SYNTHESIS OF 25-HYDROXYCHOLECALCIFEROL, THE BIOLOGICALLY EFFECTIVE METABOLITE OF VITAMIN D_3

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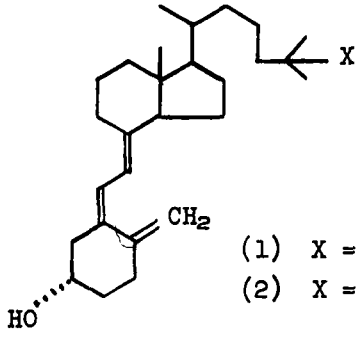
ABSTRACT

A synthesis of 25-hydroxycholecaliferol, the biologically active metabolite of vitamin D_3, is described.

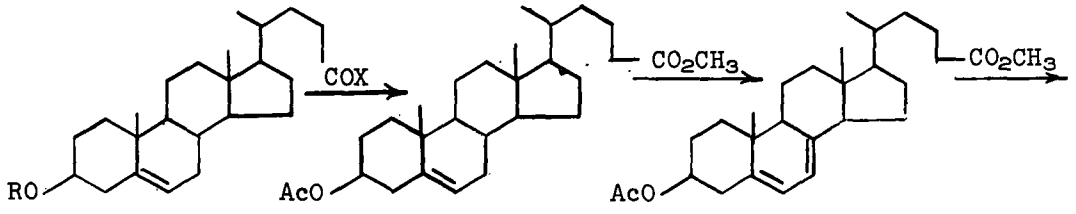
When tritium-labelled vitamin D_3 was administered to rachitic rats in physiologic amount and the intestinal mucosa fractionated into its subcellular components, some 50% of the mucosal radioactivity was found associated with the nuclear fraction.[1,2] Of this membrane-bound material, 80% or more was not vitamin D_3 but, instead, a metabolite, designated by DeLuca and co-workers as "Peak IV" because of its chromatographic behavior.[3] Blunt, DeLuca and Schnoes administered massive doses of vitamin D_3 to four hogs for 26 days and



(1) X = H
(2) X = OH

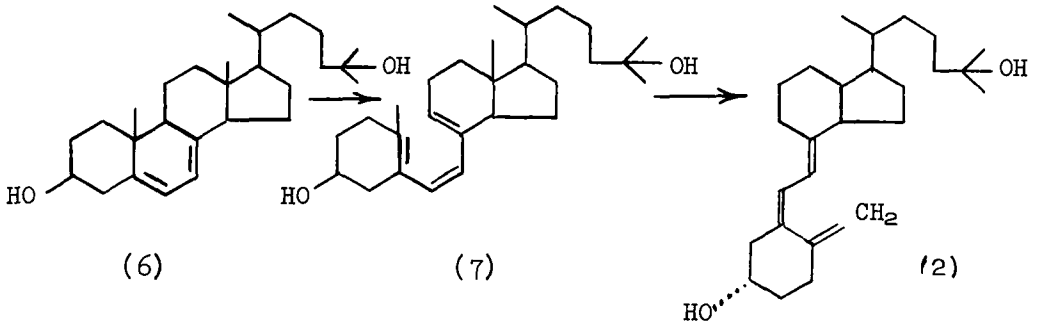
successfully isolated 1.3 mg. of the peak IV metabolite.[4,5] From its similarity to vitamin D_3 and from definitive differences in its NMR and mass-spectra, the metabolite was conclusively identified as 25-hydroxycholecaliferol (25-hydroxy vitamin D_3, 2).[5] This substance, more potent than vitamin D_3, is the biologically effective form of the vitamin and is the most potent anti-rachitic substance known.[1] The metabolite acts far more rapidly than the vitamin and is thought to induce the synthesis of calcium transport protein in the intestine and mineral mobilization systems in bone.[1] Its production appears to be altered in a number of clinical conditions associated with defects in calcium disposition.[6-8]

A number of synthetic approaches have been used to prepare vitamin D and its congeners.[9] We undertook the synthesis of the active metabolite (2), utilizing as starting material 3β-hydroxychol-5-enic acid (3a), a substance once relatively available as a by-product of oxidative cleavage of the side-chain of cholesterol.[10]



(3) (4) (5)

a R = H, X = OH
b R = Ac, X = OH
c R = Ac, X = Cl
d R = Ac, X = CHN_2



(6) (7) (2)

Preparation of 25-hydroxycholecalciferol (25-hydroxy vitamin D_3, 2) from this starting material can be visualized in two stages: elaboration of the side chain and manipulation of ring B to produce the vitamin D chromophore. The sequence of steps did not appear critical and acccordingly several variants were studied.

The hydroxycholenic acid (3a) was converted through its 3-acetate (3b) and acid chloride (3c) to the diazoketone (3d). Arndt-Eistert homologation with silver benzoate in methanol[11] proceeded smoothly to the 25-homoester (4).[12]

Since the conversion of Δ^5- to $\Delta^{5,7}$-steroids has been extensively studied,[9] we selected the procedure used earlier by Hunziker and Müllner[13] in the cholesterol series for the preparation of (5). Allylic bromination with 1,3-dibromo-5,5-dimethylhydantoin followed by dehydrobromination of the crude 7-bromosteroid with trimethylphosphite proved erratic, but the 5,7-diene (5) was obtained in about 20% yield. Chromatography over silver nitrate impregnated silica gel was customary at this point to remove starting material

and variable amounts of the $\Delta^{4,6}$-by-product although on occasion (5) could be purified by direct crystallization.

Elaboration of the side chain was completed by reaction with methylmagnesium bromide, affording 25-hydroxycholesterol[14] from (4) and 25-hydroxy-7-dehydrocholesterol (cholesta-5,7-diene-3β,25-diol, 6) from (5). Both compounds exhibited an intense singlet in the nmr at δ 1.21 for the 26- and 27-methyl groups as expected.

Irradiation[15] of (6) in benzene-ethanol for 15 minutes at 15-19° with a 100 watt Hanovia 8A 36 lamp, followed by chromatography, gave 25-hydroxyprecholecalciferol [9,10-secocholesta-5(10),6-cis,8-triene-3β,25-diol, 7] as an amorphous foam. Its structure was confirmed by its ultraviolet absorption (λ_{max} 256 mμ, ε 8300) and its nmr spectrum (AB quartet at about δ 5.72 and 5.95, J about 11 cps.,* for the protons at 6 and 7, and δ 5.52 for the proton at 9). The mass spectrum supported the molecular weight (M = 400) and lacked the intense peak at m/e 136 found both in cholecalciferol (1) and 25-hydroxycholecalciferol (2).[5] Gas-liquid chromatography of (7) afforded two peaks of similar area resulting from thermal rearrangement to the pyro and isopyro steroids as reported for cholecalciferol and its metabolite.[5]

The previtamin (7), heated to equilibrium for 3 1/2 hours at 70-75° in chloroform in a sealed tube, was isomerized

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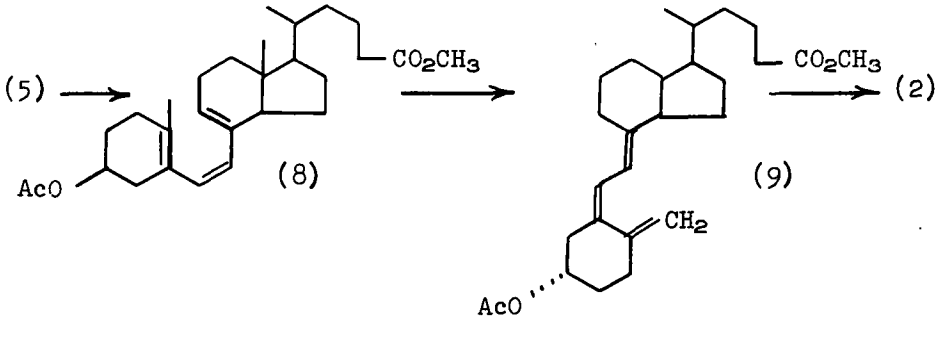
* An exact J value and chemical shift could not be assigned because the two outside weak signals of the multiplet were partly obscured by the proton at 9 and by signals from a small amount of 25-hydroxycholecalciferol (2) present in (7).

to 25-hydroxycholecalciferol (2). Purification by chromatography over Florisil and crystallization from aqueous methanol afforded (2) as a hydrate after drying under high vacuum for 25 hours at room temperature. The product exhibited ultraviolet absorption at 264 mμ and nmr and mass spectra consistent with those reported by Blunt, et al.[5] for 25-hydroxycholecalciferol isolated from hog blood. Gas-liquid chromatography afforded the same two peaks as were observed for (7), above, as expected.

The homo-ester (5) was also irradiated under the conditions described above for (6) to produce methyl 3β-hydroxy-9,10-seco-25-homochola-5(10), 6-cis, 8-trienate 3-acetate (8). After purification by chromatography the oily product exhibited the appropriate signals in the nmr (fig. 1b) and an ultraviolet absorption at 258 mμ. Thermal rearrangement of the oily ester (8) produced an equilibrium mixture of methyl 3β-hydroxy-9,10-seco-25-homochola-5-cis-7,10(19)-trienate 3-acetate (9) and (8) in a ratio of about 80:20



estimated from the nmr spectrum (fig. 1a). Finally, treatment of (9) with methylmagnesium bromide at room temperature

yielded 25-hydroxycholecalciferol (2), identical to the product obtained from (7) by the alternative sequence described above.

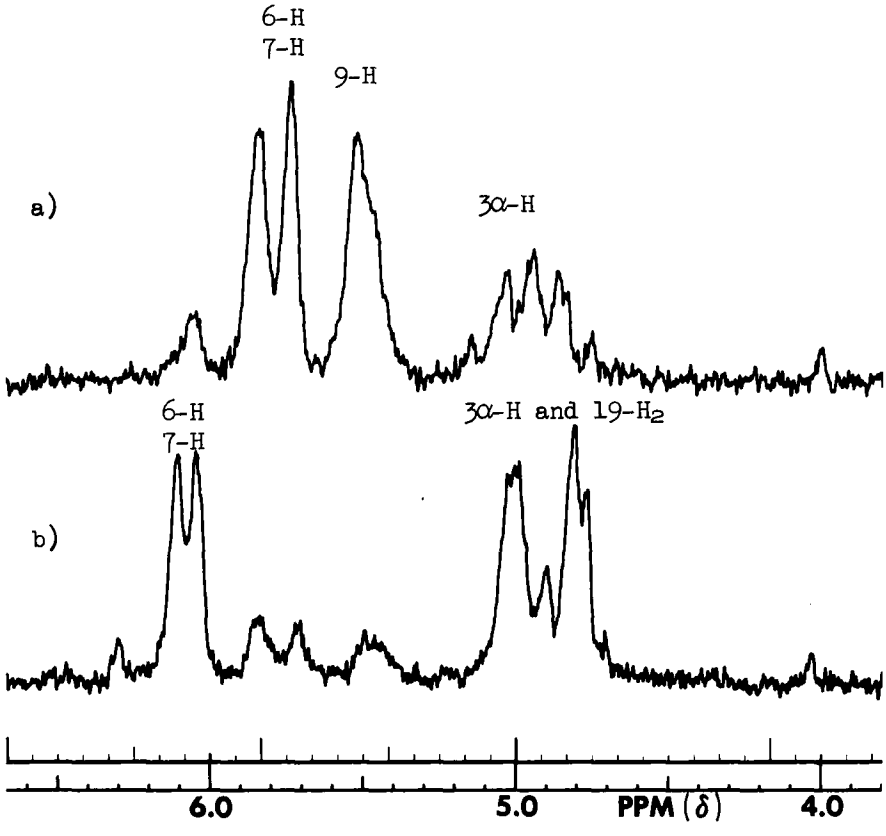


Fig. 1 a) nmr spectrum of (8); b) nmr spectrum of equilibrium mixture of (8) and (9).[16]

EXPERIMENTAL

Methyl 3β-hydroxy-25-homochol-5-enate 3-acetate (4). - To 95.8 g. of 3β-hydroxychol-5-enic acid (3a) in 600 ml. of pyridine cooled in an ice bath was added slowly with stirring 100 ml. of acetic anhydride, keeping the temperature at 10° or lower. The mixture was stirred overnight at room temperature and was diluted first with 25 ml. of water. After about 1 hour, the mixture was poured into 750 ml. of concentrated hydrochloric acid diluted to 2500 ml. with crushed ice. The crude product was filtered, washed thoroughly, and dried to give 104.3 g. of crude acetate. Ninety grams of the acetate was almost completely dissolved in 450 ml. of warm acetic acid plus 100 ml. of methylene chloride.

After filtration, the solution was allowed to cool slowly to room temperature to afford large crystals. The product was filtered, dried under suction and washed with water to give 49 g. of 3β-hydroxychol-5-enic acid 3-acetate (3b) m.p. 178-185°. An additional 12 g. was obtained from the liquors, m.p. 178-186°.

A slurry of 60 g. of 3β-hydroxychol-5-enic acid 3-acetate (3b) in 1.13 l. of benzene and 2.3 ml. of pyridine was cooled in an ice bath for 1/2 hr. during dropwise addition of 60 ml. of thionyl chloride. After 2 1/2 hrs., the solution was concentrated to near dryness, benzene was added, and the solution concentrated again and the process was repeated several times to remove all the thionyl chloride and hydrochloric acid.

The acid chloride (3c) without further purification, was dissolved in 600 ml. of benzene, filtered through glass wool and added dropwise to a stirred, ice-cold solution of diazomethane in ether (prepared from 50 g. of N-nitro-N-nitroso-N'methylguanidine, 400 ml. of 23% potassium hydroxide and about 900 ml. of ether; three 50 g. batches were prepared and combined for this run).

About 1/2 hr. after the addition was completed the reaction mixture was concentrated on the rotary evaporator. The residue (78 g., 3d), which showed a band in the infrared (at 2100 cm^{-1}) characteristic of a diazoketone, was dissolved in 700 ml. of methylene chloride and 935 ml. of methanol and stirred at room temperature. A solution of 5 g. of silver benzoate[11] in 50 ml. of triethylamine was added in about 0.5 ml. portions at intervals often enough to maintain evolution of gas. After about half of the silver benzoate solution had been added the reaction was complete as judged by thin layer analysis. Water was added and the product was extracted with methylene chloride. The extracts were washed with dil. hydrochloric acid, water and sodium bicarbonate solution and again with water. The solution was dried over sodium sulfate, filtered and concentrated to a solid. The crude ester was chromatographed through a 3 kg. Florisil column. The column was eluted by gradient elution between Skellysolve B and 10% ethyl acetate-Skellysolve B. About 65 l. of solvent was used. The fractions containing pure methyl 3β-hydroxy-25-homochol-5-enate 3-acetate (4) were combined and recrystallized from methylene chloride-methanol, yield 44 g., m.p. 110-112.5°, $[\alpha]_D$ -45° ($CHCl_3$); nmr and ir support the proposed structure.

Anal. Calcd. for $C_{28}H_{44}O_4$: C, 75.63; H, 9.97. Found: C, 75.45; H, 9.83.

Methyl 3β-hydroxy-25-homochola-5,7-dienate 3-acetate (5). - A solution of 2.22 g. of methyl 3-hydroxy-25-homochol-5-enate 3-acetate (4) in 20 ml. of petroleum ether, and 15 ml.

of benzene was heated at reflux and 0.82 g. of 1,3-dibromo-5,5-dimethylhydantoin was added. After refluxing for 1/2 hr. the solution was cooled and the precipitate of 5,5-dimethylhydantoin was filtered. The filtrate was concentrated to give the crude 7-bromo compound as a heavy oil. It was dissolved in 8 ml. of xylene (dried over a molecular sieve) and added dropwise to a refluxing solution of 2 ml. of trimethylphosphite in 10 ml. of xylene. After heating under N_2 for about 1 hr., the solvent was removed and the residue was chromatographed through Florisil. The fractions were assayed by tlc on silica gel plates impregnated with silver nitrate. The product (5) was recrystallized from methylene chloride-methanol, yield 260 mg., m.p. 130-134°, λ_{max}^{alc} 271 mμ (ε = 10,450), 282 (ε = 11,000), 293 (ε = 6,250); ir, 1735 (C=O), 1650, 1600 (C=C); nmr ($CDCl_3$), 0.62 (C-18-H_3), 0.96 (C-19-H_3), 0.98 (d, C-21-H_3), 2.08 ($COCH_3$), 3.67 (OCH_3), 5.52 (q, J=6 cps, C-6 and C-7-H_2).

Anal. Calcd. for $C_{28}H_{42}O_4$ (432.54): C, 75.97; H, 9.56. Found: C, 75.73; H, 9.47.

Cholesta-5,7-diene-3β,25-diol (6). - To a solution of 2.0 g. of the ester (5) in 100 ml. of dry ether was added 20 ml. of 3M ethereal methylmagnesium bromide. After standing at room temperature overnight ammonium chloride solution was added. The ether was removed under a stream of N_2 and the product (6) collected on a filter, washed well with water, dried and recrystallized from methylene chloride-methanol, yield 1.15 g., m.p. 185-187°, λ_{max}^{alc} 271 mμ (ε = 9,850), 281 (ε = 10,450), 293 (ε = 6,100); ir 3310, 3360 (OH), 1600, 1650 (C=C); nmr ($CDCl_3$); δ 0.66 (C-18, H_3), 0.98 (C-19, H_3), 1.0 (d, C-21, H_3), 1.21 (C-25, C-26, H_6), 5.50 (q, J=6 cps C-6 and C-7-H_2).

Anal. Calcd. for $C_{27}H_{44}O_2 \cdot 1/2\ H_2O$: C, 79.16; H, 11.07. Found: C, 79.08; H, 11.09.

Cholest-5-ene-3β,25-diol. - In similar manner, 4 g. of the ester (4) in 80 ml. of ether and 40 ml. of 3M ethereal methylmagnesium bromide afforded, after chromatography and recrystallization from acetone, 2.55 g. of 25-hydroxycholesterol,[14] m.p. 179-183°, nmr ($CDCl_3$); δ 0.71 (C-18-H_3), 1.02 (C-21-H_3), 1.22 (C-26 and C-27-H_6), 5.49 (d, C-6-H).

25-Hydroxyprecholecalciferol (9,10-secocholesta-5(10),6-cis, 8-triene-3β,25-diol, 7). - Solution of 125 mg. of cholesta-5,7-diene-3β,25-diol (6) in 125 ml. of benzene and 10 ml. of absolute ethanol was placed in a photo reactor equipped with a quartz lampwell cooled with water and a nitrogen inlet to ebulate and keep out air. The reaction mixture was cooled to 16° C. and purged with N_2. A Hanovia 8A36, 100 watt lamp was turned on for 15 min., including the 5-6 min. required for the lamp to reach full brilliance. A fast stream

of water was necessary to keep the outlet water temperature below 20° C.

The reaction mixture was then concentrated to dryness in a rotary evaporator below room temperature. The semisolid residue was triturated with 5 ml. of 35% ethyl acetate-65% Skellysolve B mixture and filtered and another 5 ml. was used for wash. The solid contained unreacted starting material and the liquor contained the product. The liquor was poured on a 40 g. Florisil column (150 mesh, tlc grade) packed wet with 35% ethyl acetate-Skellysolve B and the products were eluted with the same solvent mixture. Each fraction was 10 ml. and the product was located by spotting on a tlc plate. Fractions 15-19 were combined and evaporated to dryness on a rotary evaporator, warming with a cold water bath. A few drops of absolute ether were added and then removed under vacuum. The residue of 25-hydroxyprecholecalciferol (7) fluffed into a solid foam, yield 60 mg., λ_{max}^{alc} 256 mμ (ε = 8,300), m.s. m/e 400 (M^+), 385, 382, 380, 376, 154, 136, 118; nmr ($CDCl_3$), δ 0.71 (C-18-CH_3), 0.97 (d, C-21-H_3), 1.20 (C-25 and C-26-H_6), 1.63 (C-19-H_3), 3.90 (m, C-3-H), 5.54 (C-9-H), 5.54, 5.78, 5.89, and 6.10 (q, C-6 and C-7-H_2); glc (6 ft. OV-17 column at 230°, injection port 240°) shows two peaks with retention times of 49 and 58 min.

Methyl 3β-hydroxy-9,10-seco-25-homochola-5(10),6-cis,8-trienate 3-acetate (8). - A solution of 0.25 g. of methyl 3β-hydroxy-25-homochola-5,7-dienate 3-acetate (5) in 125 ml. of benzene was irradiated as described above. The solvent was evaporated and the residue, combined with a similar run, was chromatographed through a 60 g. Florisil column as described previously, but using 8% ethyl acetate-Skellysolve B. The seco-steroid (8) was isolated as an oil, λ_{max} 258 mμ (ε = 11,800); ir 1740 (C=O); 1435, 1375, 1360 (C-H), 1245, 1165, 1030 (C-O); nmr ($CDCl_3$) δ 0.66 (s, C-18-H_3), 0.95 (d, C-21-H_3), 1.61 (s, C-19-H_3), 1.98 (s, $COCH_3$), 3.62 (s, OCH_3), 4.93 (m, C-3-H), 5.50 (s, C-9-H), 5.7 (d, J=about 11), 5.9 (d, J=about 11). (See discussion section and Fig. 1b).

Methyl 3β-hydroxy-9,10-seco-25-homochola-5-cis,7,10(19)-trienate 3-acetate (9c). - A solution of 0.22 g. of the triene (8) in 5 ml. of chloroform was heated at 70-75° C. for 3 1/2 hrs. in a sealed tube. The solvent was evaporated and the residue was chromatographed through Florisil to give 90 mg. of oily product, λ_{max}^{alc} 264 mμ (ε = 14,450) ir 1740 (C=O), 1645, 1630 (C=C), 910, 890 (=CH_2); nmr ($CDCl_3$), 0.52 (s, C-18-H_3), 0.93 (d, C-21,H_3), 2.00 (s, CH_3CO), 3.58 (s, OCH_3), 4.71 and 4.96 (d, =CH_2), 4.85 (m, C-3-H), 6.0 (d, J=11 C-6 or C-7, H), 6.2 (d, J=11, C-6 or C-7, H). (See Fig. 1a and discussion section). A small peak at δ 0.70 indicates

that about 20% of the starting material (8) is still present. This sample was heated further in an nmr tube and no change in the ratio of 8 to 9 was observed. Thin-layer chromatography on silica gel plates impregnated with silver nitrate developed with 15% ethyl acetate-85% Skellysolve B separated the two components with the $\Delta^{5,7,10(19)}$ compound moving the slowest.

25-Hydroxycholecalciferol (2). - from cholesta-5,7-diene-3β,25-diol (6). - Five 125 mg. batches of cholesta-5,7-diene-3β,25-diol (6) were irradiated as above and combined. The residue was triturated with 10 ml. of 35% ethyl acetate-65% Skellysolve B and filtered and an additional 5 ml. of solvent mixture was used for washing. The filtrate and wash containing the previtamin (7) were combined, concentrated to dryness, dissolved in 5 ml. of chloroform, and heated for 3 1/2 hrs. at 70-75° under N_2 in a sealed flask. The solvent was evaporated and the residue was chromatographed through Florisil. The fractions which crystallized on trituration with aqueous methanol were combined and recrystallized twice from aqueous methanol to give 25-hydroxycholecalciferol (2) as a hydrate, yield 120 mg., m.p. 81-83° (sinters 75°), uv λ_{max} 264 mμ (ε = 17,400). Mass spec: same as reported by Blunt, et al.[5] NMR ($CDCl_3$), 0.55 (C-18, H_3), 0.96 (d, C-21, H_3), 1.0 (C-25 and C-26, Hε), 3.93 (m, C-3-H), 4.83 and 5.07 (C-19, H_2), 6.09 (d, J=11, C6 or 7-H), 6.23 (d, J=11, C6 or 7-H); glc under same conditions used for 7 above also shows two peaks with retention times of 49 and 58 min.

Anal. Calcd. for $C_{27}H_{44}O_2 \cdot H_2O$: C, 77.46; H, 11.48; H_2O, 4.32%. Found C, 76.73; H, 11.13; H_2O, 4.95%.

From methyl 3β-hydroxy-9(10)-seco-25-homochola-5-cis-7,10(19)-trienate 3-acetate (9). A solution of about 20 mg. of (9) in 2 ml. of 3M ethereal methylmagnesium bromide was kept at room temperature overnight. Ammonium chloride solution was added and the product was isolated with ether and chromatographed through Florisil. Recrystallization from aqueous methanol gave 4 mg. of 25-hydroxycholecalciferol (2) identical to the product described above by tlc, uv and mass spectrum.

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16. Except where otherwise noted uv spectra were run in 95% ethanol using a Carey 15 instrument, nmr in deuterochloroform on a Varian A60A spectrometer and mass spectrum on a Varian-MAT-CH-4 spectrometer equipped with a TO-4 source with ionizing voltage of 70 e.v.

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