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2D-NMR STUDIES OF A NOVEL STEROID FROM THE RED ALGA *ACANTOPHORA SPICIFERA*

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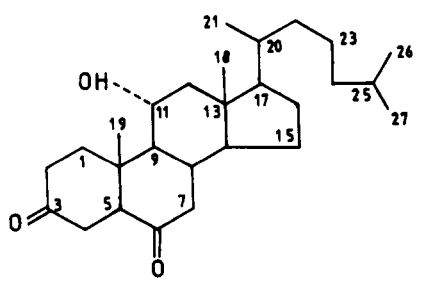
ABSTRACT.—A novel steroid has been isolated from the red alga *Acantophora spicifera*. Its structure and relative stereochemistry have been determined mainly by the use of 2D correlated nOe difference nmr spectroscopy and stereospecific coupling constants and characterized as 11α-hydroxy-5α-cholestane-3,6-dione [**1**].

During the course of broad biological screening of the extracts of marine organisms from the Indian sea coast, the MeOH extract of the red alga *Acantophora spicifera* (Vahl) Borgesen (Rhodophyta) collected from Baga, Goa, India, exhibited 100% anti-implantation activity at 200 mg/kg in female rats. LD_{50} of the active extract was above 1000 mg/kg (1). In the follow-up studies the activity was concentrated in the petroleum-ether- and $CHCl_3$-soluble fractions. 5-α-Cholestane-3,6-dione and other constituents have been previously isolated from the active fractions (2). We now report the isolation and structure of the new sterol **1** from the $CHCl_3$ extract of the air-dried alga.

RESULTS AND DISCUSSION

The sterol **1**, mp 145°, $[\alpha]^{25}D$ −11.1 ($c = 3$), was isolated by cc of the $CHCl_3$ extract on Si gel, followed by preparative tlc on Si gel and crystallization from MeOH.

The molecular formula of **1**, $C_{27}H_{44}O_3$, was determined by eims (*m/z* 416 $[M]^+$). Its ir spectrum showed strong bands at 3440, 1680, and 1700 cm^{-1} suggesting the presence of hydroxyl and carbonyl functions in the molecule. The mass spectrum of compound **1** gave a base peak at *m/z* 43 and the molecular ion peak at *m/z* 416. Moreover, the molecular formula of **1** was further determined by high resolution mass measurements of its $[MH]^+$ ion under fab conditions. The accurate mass of the $[MH]^+$ ion at *m/z* 417 was found to be 417.3369 corresponding to the molecular formula $C_{27}H_{44}O_3$ for **1**. These data suggested that the compound had a tetracyclic carbon skeleton with two carbonyl and one hydroxyl functions. The presence of the hydroxy group was further reinforced in the ^1H-nmr spectrum (400 MHz) which displayed a signal at δ 4.01. The presence of two carbonyl groups was confirmed by the ^{13}C-nmr spectrum. The signals for two carbonyl functions were at δ 208.5 and 211.1. The presence of three secondary methyls at δ 0.86, 0.87, and 0.94 and two tertiary methyls at 0.71 and 1.08, along with the spectral data given above, revealed that the compound is a sterol belonging to the cholestane series with two carbonyls and a hydroxyl.



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To fix the positions of the functional groups in compound **1**, one-dimensional ^1H and ^{13}C BB, DEPT, and 2D ^1H-^1H COSY, COSYLR, and ^1H-^{13}C XHDEPT nmr experiments were carried out. The ^1H- and ^{13}C-chemical shifts are given in Table 1.

TABLE 1. ^{13}C and ^1H NMR chemical shifts of **1** in $CDCl_3$.

Atom	δC	δH	$J_{H,H}$(Hz)
1α	37.7	2.37	m[b]
1β		2.44	m
2α	39.9	1.82	m
2β		2.81	$J_{gem}=14.1; J_{2,1}=6.5; J_{2,1}=2.3$
3	208.5		
4α	37.3	2.32	m
4β		2.60	m
5	57.9	2.68	$J_{5,4}=2.7; J_{5,4}=12.9$
6	211.1	—	m
7α	46.1	2.09	$J_{7,8}=12.9; J_{gem}=14.1$
7β		2.36	m
8	36.5	1.83	m
9	35.7	1.41	m
10	42.9[a]	—	—
11	68.7	4.01	$J_{11,12}=11.0; J_{11,12}=4.7; J_{11,9}=10.6$
12α	51.7	1.32	m
12β		2.36	m
13	43.1[a]	—	—
14	28.0	1.35	m
15α	28.1	1.05	m
15β		1.91	m
16α	24.0	1.09	m
16β		1.51	m
17	56.1	1.18	m
18	13.1	0.71	s
19	13.0	1.08	s
20	55.9	1.33	m
21	18.6	0.94	$J=6.6$
22	39.4	1.13	m
23	35.9	1.28	m
24	23.8	1.37	m
25	59.2	1.44	m
26	22.7	0.87	$J=6.6$
27	22.5	0.86	$J=6.6$

[a]Assignments are interchangeable.
[b]m indicates that the signal is overlapped with other signals.

Various DEPT experiments identified the multiplicity of each ^{13}C-nmr resonance. They indicated the presence of four quaternary carbons. Two of these were for two carbonyl functionalities, while the remaining two were aliphatic in nature. The experiments also revealed the presence of seven aliphatic methines, one hydroxy methine, ten methylenes, and five methyl carbons and suggested the molecule **1**. However, the positions of the two carbonyl and one hydroxyl functions were still undecided, and they were determined with the help of 2D-nmr experiments.

The signal at δ 4.01 in the ^1H nmr spectrum was unambiguously assigned to the spin system containing the hydroxy function. It showed cross peaks at δ 2.38, 1.41, and 1.31 only. The signals at δ 2.38 and 1.31 were due to a methylene signal from the

2D XHDEPT spectrum, whereas a methine signal was at δ 1.41. These data indicated that the carbon-bearing hydroxyl group is flanked by a methylene and a methine carbon. Moreover, in the long range COSY spectrum, the Me-18 signal at δ 0.71 showed cross peaks with the signals at δ 2.38, 1.31, and 1.18 due to H-12β, H-12α, and H-17α (W-type couplings). Since H-12β and H-12α were coupled to the proton-bearing functionality, this could only be possible if the hydroxyl group is placed at C-11. The signal at δ 1.41 was for H-9, which further showed cross-peaks at δ 2.36 and 2.09 (H-7β, H-7α).

The H_2-7 protons did not show any cross peaks with any of the protons in the 2D spectrum, clearly suggesting that there are no adjacent protons which are scalar-coupled to both the H_2-7 protons. Thus, the locus of one of the carbonyl functions is at C-6. This was confirmed by the downfield shift of C-7 in the 2D ^1H-^{13}C (XHDEPT) spectrum at δ 46.1 in the F_2 dimension and also by the coupling constants at H-7α in the ^1H-nmr spectrum (Table 1).

The two methyl signals as doublets in the ^1H nmr spectrum at 0.87 and 0.86 were assigned to Me-26 and Me-27, and these signals showed cross peaks at δ 1.44 for H-25. Similarly, connectivities were traced as an unbroken sequence from H-25 to H-14 (protons of the side chain and D ring) in **1**. The data discussed above suggested that the second carbonyl function is located in ring A in **1**. The locus of this carbonyl function was at C-3, because in the COSYLR spectrum, the H-7α signal showed cross peaks at δ 2.68 due to W type ^4J coupling with H-5. The H-5 signal showed cross peaks (Figure 1) at δ 2.60 and δ 2.32 for both the H_2-4 protons. Neither H_2-4 proton showed any cross peaks, indicating that there is an interruption at C-3. The assignment of H-5 was confirmed in the COSYLR spectrum (Figure 2) where it gave a cross peak with Me-19 at δ 1.08. The signal at δ 2.81 was assigned unambiguously to H-2β (α to the carbonyl group); it showed cross peaks at δ 2.44, 2.37, and 1.82. The signal at δ 1.82 was due to the geminal proton of C-2 in the 2D-XHDEPT spectrum and was assigned to H-2α in the ^1H-nmr spectrum, whereas the signals at δ 2.44 and 2.37 were assigned to the H_2-1 protons. Thus, the ^1H- and protonated ^{13}C-nmr signals of compound **1** were assigned. The signals for aliphatic quaternary carbons at δ 43.1 and 42.9 have not been assigned unambiguously. These signals were due to C-10 and C-13 or vice versa.

The long-range coupling interactions are known to be highly stereospecific in rigid systems. ^4J coupling follows the empirical W rule (3–5). Larger long-range ^4J coupling observed for a path having a zig-zag or W-like shape has been confirmed by double perturbation calculations (6). These coupling interactions can be used in configurational and conformational analysis if their stereospecificity is clearly demonstrated. The Me-18 signal at δ 0.71 showed cross peaks at δ 1.18, 1.32, and 2.36 for H-17α, H-12α, and H-12β only. The Me-19 signal at δ 1.08 exhibited cross peaks at δ 2.68 for H-5, thus suggesting that the A/B ring junction is trans. Because theoretical and experimental studies (7) have shown that if one of the coupled protons is a part of a methyl group, appreciable values for ^4J H-C-5-C-10-Me are confined to the geometries where the dihedral angle between H-5, C-5, C-10 and C-5, C-10, Me approaches 180°. Irradiation of the Me-19 signal at δ 0.71 showed nOe's with H-8 and H-11, whereas no nOe was observed for H-9; this suggests that the B/C ring junction is trans. Similarly, irradiation of Me-18 showed no nOe for H-14, thus confirming that the C/D ring junction is trans. The couplings to H-11 of 11.0, 10.6, and 4.7 Hz could be interpreted as $J_{11ax,12ax}$, $J_{11ax,9ax}$, and $J_{11ax,12eq}$, respectively. Irradiation of H-11 gave nOe to Me-19, Me-18, and H-8, thus suggesting that the hydroxyl group at C-11 is α-equatorially oriented.

On the basis of above studies the new sterol was characterized as 11α-hydroxy-5α-cholestane-3,6 dione [**1**].

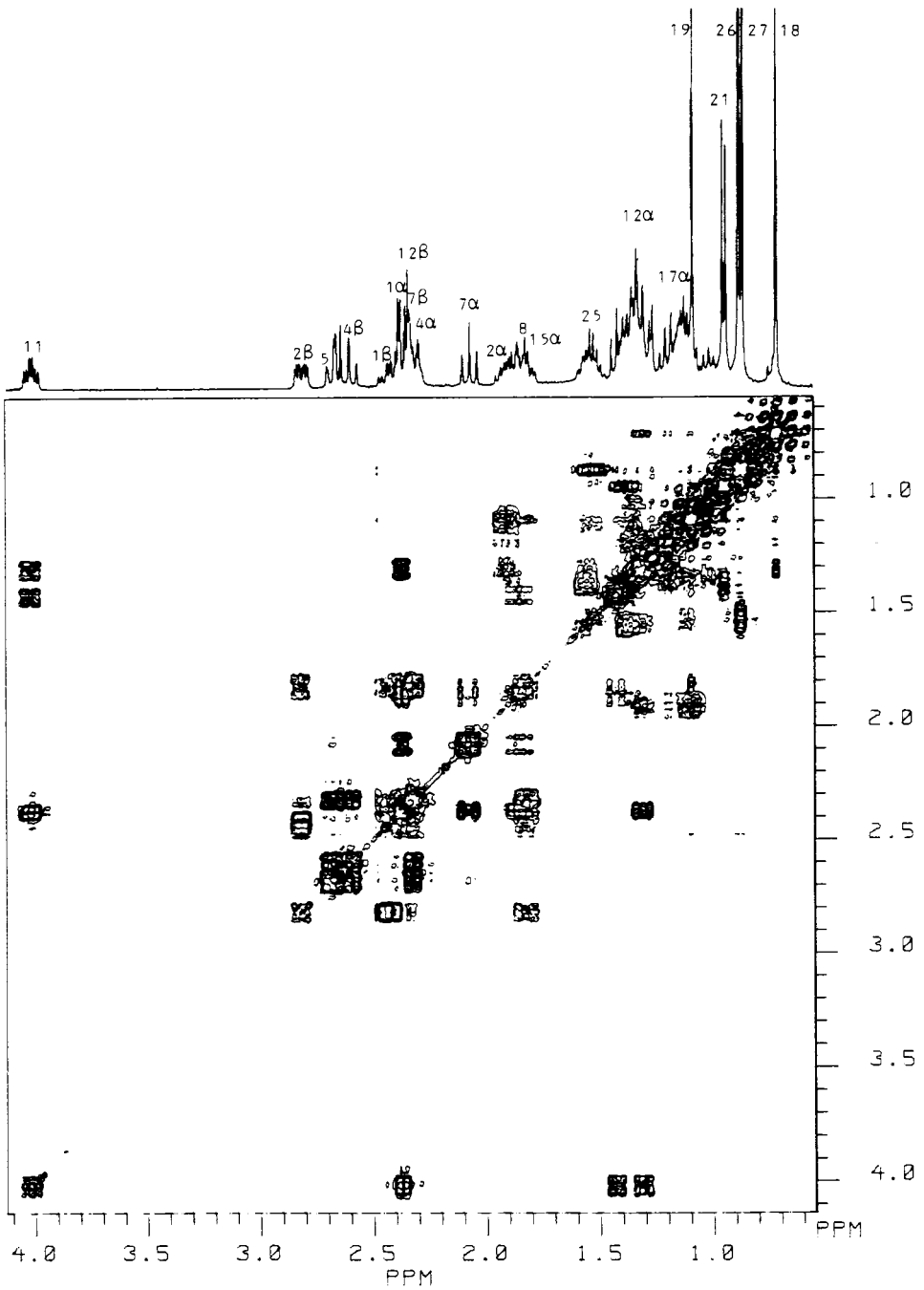

FIGURE 1. A contour plot of the 2D ^{1}H-^{1}H COSY spectrum of **1** underneath the 1D ^{1}H-nmr spectrum.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H- and ^{13}C-nmr spectra were taken on a Bruker WM-400 multinuclear Ft nmr spectrometer equipped with a 5-mm ^{1}H/^{13}C dual probehead and an ASPECT 2000 computer using DISNMR program version 860101.1. TMS was used as an internal standard, and chemical shifts are recorded in δ values. Samples for ^{1}H measurements of **1** were carried out on 0.04 M solution in $CDCl_3$. The typical Ft conditions were as follows: pulse width, 7 μsec; flip angle, 40°; sweep width, 4807.69 Hz; data points 16K; acquisition time, 1070 sec; and resolution 0.59 Hz. The 2D ^{1}H-^{1}H

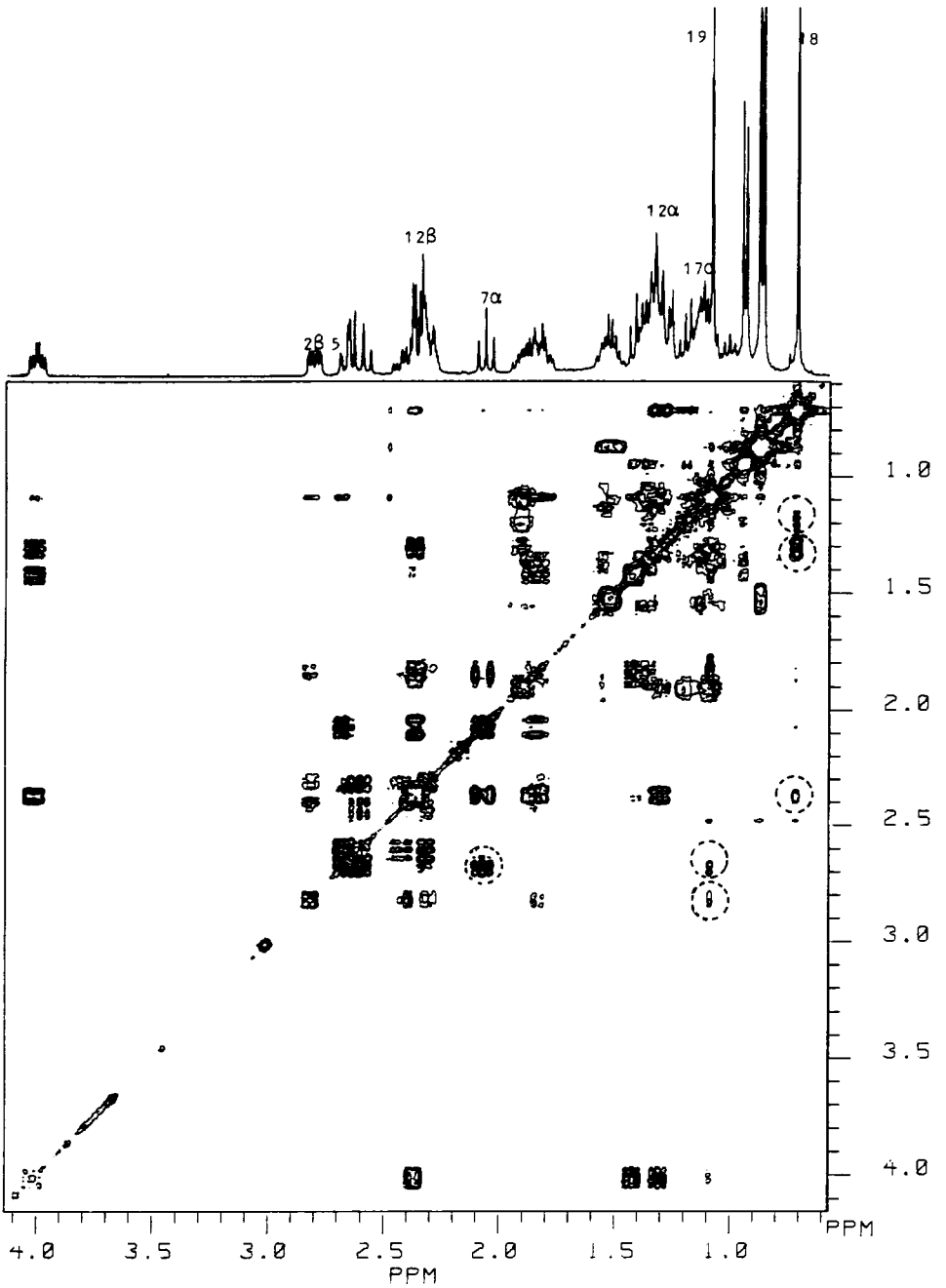

FIGURE 2. A contour plot of the 2D ^{1}H-^{1}H COSY (long range) spectrum of **1** underneath the 1D ^{1}H-nmr spectrum. The long range cross peaks are marked by dotted circles.

chemical shift correlation nmr spectrum was performed using COSY (8) with a 90° mixing pulse and COSYLR (9) using 90°-t_1-Δ-90°-Δ-t_{12} pulse sequence with a fixed delay Δ = 0.2 sec.

The ^{13}C-nmr measurements were made at 100.57 MHz on a Bruker WM 400 NMR spectrometer over 27777.78 Hz, using 32K data points for BB decoupled SFORD and DEPT (10) nmr experiments. The 2D ^{1}H-^{13}C chemical shift correlation spectra were obtained using the DEPT 2D pulse sequence (11) with ^{1}H broad band decoupling throughout the acquisition period. The interferograms were acquired over 1024 data points and 6944.44 Hz for each 256 values of evolution time. The second dimension Ft sweep

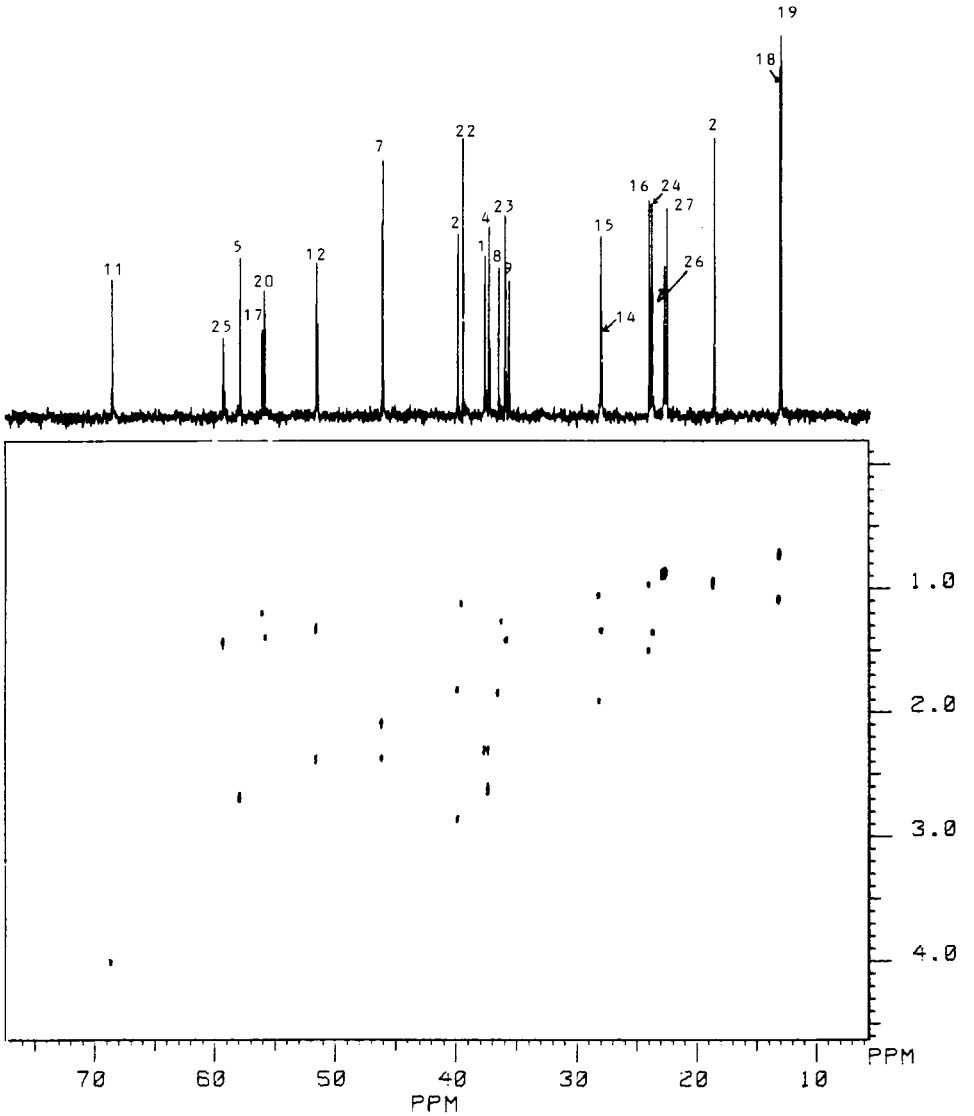

FIGURE 3. A section of the contour plot of the 2D ^{1}H-^{13}C COSY spectrum of **1**. The 1D ^{13}C-nmr spectrum of this section is shown above the 2D spectrum, giving their respective carbon assignments.

width was ± 853.24 Hz. The raw data were zero-filled, and sine bell window function was used prior to double Fourier transformation. The optical rotation of **1** was taken on a Perkin-Elmer 241 Polarimeter. The ei mass spectrum was recorded on a Jeol D-300 mass spectrometer and the high resolution mass measurement was done on VG-ZAB-SE mass spectrometer using peak matching technique.

ISOLATION.—*A. spicifera* was collected by the scientists of NIO, Goa, and an herbarium specimen is kept in NIO Herbarium. The $CHCl_3$ extracts (4 g) of air-dried seaweed *A. spicifera* (200 g) were chromatographed over SiO_2 column, and the column was successfully eluted with solvents of increasing polarity. Elution of the column with EtOAc-petroleum ether (20:80) gave a product which on further purification on preparative tlc (SiO_2) and crystallization from MeOH afforded compound **1** (60 mg), mp 145°.

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