As seen in Fig. 1 the use of Bio-Gel P-300 resulted in a good separation of the enzyme. However, the enzyme activity was gradually lost in the pooled fractions during storage (Fig. 2). Therefore, in the chromatogram of the gel permeation on Bio-Gel P-150 the enzyme peak was only partly separated from the main protein peak which was eluted from the column in the void volume (Fig. 1), but the stability of the enzyme was better than after chromatography on P-300, A corresponding stability as found on P-150 was also found in the case of P-60. The loss of stability of the enzyme was evident only if the main protein peak and the enzyme peak did not coincide (Figs. 1 and 2). This was repeatedly observed in several fractionations. The loss of the activity of the enzyme after P-300 could not be reversed by any of several attempts (for example, the use of thiols, combination with void volume fractions, addition of magnesium or zinc ions to the elution buffer or to the pooled enzyme preparation).

This result may indicate that certain most likely irreversible molecular changes had occurred in the enzyme structure, when using the particular polyacrylamide gel which has a large degree of cross-linkage and a large pore size. Evidently, this also explains why preparative polyacrylamide electrophoresis was found to be unsuitable, in spite of several repeated experiments carried out according to the recommendations provided by Shandon Scientific Company Ltd. (London, England). Also, ion exchange chromatography on hydroxylapatite [8] was found to be unsuitable in the purification of the enzyme. The high phosphate concentrations (0.07 – 0.2 M) at pH 7.5 needed for desorption inhibited the present *p*-nitrophenylphosphatase. The removal of this phosphate would have required additional chromatographic and other steps. Moreover, the degree of the purification was considered to be low when hydroxylapatite was used.

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New Tobacco Constituents — The Structures of Five Isomeric Megastigmatrienones

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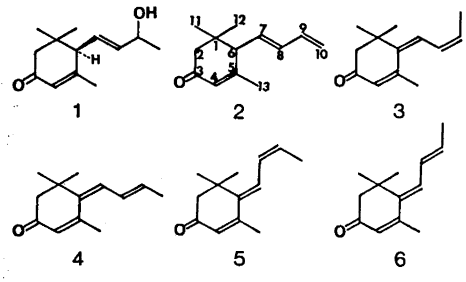
In continuing our studies of the volatile constituents of Greek tobacco (*Nicotiana tabacum* L.) we have encountered five compounds which, as judged by their mass spectra and retention times, appeared to be dehydration products of 3-oxo-α-ionol (*1*), another constituent of Greek tobacco recently discussed by us.[1] The present paper provides evidence for assigning the structures 6ξ-megastigma-4,7E,9-trien-3-one (*2*), megastigma-4,6Z,8Z-trien-3-one (*3*), megastigma-4,6Z,8E-trien-3-one (*4*), megastigma-4,6E,8Z-trien-3-one (*5*), and megastigma-4,6E,8E-trien-3-one

(*6*) to these new tobacco constituents.*

As the first step in clarifying their structures 3-oxo-α-ionol (*1*) (= 9ξ-hydroxy-megastigma-4,7E-dien-3-one) was dehydrated under mild acidic conditions to give five anhydro isomers which by means of GC–MS proved to be identical to the new tobacco compounds. The mass spectra of four of the isomers, 3–6, are nearly identical suggesting that these compounds differ only with respect to the configurations of the side chain double bonds. The mass spectrum of the fifth isomer, however, is different from these but is closely similar to that of the sole product *2* formed by thermal dehydration of 3-oxo-α-ionol (*1*) in the inlet system of the GC–MS instrument.

Subsequent preparative separations permitted the isolation of four of the five isomers from tobacco in the pure state. Further spectral data (NMR, IR, UV, MS) confirmed the above conclusions and allowed the configurations of the double bonds to be established mainly with the aid of nuclear Overhauser effects (NOE).[2]



6ξ-Megastigma-4,7E,9-trien-3-one (*2*). Neither this compound nor any of the others have previously been reported to be present in tobacco but they have been patented as tobacco additives to improve the smoke flavour.[4] The identity of the NMR, IR, and mass spectra of the natural compound with those of synthetic *2* established the complete structure except for the stereochemistry at the 6-position. Although not investigated, its chirality is assumed to be *R*, corresponding to that of 3-oxo-α-ionol (*1*)[5] and the carotenoids lutein [6,7] and α-carotene [8] which are known tobacco constituents.[9] The latter two compounds are possible precursors of 3-oxo-α-ionol (*1*). The base peak at *m/e* 134 is readily explained by expulsion of a $Me_2C(1)-C(2)$ fragment.

Megastigma-4,6Z,8Z-trien-3-one (*3*). The stereochemistry of the exocyclic double bond is *cis*(Z) judging from the positive NOE-effect on the proton resonating at δ 6.41, *i.e.* C(8)H, when irradiating at δ 2.23, *i.e.* $C(13)H_3$. The *cis*-configuration of the 8,9-double bond was supported by the IR-absorption at 730 cm^{-1} and the vicinal spin-spin coupling constant $J_{8,9} = 9.0$ Hz.

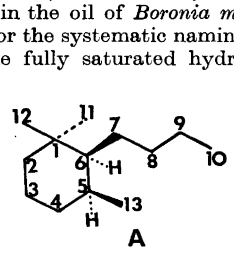
Megastigma-4,6Z,8E-trien-3-one (*4*). The intense IR-absorption at 970 cm^{-1}, the vicinal spin-spin coupling constant $J_{8,9} = 14$ Hz, and the enhancements in the NMR signals of the C(8)H when irradiating the $C(13)H_3$ were in accord with the presence of *trans* disubstituted and *cis* (Z) exocyclic double bonds, respectively.

Megastigma-4,6E,8Z-trien-3-one (*5*). A pure specimen of this isomer has as yet not been obtained either from the tobacco extract or from the synthetic mixture. Its identity follows, however, from its exhibiting a mass spectrum (GC–MS) nearly identical with those of *3*, *4*, and *6*, and by being the last of the four possible isomers.

Megastigma-4,6E,8E-trien-3-one (*6*). The *trans*-configurations of both the exocyclic and the disubstituted double bonds were apparent from the positive NOE effect noticed for the NMR signal of C(7)H when irradiating the $C(13)H_3$-group, the strong IR-absorption at 968 cm^{-1} and the vicinal spin-spin coupling constant $J_{8,9} = 14.5$ Hz.

It can be seen from Table 1 that the two compounds (*3* and *4*) which have a *cis*(Z) exocyclic double bond, exhibit $C(1)(CH_3)_2$ signals at higher field, and $C(13)H_3$ signals at lower field than found for the *trans*(E) isomer (*6*). This effect, which might be due to steric interaction from the proton in position 8, can evidently also be used for stereochemical assignments

* The large and increasing number of compounds possessing this carbon skeleton has made it desirable to adopt the name megastigmane (the α- and β-ionones were first found in the oil of *Boronia megastigma* [3]) as a basis for the systematic naming of derivatives of the fully saturated hydrocarbon A.



A

Table 1. Chemical shifts and spin-spin coupling constants of four isomeric megastigmatrienones.

Assignment	Chemical shifts (δ) of compounds			
	2	*3*	*4*	*6*
C(1)$(CH_3)_2$	0.97 1.04	1.22	1.19	1.35
C(10)H_3		1.82	1.86	1.89
C(13)H_3	1.91	2.23	2.29	2.08
C(2)H_2	2.11 2.35	2.30	2.29	2.35
C(9)H	6.30	5.73	5.89	5.94
C(4)H	5.92	5.90	5.92	5.91
C(8)H	6.10	6.41	6.55	6.78
C(7)H	5.57	6.55	6.24	6.49
C(6)H	2.57			
C(10)H_AH_B[a]	5.08 5.19			

X,Y	Coupling constants: $J_{X,Y}$(Hz) of compounds			
	2	*3*	*4*	*6*
9,10		7.0	7.0	7.0
8,10		1.5	1.5	1.5
8,9		9.0	14.0	14.5
7,8	14.0	12.0	11.0	11.5
4,13	1.5	1.5	1.5	1.0
4,7[b]	0.9	1.0	1.0	0.7
$9,10_A$	9.0			
$9,10_B$	6.0			
6,7	9.0			
6,13	0.5			
2_{gem}	17.0			
10_{gem}	2.5			

[a] H_A and H_B are *cis* and *trans*, respectively, to C(9)H. [b] Approximate values.

in the *retro*-carotenoid series. Thus published data[13] indicate that the configuration of the 6,7- and 6′,7′-double bonds of rhodoxanthin and isocatene is E.

The precursor of the compounds *2*–*6* is possibly 3-oxo-α-ionol (*1*) from which they can be prepared chemically. We consider it unlikely, however, that they are formed from *1* during the isolation procedure since they have also been detected in tobacco head space.[10] Furthermore, 3-oxo-α-ionol (*1*) was found to be stable when heated in the presence of nicotine under the distilling conditions previously described.[11]

The five constituents, *2*–*6*, occur in Greek tobacco in the amounts 0.1 ppm, 3 ppm, 15 ppm, 2 ppm, and 11 ppm, respectively, and presumably contribute considerably to the flavour of this tobacco.

Experimental. NMR, IR, UV, and mass spectra were recorded on Varian HA100D, Digilab FTS-14, Beckmann DK-2A, and LKB 9000 (70 eV) instruments, respectively. Accurate mass determinations were carried out at the Laboratory for Mass Spectrometry, Karolinska Institutet, Stockholm.

The isolation of the title compounds from fraction B5[11] will be described elsewhere.[12]

The synthesis of all compounds was performed by heating 3-oxo-α-ionol (*1*) or its acetate in the presence of $KHSO_4$ (neat, 100°) or *p*-toluenesulphonic acid (in refluxing benzene) for 2–3 h. The five isomers *2*–*6* were always found in the ratio 10:1:10:1:10, their relative retention times on a column packed with 5 % Carbowax 20 M on Chromosorb G, 3 m × 3.2 mm, isothermal at 220° were 1, 1.2, 1.28, 1.43 and 1.49. The individual components, except *3* and *5*, were obtained in the pure state by preparative gas chromatography on the column mentioned. For compounds *4* and *6* repeated column chromatography on $AgNO_3$-impregnated silica gel was also used. The physical data for each compound are given below, and in Table 1.

6ξ-Megastigma-4,7E,9-trien-3-one (*2*). MS: *m/e* 190=M^+ (6), 134 (100), 91 (67), 119 (42), 133 (28), 106 (23), 105 (17), 41 (13), 135 (12), 79 (12), 78 (11), 77 (11), 65 (11); UV:λ_{max} (EtOH) at 223 nm, shoulder at 235 nm, lit.[4] λ_{max} 225 nm; IR: (film) 1665 (s), 1630 (m) 1380 (m), 1370 (m). 1250 (m), 1008 (m), 952 (w), 908 (m), 871 (w), 833 (w) cm^{-1}; NMR ($CDCl_3$) see Table 1.

Megastigma-4,6Z,8Z-trien-3-one (*3*). *3* was obtained in the pure state from tobacco only. MS: *m/e* 190=M^+ (100), 147 (81), 91 (78), 41 (78), 133 (77), 175 (74), 148 (73), 69 (70), 105 (62), 119 (61), 122 (55), 77 (50), 43 (47), 55 (45), 79 (44); UV: λ_{max} (EtOH) 322 nm, 234 nm; IR: (film) 1665 (s), 1630 (m), 1446 (m), 1385 (m), 1366 (m), 1311 (m), 1275 (m), 1095 (w), 1064 (w), 917 (m), 730 (m) cm^{-1}; NMR: ($CDCl_3$) see Table 1. Irradiation at δ 1.82 effected line-sharpening at δ 6.41 and collapse of the quartet at δ 5.73 to a doublet (J=9 Hz). Irradiation at δ 2.23 resulted in sharpening of the multiplet at δ 5.90 to give a doublet (J=*ca.* 1 Hz), and 8 % enhancement of the

integral of the proton resonating at δ 6.41.

Megastigma-4,6Z,8E-trien-3-one (*4*). MS: as for *3*; accurate mass determination (on the natural compound) $C_{13}H_{18}O$, found 190.1369, calc. 190.1358; UV: λ_{max} (EtOH) 323 nm (ε 18 400), 231 nm (ε 10 600); IR: (film) 1660 (s), 1635 (m), 1595 (m), 1449 (m), 1375 (m), 1365 (w), 1322 (s), 1281 (m), 1260 (m), 1195 (w), 1135 (w), 1115 (w), 970 (s), 931 (m), 911 (w), 842 (w) cm^{-1}; NMR: ($CDCl_3$) see Table 1. Irradiation at δ 2.29 effected line-sharpening at δ 5.92, and 12 % and 10 % enhancement, respectively, of the integrals of the protons resonating at δ 6.55 and δ 5.92.

Megastigma-4,6E,8Z-trien-3-one (*5*). MS: obtained by GC–MS, as for *3*. This compound has so far not been obtained in the pure state either from the synthetic mixture or from tobacco.

Megastigma-4,6E,8E-trien-3-one (*6*). MS: as for 3; accurate mass determination (on the natural compound) $C_{13}H_{18}O$, found 190.1352, calc. 190.1358; UV: λ_{max} (EtOH) 322 nm (ε 26 600), 229 nm (ε 10 000); IR: (film) 1669 (s), 1630 (m), 1585 (s), 1447 (m), 1386 (m), 1369 (m), 1350 (m), 1311 (m), 1285 (m), 1265 (m), 1199 (m), 1112 (w), 1032 (w), 998 (w), 968 (s), 898 (w), 832 (w) cm^{-1}; NMR: ($CDCl_3$) see Table 1. Irradiation at δ 2.08 resulted in 12 % increase in the integral of the proton resonating at δ 6.49.

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