AN IMPROVED PROCEDURE FOR THE ISOLATION OF SUPRASTEROL₂ I AND II FROM A PHOTOCHEMICAL REACTION MIXTURE OF ERGOCALCIFEROL (VITAMIN D₂)^{1,2}

Tadashi KOBAYASHI, Sachiko YOSHIMOTO, and Mitsue YASUMURA³

*Department of Hygienic Chemistry, Kobe Women's College of Pharmacy,
Higashinada-ku, Kobe 658, Japan*

(Received February 21, 1977)

Summary An improved procedure for the isolation of suprasterol₂ I and II from a photochemical reaction mixture of ergocalciferol (vitamin D₂) and their spectral data are described in this paper. When a solution of ergocalciferol in ethanol was irradiated by UV light from a high-pressure mercury lamp, the reaction mixture gave six spots, including suprasterol₂ I and II, on the thin-layer chromatogram, while the peaks corresponding to pyro-D₂, isopyro-D₂, 5,6-*trans*-D₂, suprasterol₂ I and II were observed in the gas chromatogram obtained from a capillary column GLC (Suprasterol₂ I and II were main peaks). After purifying the mixture by column chromatography on silica gel containing 12% alumina as an adsorbent, two main fractions were isolated. The data of their spectra, TLC and GLC showed that the former fraction was suprasterol₂ II while the latter was suprasterol₂ I and that the both fractions contained the respective compound only. Both suprasterol₂ were crystallized as the 3,5-dinitrobenzoates.

The effect of wavelength on the photochemical reaction of ergocalciferol (vitamin D₂) was reported in a previous paper (1). The results showed that UV light in a range of 295–312 nm was most effective on the photochemical transformation of ergocalciferol into suprasterol₂ I and II while that in the other range had little effects on the transformation but no other special reaction occurred. In this work, the isolation of the reaction products was investigated and suprasterol₂

¹ Studies on the ultraviolet irradiation of vitamin D. Part II. Part I, see Ref. 1).

² Following abbreviations are used: pyro- and isopyro-D₂, pyro- and isopyroergocalciferol; 5,6-*trans*-D₂, 5,6-*trans*-ergocalciferol; CA, cholesteryl acetate; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; UV, ultraviolet; IR, infrared; NMR, proton nuclear magnetic resonance; TMS, tetramethylsilane for NMR and trimethylsilyl for GLC.

³ 小林 正, 吉本佐雅子, 康村満枝

I and II were isolated from a reaction mixture of ergocalciferol irradiated with an ordinary high-pressure mercury lamp as a light source.

Suprasterol₂ I and II were first isolated as allophanates from a long-term irradiated mixture of ergosterol by WINDAUS *et al.* (2). WESTERHOF and KEVERLING BUISMAN (3) also isolated them as allophanates from an irradiated mixture of ergocalciferol and then confirmed that their direct origin was not ergosterol but ergocalciferol. However, the isolation method took two or three weeks to obtain the crystals. Therefore, we investigated the use of column chromatography to find more a convenient procedure for the isolation. After trying various kinds of column chromatography, we found that the one with use of silica gel containing 12% alumina as an adsorbent according to MERMET-BOUVIER (4) gave good results for the separation among suprasterol₂ I, II and other substances. In this paper, the improved procedure and the spectral data of suprasterol₂ I and II obtained thus are described.

EXPERIMENTAL

UV absorption spectra were obtained on a Hitachi 323 spectrophotometer with ethanol as a solvent; IR spectra were obtained on a Shimadzu IR-27G infracord spectrophotometer with CCl₄ as a solvent; NMR spectra were obtained on a Varian A-60D spectrometer (60 MHz) with CDCl₃ as a solvent and tetramethylsilane (TMS) was used as an internal standard; mass spectra were recorded on a double focus high-resolution spectrometer (Japan Electron Optics JMS-01SG) equipped with a direct inlet system. Melting points were estimated in open capillary tubes and are uncorrected.

Capillary column gas-liquid chromatography (GLC) and thin-layer chromatography. Capillary column GLC and TLC were carried out according to the directions as described in the foregoing paper (1).

Ultraviolet (UV) irradiation of an ergocalciferol solution in ethanol. A solution of ergocalciferol (4 g) in ethanol (400 ml) was put in a photochemical reaction apparatus of Taika Ind. Co. and irradiated for 6 hr with a high-pressure mercury lamp (200 W) with stirring by bubbles of nitrogen gas. The temperature of the solution was kept below 20°C during irradiation and no filter solution was used. The same procedure was repeated once again on another ergocalciferol (4 g) solution in ethanol (400 ml). The irradiated solution was joined with the first solution and then evaporated to dryness under reduced pressure to obtain 8 g of slightly yellow oil.

Suprasterol₂ I. The oil obtained above was dissolved in about 20 ml of 50% ethyl ether in *n*-hexane and then applied to a column (3 × 50 cm) filled with silica gel containing 12% alumina (The mixed adsorbent was purchased from Sigma Chem. Co.) (4). The column was developed with 50% ethyl ether in *n*-hexane and each 20 ml fraction was collected. The suprasterol₂ I fractions (fractions no.37-47)

detected by the capillary column GLC and TLC were collected and evaporated to dryness under reduced pressure to obtain a colorless oil. Yield 0.6 g; UV spectrum, no absorption maximum in the range higher than 210 nm; IR (cm^{-1}), 3625 (3-OH), 1030 (3- >C-OH), 968 (22,23- H-C=C-H); NMR (δ), 0.78 (s, 18- CH_3), 0.83 (d, $J=6.1$ Hz, 26 and 27- CH_3), 0.92 (d, $J=5.5$ Hz, 21 or 28- CH_3), 1.03 (d, $J=6.1$ Hz, 21 or 28- CH_3), 3.96 (1H, m, 3-CH), 5.22 (2H, m, 22,23- CH=CH-); mass spectrum (m/e), 396 (M^+), 378 ($\text{M-H}_2\text{O}$), 271 (M-side-chain), 253 ($\text{M-side-chain-H}_2\text{O}$), 136, 118; $[\alpha]_{\text{D}}^{25} (\text{CHCl}_3) -53.2^\circ$.

Suprasterol₂ I-3,5-dinitrobenzoate. Suprasterol₂ I (0.2 g) was dissolved in a mixture of benzene (10 ml) and pyridine (10 ml) and then a solution of 3,5-dinitrobenzoyl chloride (0.4 g) in benzene (20 ml) was added to the sterol solution. After stirring for 1 hr at room temperature, the mixed solution was left overnight. The solution was mixed with 2% sodium carbonate solution (70 ml) and then extracted with peroxide free ethyl ether (50 ml $\times$ 2). The extracts were successively washed with 2% sodium carbonate solution, water, 1 N hydrochloric acid and water. After drying over anhydrous sodium sulfate, the extracts were evaporated to dryness under reduced pressure. The resulting yellow oil gave slightly yellow crystals of suprasterol₂ I-3,5-dinitrobenzoate after crystallization from acetone. Yield 0.1 g; mp 146–147°C; mass spectrum (m/e), 590.335 (M^+ , $\text{C}_{35}\text{H}_{46}\text{N}_2\text{O}_6$ requires 590.335); Anal. Calcd. for $\text{C}_{35}\text{H}_{46}\text{N}_2\text{O}_6$, C 71.16, H 7.85, N 4.74, Found, C 70.63, H 7.64, N 4.58.

Suprasterol₂ II. On the column chromatography described above in the part of suprasterol₂ I, the fractions no. 19–23 were the suprasterol₂ II fractions. The fractions were combined and then evaporated to dryness under reduced pressure to give a colorless oil. Yield 3.4 g. The resulting oil was crystallized from acetone to give colorless crystals; mp 110°C; UV spectrum, no absorption maximum in the range higher than 210 nm; IR (cm^{-1}), 3650 (3-OH), 1030 (3- >C-OH), 968 (22,23- H-C=C-H); NMR (δ), 0.78 (s, 18- CH_3), 0.83 (d, $J=5.5$ Hz, 26 and 27- CH_3), 0.92 (d, $J=4.5$ Hz, 21 or 28- CH_3), 1.02 (d, $J=6.3$ Hz, 21 or 28- CH_3), 1.52 (1H, s, 3-OH), 4.03 (1H, m, 3-CH), 5.22 (2H, m, 22,23- CH=CH-); mass spectrum (m/e), 396 (M^+), 378 ($\text{M-H}_2\text{O}$), 271 (M-side-chain), 253 ($\text{M-side-chain-H}_2\text{O}$), 136 (see Fig. 5), 118; $[\alpha]_{\text{D}}^{25} (\text{CHCl}_3) +47.8^\circ$; Anal. Calcd. for $\text{C}_{28}\text{H}_{44}\text{O}$, C 84.78, H 11.18, Found, C 84.74, H 10.98.

Suprasterol₂ II-3,5-dinitrobenzoate. Suprasterol₂ II (0.2 g) was similarly esterified according to the direction as described in the part of suprasterol₂ I-3,5-dinitrobenzoate to give slightly yellow crystals of suprasterol₂ II-3,5-dinitrobenzoate. Yield 0.1 g; mp 141–142°C; mass spectrum (m/e), 590.334 (M^+ , $\text{C}_{35}\text{H}_{46}\text{N}_2\text{O}_6$ requires 590.335); Anal. Calcd. for $\text{C}_{35}\text{H}_{46}\text{N}_2\text{O}_6$, C 71.16, H 7.85, N 4.74, Found, C 71.28, H 7.70, N 4.46.

RESULTS AND DISCUSSIONS

An UV irradiated mixture of ergocalciferol (8 g) was obtained by treating the vitamin according to the procedure described in EXPERIMENTAL. This crude mixture gave the two main and four small spots on the thin-layer chromatogram as shown in Fig. 1, while the five main peaks corresponding to the TMS ethers of

Fig. 1. Thin-layer chromatograms of ergocalciferol, photochemical reaction mixture of ergocalciferol and the fractions obtained by the column chromatography. D₂, ergocalciferol (standard); crude, photochemical reaction mixture of ergocalciferol; fr., fraction numbers.

pyro-D₂, suprasterol₂ II, suprasterol₂ I, 5,6-*trans*-D₂ and isopyro-D₂ (according to the order of retention times) were observed on the gas chromatogram as shown in the top side of Fig. 2.

In order to purify the products, the crude mixture was applied to the column chromatography as described in EXPERIMENTAL and the eluate was fractionated into each 20 ml. The fractions were divided into the groups showing same thin-layer chromatograms and then evaporated to dryness under reduced pressure. The thin-layer chromatograms and yield of the resulting groups are shown in Fig. 1 and Table 1, respectively. The two groups of fractions 19–23 and 37–47 gave the respective mono spot on the thin-layer chromatogram (Fig. 1). When the two groups were applied to the capillary column GLC according to EXPERIMENTAL, the respective mono peak was observed as shown in the middle and bottom sides of Fig. 2. The results of TLC and GLC supported that the two groups contained each an isolated compound. They were tentatively denoted as the products A (fractions 19–23) and B (fractions 37–47). Product A was crystallized

Fig. 2. Gas chromatograms of photochemical reaction mixtures of ergocalciferol, products A (fractions 19-23) and B (fractions 37-47). The numbers in the gas chromatograms mean the TMS ethers of the following compounds while CA means cholesteryl acetate (internal standard): 1, pyro-D₂; 2, supratherol₂ II (product A); 3, supratherol₂ I (product B); 4, 5,6-*trans*-D₂; 5, isopyro-D₂.

Table 1. Yield of each fraction group obtained from the column chromatography of an UV-irradiated mixture of ergocalciferol (8 g).

Fraction no.	Yield (mg)	<i>R_f</i> (TLC)
1-5	50	0.96,
6-8	48	0.96, 0.89
9-11	37	0.89, 0.84
12-15	77	0.89, 0.84, 0.75
16-18	1,279	0.75, 0.57
19-23 (product A)	3,405	0.57
24-36	2,311	0.57, 0.43
37-47 (product B)	609	0.43

from acetone while product B could not be crystallized. When they were esterified with 3,5-dinitrobenzoyl chloride, however, both the esters could be crystallized from acetone.

Neither the product A nor B gives absorption maximum above 210 nm in the UV spectra. The IR, NMR and mass spectra of the product A are practically

Fig. 3. IR spectrum of supratherol₂ II. Fig. 4. NMR spectrum of supratherol₂ II. TMS: tetramethylsilane. Fig. 5. Mass spectrum of supratherol₂ II.

same as the corresponding spectra of the product B, but the optical rotations that the product A is dextrorotatory ($[\alpha]_D^{22}$ in CHCl_3 : $+47.8^\circ$) while the product B is levorotatory ($[\alpha]_D^{22}$ in CHCl_3 : -53.2°) are quite different one another. These results showed that the products A and B should be optical isomers one another. The IR, NMR and mass spectra of the product A (suprasterol₂ II) chosen as a representative of the two compounds are shown in Fig. 3, 4 and 5, respectively. The UV spectra showing no absorption maximum above 210 nm are a characteristic of suprasterols and the IR and NMR spectra agreed with the corresponding spectra of suprasterol₃ reported by BOOMSMA (5) except the peaks derived from the differences of the side-chain structures. The molecular peaks (396) in the mass spectra of the products A and B agreed with those of suprasterol₂ and the fragment peaks of 378, 271 and 253 were observed as $\text{M}-\text{H}_2\text{O}$, $\text{M}-\text{side-chain}$ and $\text{M}-\text{side-chain}-\text{H}_2\text{O}$ in the both spectra, respectively. The fragment peaks of 136 and 118 were obtained from the fragmentation shown in Fig. 5 and the dehydration of the fragment ion, respectively. The fragmentation patterns of mass spectra agreed with those of suprasterol₃ reported by BOOMSMA (5) and OKAMURA *et al.* (6) except M^+ and $\text{M}-\text{H}_2\text{O}$ due to the differences of side-chain structures. All of the spectral data supported that the products A and B were suprasterols. WINDAUS *et al.* (2) designated for the optical isomers of suprasterol₂ that the one showing levorotatory was suprasterol₂ I while the other showing dextrorotatory was suprasterol₂ II. It was concluded from these results and designations that the products A and B were suprasterol₂ II and I, respectively. The data of their 3,5-dinitrobenzoates also supported the structures. The chemical structures of suprasterol₂ I and II are shown in Fig. 6.

Fig. 6. Chemical structures of suprasterol₂ I and II.

The column chromatography with use of either alumina or silica gel alone as an adsorbent had been first investigated for this purpose, but satisfactory separation between suprasterol₂ I and II was not obtained although various kinds of developing solvents were used. Then, we noticed the mixed adsorbent of silica gel containing 12% alumina reported by MERMET-BOUVIER (4). Since the adsorbent gave good separations to the photochemical isomers obtained from the UV irradiation of ergosterol, it was expected to be useful for the separation of supra-

sterols, too. The results were quite satisfied as mentioned above. Therefore, the method is presently proposed as an improved one for the isolation of supra-sterol₂ I and II from the photochemical reaction mixture of ergocalciferol.

BAKKER *et al.* (7) isolated the four novel reaction products, tentatively denoted as the compounds I₁, I₂, II₁ and II₂ [Their chemical structures are referred to in Fig. 1 of a previous paper (1)], besides supra-sterol₃ I and II from the UV irradiated mixture of cholecalciferol. However, the compounds could not be isolated in this experiment because they were too small to isolate. The reason is not understood, but it may be due to the difference of irradiation conditions. The investigations to clarify this problem will follow in future.

The authors wish to thank Miss H. Endo, Miss M. Sugiura and Miss K. Saiki of Kobe Women's College of Pharmacy for making elemental analysis, NMR and mass spectra measurements.

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