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THE STRUCTURE OF CAROTATOXIN, A NATURAL TOXICANT FROM CARROT[1]

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Abstract—A substance has been isolated from extracts of the common carrot, *Daucus carota*, which is toxic to the bioassay organism *Daphnia magna* and to mice. Physical and chemical evidence showed this compound, termed carotatoxin, to be *trans*-1,10-heptadecadiene-5,7-diyn-3-ol (III).

IN THE course of an investigation of naturally occurring toxic substances in food, extracts from a number of common vegetables were examined for their toxicity to an indicator organism *Daphnia magna* Straus.[2] Among the most toxic was the extract from ordinary carrot, *Daucus carota* L.

Initial extraction of the vegetable with acetone was followed by reextraction into hexane—the toxin was found to be soluble in acetone, hexane and benzene but insoluble in water. The hexane solution was subjected to TLC on silicic acid, equal segments of the chromatogram were eluted, and the eluates were bioassayed.[2] The area containing the toxin was rechromatographed to provide a toxic fraction which appeared to consist of a single pure compound, homogeneous to gas chromatography, which could be collected from the chromatograph with its physical and biological properties unchanged. The trivial name "carotatoxin" was applied to the substance.

The quantity of toxin in fresh carrots appeared to be very small, amounting to between 10 and 20 ppm. A kilogram of carrot root supplied only about 2 mg of toxin after repeated purification. In addition to its effect on *Daphnia*, however, the compound was found to produce pronounced neurotoxic symptoms upon injection into mice, and a very rough LD_{50} value of 100 mg/kg was observed in this species.

IR spectra of the toxic fractions isolated by both forms of chromatography were identical (Fig. 1). The most singular absorption occurred at 2260 cm^{-1} and clearly indicated either a C≡N or asymmetrical disubstituted C≡C. Methylene (2940, 2873 and 1465 cm^{-1}) and C-methyl (2962 and 2872, 1380 cm^{-1}) were present, and the intensity of the 720 cm^{-1} absorption indicated a chain of 4 to 6 methylene groups. The strong absorptions at 3400 cm^{-1} and 1115 cm^{-1} indicated the probable presence of a secondary hydroxyl group.

It appeared that several carbon–carbon double bonds were present. A terminal (vinyl) C=C was suggested by absorptions at 3080, 1640, 983 and 926 cm^{-1}, although the latter two bands represented some shift to frequencies higher than normal. The presence of an adjacent (allylic) hydroxyl might account for the abnormality, however,

[1] Presented before the Division of Agricultural and Food Chemistry, 151st National Meeting of the American Chemical Society, Phoenix, Ariz., Jan. 1966. This work was supported in part by a Research Grant (EF-00306) from the U.S. Public Health Service, Bureau of State Services (Environmental Health).

[2] D. G. Crosby and R. K. Tucker, *Abst. 151st Meeting, American Chemical Society* p. A61. Pheonix (1966).

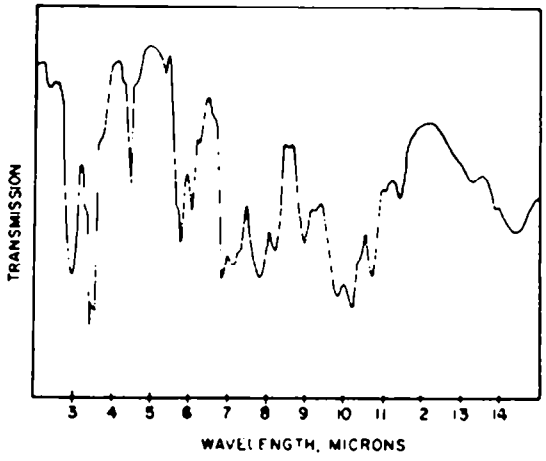


FIG. 1. IR spectrum of carotatoxin.

and this was confirmed in the spectrum of a model, 1-pentene-3-ol ($\nu_{c=c}$ 3080, 1640, 983, 926 cm^{-1}). The typical absorption exhibited by an internal, non-conjugated *trans* C=C appeared at 968 cm^{-1}; this band has been used previously for identification of naturally occurring acetylenes.[8] Absorption characteristic of the *cis* configuration (690 cm^{-1}) was not observed. The above assignments accounted for all of the major absorption bands in the carotatoxin spectrum.

A high-resolution mass spectrum showed a pronounced parent peak at *m/e* 244·1856. All but one of the six most probable empirical formulas derived from this figure contained nitrogen, but an extensive examination of major fragments failed to yield any whose exact masses indicated the presence of this element. Tentative formulas for a number of these fragments were determined (Table 1); their most

TABLE 1. PROPOSED STRUCTURES FOR MAJOR CAROTATOXIN FRAGMENTS

Observed Mass	Probable Composition (Mass)	Proposed Structure
244·1856	$C_{17}H_{24}O$ (244·1827)	$CH_3(CH_2)_4CH(OH)CHCH_2C{\equiv}C{-}C{\equiv}C{-}CH_2CHCH{=}CH_2$ III
226·1721	$C_{17}H_{22}$ (226·1725)	$CH_3(CH_2)_4CH{=}CHCH_2C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}CH{=}CH_2$ IV
155·0865	$C_{12}H_{11}$ (155·0861)	$^{+}CH_2CH{=}CHCH_2C{\equiv}C{-}C{\equiv}C{-}CH{=}CH{-}CH{=}CH_2$ V
119·0496	C_9H_7O (119·0497)	$^{+}C{\equiv}C{-}C{\equiv}C{-}CH_2CH(OH)CH_2$ VI
159·0811	$C_{11}H_{11}O$ (159·0810)	$^{+}CH_2CH{=}CH{-}C{\equiv}C{-}C{\equiv}C{-}CH_2CH(OH)CH{=}CH_2$ VII
141·0715	$C_{11}H_9$ (141·0704)	$^{+}CH_2CH{=}CHC{\equiv}C{-}C{\equiv}C{-}C{\equiv}CH{=}CH{-}CH{=}CH_2$ VIII
103·0549	C_8H_7 (103·0548)	$^{+}CH_2CH{=}CH{-}C{\equiv}C{-}C{\equiv}C{-}CH_2^{+}$ IX

[8] J. D. Bu'Lock, *Prog. Org. Chem.* **6**, 86 (1964).

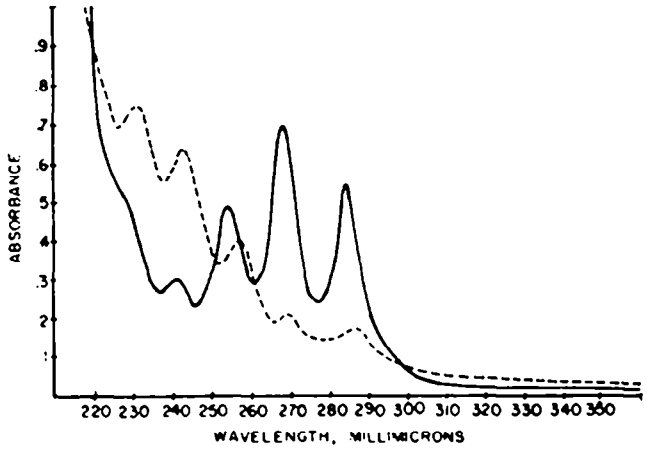


FIG. 2. UV spectrum of carotatoxin (- - -) and anhydrocarotatoxin (———).

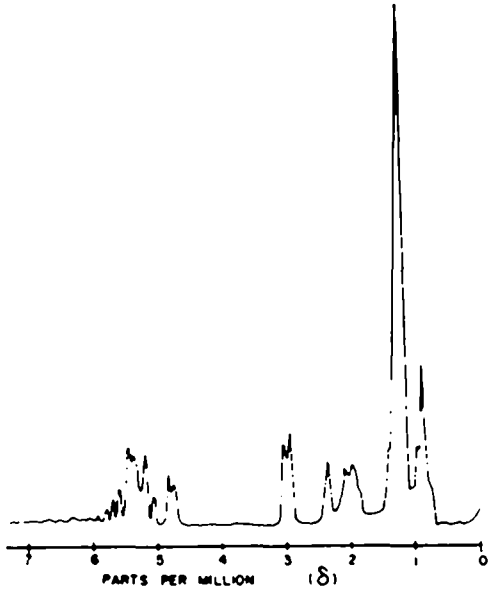


FIG. 3. NMR spectrum of carotatoxin.

characteristic feature was a very high C—H ratio which required the presence of multiple unsaturation. In the absence of nitrogen atoms, the toxin was characterized as a substituted polyacetylenic alcohol. On this basis, $C_{17}H_{24}O$ (mass 244·1872) was considered to represent the formula of carotatoxin. A major fragment of mass 226·1725 ($C_{17}H_{22}$ = 226·1721) suggested that dehydration of the secondary OH group responsible for the IR bands had occurred.

The UV spectrum of carotatoxin (Fig. 2) exhibited strong absorptions at 231 mμ, 243 mμ and 257 mμ characteristic of two conjugated triple bonds;[4] the IR absorption at 2660 cm^{-1} would be consistent with this feature if the diacetylene were asymmetrically substituted. Dehydration of the toxin with a catalytic amount of *p*-toluene sulfonic acid in benzene caused a major change in the UV spectrum (Fig. 2): the

[4] F. Bohlmann and H. J. Mannhardt, *Fortschr. Chem. Org. Naturstoffe* **14**, 1 (1957).

new absorptions at 254 mμ, 268 mμ and 284 mμ were similar to those characteristic of two triple and one double bond in conjugation,[4] although spectra representing the case of two triple and two adjacent double bonds conjugated (C≡C—C≡C—C=C—C=C) have not been reported previously. The spectrum of anhydrocarotatoxin differed markedly in λ_{max} and shape from these representing

$$C{=}C{-}C{\equiv}C{-}C{\equiv}C{-}C{=}C$$

and

$$C{=}C{-}C{=}C{-}C{=}C{-}C{=}C{-}C{=}C$$

Catalytic microhydrogenation of carotatoxin resulted in the absorption of 5·6 molar equivalents of hydrogen corresponding to the saturation of two double and two triple bonds. The resulting product exhibited an IR spectrum virtually identical with that of synthetic 2-heptadecanol. However, the IR spectra and m.ps of most secondary heptadecanols may be anticipated to be very similar;[5] the position of the OH consequently was fixed by a mass spectrum. The observed spectrum contained few peaks, and, characteristic of many saturated alcohols, no parent peak was observed at *m/e* 256 corresponding to $C_{17}H_{36}O$. However, a strong peak at *m/e* 238 represented the expected dehydration product $C_{17}H_{34}$. The *m/e* 227 peak corresponding to a $C_{14}H_{29}CHOH$ fragment, and the *m/e* 59 peak for the C_2H_5CHOH fragment clearly identified the hydrogenated toxin as 3-heptadecanol.

From the above evidence, a partial structure could be formulated for carotatoxin. The terminal vinyl (IR), secondary (allylic) hydroxyl in the 3-position (IR and mass spectrum), and the conjugated diacetylene (IR, UV) placed to conjugate also with the double bond generated upon dehydration (UV) strongly supported the structure I.

$$R{-}C{=}C{-}C{=}C{-}CH_2\underset{\displaystyle OH}{CH}CH{-}CH_3 \qquad (R = C_9H_{17})$$

I

The C_9H_{17} moiety represented by R must contain the remaining vinyl group. From the close similarity of the IR spectrum of the hydrogenated toxin to that of an authentic secondary n-heptadecanol, particularly with respect to C-methyl absorption, the carbon chain appeared to be unbranched. Despite the small quantity of sample available, a time-averaged, 13-scan NMR spectrum (Fig. 3) confirmed the presence of a single C—CH_3 (9·10 ppm) connected through a chain of 5 CH_2 groups (8·75 ppm, 7·98 ppm) to the vinyl carbon. No acetylenic proton was indicated. Although the protons associated with the two different vinyl groups gave rise to a poorly resolved and complex spectrum in the 4–5·5 ppm region, the band centered at 7·02 ppm has been suggested to result from the methylene protons of the C=C—CH_2—C≡C sequence.[6] Integration of the spectrum also confirmed the total of 24 protons, and exchange with D_2O confirmed the secondary hydroxyl group (7·62 ppm).

The structure R = II appeared to be consistent with the IR, UV and NMR data. In order to verify the location of the internal double bond, carotatoxin was oxidized

$$\underset{\displaystyle t}{CH_3CH_2CH_2CH_2CH_2CH_2CH{-}CHCH_2}—$$

II

[4] F. L. Breusch and S. Sokullu, *Chem. Ber.* **86**, 678 (1953).

[6] R. M. Silverstein and G. C. Bassler, *Spectrometric Identification of Organic Compounds* p. 88. Wiley, New York (1963).

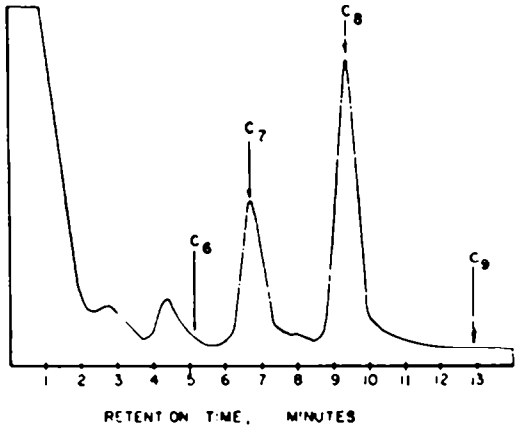
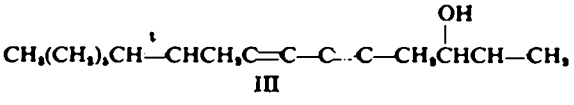


FIG. 4. Gas chromatogram of acids from carotatoxin oxidation. Arrows indicate retention times of standard acids.

in acetone with an alkaline solution of potassium permanganate, and the acidic products were subjected to gas chromatography. As shown in Fig. 4, a suitable Carbowax column clearly resolved a mixture of the lower normal aliphatic acids used as standards. The oxidation of carotatoxin resulted in a mixture of n-heptanoic and n-octanoic acids, according to retention times; each constituent was isolated from the effluent gas stream and identified by comparison of its detailed IR spectrum with that of a pure standard. Although the spectra were almost identical, n-heptanoic acid exhibited absorptions at 760 cm^{-1} and 818 cm^{-1} which were entirely absent in n-octanoic acid. The latter exhibited similar absorption at 788 cm^{-1} and 840 cm^{-1} not observed in the C_7 acid. The existence of the appropriate bands in the spectra of the isolated acids on three separate occasions confirmed their identity.

A C_7 saturated chain in R would explain the presence of the octanoic acid in the oxidation mixture but would be inconsistent with the UV spectra of carotatoxin and anhydrocarotatoxin; the necessary absorptions characteristic of a double bond and two triple bonds conjugated (near 230, 238, 251, 264 and 280 mμ)[4] and those characteristic of the C=C—C≡C—C≡C—C=C—C=C (near 252, 267, 281, 296, 316 and 337 mμ)[4] which could be anticipated upon dehydration of the toxin were not those observed. However, it is quite possible that the isolated double bond in the Δ^{10} position might have shifted into such conjugation during the alkaline oxidation.

Despite the equivocal results of the oxidation experiments, II remains the most satisfactory representation of the C_9H_{17} portion of the toxin molecule. Combination of segments I and II reveals carotatoxin to be *trans*-1,10-heptadecadiene-5,7-diyn-3-ol (III).



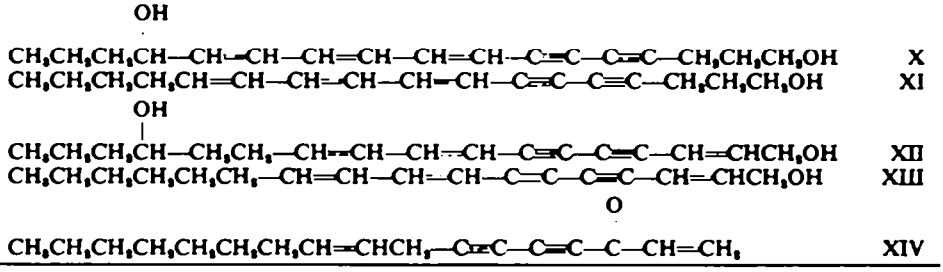
III

Partial isomerization of the Δ^{10} double bond to bring it into conjugation would help to explain several of the carotatoxin fragments observed in the mass spectrum (Table 1). Normal dehydration of carotatoxin (III) would lead to anhydrocarotatoxin (IV), and the expected allylic fission at C_{12} would result in V. The similar

fragment (C_8H_8) to be expected from fission at C_8 was not sought, but a peak corresponding to it, without dehydration, was observed (VI). Isomerization of the isolated Δ^{10} double bond of III, followed by allylic cleavage, would produce VII; either prior or subsequent dehydration would yield VIII which, with the anticipated β-fission at C_3, would result in IX.

In recent years, a relatively large number of acetylene derivatives have been found to occur in higher plants.[3,4,7] Although the majority of them have been isolated from the family Compositae, plants of the Umbelliferae, to which the carrot belongs, have yielded several particularly interesting substances.[3,8]

In 1953, the isolation of toxic principles from two of the most poisonous plant species known—*Cicuta virosa* and *Oenanthe crocata* was described.[9] Four of the five substances identified were C_{17} alcohols containing two conjugated acetylenic bonds (X-XIII). The diols cicutoxin (X) and oenthetoxin (XII) were shown to be extremely poisonous.[9,10] Another closely related diacetylene, falcarinone (XIV), has been

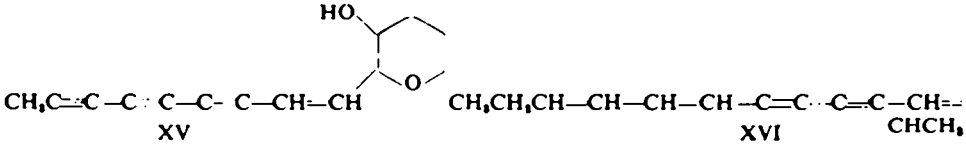


X
XI
XII
XIII
XIV

isolated from other umbelliferous species including *Falcaria vulgaris*, *Oenanthe pimpinelloides*, *Sium sisarum*, *Carum carvi and Aegopodium podagraria*.[8]

The close structural similarity of carotatoxin to falcarinone is apparent. However, the facile dehydration of the former and its conversion to heptanoic acid upon oxidation are sufficient in themselves to differentiate the two types. Falcarinone as well as the other C_{17} diacetylenes also have been described as very unstable, while carotatoxin in solution could be stored exposed to daylight and room temperature for several weeks without apparent change and recovered in a pure state after both GLC and TLC.

Except for the constituents of *Cicuta virosa* and *Oenthe crocata*, only two other polyacetylenes from higher plants appear to have been recognized for their toxicity to animals. It has been shown recently[11] that the fish poison from the Amazon species *Ichthyothere terminalis* possesses the structure XV, and "aethusin" (XVI) has been isolated[12] from the Fool's Parsley (*Aethusa cynapium*) reported to have been responsible



XV XVI

[7] F. Bohlmann, *Fortschr. Chem. Forsch.* **4**, 138 (1962).
[8] F. Bohlmann, C. Arndt, H. Bornowski and K. -M. Kleine, *Chem. Ber.* **94**, 958 (1961).
[9] E. F. L. Anet, B. Lythgoe, M. H. Silk and S. Trippett, *J. Chem. Soc.* 309 (1953).
[10] E. G. C. Clarke, D. E. Kidder and W. D. Robertson, *J. Pharm. Pharmacol.* **1**, 377 (1949).
[11] S. C. Cascon, W. B. Mors, B. M. Tursch, R. T. Alpin and L. J. Durham, *J. Amer. Chem. Soc.* **87**, 5237 (1965).
[12] F. Bohlmann, C. Arndt, H. Bornowski and P. Herbst, *Chem. Ber.* **93**, 981 (1960).

for many instances of human poisoning. It is of interest, however, that a significant proportion of the genera reported to contain C_{17} diacetylenes—*Cicuta, Oenanthe, Sium, Hedera,* and *Aralia* are listed as poisonous in the comprehensive work by Kingsbury.[13]

According to that authority, Wild Carrot or Queen Anne's Lace (also *D. carota)* has been suspected of causing mild intoxication in horses and cattle which fed upon it. It also has been purported to act as a stimulant, diuretic and carminative.[14] No such claims have been made for the domesticated carrot, however, and carotatoxin represents the first polyacetylene to have been reported from this species.

EXPERIMENTAL

IR spectra: a Perkin-Elmer Model 221 IR Spectrophotometer and a Perkin-Elmer Model 337 IR Spectrophotometer, each equipped with a 4X microbeam condenser; UV spectra; a Beckman DK-2A Recording Spectrophotometer; NMR data: in CCl_4 soln on a Varian A-60 NMR Spectrometer using micro cuvettes and a Varian Computer of Average Transients; and mass spectra: on AEI MS-9 and Consolidated Model 110B instruments.

Most of the gas chromatographic analyses were conducted on an F and M Model 720 temp-programmed instrument equipped with a thermal conductivity detector; except where noted, a 2′ column of 5% Dow 11 silicone oil on 60/80 mesh Chromosorb W (HMDS-treated) and a helium flow of 100 ml/min were employed. Collections were made by inserting the small end of a Pasteur pipette into the effluent gas stream through a small neoprene septum as the appropriate fraction emerged. The injector port temp did not exceed 220°, and the detector, maintained at 285° for analytical work, was turned off during collections to avoid decomposition of the toxin.

Detection of toxicity. Samples of the suspected toxin were dissolved in redistilled acetone, and 100 μl aliquots were injected below the surface of 50 ml of deep-well tap water containing 1 ppm of non-ionic surfactant (Tween 20) in which 25 pre-starved first-instar female *Daphnia magna* were swimming. After periods up to 26 hr, the number of immobilized animals was counted as a measure of toxicity.

Extraction and detection of carotatoxin. Fresh market carrots (1·8 kg) were extracted in a Waring blendor with three 1 liter portions of redistilled acetone. After filtration, the acetone extract was evaporated under vacuum on a rotary evaporator and the remaining aqueous layer was extracted with several 250 ml portions of redistilled hexane. The combined hexane layers were washed with water, dried over Na_2SO_4, evaporated to about 50 ml, and stored in the cold to avoid decomposition of the toxin.

The hexane soln (1 ml) was applied to a thin-layer chromatoplate (200 mm square) of Merck Silica Gel G and chromatographed in hexane:acetone (2·5:1). The plate was dried, and the adsorbent layer was divided into 5 equal segments between the origin and solvent front. Each segment was scraped into a centrifuge cone, eluted with 5 ml acetone, centrifuged, the supernatent decanted and evaporated to 500 μl, and an aliquot bioassayed. Maximum toxicity resided in fraction 3 (*Rf* 0·5).

More of the original hexane extract was chromatographed in a similar manner on a number of plates, and an aliquot of their combined fraction 3 was rechromatographed on Silica Gel G in pet ether:ether (2:1). Division of this plate into 7 segments followed by elution and bioassay showed toxicity to reside almost exclusively in fraction 4. Spraying a plate with 1% $KMnO_4$ in 2% Na_2CO_3aq gave an immediate yellow band in this region (*Rf* 0·5). Chromatography of the remaining "fraction 3" provided several mg of material for initial measurement of IR and UV spectra.

Gas chromatography. A sample of toxin purified by TLC was examined on the gas chromatograph at a linear program rate of 7·5°/min. A single product was obtained at an elution temp of 192°; it was collected as a yellow oil and found to be highly toxic to *Daphnia.* Further quantities were isolated in this way directly from the original hexane extract and exhibited UV and IR spectra identical to those of isolates from thin-layer plates.

Dehydration of carotatoxin. The toxin (2·0 mg) was dissolved in 110 ml redistilled benzene and

[13] J. M. Kingsbury, *Poisonous Plants of the United States and Canada.* Prentice-Hall, Englewood Cliffs, N.J. (1964).

[14] J. E. Meyer, *The Herbalist.* Hammond, Hammond, Ind. (1934).

50 mg *p*-toluenesulfonic acid was added. The soln was boiled for 10 min, cooled, extracted with three 20 ml portions of 2% $NaHCO_3$aq, washed with water, and dried over Na_2SO_4. The solvent was removed under vacuum, and the residue was dissolved in MeOH (UV λ_{max} 254, 268, 284 mμ). Evaporation of the MeOH soln onto KBr permitted measurement of the IR spectrum; the original features of the carotatoxin spectrum were present, but the OH bands at 3400 and 1115 cm^{-1} no longer were present.

Hydrogenation of carotatoxin. The reaction was conducted in a Pyrex tube fitted with a sidearm closed by a neoprene septum, an inlet and outlet for H, and a 5 ml burette calibrated to 0·01 ml. EtOH (6 ml) and 10 mg 10% Pt on powdered charcoal were placed in the tube, stirred magnetically, and the system was purged with H and closed after 2 min. The temp was held constant with a water bath. The burette, filled with Hg, was adjusted until the internal press equaled that of the external atm, and a soln of 5·50 mg carotatoxin in 500 μl benzene was injected into the catalyst chamber through the septum. No further uptake of H was observed after 30 min. and the corrected volume represented 5·6 moles of consumed H/mole of carotatoxin.

The reaction mixture was filtered, concentrated, injected onto the gas chromatograph, and the two components were collected. The first to be eluted (165°) was a colorless liquid; its elution temp and IR spectrum were identical with those of an authentic speciment of n-heptadecane except for the presence of an IR band at 1700 cm^{-1} apparently due to an impurity.

The second component (175°) was collected as a white, waxy solid. m.p. 44–46°, ν_{max}: 3340, 2925, 2860, 1470, 1380, 1348, 1138, 940, 724 cm^{-1}. The observed m.p. was in the range of those exhibited by isomeric n-heptadecanols:[6] 2-isomer, m.p. 44–45·5°; 3-isomer, 45·6–47·5°; 4-isomer, 44·7–46·1°; 5-isomer, 45·8–46·7°; 6-isomer, 46·4–47·4°.

Oxidation of caratotoxin. Redistilled acetone (50 ml), 40 μl of 20% NaOHaq, 1 g powdered $KMnO_4$ and 30 mg carotatoxin were stirred and boiled under reflux for 6 hr. The mixture was filtered, and the dark residue was washed with acetone followed by cold and then hot 5% $NaHCO_3$aq. The washings were combined, the acetone evaporated, and the remaining soln was acidified and extracted with ether. The dried ether extract (Na_2SO_4) was concentrated to 1 ml under vacuum and subjected to gas chromatography on a 2′ column of 5% Carbowax 20 M on 40/60 mesh Chromosorb P. Retention times were compared with those of pure n-pentanoic to n-nonanoic acids. The two acids resulting from the oxidation were collected from the effluent stream, rechromatographed, recollected, and their spectra measured as liquid films on NaCl windows.

2-Heptadecanol. Recrystallized 2-heptadecanone (Aldrich Chemical Co.) (1·0 g, 0·004 mole) was dissolved in 150 ml redistilled MeOH, 0·33 g (0·087 mole) of $NaBH_4$ was added, and the soln was boiled under reflux for 2 hr. After cooling, solvent was removed on a rotary evaporator, the residue was dissolved in 4 ml hot MeOH, decanted from the insolubles, and allowed to crystallize at 4°. Recrystallization from abs MeOH resulted in an almost quantitative yield of white waxy solid, m.p. 52–53°, which was homogeneous on gas chromatography. ν_{max}: 3300, 2910, 2850, 1470, 1120, 940 and 720 cm^{-1}.

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