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Anticomplementary Activity of Ergosterol Peroxide from *Naematoloma fasciculare* and Reassignment of NMR Data

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A very high content (at least 0.23%) of ergosterol peroxide was isolated from *Naematoloma fasciculare* Karst. Not only ergosterol peroxide but also ergosterol showed very strong anticomplementary activity on the classical pathway, the IC_{50} values being 5.0 μM and 1.0 μM, respectively. The ^{1}H and ^{13}C NMR data of ergosterol peroxide were revised and completely assigned by DEPT, ^{1}H-^{1}H COSY, HMQC and HMBC correlations.

Key words : *Naematoloma fasciculare*, Anticomplementary activity, Ergosterol, Ergosterol peroxide, ^{1}H NMR, ^{13}C NMR, HMBC

INTRODUCTION

The complement system consists of over 20 serum proteins which are activated by a cascade mechanism of classical (CP) or alternative pathway (AP). It plays an important role in host defence, inflammation, and allergic reactions (Kimball, 1990; Kuby, 1994). The activation of the complement releases $C3_a$, $C4_a$, and $C5_a$, and they result in organ transplant rejection (Miyagawa *et al.*, 1988), asthma, allergy, atopy's dermatitis (Rother *et al.*, 1985; Strunk *et al.*, 1988; Alexander *et al.*, 1988; Mcgeer *et al.*, 1995), arthritis (Sato *et al.*, 1993; Corvetta *et al.*, 1992), systemic lupus erythematosus (Takematsu *et al.*, 1991) and adult respiratory distress syndrome (Wortel *et al.*, 1993).

Recently, we screened anticomplementary activity of the extracts of various natural products and found an active substance from *Naematoloma fasciculare*, a toxic mushroom which grows on the stumps of old trees in tufts and widely distributed in the world (Lee, 1988). It was elucidated as 3β-hydroxy-5,8-epidioxy-ergosta-6,22-diene (ergosterol peroxide).

Ergosterol and ergosterol peroxide are mainly isolated from fungi (Takaishi *et al.*, 1992) and lichens (Takaishi *et al.*, 1972), and have antiviral (Lindequist *et al.*, 1989) or antitumor action (Kahlos *et al.*, 1989). However their effects on the complement system have not been reported so far. In this paper, we report that ergosterol and erogsterol peroxide have strong anti-complementary activity. In addition, ^{13}C NMR data of ergosterol peroxide in the literature (Kahlos *et al.*, 1989; Takaishi *et al.*, 1991; Yu *et al.*, 1994) reported have not been correlated. In additon, further reliable evidence for each set of data has not been presented for any of them. Therefore we also propose fully assigned ^{1}H and ^{13}C NMR data by 2D NMR techniques.

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MATERIALS AND METHODS

NMR: Bruker AMX-400; Mps: Electrothermal 9100 (uncorr); Optical rotation: JASCO DIP-370; EIMS: Varian Mat 212MS; Column chromatography was carried out on Kieselgel 60 (70-230 mesh, Merck). ^{1}H NMR and ^{13}C NMR chemical shifts were referenced to $CDCl_3$ (7.24 ppm and 77.00 ppm, respectively). The ergosterol used for testing on anticomplement assay was purchased from Sigma Co. and recrystallized in MeOH.

Plant material

Naematoloma fasciculare was collected in the mountain in Taejon, Korea, in October, 1995 and identified by professor Kwang-Soo Shin., Dept. of Microbiology, Taejon Univ., Taejon, Korea.

Preparation of samples for Anticomplementary activity assay

Each sample was dissolved in acetone and diluted with buffer (final concentration of acetone, 1.0%).

Determination of anticomplementary activity on classical pathway of complement system

Anticomplementary activity was determined by the modified method of Mayer (Kabat and Mayer, 1961) as described previously (Oh *et al.*, 1996). A diluted solution of normal human serum (80 μl) was mixed with gelatin veronal buffer (80 μl) with or without a sample. The mixture was preincubated at 37°C for 30 min and sensitized sheep red blood cell (40 μl) was added. After incubation under the same conditions, it was centrifuged (800×g, 4°C, 4 min) and optical density of supernatant (100 μl) was measured at 405 nm. Anticomplementary activity was determined as a mean of triplicates.

Extraction and isolation

The dried *Naematoloma fasciculare* (10 g) was cut into pieces and extracted with MeOH (3×1 l). The MeOH extract was concentrated under reduced pressure to give a residue (940 mg). The residue was partitioned between EtOAc and water, and the EtOAc layer (530 mg) was chromatographed over a series of silica gel columns using $CHCl_3$-MeOH (10:1) and hexane-EtOAc (2:1) according to the activity-guided separation method to give ergosterol peroxide (23 mg).

Ergosterol peroxide (3β-hydroxy-5,8-epidioxyergosta-6,22-diene)

Needles, mp 179-182°C, EI-MS (*m/z*) 428 $[M^+]$, 410 $[M-H_2O]^+$, 396 $[M-O_2]$, 392, 376, 249, 107, 69. ^1H NMR (400 MHz, $CDCl_3$) see Table I; ^{13}C NMR (100 MHz, $CDCl_3$) see Table II.

RESULTS AND DISCUSSION

Compared with other natural sources (at most 0.001%) (Takaishi *et al.*, 1992; Takaishi *et al.*, 1972; Kahlos *et al.*, 1989; Takaishi *et al.*, 1991; Yu *et al.*, 1994), a very large amount of ergosterol peroxide (0.23%) was isolated from *Naematoloma fasciculare* Karst.

^1H-^1H COSY, DEPT, HMQC and HMBC were measured for detailed analyses of ^1H and ^{13}C NMR data.

In the ^1H and ^{13}C NMR, the methyl proton signals of ergosterol peroxide showed good signal separation because it has $\triangle^{22}$ unsaturation, and all these data were consistent with the literature (Gunatilaka *et al.*, 1981; Kahlos *et al.*, 1989; Takaishi *et al.*, 1991; Yu *et*

Table I. ^1H NMR Data of Ergosterol Peroxide ($CDCl_3$)

	Yu *et al.* (1994)	Reassignment[a]
H-1	-	1.71 (dd, 13.5, 3.1), 1.98 (dd, 13.5, 3.1)
H-2	-	1.55 (dd-like), 1.85 (dd-like)
H-3	3.97 (m)	3.98 (m)
H-4	-	1.94 (dd-like), 2.11 (dd-like)
H-6	6.24 (d, 8.4)[b]	6.51 (d, 8.6)
H-7	6.50 (d, 8.4)[b]	6.25 (d, 8.6)
H-9	-	1.50 (dd-like)
H-11	-	1.22 (m), 1.53 (m)
H-12	-	1.25 (m), 1.96 (m)
H-14	-	1.57 (m)
H-15	-	1.40 (m), 1.65 (m)
H-16	-	1.35 (m), 1.80 (m)
H-17	-	1.24 (m)
H-18	0.82 (s)	0.83 (s)
H-19	0.88 (s)	0.89 (s)
H-20	-	2.03 (m)
H-21	1.00 (d, 6.6)	1.00 (d, 6.6)
H-22	5.23 (dd, 15.3, 7.0)[c]	5.15 (dd, 15.2, 7.7)
H-23	5.14 (dd, 15.3, 7.8)[c]	5.22 (dd, 15.2, 8.2)
H-24	-	1.85 (m)
H-25	-	1.50 (m)
H-26	0.82 (d, 6.3)	0.82 (d, 6.7)
H-27	0.83 (d, 6.5)	0.84 (d, 6.7)
H-28	0.91 (d, 6.8)	0.91 (d, 6.7)

Figures in parenthesis are coupling constants in Hz.
[a]It was carried out by means of HMQC, ^1H-^1H COSY and HMBC.
[b,c]The assignments indicate that data of the same alphabet were reversed.

Table II. ^{13}C NMR Data of Ergosterol Peroxide ($CDCl_3$)

	Kahlos *et al.*	Yu *et al.*	Reassignment[a]
C-1	39.4[b]	34.7	35.06
C-2	30.1	30.2	30.47
C-3	66.3	66.5	66.84
C-4	51.2[b]	37.0	37.29
C-5	79.4[c]	79.5[b]	83.10
C-6	130.7[d]	130.8[c]	135.80
C-7	135.4[d]	135.5[c]	131.12
C-8	82.7[c]	82.2[b]	79.82
C-9	34.7[b]	51.7[d]	51.43
C-10	36.9[b]	37.0	37.33
C-11	20.9e	20.7[e]	23.77
C-12	39.4	39.4	39.70
C-13	44.6	44.6	44.93
C-14	51.7	51.1[d]	52.05
C-15	28.6[e]	23.4[e]	21.01
C-16	23.4[e]	28.7	29.05
C-17	56.3	56.2	56.55
C-18	12.9	12.9	13.25
C-19	18.7	18.2	18.57
C-20	39.7	39.8	40.14
C-21	19.6[e]	20.9	21.26
C-22	132.3[f]	135.2	135.58
C-23	135.2[f]	132.3	132.40
C-24	42.8	42.8	43.14
C-25	33.0	33.1	33.44
C-26	19.9[e]	19.7	20.03
C-27	20.7[e]	20.0	20.34
C-28	17.5	17.6	17.95

[a]It was carried out by means of HMQC, ^1H-^1H COSY and HMBC.
[a-e]The assignments indicate that data of the same alphabet was reported to be exchangeable in each article.

al., 1994) (Table I and II). Especially, the correlations from these methyl proton signals to carbon signals through two and three bonds showed very strong cross peaks in the HMBC, which played an important role in signal assignment.

In the ^{1}H-^{1}H COSY spectrum, the signal of the methine proton attached to carbon bearing a hydroxyl group at δ 3.98 (H-3) was coupled with two methylene proton signals at δ 1.94 and 2.11 (H-4) and at δ 1.55 and 1.85 (H-2), and then the methylene proton signals at δ 1.55 and 1.85 (H-2) were coupled with methylene proton signals at δ 1.71 and 1.09 (H-1). But in fact, almost all of the methine and methylene proton signals were overlapped, like other sterols, and therefore it was ambiguous and difficult to distinguish each signal only with the ^{1}H-^{1}H COSY. However, in the HMBC correlations (Fig. 1), these three methylene proton signals were correlated to the methine carbon signal at δ 66.84 (C-3), and the methylene carbon signal at δ 35.06 (C-1) was correlated with the methyl proton signal at δ 0.89 (H-19). The methylene proton signals at δ 1.94 and 2.11 (H-4) were clearly correlated to two carbon signals at δ 83.10 (C-5) and 135.80 (C-6) in 5α,8α-epidioxy system. The methyl proton signal at δ 0.89 (H-19) was long-range-correlated to the methyne carbon signal at δ 83.10 (C-5) to which the proton signals at δ 1.94 and 2.11 (H-4) were correlated, but not to the signal at δ 79.82 (C-8). The methyl proton signal was also correlated to the methine carbon signal at δ 51.43 (C-9), but not to the signal at δ 52.05 (C-14) which correlated with the methyl proton signal at δ 0.83 (H-18). Also an olefinic proton signal in 5α,8α-epidioxy system at δ 6.25 (H-7) was correlated to the carbon signals at δ 51.43 (C-9) and 52.05 (C-14), while the other olefinic proton signal at δ 6.51 (H-6) was correlated to carbon signals at δ 37.29 (C-4) and 37.33 (C-10) (Fig. 2). These results indicated that the carbon signal assignment was mistakenly reported in two articles (Kahlos *et al.*, 1989 and Yu *et al.*, 1994). That is, the carbon chemical shifts of C-5 and C-8 were inverted, and C-9 and C-14 also were inverted, respectively, and the proton chemical shifts of H-6 and H-7 were also inverted.

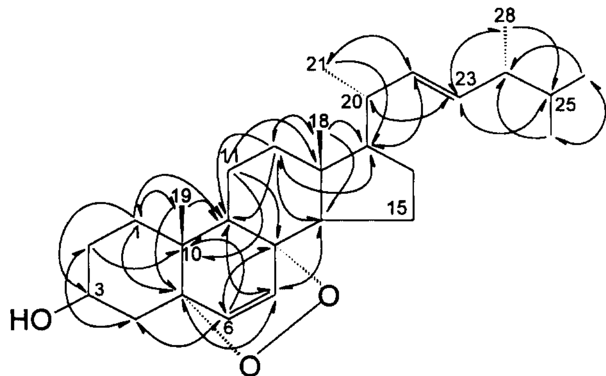

Fig. 1. The long range correlations of ergosterol peroxide through three bond connections observed in HMBC. All proton signals were correlated to carbon signals through two bond connections except H-3, H-16 and H-17. The arrow indicate correlation from proton to carbon.

The methyl proton signal at δ 0.89 (H-19) was correlated to the methine carbon signal at δ 51.43 (C-9) in the HMBC, and the methine proton signal at δ 1.50 (H-9) was coupled with the methylene proton signals at δ 1.22 and 1.53 (H-11) in the ^{1}H-^{1}H COSY (Fig. 4). Both of the methyl proton signals at δ 0.83 (H-18) and 1.00 (H-21) were correlated to the carbon signal at δ 56.85 (C-17), and the methine proton signal at δ 1.24 (H-17) which was assigned by the HMQC (Fig.

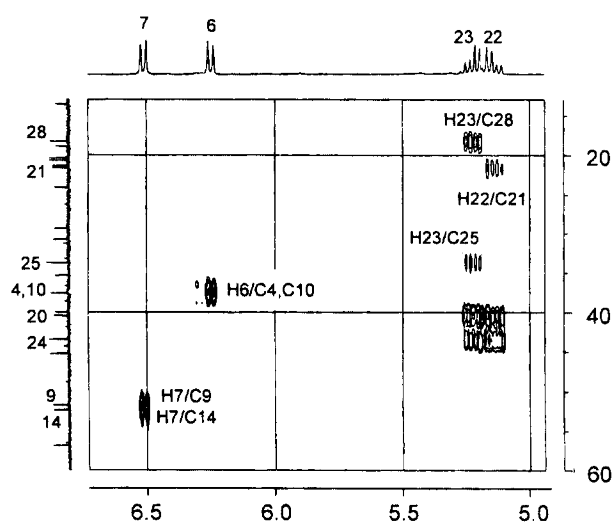

Fig. 2. HMBC spectrum of ergosterol peroxide.

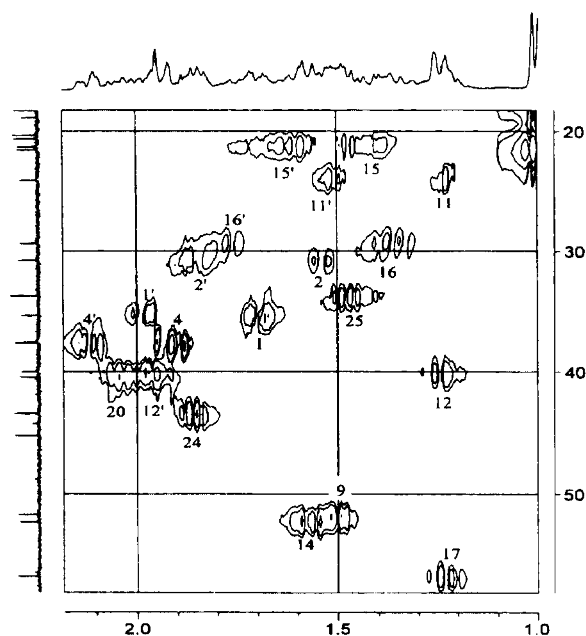

Fig. 3. HMQC spectrum of ergosterol peroxide.

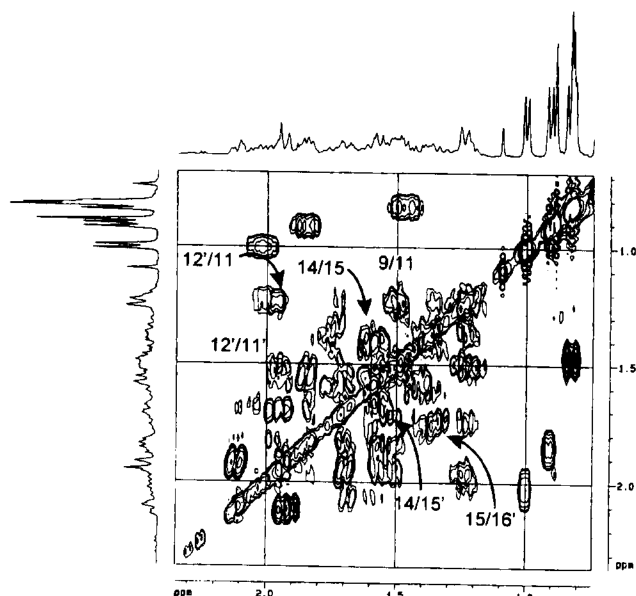

Fig. 4. ^{1}H-^{1}H COSY spectrum of ergosterol peroxide.

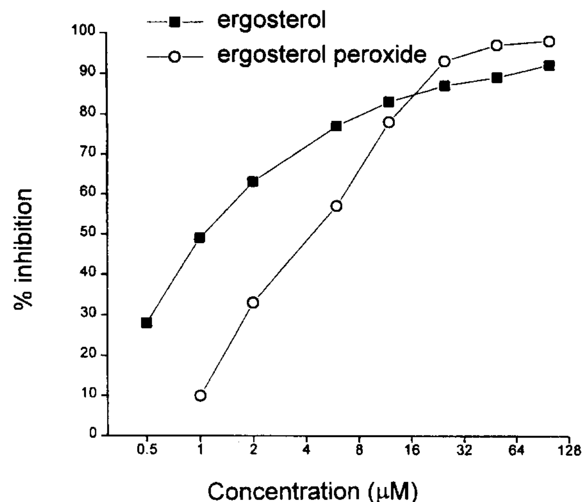

Fig. 5. Anticomplementary activity of ergosterol and ergosterol peroxide. Rosmarinic acid was used as a positive control (IC_{50}=180 μM).

3) was coupled with the methylene proton signals at δ 1.35 and 1.80 (H-16). Moreover, H-16 was coupled with the methylene proton signals at δ 1.40 and 1.65 (H-15) which were coupled with the methylene proton signals at δ 1.57 (H-14) in the ^{1}H-^{1}H COSY (Fig. 4). From the above results of H-11 and H-15 and the HMQC (Fig. 3), the carbon chemical shifts of C-11 and C-15 were assigned to δ 23.77 and 21.01, respectively. Thus these assignments in all literature (Kahlos *et al.*, 1989; Takaishi *et al.*, 1991; Yu *et al.*, 1994) have to be corrected. That was also supported by the correlations of H-11 to C-9, C-12 and C-13, and H-15 to C-14 and C-16 in the HMBC spectrum.

In general, the carbon chemical shifts of C-11 and C-15 in sterol analogues are 21.0±0.5 and 23.5±0.5 ppm, respectively. However these data of ergosterol peroxide were unexpectedly inverted compared to the chemical shifts of other sterol analogues as well as ergosterol. Therefore they have to be carefully assigned in 5α,8α-epidioxy systems of steroids.

In addition, it was also found that the assignment of C-22 and C-23 had been reversed in the literature (Kahlos *et al.*, 1989). An olefinic proton signal at δ5. 15 (H-22) was correlated to the methyl carbon signal at δ21.26 (C-21), while the other olefinic proton signal at δ 5.22 (H-23) was correlated to the methyl carbon signal at δ 17.95 (C-28) in the HMBC spectrum (Fig. 2). Thus the signanls of H-22 and H-23 were assigned as δ 5.15 and 5.22, respectively, by HMQC. On the other hand, these two proton signals had been reported to have the same chemical shift values (Takaishi *et al.*, 1991; Gunatilaka *et al.*, 1981) or be exchanged with each other (Yu *et al.*, 1994). That was also supported by the couplings of the proton signals of H-22 and H-23 with the proton signals at δ 2.03 (H-20) and 1.85 (H-24), respectively, in the ^{1}H-^{1}H COSY.

On the basis of these data and HMQC, all the remaining proton signals were completely assigned as shown in Table I.

Ergosterol, as well as ergosterol peroxide showed a strong anticomplementary activity on classical pathway (Fig. 5). Ergosterol ($IC_{50}=1.0\times10^{-6}$ M) was shown to be more active than ergosterol peroxide ($IC_{50}=5.0\times10^{-6}$ M). On the other hand, both of them had no significant anticomplementary activity on the alternative pathway (data not shown).

In the above anticomplementary activity test, acetone, in which both of them are soluble, was used as a solvent for dissolving samples before addition to the reaction buffer. Ergosterol peoxide was very soluble but ergosterol was very slightly soluble in DMSO. The anticomplementary activity of ergosterol peroxide using DMSO as a solvent was similar ($IC_{50}=4.9\times10^{-6}$ M) with above result using acetone.

Sometimes, it has been noted that the bioactivity of extracts or crude fractions of which bioactive principles were nonpolar substances was decreased as it was purified to a single component by means of an activity-guided separation method in which DMSO was used as a general solvent for dissolving sample. Similarly, the reason that the anticomplementary activity of ergosterol has not been reported, even though ergosterol is a major component of fungi and lichens, might be related to its solubility against DMSO.

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