Purification of Viscotoxin A*ox2* from the European Mistletoe (*Viscum album* L.) by Chromatography on DEAE-Cellulose

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The European mistletoe, *Viscum album* L., contains a number of pharmacologically active, small, basic proteins. A mixture of these proteins has been isolated under the name of Viscotoxin.[1,2] Separation of the native Viscotoxins has hitherto been only partly successful, but better results were obtained with derivatives of the proteins obtained by performic acid oxidation. Thus chromatography on phosphate cellulose yielded the pure protein Viscotoxin A*ox3*,[3] for which the amino acid sequence has been determined.[4] This report describes the purification of another oxidized Viscotoxin called Viscotoxin A*ox2*.

Materials and methods. DEAE-cellulose, Whatman DE11, was successively washed with HCl (0.1 M), water, NaOH (0.1 M), water, HCl (0.1 M), water, and stored in water containing 2 % butanol as a preservative. Sephadex G-25 fine was obtained from Pharmacia AB, Uppsala, Sweden. All chemicals used were of reagent grade. 2 % butanol was routinely added as a preservative to all buffer solutions, except the 2 % acetic acid, used for elution of Sephadex columns in desalting experiments. For amino acid analysis the proteins were hydrolyzed with constant boiling HCl as described by Hirs *et al.*[5] and the amino acids determined with an automatic amino acid analyzer according to Spackman *et al.*[6] as modified by Samuelsson,[7] using norleucine and α-amino-β-guanidinopropionic acid as internal standards.

Chromatography of Viscotoxin Aox2. The preparation of performic acid oxidized material from the most retarded peak obtained in cellulose phosphate chromatography of crude Viscotoxin has been described previously.[3] Chromatography of this material on cellulose phosphate yields two peaks which are only partly resolved. They are denoted peaks 1 and 2 in Fig. 5 of Ref. 3. 47 mg of material recovered from the combined peaks 1 and 2 was dissolved in 1 ml 0.02 M TRIS-buffer pH 8.8. and the solution applied to a column of DEAE-cellulose (10 × 200 mm), previously equilibrated with 0.02 M TRIS-buffer pH 8.3. The column was eluted with a gradient of NaCl in the same buffer, linearly increasing from 0 to 0.15 M in 1000 ml. The gradient was obtained by the arrangement described by Parr.[8] Fractions of 3 ml were collected and their protein content was determined from their optical density at 280 mμ. The result is presented in Fig. 1.

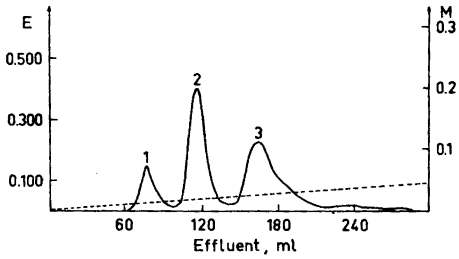

Fig. 1. DEAE-cellulose chromatography of material from peaks 1 and 2 in Fig. 5 of Ref. 3. Viscotoxin A*ox2* was recovered from peak 2. —— = optical density at 280 mμ. ---- = calculated NaCl gradient.

Fractions corresponding to the three peaks were pooled, desalted on Sephadex G-25 and freezedried. Electrophoresis in Sephadex G-25 of the material recovered from peak 2 showed only one band with the same mobility as Viscotoxin A*ox2*.[3] The yield of Viscotoxin A*ox2* was 29 mg (60 %). The amino acid composition is presented in Table 1.

The molecular weight of Viscotoxin A*ox2* calculated from the amino acid composition is 5126 including the terminal molecule of water and assuming that the second carboxyl group of aspartic and glutamic acid is free. The number of spots in a peptide map shows that the molecular weight cannot be a multiple of this figure.

Table 1 also gives the amino acid composition of Viscotoxin A*ox3*, the amino acid sequence of which has been determined.[4] Viscotoxin A*ox2* contains one molecule each of glutamic acid, valine, and phenylalanine, which are not present in Viscotoxin A*ox3*. The content of serine and glycine is higher while the amount of lysine, threonine, proline, alanine, and leucine is lower. Other amino acids are present in the same amount, and

Table 1. Amino acid composition of Viscotoxin A*ox2*. The results are expressed as number of residues per molecule.

Amino acid	Found	Nearest integer	Amino acid composition of Viscotoxin A*ox3* [4]
Lysine	3.01 [a]	3	4
Arginine	2.75 [a]	3	3
Cysteic acid	6.25 [a]	6	6
Aspartic acid	4.07 [a]	4	4
Threonine	3.98 [b]	4	5
Serine	6.93 [b]	7	5
Glutamic acid	0.83 [a]	1	—
Proline	3.08 [a]	3	5
Glycine	5.10 [a]	5	4
Alanine	1.99 [a]	2	3
Valine	0.34 [b]	1	—
Isoleucine	2.83 [b]	3	3
Leucine	1.11 [b]	1	2
Tyrosine	1.87 [b]	2	2
Phenylalanine	0.82 [a]	1	—

[a] Mean value of 2 determinations.
[b] Value extrapolated to zero time or maximum value as calculated from analysis of samples hydrolyzed for 24 h and 72 h.

the total number of amino acid residues is 46 in both substances.

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Ageratone and Dihydroageratone, New Benzofuran Derivatives from *Ageratum houstonianum* Mill.

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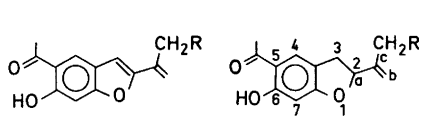
The genera *Ageratum* and *Eupatorium* which are botanically closely related have also been shown to be chemically related since both *Ageratum* [1,2] and *Eupatorium* [3] species contain chromenes.

Benzofuran derivatives such as euparin (*1*) [4] and hydroxytremetone (*2*) [5] are also present in *Eupatorium* species. We now wish to report the isolation of two new acetates, ageratone (*3*) and dihydroageratone (*4*) from the roots of *Ageratum houstonianum* Mill. Their structural relationship to euparin (*1*) and hydroxytremetone (*2*) further confirms the chemical relationship between the two genera.

Ageratone (2-acetoxyisopropenyl-5-acetyl-6-hydroxybenzofuran) (*3*) (m.p. 122—124°C). The IR spectrum (KBr) indicates the presence of an acetate ester (1735 cm^{-1}) and a hydrogen-bonded aromatic carbonyl group (1632 cm^{-1}). This is confirmed by



1 R = H
3 R = OAc

2 R = H
4 R = OAc

the NMR spectrum (Table 1) which has two sharp three proton singlets at τ 7.88 (acetate methyl) and 7.32 (aromatic acetyl methyl). The hydrogen bonded hydroxyl proton resonates as a sharp singlet at τ −2.15.

Moreover, the NMR spectrum reveals the presence of two aromatic protons at τ 2.18 and 3.31, two well separated methylene protons at τ 3.90 and 4.37 and the furanoid proton at τ 2.93. Finally the methylene protons of the acetate carrying carbon atom resonate as a two proton