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Identification and quantification of 18-nor- and 19-norditerpenes and their chlorinated analogues in samples of sediment and fish

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ABSTRACT

The norditerpenes 18-norabieta-8,11,13-triene and 12,14-dichloro-18-norabieta-8,11,13-triene, corresponding to decarboxylation of dehydroabietic acid and 12,14-dichlorodehydroabietic acid, respectively, were isolated from environmental samples of sediment and fish from the Gulf of Bothnia. Identification was unambiguously accomplished by comparison with synthetic reference compounds whose structures were established by NMR. The synthetic product was a mixture of the nor epimers at C-4 in the ratio of *ca.* 1:3, and NMR showed that the 18-nor epimer was the major component. Whereas the 18-nor epimer was the dominant C_{19} hydrocarbon in the sediment samples, the 19-nor compound dominated the C_{19} hydrocarbon fraction in the samples of fish. These C_{19} terpenes are probably environmental transformation products of the corresponding dehydroabietic acids. The 18-nor hydrocarbon was quantified in sediment samples, which were also analysed for a number of other compounds representative of alicyclic and aliphatic components of bleachery effluents. The 19-nor hydrocarbon was conclusively identified, although not quantified in samples of fish. 12,14-Dichloro-18-norabieta-8,11,13-triene was identified in samples of sediment and fish which also contained the 19-nor compound. The presence of both hydrocarbons in fish was consistent with the experimentally determined estimates of their bioconcentration potential by reversed-phase HPLC. A number of other norabietanes and bisnorabietanes were tentatively identified in both sediment and fish samples, together with their dehydrogenation products, and a hypothetical scheme relating these to dehydroabietic acid is proposed. Attention is directed to the value of procedures including open-column chromatography on silica gel, gel permeation chromatography and mild chemical treatment for preparing and pretreatment of samples before identification. It is emphasized that analytical procedures should be directed to the specific structure of the analyte and will depend on the nature of interfering compounds in the samples. The search for universal methods that are applicable to structurally diverse analytes may be unrealistic.

INTRODUCTION

There are several problems in determining the environmental impact of effluents from industrial processes which contain a large number of components with diverse structures. In the specific case of bleachery effluents, the problem is particularly severe. The large number of compounds encountered

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has resulted in extensive efforts being directed to their identification and quantification. Ideally, all of these compounds should be identified individually so that their persistence, their biological effect and their distribution can be rationally evaluated [1].

Analysis of cyclohexane-extractable organically bound chlorine (EOCl) has been used to monitor the distribution of compounds putatively originating from bleachery effluents in sediments and biota [2]. A previous study [3] was directed to identifying

the components of these extracts from contaminated sediment samples, but resulted in identification of only *ca.* 10% of the total organochlorine content in terms of specific compounds. Further effort has now been devoted to this problem and attention directed to procedures for fractionating the extracts prior to attempted identification of their components.

In this investigation, a hitherto unidentified group of chlorinated hydrocarbons was isolated and identified by comparison with authentic synthetic compounds. The assessment of the distribution of these compounds in environmental samples has therefore now become possible: their concentration in sediment samples was compared with those of other alicyclic and aliphatic compounds originating in bleachery effluents. As these compounds appear not to have been described previously as environmental contaminants, their octanol–water partition coefficients were measured by a surrogate HPLC procedure.

EXPERIMENTAL

Source of samples

Effluent samples were chlorination-stage (C-stage) and extraction-stage (E-stage) bleach liquors, and were kept at 4°C after collection. Samples of sediment and perch (*Perca fluviatilis*) were collected from the Gulf of Bothnia within *ca.* 5 km of the discharge of bleachery effluents. The sediment was kept in filled screw-capped jars at 4°C before use; the moisture content was determined from the mass loss after heating at 105°C for 12 h and the organic carbon from the mass loss from acidified dried samples after ignition at 550°C for 5 h. The fish samples were wrapped in aluminium foil and kept frozen at −20°C. They were cut into fillets before analysis.

Chemicals

Dehydroabietic acid was purchased from Helix (Richmond, BC, Canada) and retene from ICN Biomedicals (Costa Mesa, CA, USA). 12,14-Dichlorodehydroabietic acid was synthesized by modification [3] of a published procedure. The following reagents were purchased from the sources given in parentheses: phosgene (20% solution in toluene) (Fluka, Buchs, Switzerland), 2-thiopyridone N-oxide, 4-dimethylaminopyridine and tri-*n*-butylstan-

nane (Aldrich, Steinheim, Germany) and α,α′-azo-isobutyronitrile (Jansen, Beerse, Belgium). Solvents were obtained from Burdick and Jackson (Muskegon, MI, USA).

Synthesis of reference compounds

The structures of the 18{19}-norditerpenes investigated in this study are shown in Fig. 1. It should be noted that the prefix "z-nor" implies replacement of the original substituent at position z (in this case CO_2H) with H. We have used the standard terpene numbering system throughout, and designated the compounds as derivatives of abietane. In *Chemical Abstracts*, however, these compounds are designated as octahydrophenanthrenes with a numbering system different from that used for sterols and terpenes; for example, 18{19}-norabieta-8-11,13-triene would be 1,4a-dimethyl-7-(1-methylethyl)-1,2,3,4,4a,9,10,10a-octahydrophenanthrene.

The synthesis of 18{19}-norabieta-8,11,13-triene and 12,14-dichloro-18{19}-norabieta-8,11,13-triene was carried out by the decarboxylation of the corresponding dehydroabietic acids using a published procedure [4]. Briefly, this consisted of the preparation of the acid chlorides from the corresponding carboxylic acids with phosgene in benzene at room temperature, reaction with 2-thiopyridone N-oxide to form the ester, followed by reduction with tributyltin hydride in the presence of the radical initiator α,α′-azoisobutyronitrile. The products were separated from small amounts of remaining organotin compounds by chromatography, first by elution from silica gel with hexane followed by elution from
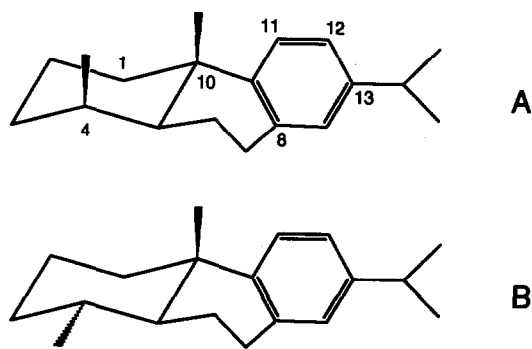

Fig. 1. Structural formulae of (A) 18-norabieta-8,11,13-triene and (B) 19-norabieta-8,11,13-triene.

neutral alumina with hexane. All reactions were carried out in a stream of dry nitrogen. It should be noted that all the reagents are either toxic, potentially explosive or both. The products were initially characterized by GC–MS analysis and consisted of a mixture of the diastereoisomers at C-4 (18-nor and 19-nor compounds). These could readily be separated on a 15-m DB-5 capillary column, but no attempt was made to separate them on a preparative scale. For quantification, the GC response was calculated from the concentrations of the respective epimers.

Identification of 18{19}-norabieta-8,11,13-trienes in sediment cyclohexane-extractable organic chlorine (EOCl) extracts

Cyclohexane-EOCl extracts were prepared from contaminated sediment samples as described previously [3]. Fractionation was carried out on silica gel (Kieselgel 60, 70–230; mesh; Merck). Elution with hexane yielded a neutral fraction in which the 19-norabieta-8,11,13-triene was initially identified.

Extraction of water samples

Samples of water (1 l) were treated as follows: 50 μl of a solution of 5-α-cholestane (1 mg/ml) in benzene were added as a surrogate, the pH of the solution was adjusted to pH 12 and the mixture was extracted twice with hexane–*tert.*-butyl methyl ether (7:3, v/v) (100 ml). The organic phase was washed twice with a saturated solution of NaCl (50 ml), the organic phase removed, dried (Na$_2$SO$_4$), the solvent removed and the residue dissolved in hexane (1 ml). The hexane solution was applied to a Kieselgel 60 (70–230 mesh) silica gel column (30 × 6 mm I.D.) in a Pasteur pipette. The hexane eluate was evaporated to dryness and the residue was dissolved in hexane (1 ml) containing biphenyl (25 μg/ml) as internal standard. Analysis was carried out by GC using a Hewlett-Packard HP 5890 instrument and with splitless injection (the split closed for 45 s) and flame ionization detection. The following temperature programme was used: 45°C for 1 min isothermal, increased at 15°C/min to 300°C, which was held for 10 min. The injector temperature was 240°C and the detector temperature 300°C.

Extraction of sediment samples

Extraction with water-miscible solvents. Samples of sediment (*ca.* 5 g wet mass) were weighed into 10-ml glass tubes fitted with PTFE-lined screw-caps and 5 ml of each of the following solvents were added: tetrahydrofuran, acetonitrile, 2-propanol or dimethylformamide. The samples were kept in an ultrasonic bath for 10 min, shaken gently overnight by inversion, centrifuged (1000 *g*) and the organic phase removed. The extraction was repeated with a further 5-ml portion of solvent, the extracts were combined and 50 μg of the following were added as surrogate standards: for neutral compounds, cholestane; for diterpenes, podocarpic acid O-ethyl ether (prepared from the corresponding acid with diethyl sulphate in alkaline medium and crystallized from acetonitrile); for alkanoic acids, 9,10-dibromooctadecanoic acid (prepared by bromination of 9-octadecenoic acid in CCl$_4$); and for sterols and triterpenes, 5-α-cholestan-3-β-ol.

The extracts were then treated as follows: the extracts from tetrahydrofuran, acetonitrile or 2-propanol were concentrated under a stream of nitrogen to a volume of *ca.* 1 ml, diluted to 7 ml with saturated NaCl solution and extracted three times with 1.5 ml of hexane–*tert.*-butyl methyl ether (7:3, v/v). The combined extracts were washed with saturated NaCl solution, the organic phase dried (Na$_2$SO$_4$) and the solvent removed under a stream of nitrogen.

The dimethylformamide extract was diluted to 80 ml with saturated NaCl solution and extracted twice with 25 ml of hexane–*tert.*-butyl methyl ether (7:3, v/v), the combined organic phases were washed twice with saturated NaCl solution, the organic phase was removed and dried (Na$_2$SO$_4$) and the solvent removed under a stream of nitrogen.

Extraction with water-immiscible solvents. Extraction with benzene was carried out by two methods: (i) Soxhlet extraction of freeze-dried samples (*ca.* 10 g) with benzene for 3 h and (ii) Dean and Stark extraction of wet samples with benzene; when all the water had been removed, extraction was continued for 2 h. Surrogate standards were added to the benzene extracts, which were then concentrated in vacuum and chromatographed as follows.

The concentrates prepared above were dissolved in 2 ml of cyclohexane and chromatographed on a column of silica gel (Kieselgel 60, 70–230 mesh).

Neutral compounds were eluted with hexane [fraction (a)] and other compounds with *tert.*-butyl methyl ether [fraction (b)]. Fraction (a) was concentrated under a stream of nitrogen to a volume of 2 ml. Elemental sulphur was removed by shaking with an aqueous solution of tetrabutylammonium sulphite (1 ml) and 2-propanol (1 ml) [5]. The organic phase was dried (Na_2SO_4) and used for quantification by the GC procedure described above. Fraction (b) was concentrated under a stream of nitrogen, methylated with a solution of diazomethane in diethyl ether and concentrated. The concentrate was acetylated overnight at room temperature with equal volumes of acetic anhydride and pyridine (50 μl). The reaction mixture was dissolved in hexane, pyridine was removed by washing with 0.5 M HCl (4 ml) and acetic anhydride was hydrolysed by shaking with 0.8 M K_2CO_3 solution (4 ml). The hexane solution was dried (Na_2SO_4) and chromatographed on a column (4 × 1 cm I.D.) of silica gel (Kieselgel 60, 70–230 mesh). Elution was carried out with hexane–*tert.*-butyl methyl ether (7:3, v/v), the solvent removed under a stream of nitrogen and a solution of biphenyl in hexane (25 μg) was added as internal standard. GC analysis was carried out as described above.

For mass spectrometric identification, the extracts were further purified by gel-permeation chromatography (GPC) as follows: the solvent was removed under a stream of nitrogen and the residue was dissolved in tetrahydrofuran (70 μl) and applied to a column of PL-gel (particle size 10 μm, porosity 500 Å, 600 × 10 mm I.D.). The column was eluted with tetrahydrofuran at a flow-rate of 1 ml/min. Detection was carried out at 280 nm and the fraction eluting at the same time as the authentic standard was collected. The sample was concentrated, dissolved in hexane, chromatographed on a short column of neutral alumina (Merck), eluted with hexane or benzene and the solutions obtained were used for GC–MS analysis.

Extraction of fish samples

Muscle tissue was removed from fish and samples (*ca.* 35 g) were treated by two different procedures. (i) Samples were freeze-dried and the residue was Soxhlet extracted with benzene. (ii) Samples were mixed with portions of solid carbon dioxide and mixed in a blender to obtain a fine flour, then the

CO_2 was removed by sublimation at $-20°C$ [6]. The product was either freeze-dried and Soxhlet extracted with benzene, or the wet sample Dean and Stark extracted with benzene.

The benzene extracts containing *ca.* 250 mg of lipid material were treated as follows. Benzene was removed in a stream of nitrogen and the residue was dissolved in *tert.*-butyl methyl ether (1 ml). A 2 M solution of sodium methoxide in methanol (300 μl) was added and the mixture was shaken for 5 min at room temperature. The reaction was terminated by adding 0.5 M HCl (5 ml) and extracted twice with 1.5-ml portions of hexane–*tert.*-butyl methyl ether (7:3, v/v). The combined organic extracts were washed with water (3 ml), dried (Na_2SO_4) and the solvent was removed. The residue was dissolved in hexane (2 ml) and chromatographed on a column of silica gel (5 × 1 cm I.D.); the desired hydrocarbons were eluted with hexane and purified further by the methods used for the sediment samples, *i.e.*, GPC using tetrahydrofuran followed by chromatography on neutral alumina and elution with hexane. The resulting eluate was concentrated and used for GC–MS analysis.

Gas chromatographic–mass spectrometric and nuclear magnetic resonance analysis

GC–MS analysis was carried out as described previously [7].

NMR spectra were recorded on a JEOL GX400 spectrometer (399.65 MHz for ^1H and 100.40 MHz for ^{13}C) equipped with a standard 5-mm diameter C/H probe. The samples were prepared in $C_6{}^2H_6$ or C^2HCl_3 at a concentration of 20 mg/ml. All spectra were acquired using standard pulse sequences supplied by JEOL. The ^1H spectra were referenced to internal tetramethylsilane and the ^{13}C spectra referenced to the central line of the $C_6{}^2H_6$ signal (δ 128.00) or C^2HCl_3 (δ 77.00).

The ^1H spectra were acquired using the following conditions: spectral width 8.8 kHz, 32K data points, a pulse duration of 10 μs (30°) and a cycle time of 6 s. After sixteen scans the data were zero filled once and processed with a Gaussian window (-0.23 Hz line broadening). The unoptimized NOE difference spectra were acquired using the following conditions: spectral width 8.8 kHz, 32K data points, a pulse duration of 10 μs (30°), an irradiation time of 5 s with a total cycle time for the

NOE–normal spectrum pair of 20 s. After 64 scans the data were zero filled once and processed using an exponential window (1.0 Hz line broadening).

The $\{^1H\}^{13}C$ spectra were acquired using the following conditions: spectral width 25 kHz, 32K data points, a pulse duration of 4.7 μs (25°) and a cycle time of 2 s. After 1000 scans the data were zero filled once and processed with an exponential window (1.0 Hz line broadening). Accepting the differences in pulse durations required, the DEPT-90 and DEPT-135 spectra (256 scans each) were acquired and processed in the same way as the $\{^1H\}^{13}C$ spectrum.

Octanol–water partition coefficient

The octanol–water partition coefficient (P_{ow}) was estimated by a surrogate procedure using HPLC [8]. A Nucleosil C_8 column (150 × 10 mm I.D., 5 μm particle size) and a mobile phase of phosphoric acid (0.05 mol/l)–methanol (1:3, v/v) at a flow-rate of 1 ml/min were used; the UV detector was set at 280 nm. The following compounds were used as calibration standards: formamide, benzene, toluene, naphthalene, biphenyl, 1,2,4-trichlorobenzene, phenanthrene, fluoranthrene and DDT. Values for the capacity factors were calculated and from them values of P_{ow}; these values were then used to estimate bioconcentration factors (*BCF*) using the equation of Mackay [9].

RESULTS

Structure of authentic compounds

The mass spectrum of the synthetic 18{19}-norabieta-8,11,13-triene had a parent ion at *m/z* 256 corresponding to $C_{19}H_{28}$ and that of 12,14-dichloro-18{19}-norabieta-8,11,13-triene had a parent ion at *m/z* 324 corresponding to $C_{19}H_{26}Cl_2$ and with a ratio in the intensity of the peaks at 324 and 326 of 100:70. In both products, the two epimers were present in the ratio of *ca.* 3:1 (the minor product eluting first), but no attempt was made to separate these on a preparative scale.

The signals in the ^{13}C NMR spectrum of the synthetic mixture of 18-nor- and 19-norabieta-8,11,13-trienes were assigned using $\{^1H\}^{13}C$, DEPT-90 and DEPT-135 spectra to determine the chemical shifts and the number of protons attached to each carbon. The reported spectra are those observed in $C_6{}^2H_6$ solution.

18-Norabieta-8,11,13-triene (major component): δ 147.14 (C-9), 145.39 (C-13), 135.04 (C-8), 126.94 (C-14), 124.29 (C-11), 123.77 (C-12), 44.79 (C-5), 39.00 (C-3), 37.53 (C-10), 34.25 (C-4), 34.00 (C-15), 33.40 (C-1), 30.68 (C-7), 25.74 (C-20), 24.94 (C-2), 24.30 (C-16,17), 18.37 (C-6), 15.28 (C-19).

19-Norabieta-8,11,13-triene (minor component): δ 145.75 (C-9), 145.44 (C-13), 135.23 (C-8), 126.89 (C-14), 124.60 (C-11), 123.64 (C-12), 49.31 (C-5), 38.55 (C-3), 37.27 (C-10), 36.47 (C-1), 34.00 (C-15), 31.80 (C-4), 30.03 (C-7), 24.30 (C-16,17), 22.93 (C-18), 22.43 (C-2), 21.70 (C-6), 20.64 (C-20).

The δ 1.23–0.86 region of the ^1H spectrum of the $C_6{}^2H_6$ solution of this mixture contained only methyl groups. The methyl group at C-10 of 18{19}-norabieta-8,11,13-triene is a singlet. Irradiation of the major component singlet methyl produced a 3.5% NOE on the corresponding C-4 methyl signal (δ 0.94), indicating that the two groups are spatially close, and thereby confirming the major components as the 18-nor epimer. Irradiation of the minor singlet methyl produced no measurable NOE interaction with the corresponding C-4 methyl signal (δ 0.85) which is consistent with the structure of the 19-nor epimer. These spectra are shown in Fig. 2.

The NMR spectrum for the dichloronorabietatriene was significantly more complex than that of the parent hydrocarbon owing to signals from unseparated impurities, and no attempt was made to provide a complete interpretation on the basis of ^{13}C NMR spectrum. In the critical region of the ^1H spectrum where the methyl resonances occurred, there was sufficient similarity to those of the nonchlorinated compound to support the structural assignments and to show that the major component was the 18-nor epimer. This similarity is illustrated in Fig. 3 for the spectra of the compounds in C^2HCl_3.

Identification and quantification in environmental matrices

For conclusive identification of the hydrocarbons in the sediment samples, it was necessary to remove interfering compounds as far as possible. A combination of silica gel and gel permeation chromatography was used and the value of these is illustrated in Fig. 4. For biota, the additional transesterification step with sodium methoxide was valuable and in addition enabled larger amounts of material to
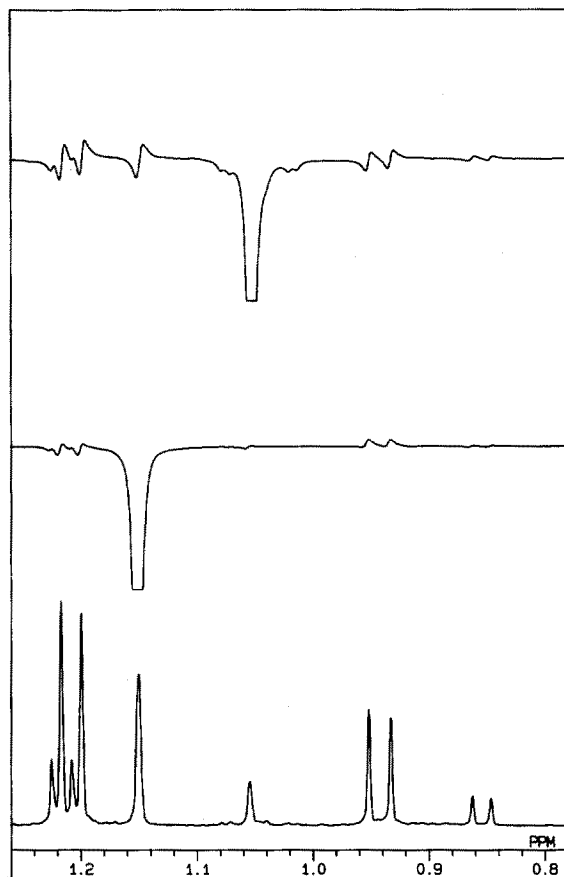
Fig. 2. ^{1}H normal NMR spectrum in $C_6{}^2H_6$ solution of mixture of synthetic 18-nor and 19-nor epimers (bottom) and NOE on the C-10-methyl of 18-norabieta-8,11,13-triene (middle) and 19-norabieta-8,11,13-triene (top).

Fig. 3. ^{1}H normal NMR spectra in C^2HCl_3 solution of 18-norabieta-8,11,13-triene (bottom) and 12,14-dichloro-18-norabieta-8,11,13-triene (top). The peaks marked with asterisks correspond to unknown impurities.

be analysed. A comparison of the mass spectra of authentic 18-norabieta-8,11,13-triene and the compounds isolated from the sediment and fish samples is shown in Fig. 5. A similar comparison for 12,14-dichloro-18-norabieta-8,11,13-triene is shown in Fig. 6.

Both epimers were found in environmental samples, and for quantification it was assumed that the GC response of both epimers was equal. The concentrations of 18-nor-abieta-8,11,13-triene in samples of extraction stage bleachery effluents ranged from 3 to 6 μg/l, whereas levels of the corresponding 12,14-dichloro-18{19}-nor compound were undectable. The extracts from the wet sediment samples
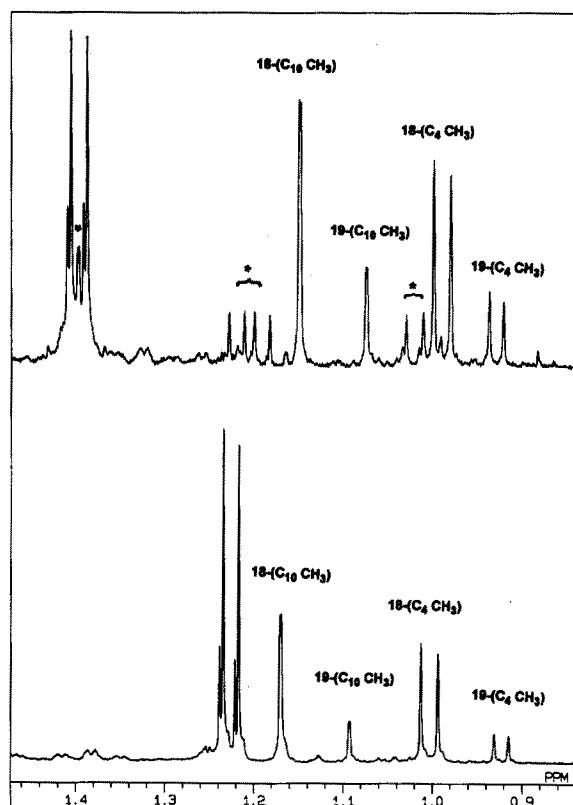
were generally dark coloured, presumably owing to the presence of humic substances, whereas the benzene extracts were light yellow. The concentrations of 18-norabieta-8,11,13-triene and its 12,14-analogue are given in Table I together with those of a number of compounds traditionally associated with bleachery effluents; these values were obtained using surrogate standards and their accuracy is calculated to be *ca.* 10%.

Monochloro compounds analogous to 12,14-dichloro-18{19}-norabieta-8,11,13-triene were found in sediment samples, although as reference compounds were not available quantification was not possible. The two isomers could be distinguished on
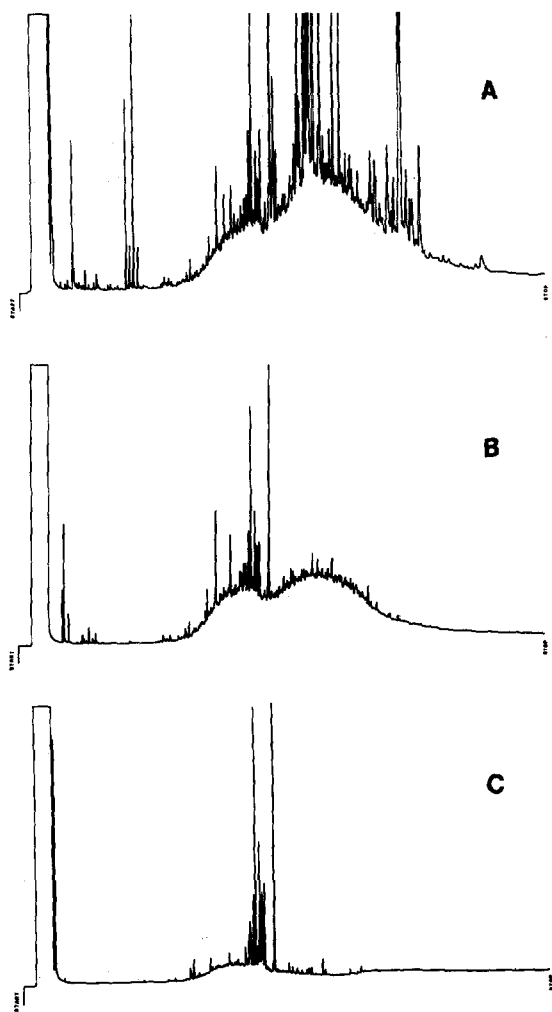
Fig. 4. Illustration of the value of silica gel chromatography (B) followed by gel permeation chromatography (C) on a crude sediment extract (A).

Octanol–water partition coefficient

The logarithms of the octanol–water partition coefficients for 18-norabieta-8,11,13-triene and 12,14-dichloro-19-norabieta-8,11,13-triene determined by HPLC were greater than that for DDT (6.2) and were estimated to have approximate values of 8.1 and 9.4, from which bioconcentration factors (log BCF) of 6.8 and 8.1 were calculated.

DISCUSSION

To simplify the discussion, the structures of the diterpene-related hydrocarbons derived from dehydroabietic acid and structurally related to the 18{19}-norabieta-8,11,13-trienes are shown in Fig. 10.

The structures of the 18{19}-nor hydrocarbons were supported by their mass spectra, and the configurations at C-4 were confirmed by the results of the NOE experiment after irradiation of the singlet methyl groups at δ (0.94 (18-nor epimer) and at δ 0.85 (19-nor epimer) in $C_6{}^2H_6$ solution (Fig. 2). Comparison of the ^{13}C chemical shifts of the methyl groups at C-4 and C-10 with those of abieta-8,11,13-trienes reported in the literature [10] strongly supports the assigned stereochemistry. The configuration of the 12,14-dichloro-18(19)-norabieta-8,11,13-trienes was based on the similarity of the ^1H spectra in C^2HCl_3 (Fig. 3) to those of the non-chlorinated hydrocarbons.

Identification of the C-19 nor hydrocarbons and their dichlorinated analogues in the environmental matrices was confirmed by comparison of the mass spectra with that of the authentic compound (Figs. 5 and 6). Conclusive identification was critically dependent on the preparation of samples free from interfering impurities. Whereas the analysis of water samples was essentially straightforward, the other two environmental samples illustrated the need to take into consideration both the nature of the analyte and the type of matrix. Analysis of samples of sediment and biota illustrated the need for different clean-up procedures. Sediment samples were contaminated with large amounts of elemental sulphur, which was readily removed with tetrabutylammonium sulphite, and silica gel was effective in removing the more polar fatty acids and sterols. It was the subsequent application of GPC, however, that effectively removed interfering hydrocarbon

the basis of their different GC retention times whereas the configuration at C-4 could not be determined; the mass spectra of the two compounds, arbitrarily designated A and B, are shown in Fig. 7. In addition, a number of other hydrocarbons were isolated from sediment and fish samples and were tentatively identified from their mass spectra presented in Fig. 8. A comparison of the mass spectrum of authentic retene with that of the compound isolated from sediment samples is given in Fig. 9.
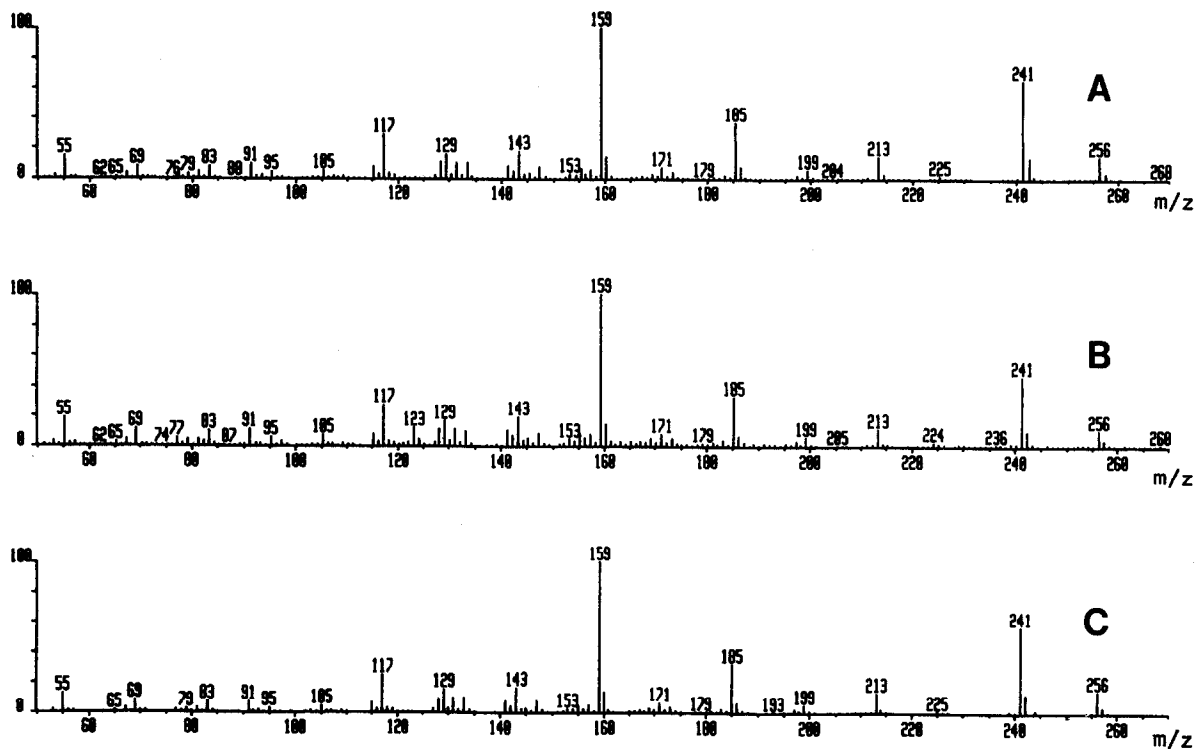
Fig. 5. Comparison of the mass spectra of authentic 18-norabieta-8,11,13-triene (bottom) and the products isolated from a fish sample (middle) and from a sediment sample (top).
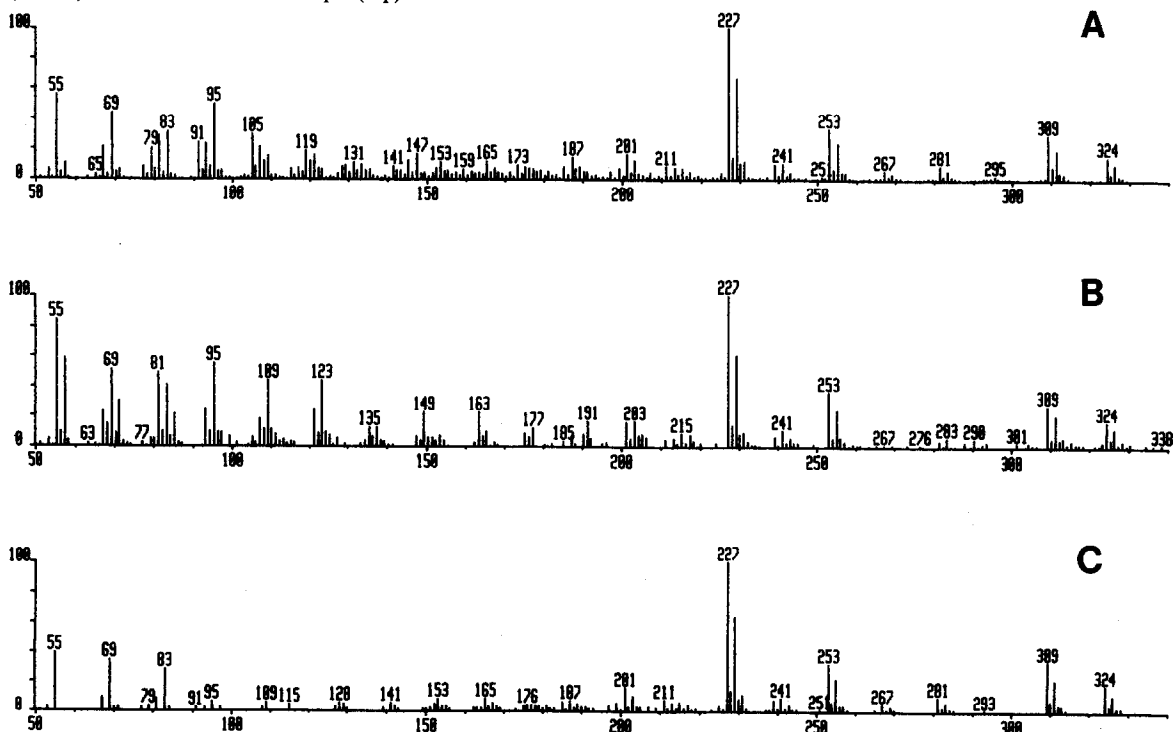

Fig. 6. Comparison of the mass spectra of authentic 12,14-dichloro-18-norabieta-8,11,13-triene (bottom) and the products isolated from a fish sample (middle) and from a sediment sample (top).

TABLE I

CONCENTRATIONS OF SELECTED ORGANIC COMPONENTS OF SEDIMENT SAMPLES FROM TWO LOCALITIES

Sample	Extraction	Concentration (mg/g organic C)[a]							
		nor	Cl_2-nor	DHA	Cl_2-DHA	C_{18}	Cl_2-C_{18}	β-sito	betulin
A	Acetonitrile	40	4.1	160	24	19	17	690	280
	Dimethylformamide	30	2.4	90	17	22	17	420	200
	2-Propanol	50	4.6	250	61	46	36	1400	260
	Tetrahydrofuran	120	5.3	530	130	51	39	1500	280
	Dean-Stark:benzene	50	4.6	200	55	22	14	1150	150
	Soxhlet:benzene	50	4.6	300	61	35	24	1000	180
	Soxhlet:tetrahydrofuran	20	2.5	480	170	75	46	730	85
B	Acetonitrile	650	6.1	580	18	67	43	2300	5500
	Dimethylformamide	450	5.0	160	6	49	18	2000	5700
	2-Propanol	780	15	780	36	128	55	3900	8100
	Tetrahydrofuran	1000	17	2200	120	274	130	4400	8000
	Dean-Stark:benzene	870	11	690	41	62	27	3300	5500
	Soxhlet:benzene	840	11	760	43	135	78	2900	5200

[a] nor = 18-Norabieta-8,11,13-triene; Cl_2-nor = 12,14-dichloro-18-norabieta-8,11,13-triene; DHA = dehydroabietic acid; Cl_2-DHA = 12,14-dichlorodehydroabietic acid; C_{18} = octadecanoic acid; Cl_2-C_{18} = 9,10-dichlorooctadecanoic acid; β-sito = β-sitosterol.
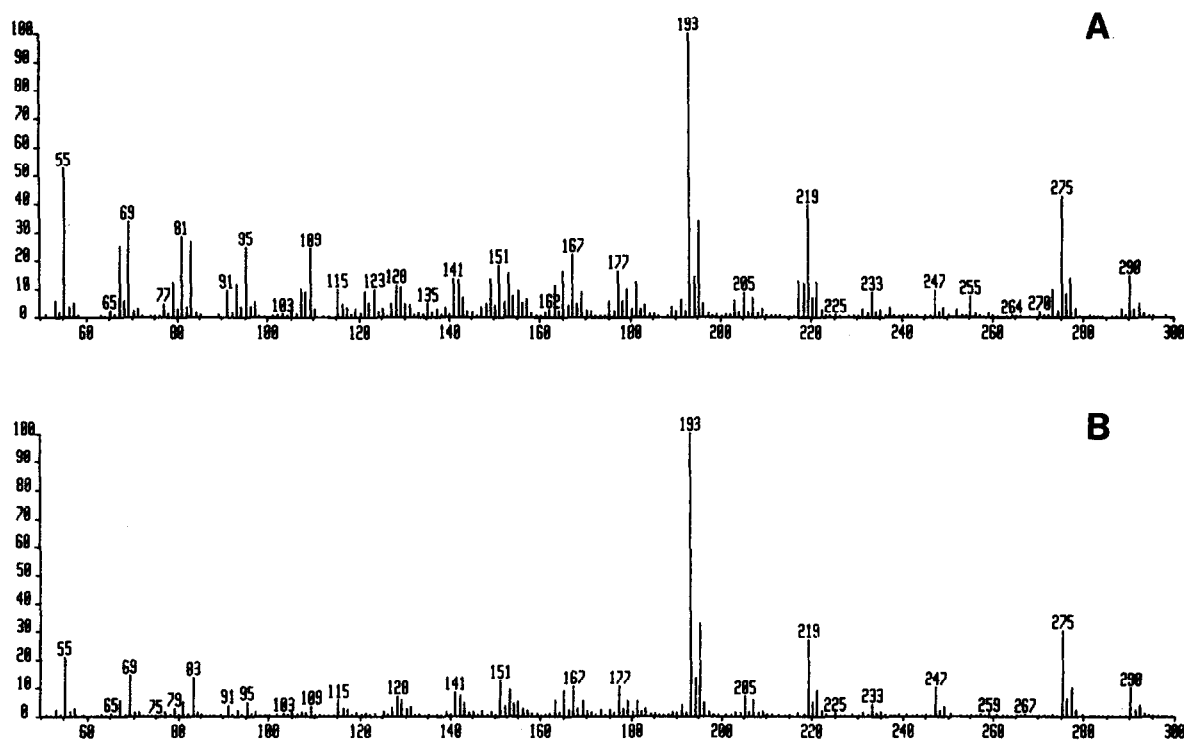

Fig. 7. Mass spectra of the isomeric 12{14}-monochloro-18{19}-norabieta-8,11,13-trienes isolated from sediment samples and arbitrarily designated A and B.
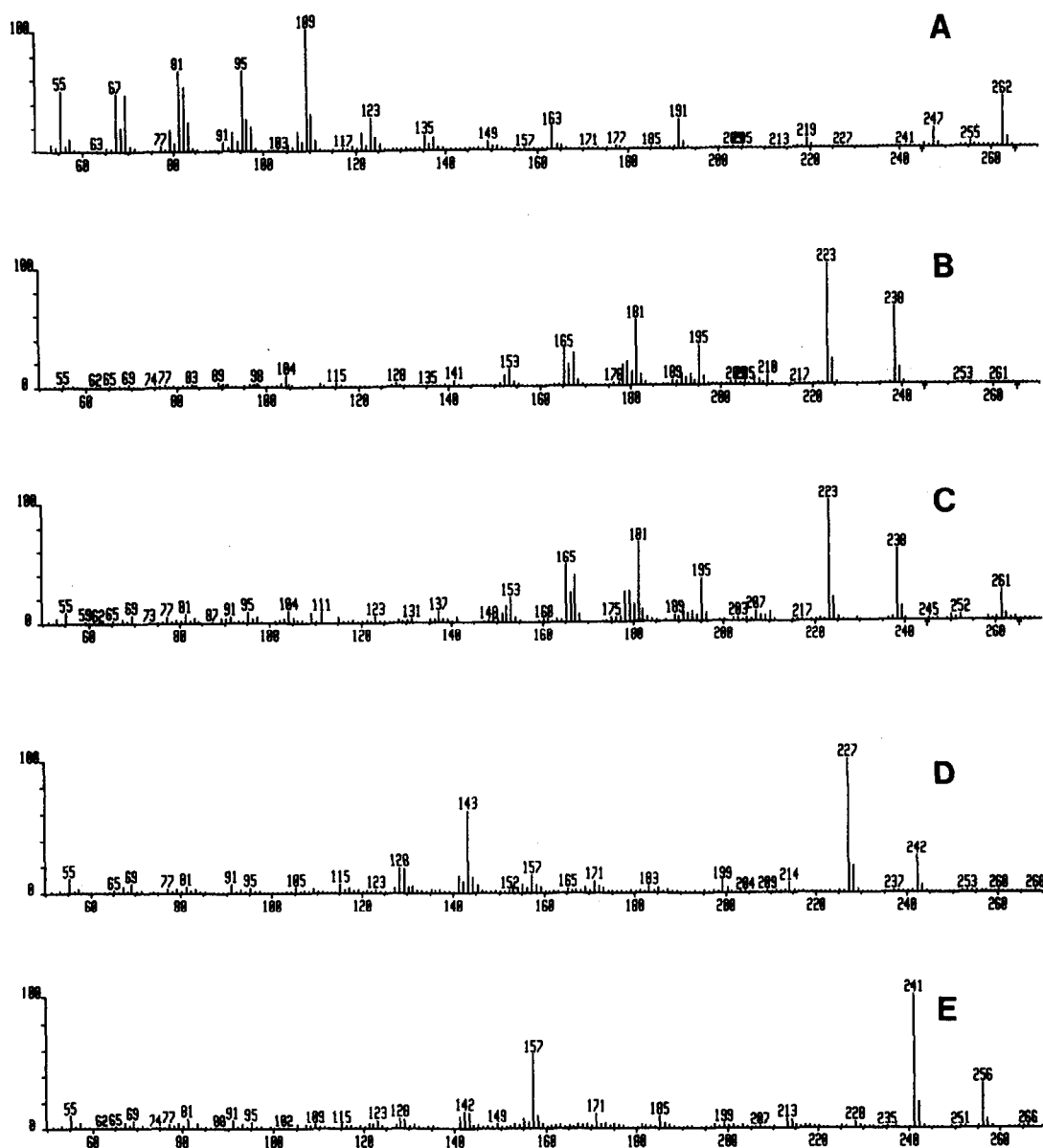
Fig. 8. Mass spectra of diterpene-related hydrocarbons isolated from sediment samples, (A) 18{19}-norabietane (**IV**) and (B) 10,18{19}-bisnorabieta-5,7,9(10),11,13-pentaene (**V**) and from fish, (C) 10,18{19}-bisnorabieta-5,7,9(10),11,13-pentaene (**V**), (D) 10,18{19}-bisnorabieta-8,11,13-triene (**VI**) and (E) 1-methyl-10,18{19}-bisnorabieta-8,11,13-triene (**VII**).

components and allowed the unambiguous mass spectrometric identification of the norabietatrienes (Fig. 4). Whereas in the present study no systematic effort was made to evaluate the extraction procedures for biota, as that involving homogenenization with solid CO_2 was both expedient and effective,

clean-up of extracts from biota presented an entirely different problem from that of sediments. The major interfering compounds were lipids and a variety of procedures have been proposed for their removal [11–13]. Although no systematic examination was carried out in the present investigations, it
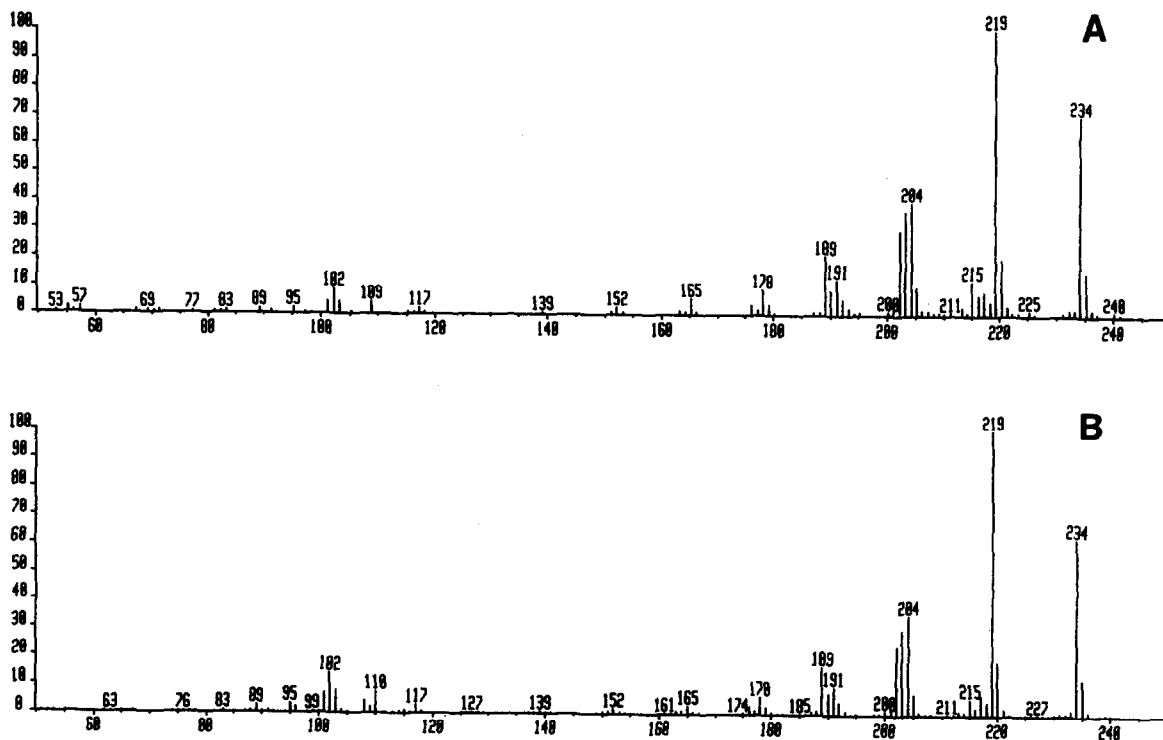
Fig. 9. Comparison of the mass spectrum of retene (**VIII**) (bottom) with that of the compound isolated from sediment samples (top).
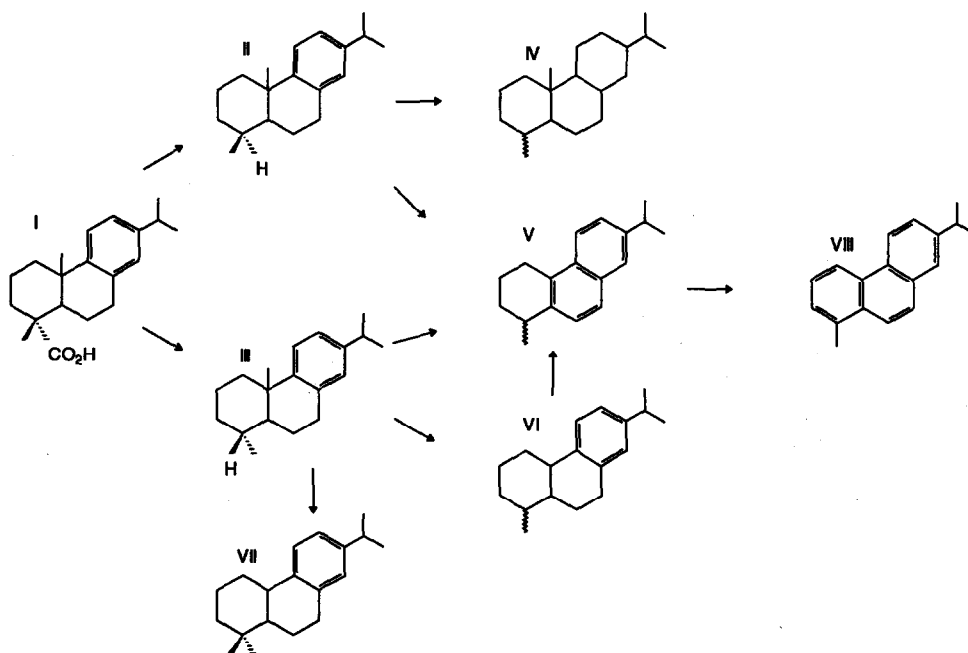

Fig. 10. Hypothetical scheme showing the relationship to dehydroabietic acid of the hydrocarbons isolated from sediment and fish samples: compounds **II**, **IV**, **V** and **VIII** were isolated from sediments and **II**, **III**, **V**, **VI** and **VII** from fish.

was found that transesterification of the lipids with sodium methoxide was effective in forming long-chain carboxylic acid esters which were retained on silica gel columns while the hydrocarbons were effectively eluted with hexane. This procedure reduced the level of interfering compounds, even in relatively large fish samples, sufficiently to facilitate the use of GPC at a later stage without overloading the column.

For analysis of the nor hydrocarbons, benzene was chosen for Soxhlet extraction of dried samples of both sediments and fish on account of its successful application to the extraction of polycyclic aromatic hydrocarbons from air particulates [14]. On the other hand, it was clear that the extraction of wet sediment samples with water-miscible solvents was superior (Table I). This is not necessarily true in all circumstances, however, as the use of tetrahydrofuran for Soxhlet extraction was almost twice as effective as that of benzene for the acidic components (chlorinated and unchlorinated dehydroabietic acids and octadecanoic acids), whereas it was clearly less effective for the hydrocarbons. Tetrahydrofuran, which was used advantageously with wet sediment samples, was not suitable for samples of biota, however, as this solvent simultaneously extracted large amounts of undesirable compounds, particularly lipids. Benzene extracts prepared from biota were therefore used throughout this investigation. Although extraction of 18-norabieta-8,11,13-triene and the corresponding dichloro compound from the sediment samples was most effectively accomplished with tetrahydrofuran, it should be emphasized that no single solvent or procedure is likely to be optimum for more than a limited structural range of compounds. Efforts to apply a single methodology to the analysis of a wide range of analytes may therefore encounter serious difficulties, although attempts using spiking to assess recovery have achieved moderate success [15].

The fact that only 18-norabieta-8,11,13-triene, and not the chlorinated analogues, was present in the effluent sample suggested that the chlorinated compounds were produced in the environment by stereospecific decarboxylation of the precursor 12,14-dichlorodehydroabietic acid, and that this reaction was probably mediated by anaerobic bacteria. This is consistent with the observation that the concentration of a compound tentatively identified as 18-norabieta-8,11,13-triene acid increased after anaerobic biological treatment [16]. Presumably the microbial reaction is stereospecific so that only the 18-nor compound, with the same configuration as dehydroabietic acid itself, is found in environmental samples in contrast to the radical decarboxylation used for chemical synthesis, which produced ca. 25% of the 19-nor epimer. The 18-nor configuration is also consistent with the structure of the fossil resin hydrocarbon fichtelite that has been established as 18-norabietane [17]. The situation with fish samples was more complex as the principal non-chlorinated nor hydrocarbon was the 19-nor epimer, whereas for the dichlorinated analogue the 18-nor epimer was dominant. Further speculation on the reasons for this is not merited on account of the interacting factors of uptake by fish and metabolism before or after ingestion.

The other compounds that were determined in the sediment samples were chosen to represent a range of alicyclic and aliphatic compounds known to be present in bleachery effluents, and therefore putatively present in contaminated sediments. Particularly for sample B, the concentration of 18-norabieta-8,11,13-triene was comparable to that of dehydroabietic acid itself, although the concentrations of 12,14-dichloro-18-norabieta-8,11,13-triene were lower than those of 12,14-dichlorodehydroabietic acid.

The quantification of the nor hydrocarbons in fish samples presented severe problems because a suitable surrogate standard was not avilable; cholestane clearly cannot be used in sterol-rich extracts from fish. On the other hand, the samples were sufficiently free from interfering impurities to permit unambiguous mass spectrometric identification. To avoid possible misinterpretation, it should be pointed out that the total concentrations of these chlorinated nor compounds found in the environmental samples were not sufficiently high for them to make a numerically significant contribution to the total organic chlorine in the samples, maximally comparable to that of the chlorinated dehydroabietic acids [3].

At present, no data on the toxicity of these neutral compounds is avilable, although their potential for significant bioconcentration is suggested by the estimated values of P_{ow} and hence BCF. The possi-

bility of bioconcentration is unequivocally supported by the identification of both 18(19)-norabieta-8,11,18-triene and the 12,14-dichloro-18-nor compound in muscle samples of fish captured from areas subject to contamination of bleachery effluents. As data on persistence and toxicity are not currently avaible, and require access to pure samples of both enantiomers, it is not possible to provide any estimate of the environmental hazard of these compounds. It is clear, however, that their concentrations in sediment samples are at least as significant as those of dehycroabietic acid and its chlorinated analogues.

A number of other neutral hydrocarbons were isolated for which no authentic samples were available and quantification was therefore not possible. The structures of these compounds were tentatively determined, however, by interpretation of their mass spectra and by comparison with some spectra published in the literature. The following groups of compounds were isolated.

(i) Monochloro compounds corresponding to 12,14-dichloro-18{19}-norabieta-8,11,13-triene were isolated from sediment samples (Fig. 7); these were presumably the 12- and 14-chloro compounds, although it could not be established in this study which isomer was present.

(ii) A range of non-chlorinated hydrocarbons was isolated from both fish and sediment samples and all of these are presumably derived from dehydroabietic acid; a hypothetical scheme illustrating their structural relation is given in Fig. 10.

(a) The totally reduced 18{19}-nor compound (**IV**) was isolated from sediment samples and on the basis of its mass spectrum (Fig. 8A) [18] was identified as fichtelite or its C-4 epimer.

(b) Compounds in which ring B is aromatic with loss of the methyl group at C-10 and decarboxylation of the carboxylic acid group from C-4 (**V**) were isolated both from sediment (mass spectrum in Fig. 8B) and fish samples (mass spectrum in Fig. 8C). These compounds have been isolated previously from sediment samples [19,20] and may plausibly be formed from the diterpenes which dominate such samples.

(c) The completely dehydrogenated compound retene (**VIII**) 1-methyl-7-(1-methylethyl)phenanthrene (mass spectrum in Fig. 9) was unambiguously identified in sediment samples.

(d) The dominant hydrocarbon in fish was a C_{18} compound (**VI**) corresponding to the loss of one additional methyl group from the 18{19}-norabietanes (mass spectrum in Fig. 8D).

(e) A C_{19} hydrocarbon isomeric with the 18{19}-norabietatrienes was isolated from fish samples (mass spectrum in Fig. 8E) and was tentatively assigned the structure **VII**.

In addition, two other groups of non-chlorinated hydrocarbons were isolated. Substantial amounts of compounds corresponding to reduction of the carboxyl group of abietic acid to methyl, and with unsaturation at various positions in rings B and C, were found in sediment samples. These presumably originate from pimaric acid; the mass spectra were identical with those published [21] although the structures appear not to have been unambiguously established. The sesquiterpene calamenen, which has already been identified in bleachery effluents [22], was a significant component in all sediment samples.

The chlorinated compounds identified in these investigations clearly arise from precursors formed during the production of pulp by conventional technologies using molecular chlorine. In a wider context, however, it should be appreciated that a structurally diverse range of non-chlorinated hydrocarbons including many of those isolated in this study are widely distributed in fossil wood, ambers, coal and amber [23] and in deep-sea sediments [19,20]. In all instances, these diterpene-related hydrocarbons originate from plant material during fossilization by a number of reactions, including decarboxylation, reduction and dehydrogenation. This appears, however, to be the first time such compounds have been isolated from biota. It should be clearly appreciated, however, that the chlorinated compounds are the result of industrial activity and that their ultimate fate will depend on the degree to which they may be dechlorinated, degraded or transformed by microbial reactions. In a wider perspective, it may be noted that 24-nor- and 28-nor-triterpenes which have been isolated from marine sediments [24] may be derived from plant triterpenoids by decarboxylation of the corresponding carboxylic acids by reactions formally analogous to those resulting in the formation of the 18-nor- and 19-nor-diterpenes.

CONCLUSIONS

The investigation has illuminated and provided further illustration of three important principles of environmental analysis.

(i) The value of effective clean-up procedures for the preparation of samples before attempted identification of new compounds in environmental samples, and the need for these to be adapted to the structure of the specific compounds to be examined. Even for the neutral hydrocarbons examined in this investigation, the combination of various types of chromatography was more flexible than use of drastic procedures involving, for example, strong acids and alkalis, and avoided the formation of artefacts resulting from chemical reactions with the analytes.

(ii) Access to synthetic reference compounds played an important role in this investigation by providing authentic samples for identification and making possible their quantification. In this case, identification required the use of both MS and NMR as the two epimers had identical mass spectra. The availability of the synthetic compounds also made possible the determination of their physico-chemical properties. A programme in synthetic chemistry was therefore a valuable adjunct to the purely analytical activity in this laboratory.

(iii) The application of the procedures to samples of sediment and fish resulted in the unambiguous identification of a novel group of chlorinated aromatic hydrocarbons which are presumably transformation products of compounds formed during production of bleached pulp by conventional procedures using molecular chlorine.

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