Pergamon

Tetrahedron Vol. 50, No. 30, pp. 9175-9180, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4020/94 $7.00+0.00

0040-4020(94)00515-X

Structural Determination and Biosynthetic Origin of Two Ester Derivatives of Okadaic Acid Isolated From *Prorocentrum lima.*

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Abstract: The dinoflagellate *Prorocentrum lima* is responsible for the production of the toxins involved in the red tide phenomenon known as diarrhetic shellfish poisoning (DSP). In this paper we report on the isolation, structural elucidation and biosynthetic origen of two ester derivatives of okadaic acid on the basis of their spectroscopical data. Its biosynthetic origin was indicated by addition of labelled sodium [1-$^{13}C_1$], [2-$^{13}C_1$] and [1,2-$^{13}C_2$] acetate to artificial cultures of the dinoflagellate.

Diarrhetic shellfish poisoning (DSP) has only been recognized as a red tide phenomenon since 1978. It is probably explained by the similarity of the symptoms with gastroenteritis associated with the ingestion of polluted shellfish. The toxins responsible for this phenomenon are okadaic acid and its derivatives, which are produced by dinoflagellates of the genera *Dinophysis* and *Prorocentrum*[1]. DSP-outbreaks occur regularly and the correlation between the occurrence and concentrations of these species with the toxicity of mussels on the Spanish coasts, has been established[2]. This class of polyether toxins have received wide interest not only for their chemical structures but also for their pharmacological properties. Thus, it has been demonstrated that OA is a potent and highly selective inhibitor of protein-phosphatases as well as a potent tumour-promoting agent[3].

We have recently published the complete proton and carbon NMR study of okadaic acid **3** which has been obtained from the unialgal culture of the dinoflagellate *Prorocentrum lima* carried out in our laboratory[4]. From two different strains of *P. lima* (see experimental part), we have isolated two new toxins which have been identified as ester derivatives of okadaic acid on the basis of spectroscopical methods.

Compound **1** showed a molecular ion at m/z 943 (MH^+) in the FAB mass spectrum, thioglycerol being the most suitable matrix. The ions formed by simple fragmentation of the molecular ion were as follows: at m/z 925 a fragment formed by the loss of water (MH^+-H_2O) and another at m/z 805 originated from an ester fragmentation (MH^+-$C_9H_{15}O$), which was identical with the one observed as a molecular ion for okadaic acid. Compound **1** showed in the I.R. spectrum bands centred at 3500 and 1730 cm^{-1} for hydroxy and ester groups, respectively. The UV(EtOH) had a maximum at 264 nm (ε=14.822) due to the presence of a conjugated diene system.

Compound **1**, showed a ^{1}H-NMR spectrum clearly related with that from okadaic acid. Thus, the presence of three secondary methyl groups was clear at δ 0.92 (Me-44), 1.03 (Me-41) and 1.05 (Me-43); a methyl group on a quaternary carbon bearing an oxygen atom at δ 1.39 (Me-39) and a methyl group on a double bond at δ 1.73 (Me-40). The olefinic proton signals were identified at δ 5.32 (H-9), those from the trans double bond C_{14}-C_{15} at δ 5.51 and 5.58 (H-14) and those from the methylene H-42 at δ 5.04 and 5.37. In

addition to these signals, there were two isolated olefinic protons centred at δ 5.38 and 5.45, two olefinic methyl groups at δ 1.78 and 1.82, and an allylic methylene group centred at δ 2.39. Finally, the spectrum showed a cluster of protons around δ 3.5 and 4.08 represented by 9 -O-CH- and 3 -O-CH_2- groups, which implies that in this region, compound **1** has two methylene groups more than okadaic acid.

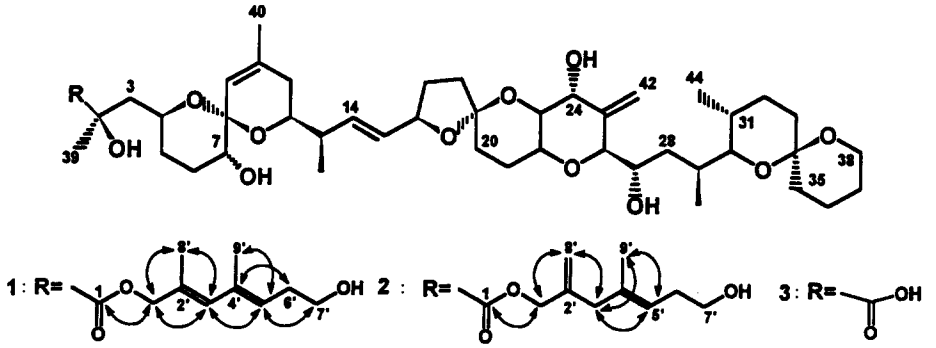

Figure 1.- Compounds **1**, **2** and **3**. Arrows indicate significant HMBC correlations for the ester side-chain of compounds **1** and **2**.

The ^{13}C-NMR spectrum showed the presence of 53 carbon signals and the DEPT experiment established that 13, 19 and 7 were methine, methylene and methyl, groups respectively. The seven quaternary carbons could be identified by their absence in the normal DEPT spectrum in comparison with the proton decoupled ^{13}C-NMR spectrum. The DEPT sequence identified the carbons C-42 (the only olefinic carbon signal with a triplet multiplicity) and C-1 (which could be recognized by its characteristic downfield shift). In order to extend the proton and carbon assignments, the DQF-COSY and the HMQC experiments were carried out. The connectivities observed in these experiments combined with those observed in okadaic acid made it possible to assign most of the proton and carbon chemical shifts in the molecule. Thus the 10 -O-CH- carbons could be assigned as follows: C-4 (δ 69.0); C-7 (δ 72.0); C-12 (δ 71.3); C-16 (δ 79.5); C-22 (δ 70.0); C-23 (δ 77.1); C-24 (δ 71.3); C-26 (δ 85.3); C-27 (δ 65.1) and C-30 (δ 75.5). In addition, the methylene groups C-38 at δ 60.7 and C-42 at δ 112.8, and the olefinic carbons C-9 (δ 122.1), C-14 (δ 135.9) and C-15 (δ 131.5) were also identified. In the identification of carbons that could not be assigned by using the HMQC experiment, the HMBC experiment was applied. Thus, the C-O quaternary centre at C-2 (δ 75.8) was identified directly by its HMBC correlations with H-3 and Me-39; C-8 (δ 96.6) with H-9 and C-19 (δ 106.2) with H-17, whereas carbon C-34 (δ 96.0) was assigned on the basis of its being the only remaining C-O quaternary centre. The connections between C-12, C-13 and C-14 and Me-41 (δ 16.3); between C-28, C-29 and C-30 and Me-43 (δ 16.6); and between C-30, C-31 and C-32 and Me-44 (δ 11.1) established the chemical shift assignments for the secondary methyl groups . The observed correlations helped us to confirm the proton and carbon assignments made for compound **1** (Table 1).

The additional signals observed in the proton NMR spectrum with respect to okadaic acid were assigned as follows: in the DQF-COSY experiment the proton signals at δ 2.39 (H-6'), 3.62 (H-7') and 5.38 (H-5') were found to be connected in the sequence: 5.38-2.39 and 2.39-3.62 and no further coupling of the 2.39/3.62 was observed. However, the most valuable information obtained from this experiment was that observed for long-range connectivities. Thus, the isolated olefinic signal at δ 5.95 (H-3') was long-range correlated with the methyl group at δ 1.82 (Me-8'), with the methylene group at δ 4.52 and 4.72 (H-1') and

Atoms	Compound 1 ^1H	Compound 1 ^{13}C	Compound 2 ^1H	Compound 2 ^{13}C	Atoms	Compound 1 ^1H	Compound 1 ^{13}C	Compound 2 ^1H	Compound 2 ^{13}C
1	-	176.6	-	176.5	28	0.98; 1.30	35.7	0.98; 1.33	35.7
2	-	75.8	-	75.8	29	1.95	31.5	1.95	31.5
3	1.69; 2.02	44.5	1.79; 2.08	44.5	30	3.28	75.5	3.28	75.5
4	3.98	69.0	4.01	69.0	31	1.79	27.8	1.98	27.8
5	1.34; 1.40	32.1	1.44; 1.78	32.2	32	1.96; ?	26.8	1.75; 1.82	26.8
6	1.71; 1.86	26.9	1.71; 1.88	26.9	33	?	30.8	?	30.8
7	3.37	72.0	3.39	72.0	34	-	96.0	-	96.0
8	-	96.6	-	96.6	35	?	36.3	?	36.3
9	5.32	122.1	5.35	122.1	36	?	19.2	?	19.2
10	-	139.2	-	139.2	37	1.50; 1.89	25.9	1.60; 1.90	25.9
11	1.87; 1.93	33.2	1.91, 2.04	33.3	38	3.50; 3.64	60.7	3.52; 3.70	60.7
12	3.66	71.3	3.68	71.3	39	1.39	27.7	1.41	27.6
13	2.30	42.2	2.30	42.3	40	1.73	23.4	1.74	23.5
14	5.58	135.9	5.60	136.1	41	1.03	16.3	1.03	16.2
15	5.51	131.5	5.50	131.4	42	5.04; 5.37	112.8	5.08; 5.41	112.9
16	4..50	79.5	4.45	79.5	43	1.05	16.6	1.06	16.6
17	1.61; 2.17	31.1	1.63, 2.17	31.1	44	0.92	11.1	0.92	11.1
18	1.83; 2.04	37.6	1.64; 2.02	37.6	1'	4.52; 4.72	71.9	4.56; 4.66	66.9
19	-	106.2	-	106.2	2'	-	129.7	-	141.6
20	?	33.2	?	33.3	3'	5.95	133.3	2.8	44.8
21	1.81; 1.88	27.8	1.81; 1.87	27.8	4'	-	135.0	-	135.0
22	3.58	70.0	3.58	70.0	5'	5.38	127.4	5.29	124.1
23	3.46	77.1	3.44	77.6	6'	2.39	32.2	2.28	32.0
24	4.10	71.3	4.12	71.3	7'	3.62	62.6	3.68	62.7
25	-	144.2	-	144.2	8'	1.82	16.0	5.02; 5.14	114.8
26	3.93	85.3	3.90	85.3	9'	1.78	17.2	1.61	16.2
27	4.08	65.1	4.08	65.1					

Table 1.- ^{1}H and ^{13}C-NMR Chemical Shift Data ($CDCl_3$) for Compounds **1** and **2**.

with the olefinic proton at δ 5.38 (H-5'). Moreover, this last proton was long-range correlated with the methyl group at δ 1.78 (Me-9'). These correlations established the presence of an $HOCH_2$-CH_2-CH=C(Me)-CH=C(Me)-CH_2O moiety in the ester chain. The observed proton signals were correlated with their carbon signals in the HMQC experiment. The position of this moeity in compound **1** was easily established on the basis of the observed HMBC correlation between H-1' and the carbon signal from the carboxyl group of okadaic acid C-1 (δ 176.6) (Figure 1). This experiment also confirmed the structure proposed on the basis of the connectivities observed in the DQF-COSY experiment. Thus, the proton signals at δ 4.52 and 4.72 (H-1') were correlated with the carbon signals at δ 129.7 (C-2'), δ 133.3 (C-3') and δ 16.0 (Me-8'); the proton signal at δ 5.95 (H-3') was correlated with the carbon signals at δ 71.9 (C-1') , δ 16.0 (Me-8'), δ 127.4 (C-5') and δ 17.2 (Me-9'); finally the proton signal at δ 2.39 (H-6') was correlated with the carbon signals at δ 62.6 (C-7'), δ 127.4 (C-5') and δ 135.0 (C-4') (Fig. 1). The E geometry of the double bonds in the ester chain was assured by the correlation observed in the ROESY experiment between the olefinic protons H-3' and H-5' with the methyl groups Me-8' and Me-9', respectively. These correlations established that compound **1** is the 7'-hydroxy-2',4'-dimethyl-hepta-2'(E),4'(E)-dienylokadaate.

Compound **2** proved to be an isomer of compound **1** on the basis of its molecular ion at m/z 943 (MH^+), the main observed fragmentation being identical with those observed for compound **1** (see experimental part). This compound also showed in the I.R. bands centred at 3500 and 1730 cm^{-1} for hydroxy and ester groups. Their proton and carbon NMR spectra were closely related to those obtained for compound **1**, according to which compound **2** should also be an ester derivative of okadaic acid (Table 1).

The structure of the ester chain was established as follows. The ^{1}H-NMR spectrum showed as a new signals in addition to those derived from okadaic acid, three methylene groups centered at δ 2.28; 2.80, 3.69 together with two methylene groups showing A-B systems at δ 4.56/4.66 and δ 5.02/5.14. The presence of an olefinic proton at δ 5.29 and a methyl group at δ 1.61 was also established. In comparison with the ^{13}C-NMR spectrum of okadaic acid, compound **2** showed the presence of four aliphatic methylene groups, two of them bearing an oxygen atom, one olefinic methylene and one methyl groups.

The DQF-COSY experiment established the connectivities between three proton signals in the following sequence 5.29 (H-5') - 2.28 (H-6') and 2.28 (H-6') - 3.68 (H-7'), together with the long range connectivities between 5.02 (H-8') - 2.80 (H-3') and between 2.80 (H-3') - 1.61 (Me-9'). The only remaining AB system at δ 4.56 and 4.66 was assigned to the methylene group H-1'. On the basis of these connectivities and the correlation observed in the HMQC experiment, the carbon chemical shift assignment was established as follows δ: 66.9 (C-1'); 44.8 (C-3'); 124.1 (C-5'); 32.0 (C-6'); 62.7 (C-7'); 114.8 (C-8') and 16.2 (Me-9'). As in compound **1**, the HMBC experiment helped us to establish the structure and position of this moiety. Thus, it showed the correlation between the carboxylic carbon signal C-1 (δ 176.5) and the proton signals from H-1' at δ 4.56 and 4.66, which were also correlated with the quaternary olefinic carbon C-2' (δ 141.6) and the olefinic methylene group C-8' (δ 114.8). The main difference with respect to compound **1** was the presence of an isolated double allylic methylene group H-3' instead of an olefinic proton, which was assured by its HMBC correlations with Me-9', C-8', C-5', C-4' and C-2'. All the other observed correlations shown in Table 1 were in accordance with the presence of a 7'-hydroxy-4'-methyl-2'-methylen-hept-4'(E)-enyl ester chain in compound **2**.

A variety of C_6 and C_7 diol esters, compound **2** being one of them, have been reported from cultures of *P. lima* and *P. concavum*, although their complete spectroscopical data have not been published[5]. In order to clarify the biosynthetic origin of these unique carbon side chains sodium [1-^{13}C], [2-^{13}C] and [1,2-$^{13}C_2$] acetates were administered to cultures of PL-2 strain of *Prorocentrum lima*. The analysis of the ^{13}C-NMR spectra of **2** after administration of precursors, which is summarized in Figure 2, established that the biosynthetic backbone-carbon origin of okadaic acid is identical to that observed for DTX-1[6], as expected .

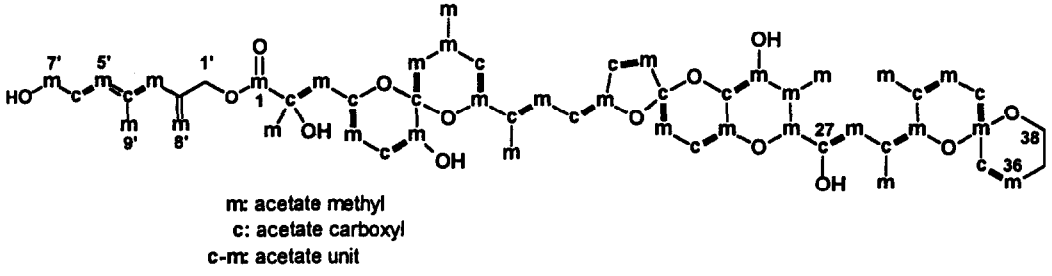

Figure 2

For the ester side-chain, the ^{13}C-NMR spectra clearly showed distinctive enriched peaks at C-4' and C-6' from feeding experiments with sodium [1-^{13}C] acetate and at C-3', C-5', C-7', C-8' and C-9' from feeding experiments with [2-^{13}C] sodium acetate, while carbon signals for C-1' and C-2' did not show distinctive enrichment in the above experiments. Moreover, the ^{13}C-NMR spectrum of compound **2** labelled from sodium [1,2-$^{13}C_2$] acetate showed that the carbon signals from C-3', C-4', C-5' and C-6' were flanked by two strong satellite signals which, in accordance with the carbon-carbon coupling constants, established the definite incorporation of two acetate units between carbons C-3'/C-4' and C-5'/C-6'. In addition, the carbon signals from

C-1', C-2', C-7', C-8' and C-9' appeared flanked by weak satellites signals, although the intensities for carbons C-2' and C-1' were significantly lower than for the others. Carbons C-7' and C-9' were derived from C-2 carbon of acetate and were attached to carbons that have been derived from C-1 carbon of intact acetate units, as observed for the secondary methyl groups as well as for the carboxylic carbon C-1 in DTX-1[6]. On the other hand, a detailed analysis of the splittings observed for fragment C-1'/C-8' showed that carbon signals from C-1' and C-8' appeared flanked by two weak satellite signals, whereas the carbon signal from C-2' was flanked by four, with constant coupling values identical to those observed for C-1' and C-8' (Figure 3). In accordance with these results, this carbon side-chain is biosynthesized by the condensation of two acetate units, with the branching methyl groups derived from C-2 of acetate and with a final C_2 unit which did not showed distinctive enrichment. This situation is similar to that found for fragment C-27/C-38 in DTX-1[6].

Pharmacological experiments carried out in rat myometrium showed that the naturally occurring ester was as active as the free acid, although the period of latency (i.e., the lag between drug addition and the appearance of the response) was significantly higher for the ester and the response was slower than the one observed with okadaic acid[7].

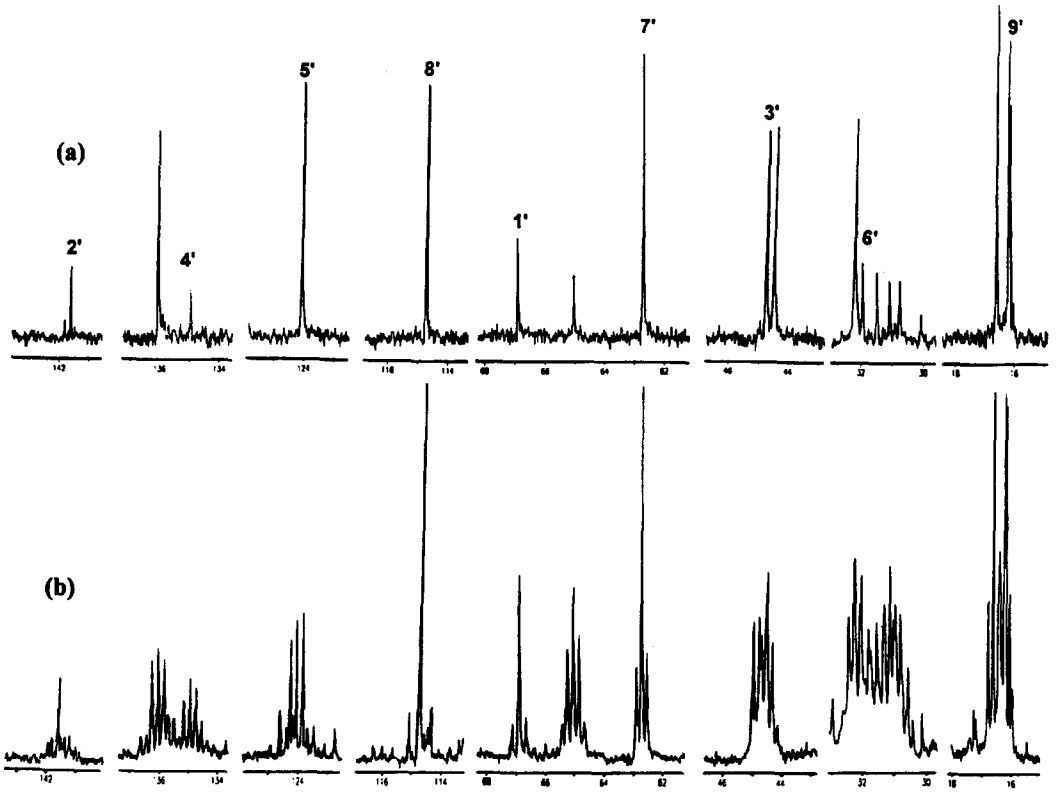

Figure 3: ^{13}C-NMR carbon signals for fragment C-1'/C-9' of compound **2** resulting from a) feeding of sodium [2-$^{13}C_1$] acetate and b) feeding of sodium [1-2-$^{13}C_2$] acetate. $J_{C\text{-}C}$ (Hz): C-1'/C-2'= 47Hz; C-2'/C-8'= 72Hz; C-3'/C-4'= 40Hz; C-5'/C-6'= 43Hz; C-6'/C-7'= 3 Hz; C-9/C-4'= 35Hz.

EXPERIMENTAL PART

Culture and extraction: Cultures of two different strains of the dinoflagellate *Prorocentrum lima* coded PL-2 and PL-3 were carried out. A typical culture experiment involved the inoculation of 20 L of a Guillard K medium with 10 L of culture of *P. lima*. This culture was allowed to grow in a 40 L tank, with constant white fluorescent illumination at 25°C for three weeks. After a period of culture, the *P. lima* cells were harvested by continuous centrifugation at 7.000 r.p.m. The cells were sonicated and extracted with acetone. The solvent was evaporated and the resultant extract chromatographed.

Isolation of compound 1: The organic extract obtained as described above from a PL-3 *P. lima* culture was chromatographed by gel filtration and the column was eluted with a mixture of $CHCl_3$: MeOH: n-Hex (1:1:2). The toxic fraction was chromatographed over a medium pressure reverse-phase Lobar LiChroprep RP-18 column with MeOH : H_2O (85:15) as eluent and 50 fractions of 3 ml each were collected. Compound 1 was localized in fractions 40-49, which were combined, and the solvent was evaporated. Its final purification was achieved by using the same solvent system on a μ-Bondapak C-18 column HPLC reverse-phase chromatography.

Compound 1: White amorphous powder, $[\alpha]_D$= +21.42° (c, 0.49, $CHCl_3$). FAB MS m/z: 943 $[MH^+]$; 925 $[MH^+-H_2O]$; 805 $[MH^+-C_9H_{15}O]$; 769 $[MH^+-(C_9H_{15}O + H_2O)]$; UV(EtOH) λ_{max} nm (ε): 263. IR $(CHCl_3)\nu_{max}$ cm^{-1}: 3487 (OH), 1735 (ester), 1453, 1382, 1262. ^{1}H- and ^{13}C-NMR (Table 1).

Isolation of compound 2: Due to the fact that compound 2 showed a chromatographic behaviour identical with that observed for compound 1, the organic extract obtained from a PL-2 *P. lima* culture, was chromatographed following the same protocol that was used for compound 1. Thus, compound 2, was localized in about the same fractions during the Sephadex LH-20 and medium pressure reverse-phase chromatographies and its final purification was also achieved by using a μ-Bondapak C-18 column HPLC, to obtain pure compound 2.

Compound 2: white amorphous powder.$[\alpha]_D$= +17.33° (c, 0.15, $CHCl_3$). FAB MS m/z: 943 $[MH^+]$; 925 $[MH^+-H_2O]$; 805 $[MH^+-C_9H_{15}O]$; 769 $[MH^+-(C_9H_{15}O + H_2O)]$. IR $(CHCl_3)\nu_{max}$ cm^{-1}: 3477 (OH), 1735 (ester), 1457, 1382, 1262. ^{1}H- and ^{13}C-NMR (Table 1)

Administration of Labelled Compounds to *P. lima*: In a typical labelling experiment, 10 x 3 l flasks of bacterial-free unialgal culture of PL-2 *P. lima* were grown at 25 °C under constant illumination in Guillard K medium. Ten days after inoculation, each flask was fed with labelled [1-^{13}C] or [2-^{13}C] or [1,2-^{13}C] sodium acetate (0.67 mmol/l). The culture was grown for a further 10 days. The cells were filtered, then extracted with $CHCl_3$:MeOH (1:1) and 0.5 mg of 2 were isolated.

Acknowledgements: M.N. acknowledges financial support from the Plan Nacional de Investigaciones Farmacéuticas Far 91-0827. A. P. thanks the M.E.C. for a fellowship. The authors thank I. Bravo from IEO (Vigo) for a *P. lima* culture.

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(*Received in UK* 29 *April* 1994; *revised* 8 *June* 1994; *accepted* 10 *June* 1994)