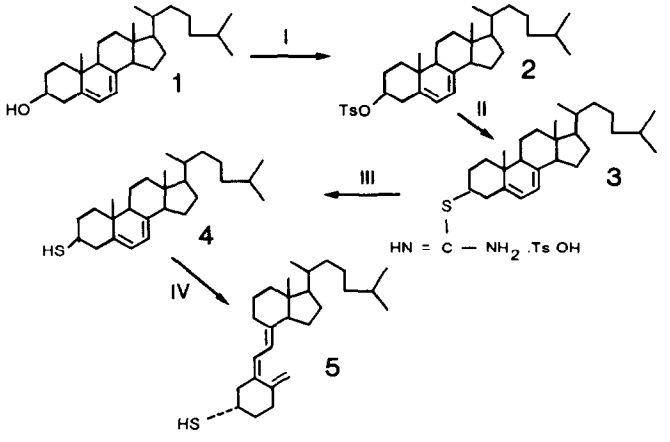

Fig. 1. Synthesis of 3β-thiovitamin D_3 from 7-dehydrocholesterol.

vitamin D metabolite tested (Fig. 6) is listed in order of decreasing ability to displace radiolabeled 1,25-dihydroxyvitamin D_3 from a chicken intestine cytosol receptor preparation: 1α,25-dihydroxyvitamin D_3 (1.5×10^{-10} M), 1α-hydroxyvitamin D_3 (1.1×10^{-7} M), 25-hydroxyvitamin D_3 (2.2×10^{-7} M), 24(R),25-dihydroxyvitamin D_3 (6.6×10^{-7} M), 25(S),26-dihydroxyvitamin D_3 (1.8×10^{-6} M), vitamin D_3 ($\geqslant 2.5 \times 10^{-4}$ M), and 3β-thiovitamin D_3 ($\geqslant 2.4 \times 10^{-4}$ M). As shown in Table 1, the activity of 3β-thiovitamin D_3 **5**, determined by measuring the concentration of vitamin D-induced calcium binding protein (CaBP) in a duodenal organ culture system, was zero at a concentration as high as 2×10^{-6} M.

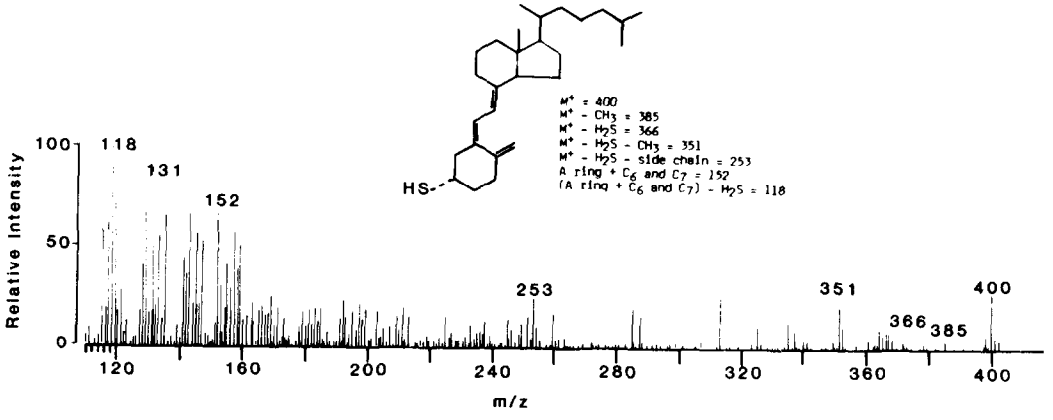

Fig. 2. Mass spectrum of 3β-thiovitamin D_3.

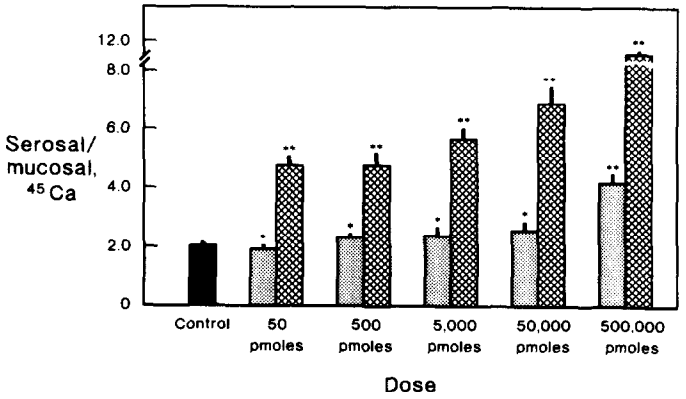

Fig. 3. Intestinal calcium transport in everted duodenal sacs 24 h after the administration of varying doses of 3β-thiovitamin D_3 (stippled bars) or vitamin D_3 (crosshatched bars) to rats raised on a vitamin D-deficient, low-calcium diet. Data are expressed as mean ± SEM. (*) P = not significant; (**) $P \leq 0.001$.

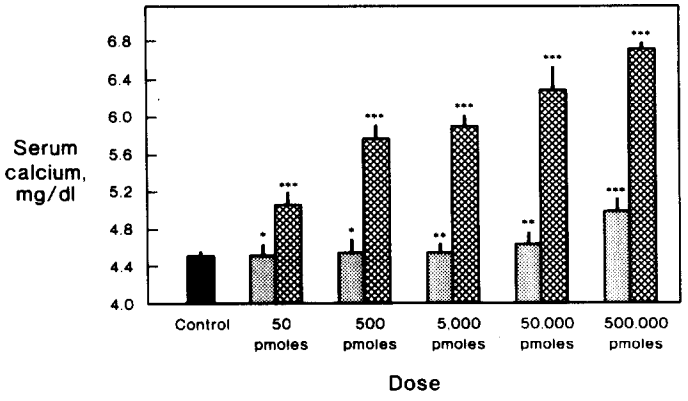

Fig. 4. Serum calcium values (mg/dl) 24 h following the administration of various doses of 3β-thiovitamin D_3 (stippled bars) or vitamin D_3 (cross-hatched bars) to hypocalcemic vitamin D-deficient rats. Data are expressed as mean ± SEM. (*) P = not significant; (**) $P \leq 0.03$; (***) $P < 0.001$.

Further, this vitamin showed no antagonist activity toward the response elicited by 1α,25-dihydroxyvitamin D_3 even at a concentration 2000-fold higher than the native hormone.

DISCUSSION

The synthesis of 3β-thiocholesta-5,7-diene was first described by Strating and Backer[1]. They prepared the sulphur analogue from cholesterol via cholesterol thiocyanate and 7-dehydrocholesterol thiocyanate. Reduction with lithium aluminum hydride afforded the 3β-thio-cholesta-5,7-diene.

Bernstein and Sax reported a similar scheme converting thiocholesterol to the thiobenzoate and the thiobenzoate-cholesta-5,7-diene [2]. Hydrolysis with either potassium carbonate or potassium hydroxide in ethanol gave the 3β-thio-cholesta-5,7-diene which was irradiated in ether with a Hanovia ultraviolet lamp. The assumption was made that irradiated

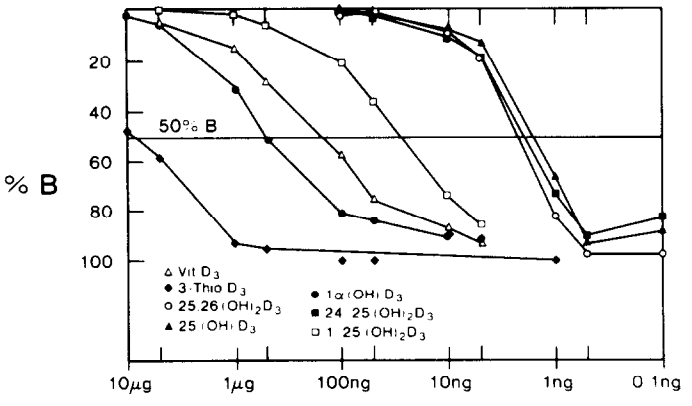

Fig. 5. Ability of various vitamin D_3 compounds to displace radiolabeled 25-hydroxyvitamin D_3 from rat plasma vitamin D binding protein.

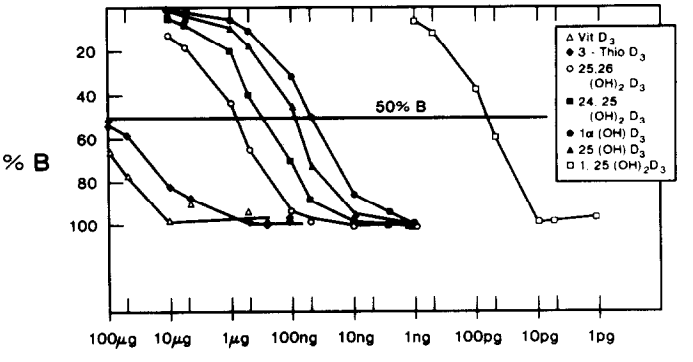

Fig. 6. Ability of various vitamin D_3 compounds to displace radiolabeled 1,25-dihydroxyvitamin D_3 from chicken intestine cytosol receptor preparation.

Table 1. Activity of 3β-thiovitamin D_3 in the induction of CaBP in the organ-cultured duodenum*

$1\alpha,25(OH)_2D_3$	3β-Thiovitamin D_3	CaBP
nM	M	μg/100 mg duodenum
0	0	0
0	2×10^{-8}	0
0	2×10^{-7}	0
0	2×10^{-6}	0
1	0	15.5 ± 2.4
1	2×10^{-8}	16.7 ± 1.9
1	2×10^{-7}	15.0 ± 0.8
1	2×10^{-6}	14.8 ± 0.9

*CaBP was determined in the embryonic chick duodena after various concentrations of vitamin D homologue compounds were separately introduced into the medium. The details of this procedure are described elsewhere [7]. Values: $\bar{X} \pm$ SE; 8 duodena/group.

thio-7-dehydrocholesterol gave, among other products, thiovitamin D_3. Some evidence for this product formation was an ultraviolet absorption curve approximately the same as irradiated 7-dehydrocholesterol.

The crude irradiation product mixture was fed to chicks at a dose of 5 mg/kg of diet. A chick bone ash test showed that the irradiation product of thio-7-dehydrocholesterol had no vitamin activity. Strating reported similar results from their irradiated thio-7-dehydrocholesterol [4].

In order to isolate and characterize a thiovitamin of D_3 for the first time, we utilized the improved synthesis of 3β-thio-7-dehydrocholesterol of Keverling Buisman and Westerhof[3]. Cholesta-5,7-diene-3β-ol was esterified to the tosylate, which was reacted with thiourea to give the isothiouronium-*p*-toluene sulphonate. Alkaline hydrolysis gave the 3β-thiocholesta-5,7-diene **4**. The mass spectrum and NMR spectrum of this steroid are reported for the first time.

Optimum conditions for photolysis of the provitamin **4** were determined by sampling the reaction mixture at regular time intervals and at various wavelengths and analyzing these aliquots by HPLC. Irradiation at $\lambda = 300$ nm for 3 min gave the previtamin in optimal yields. Thermolysis of the crude photolysate was performed under gentle conditions. Refluxing in ethanol for a short time gave decomposition products. Gentle refluxing in ether (37°C) over 72 h, under continuous nitrogen atmosphere gave the thiovitamin in good yield. The thiovitamin photolysate mixture was then purified on HPLC. The thiovitamin undergoes decomposition upon concentration to dryness. If all solvent is removed, the u.v. spectrum of the vitamin is slightly altered, and the HPLC elution volume is changed. The thiovitamin must be maintained in minimal solvent at all times.

We report for the first time the characterization of a pure thiovitamin of D_3. We were successful in obtaining mass spectral and u.v. spectral data of thiovitamin **5** although several attempts to obtain an NMR spectrum failed due to the unusual instability of **5**. The biological properties of **5** were tested in vitamin D deficient rats maintained on a low calcium diet. The thiovitamin was capable of mobilizing bone calcium, and eliciting an increase in intestinal calcium transport, only at the highest dose tested. It bound poorly to vitamin D binding protein and the intestinal cytosol receptor. The thiovitamin showed no induction of calcium binding protein and exhibited no antagonistic effect to $1,25(OH)_2D_3$ action in the organ-cultured chick duodenum.

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