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High-performance Liquid Chromatographic/Electrospray Ionization Mass Spectrometric Screening for Polyphenolic Compounds of *Epilobium hirsutum*—The Structure of the Unique Ellagitannin Epilobamide-A

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A screening method based on high-performance liquid chromatography (HPLC) interfaced to electrospray ionization mass spectrometry (ESI-MS) with detection of negative ions was devised and the conditions for assaying the complex mixture of gallotannins, ellagitannins and flavonoids of aqueous ethanolic *Epilobium hirsutum L.* whole plant extract were established. Twenty-eight polyphenolic compounds were tentatively identified and one unique ellagitannin with an odd molecular mass was detected in the extract. This compound was taken as the target and isolated by means of consecutive polyamide and Sephadex LH-20 column fractionation; repeatedly, the isolation and purification were monitored by HPLC/ESI-MS. The compound was isolated and subsequently identified as valoneic acid amide bilactone (epilobamide-A). The parent acid, known as valoneic acid bilactone, was also isolated and mass and ^{1}H and ^{13}C NMR spectra were recorded and completely interpreted. Eventually, all relevant structures were elucidated by established methods of structure analysis and confirmed by 1D and 2D NMR spectroscopy. Further, ESI-MS with in-source collision-induced dissociation of the two valoneic acid derivatives and the formation of distonic radical anions is discussed, together with oxidation processes during fragmentation. © **1997 by John Wiley & Sons, Ltd.**

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INTRODUCTION

One of the most obvious obstacles for an efficient search for new potentially useful compounds in natural sources is the lack of a rapid and sensitive screening approach. In extracts of plant material, the broad range of compounds comprising very different types of structures made separation and fast identification even on a tentative basis, almost impossible. It was the advent of high-performance liquid chromatographic/mass spectrometric (HPLC/MS) techniques[1] which opened up a new means to search rapidly for apparently new structures in a vast majority of known compounds.

There have been a few reports describing approaches employing reversed-phase HPLC interfaced to thermospray and, more recently, electrospray ionization (ESI) MS either to characterize plants by means of the content of known natural compounds[2] in a specific class or to search for new compounds.[3] An efficient screening of the gallotannins of *Quercus infectoria*, *Rhus semialata* and *Caesalpinia spinosa* by HPLC/ESI-MS with detection of negative ions has been described.[4] Even though in general only the molecular ion is formed, the technically simple induction of fragmentation by in-source collision-induced dissociations (CID) allows the aglycone and the content of sugar units to be determined very efficiently. In this paper, we report on the application of this technique to the assay of the constitutive polyphenolic compounds in the aqueous ethanolic whole plant extract of *Epilobium hirsutum.* This plant, known in Egypt as 'Siekh,' grows wild in marshy habitats and is used in folk medicine for treating prostatic diseases.[5,6] Previous phytochemical investigations of the polyphenolic compounds of the plant led to the characterization of gallic, 3-methoxygallic and protocatechuic acids[5–8] together with the flavonols kaempferol, quercetin and myricetin and some of their *O*-glycosides. The goal of this study was to optimize the conditions of the reversed-phase HPLC/ESI-MS technique to achieve

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sufficient resolution and reproducibility for the analysis of the polyphenolic compounds. These include not only gallotannins, but also ellagitannins and flavonol *O*-glycosides. Furthermore, the search for possibly biologically active compounds with unknown structures should be pursued in an efficient way which ultimately should be generalized to a generic strategy applicable to the many plants of relevance in Egyptian folk medicine. The traditional analytical procedures are far to time consuming and costly.

We describe here an approach to separate compounds in the main extracts, which led to the tentative identification of most of the 28 well resolved constituents. Furthermore, we describe the isolation and structure elucidation of the unique valoneic acid amide bilactone (epilobamide-A) (**1**) and the already known related acid valoneic acid bilactone (**2**), the ^{1}H and ^{13}C NMR spectra of which were measured and completely interpreted.

The new compound **1** represents the first reported natural example of an ellagitannin amide. Phenolic acid amides in general are very rare in plants; only the amides of hydroxylated truxinic and truxillic acids have been reported to occur in graminaceous cell walls[9] and in *Verbesina caracas.*[10]

EXPERIMENTAL

Plant material

Whole plant, as a softly hairy herb with erect leafy stems, was collected from the marshy habitats around Port Said in October 1992 and identified by Professor L. Boulos at the National Research Centre, Cairo, Egypt.

Extraction and purification

Fresh plant material was extracted with hot EtOH–H_2O (4:1). The EtOH was evaporated under vacuum and the extract (H_2O solution) was extracted with *n*-hexane to remove the chlorophyll. The resulting solution was applied to a Polyamide 6S chromatographic column (Riedel-de Haën, Seelze Hannover, Germany) and eluted with H_2O followed by H_2O–EtOH mixtures of decreasing polarity to yield 13 major fractions (I–XIII). Compound **1** (60 mg) was isolated from fraction XII as an off-white amorphous powder through repeated precipitation by diethyl ether from MeOH, followed by column chromatography on Sephadex LH-20 using EtOH as eluent and then crystallization from MeOH. Compound **2** was isolated from fraction XIII through two successive column chromatographic stages on Sephadex LH-20 using EtOH and BuOH–H_2O (saturated top layer) as eluents, respectively. Pure **2** *ca* 185 mg) was obtained as an off-white amorphous powder by crystallization from MeOH. Paper chromatography was carried out on Whatman No. 1 paper using the following solvent systems: (1) HOAc-6 (HOAc–H_2O, 3:50); (2) HOAc-60 (HOAc–H_2O, 30:20); (3) BAW (*n*-BuOH–HOAc–H_2O, 4:1:5, top layer); (4) C_6H_6–*n*-BuOH–H_2O–pyridine (1:5:3:3, top layer); and (5) *t*-BuOH–HOAc–H_2O (6:1:6, top layer). Solvents 1 and 3 were used for paper chromatography on Whatman No. 3 MM paper and solvents 3 and 4 for sugar analysis.

HPLC/MS

Two LC/MS systems were used. The first instrument consisted of a low-pressure gradient HPLC system comprising a Model 480 pump (Gynkothek, Germering, Germany) coupled to a MAT95 sector field mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an ESI II ion source [Fig. 1(A)]. The sprayer was slightly modified to accommodate the fused-silica capillary from the splitter. The eluent from HPLC column was split using a stainless-steel tee-piece. The splitting ratio was adjusted by means of two fused-silica capillaries, one (30 cm × 75 μm i.d.) leading into the sprayer and, as restriction, a second capillary (2 cm × 75 μm i.d.) leading to waste or to a fraction sampler, if required. Optimization was based on the stability of the spray and the intensity of the mass spectral signal. The second instrument incorporated a Finnigan MAT 4600 mass spectrometer equipped with a laboratory-built ESI source, which was described in detail previously;[11] the complete set-up is shown in Fig. 1(B). This instrument was used for the analysis of the fractions obtained from polyamide column chromatography and generally for orientating experiments. Only when higher resolution and sensitivity were of importance was the MAT95 employed. In all experiments, *ca* 5 μl min^{-1} methanol as sheath liquid was used in a concentric flow around the innermost capillary. SF_6 served as the sheath gas (9 ml min^{-1}) to prevent discharging and the formation of a micro plasma, which

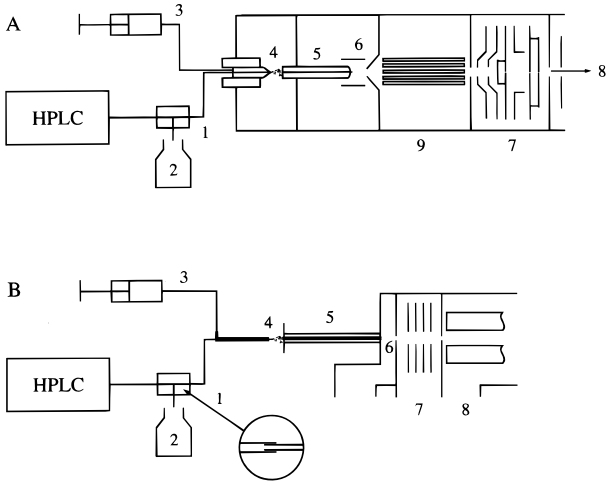

Figure 1. Schematic diagrams of (A) MAT95 and LC/ESI-MS interface (B) Finnigan 4600 LC/MS interface. 1–Splitter; 2–waste; 3–syringe for sheath flow; 4–sprayer; 5–desolvation capillary; 6–tube lens (fragmentation region); 7–source lens stack; 8–to analyser; 9–octapole. Experimental conditions are given in the text.

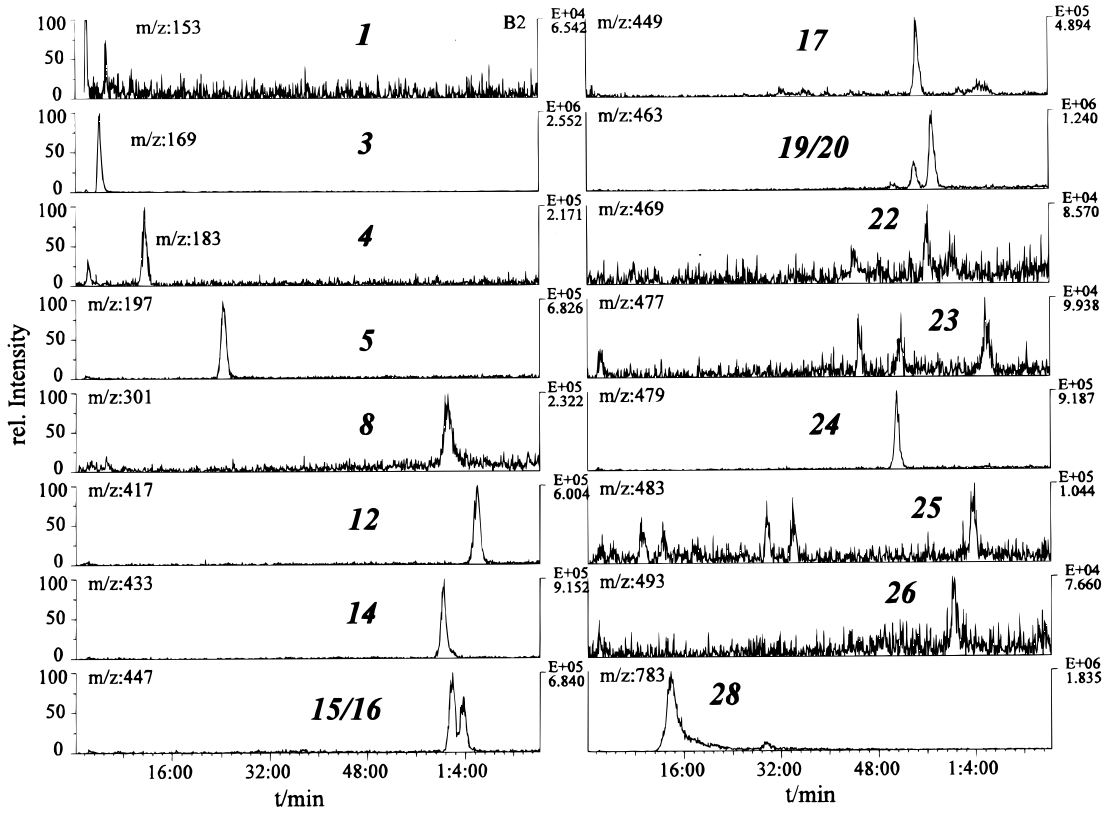

Figure 2. Mass chromatograms of $[M-H]^-$ ions from selected compounds in the crude ethanolic extract; the selection covers major and minor compounds. The numbers refer to the compounds listed in Table 1.

may happen when negative ions are formed. The conditions for infusion of pure samples are the same as described, except that the HPLC system was replaced by a Harvard infusion pump delivering a 1–0.5 μl min^{-1} sample flow.

HPLC conditions for HPLC/ESI-MS analysis

A binary gradient with the following time program was used, where the solvents are (A) H_2O–HOAc (98:2) and (B) MeOH–H_2O–HOAc (80:18:2): 0–5 min, 5% B; 5–50 min, increased to 41% B, held for 15 min; 65–80 min, increased to 100% B, held for 5 min; 85–90 min, decreased to 5% B, held for 10 min. The flow rate was 0.2 ml min^{-1} and the injection volume was 10 μl. The columns (100 × 2 mm i.d.) were filled with 5 μm Nucleosil 120 C_{18} and for intermediate UV detection at 280 nm a Spectra-Physics detector was used.

NMR spectrometry

A JEOL GX 400 spectrometer, operated at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR, was used with a superconducting magnet and a 5 mm dual probe head for ^1H and ^{13}C analysis. Data processing was performed with LABONE software from NMRi (Syracuse, NY, USA).

IR spectroscopy

IR spectra were measured on a Model 2000 FTIR spectrometer (Perkin-Elmer, Überlingen, Germany) as standard KBr disks measured against a pure KBr reference disk in a sample shuttle. The sample, detector and interferometer were purged with dried CO_2-free air.

Compounds

Compound 1. Compound **1** was isolated as an off-white amorphous powder. $R_F \times 100$ values: 46.8 and 50.17 using solvent systems 2 and 3, respectively. UV_{max} (MeOH): 215, 256 and 361 nm. Retention time: 49.85 min under the same HPLC conditions as for HPLC/ESI-MS analysis and it also gave a retention time of 21.8 min using a 5 μm Lichrospher RP 18 E column (250 × 4 mm i.d.) with a binary gradient program: 0–100% B in 30 min, held for 5 min, then 100–0% B in 5 min; solvent B = MeOH, solvent A = H_2O–H_3PO_4 (999:1); pump flow rate, 0.5 ml min^{-1}; UV detection at 280 nm; injection 20 μl. ^{13}C NMR and IR: see Results and Discussion; Table 2. ^1H NMR (400 MHz, DMSO-d_6, at room temperature), δ ppm: 7.5 (s, 1H, H-4), 7.37 (AB system, 2H, NH_2protons), 6.93 (s, 1H, H-6′) and 6.88 (s, 1H, H-9).

Compound 2. Compound **2** was isolated as an off-white amorphous powder. $R_F \times 100$ values: 44.68 and 72.0

Table 1. Tentatively assigned structures based on preliminary data, mainly HPLC/ESI-MS and chromatographic information (all the structures were confirmed later correctly)

Component No.	Molecular ions $[M-H]^-$ (*m/z*)	Preliminary identification
1	153	Protocatechuic acid
2	163	*p*-Coumaric acid
3	169	Gallic acid
4	183	Methyl gallate
5	197	Methoxymethyl gallate
6	275	—
7	285	Kaempferol
8	301	Quercetin
9	301	Ellagic acid
10	317	Myricetin
11	331	Monogalloylglucose
12	417	Kaempferol *O*-pentoside
13	431	Kaempferol *O*-rhamnoside
14	433	Quercetin *O*-pentoside
15	447	Quercetin *O*-rhamnoside
16	447	Kaempferol *O*-hexoside
17	449	Myricetin *O*-pentoside
18	461	Kaempferol *O*-glucuronide
19	463	Myricetin *O*-rhamnoside
20	463	Quercetin *O*-hexoside
21	468	Compound **1**
22	469	Valoneic acid bilactone, **2**
23	477	Quercetin *O*-glucuronide
24	479	Myricetin *O*-hexoside
25	483	Digalloylglucose
26	493	Myricetin *O*-glucuronide
27	635	Trigalloylglucose
28	783	Ellagitannin

using solvent systems 2 and 5, respectively. UV_{max} (MeOH): 215, 256 and 362 nm. Retention time: 21.84 min under the same HPLC conditions as for HPLC/ESI-MS. IR: see Results and Discussion. ^{13}C NMR: see Table 2. ^1H NMR (400 MHz, DMSO-d_6, at room temperature), δ ppm: 7.49 (s, 1H, H-4), 6.99 (s, 1H, H-6′) and 6.93 (s, 1H, H-9).

RESULTS AND DISCUSSION

The aqueous ethanolic whole plant extract of *E. hirsutum* contains a complicated mixture of gallotannins, ellagitannins and flavonoid glycosides, as was shown previously by preliminary 2D paper chromatography screening. The search for new, potentially biologically active compounds becomes much more efficient after sorting out all the known structures in that mixture. Therefore, the application of reversed-phase HPLC combined with ESI-MS is the most promising technique; aiming at polyphenolic compounds, the detection of negative ions will yield the most abundant signals. The same technique can then also be used to follow the isolation steps until the pure polyphenolic compound is obtained.

Optimum HPLC/MS conditions were established under the premises that (a) the separation of several classes of compounds should be possible in general, (b) the polyphenolic compounds should be separated and (c) the system must be robust and yield reproducible results (retention times, intensity distributions) over an extended time period to allow tentative identification based on the limited information from HPLC, ESI-MS (generally $[M-H]^-$) ions and fragmentation by CID, when necessary.

The ion source used contains a stainless-steel capillary for desolvation, which was set to 250 °C for desolvation without thermal fragmentation. An ESI voltage of −3 kV above acceleration potential and a CID voltage of typically −40 V (tube lens to octapole) gave a stable spray and high molecular ion abundance, giving the best detection power achievable. The use of 2% HOAc as one of the HPLC eluent components proved to be beneficial for both the chromatographic resolution and ion formation efficiency. During the analysis of the extracts, $[M-H]^-$ ions were detected as base peaks often without any further fragment for all metabolites. However, adducts with chlorine $[M+Cl]^-$, dimeric ions $[2M-H]^-$ and doubly charged $[M-2H]^{2-}$ ions were also observed in some instances. Such observations were taken as evidence that this class of compound generally forms $[M-H]^-$-type ions as expected. Which of these ions appear together with the common $[M-H]^-$ ion depends on the concentrations, the desolvation potential and the concentration of Cl^- in the system. In Fig. 2, the mass chromatograms of the quasi-molecular ions of the most prominent components are shown. The chromatograms revealed the presence of 28 polyphenolic components, the majority of which were shown to be of known structures on the basis of their $[M-H]^-$ ions and their ESI mass spectra. The results are given in Table 1.

In Fig. 3, the separation of the most abundant polyphenolic compounds contained in the polyamide column fractions XII and XIII is shown using the already described HPLC conditions and detection by ESI with the Finnigan MAT 4600 mass spectrometer. The temperature of the desolvation capillary was set to 75 °C (the capillary in this source is longer and has a smaller inner diameter), whereas all the other conditions were essentially unchanged. Compounds **1** and **2** were detected as $[M-H]^-$ and $[2M-H]^-$ ions as shown in the 2D pattern[12] of the HPLC/ESI-MS analysis Figs 3 and 4). In this representation, intensities are displayed as dark spots (on screen the coding is based on the brightness of the colours), hence it is straightforward to find new and unexpected compounds even in complex mixtures. After having identified the new compounds, they were isolated as described and the purified compounds were studied further.

Compound **1** has a retention time close to that exhibited by the co-existing ellagitannin valoneic acid bilactone (**2**), but possesses an odd molecular mass (M_r) of 469 ($[M-H]^- = 468$). In the next step, compounds **1** and **2** were desorbed in two different successive fractions (with 90% and 100% ethanol, 2D patterns of ESI-MS, Figs 4 and 5) from a polyamide column using the aqueous ethanolic whole plant extract. They were then isolated from the fractions through a series of

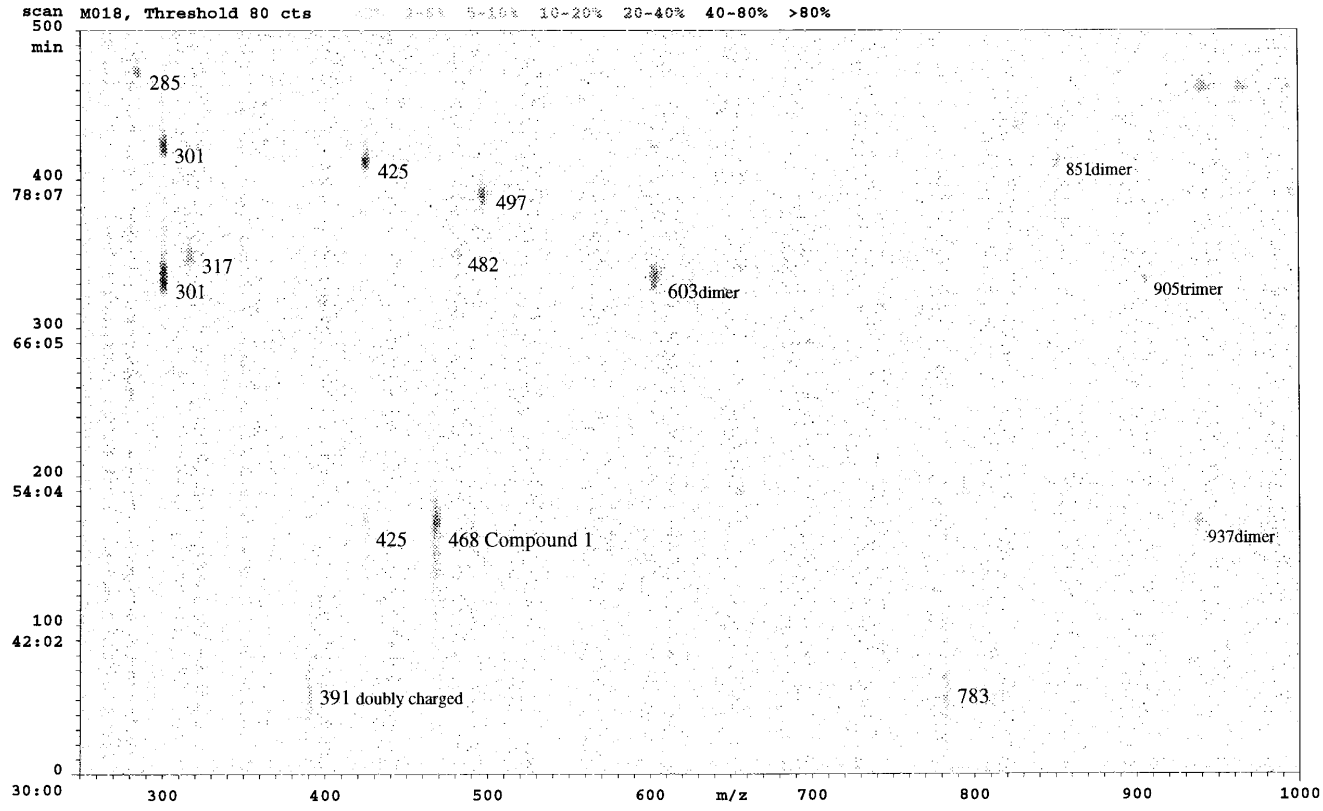

Figure 3. 2D results for extract XII from *E. hirsutum* containing compound **1**. The dark spots indicate signals from compounds in the extract; the *m/z* values are given. The assigned compounds are listed in Table 1. Separation conditions are given in the Experimental section.

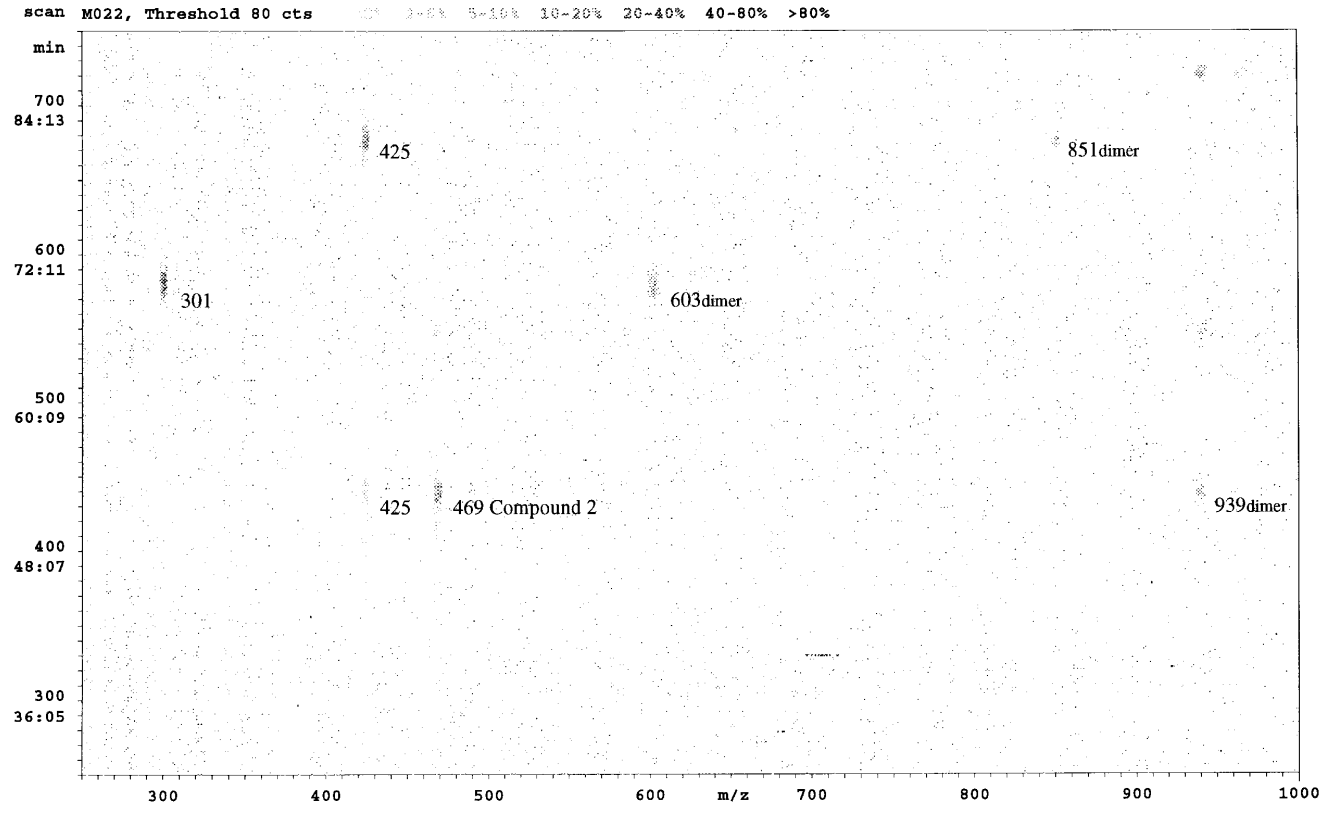

Figure 4. 2D results for extract XIII from *E. hirsutum* containing compound **2**. The dark spots indicate signals from compounds in the extract; the *m/z* values are given. The assigned compounds are listed in Table 1. Separation conditions are given in the Experimental section.

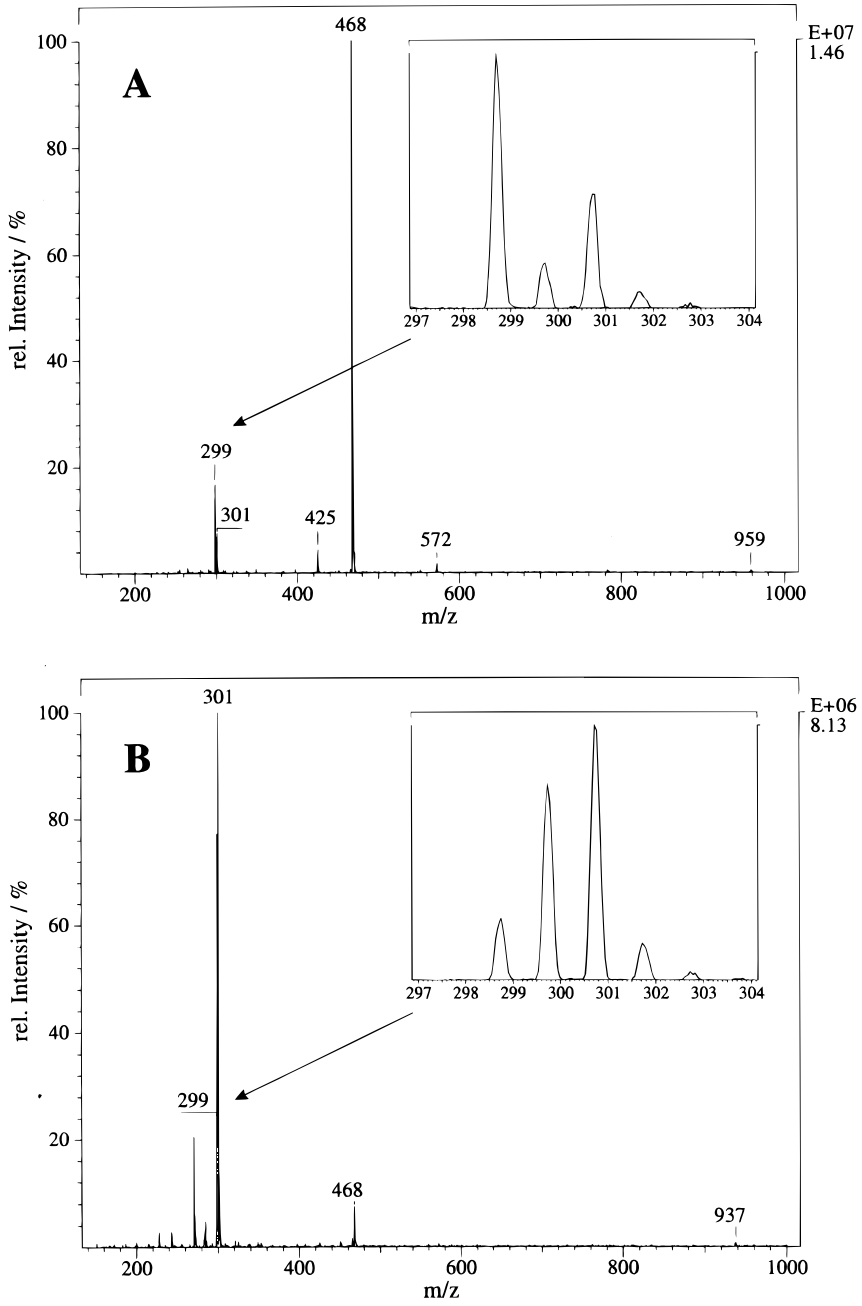

Figure 5. ESI-MS of epilobamide A. The spectrum at the top (A) was acquired with a moderate collision voltage, thus giving the $[M-H]^-$ and $[2M-H]^-$ ions and some fragmentation; the bottom spectrum (B) was acquired with a high CID voltage and the fragments are now base signals. The variation of the intensity distribution among the three signals of the aglycone is shown in the two insets.

Sephadex LH-20 fractionations and crystallizations from methanol.

The electrospray data showed an $[M-H]^-$ ion at m/z 468, which corresponds to an M_r of 469, proving the incorporation of a nitrogen atom in structure **1**, a $[2M-H]^-$ signal at m/z 937 or $[2M-2H+Na]^-$ at m/z 959 and fragments upon CID at m/z 429 (loss of the amide group, indication of a free amide moiety in the structure) and m/z 301, 300, 299 and 271.

On paper chromatography, compound **1** appeared as a dull purple spot in short-wavelength UV light and gave an intense blue $FeCl_3$ reaction, negative nitrous acid test, specific for ellagitannins,[13] and negative KIO_3 test, specific for gallotannins.[14] On acid hydrolysis (aqueous methanolic 2—HCl for 15 min), **1** yielded valoneic acid bilatone (**2**) (chromatographic properties, UV data, ESI-MS and ^1H NMR). These results, together with the UV spectral data (two absorption maxima, in MeOH at 256 and 362 nm), suggested **1** to be the amide derivative of valoneic acid bilatone (**2**). The FT-IR spectrum of **1** supported this conclusion, showing strong absorption bands at ν_{max} 821.1 (C—O—C, sym. str. in lactone ring), 1049.2 (Ar—O—Ar, sym. str.), 1108.0 (C—O—C, asym. str.), 1244.4 (Ar—O—Ar, asym. str.), 1296 (C—O, str.), 1346.0 (O—H, deformation), 1609.0 and 1623.0 (amide NH_2,

Table 2. ^{3}C NMR data for the two valoneic acid derivatives (for complete discussion, see text): δ (ppm) with coupling constants (J, Hz) in parentheses

Carbon	1	2
7′	165.90dd (5, 3)	166.09d (5)
10	159.02d (5)	159.43d (4)
5	158.96d (5)	159.33d (4)
8	148.20d (3)	149.64d (4)
3	148.07d (3)	148.73d (3)
5′	143.38d (3)	143.16d (3)
7	140.20d (7)	140.83d (7)
2	139.55d (7)	139.78d (8)
3′	138.89d ($0 < J < 1$)	139.74d ($0 < J < 1$)
2′	137.52d (10)	139.37d (8)
1a	136.67d ($0 < J < 1$)	136.80d ($0 < J < 1$)
6a	136.32d ($0 < J < 1$)	136.38d ($0 < J < 1$)
4′	132.53d (10)	135.35d (10)
1′	118.73dd (6, 3)	114.80d (2)
9b	114.41d (8)	114.10d (8)
4b	111.77d (8)	112.14d (8)
4	110.44d (163.4)	110.03d (162)
6′	108.50d (164.9)	108.55d (163)
9	107.10d (161.9)	108.52d (163)
4a	108.17d ($0 < J < 1$)	108.29d (2)
9a	107.20d ($0 < J < 1$)	107.01d (2)

deformation), 1657.0 (amide C=O, str.), 1730.0 (C=O, str.) 3370.6 (amide N—H, sym. str.) 3482.7 (O—H, str.) and 3568.0 (amide N—H, asym. str.) cm^{-1}.

In the next step, the proposed structure of **1** was further confirmed by ^{1}H NMR analysis. The spectrum (400 MHz, DMSO-d_6, at room temperature) showed three distinct aromatic proton resonances appearing as sharp singlets at 7.5, 6.93 and 6.88 ppm, which were assigned to the aromatic protons, H-4, H-6′, and H-9, respectively. The spectrum also showed an additional proton resonance which appeared as an AB system at 7.37 ppm and integrated to two protons. This resonance was changed to a broad doublet on remeasurement at high temperature and disappeared completely on addition of a small drop of trifluoroacetic acid to the DMSO-d_6 solution, thus proving that it belongs to the exchangeable NH_2 protons, whose attachment site was proved to be at the carbonyl group of the oxygallic moiety in the molecule of **1** by a long-range proton decoupling experiment.

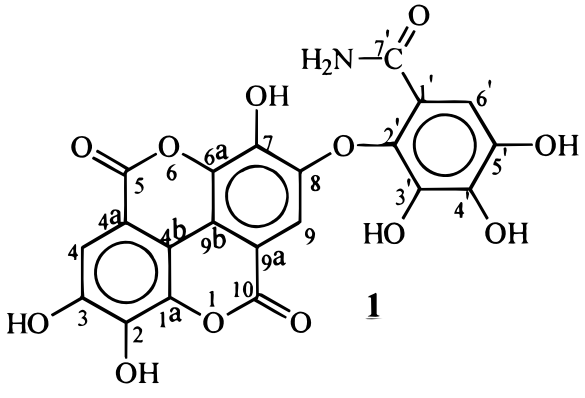

Figure 6. Structure of epilobamide A. The numbering refers to the NMR discussion in the text.

The ^{13}C NMR spectrum (100 MHz, DMSO-d_6, at room temperature) of **1** confirmed the structure. It shows 21 individual carbon resonances. The presence of the monodehydroellagic acid moiety was evident from the two typical carbon resonances at 159.02 and 158.96 ppm, attributable to the carbons of the two nonequivalent lactone carbonyl groups in this moiety. The resonances of the remaining carbons of this moiety were then assigned by comparison with the chemical shifts of the corresponding carbon resonances in the ^{13}C NMR spectrum of ellagic acid itself[15] and with help of the substituent rules to calculate the chemical shifts for carbon resonances of 8-mono substituted ellagic acid. The spectrum of **1** also had the carbonyl amide resonance at 165.9 ppm, thus proving the presence of the amino group in the 2′-oxygallic moiety. Other resonances of the 2′-oxygallamide moiety were then assigned by direct comparison with the resonances of the corresponding carbons in the oxygallic moiety of dehydrodi- or dehydrotrigallic acid.[16] These assignments were further confirmed through measurements of gated decoupled ^{13}C NMR (Table 2), HETCOR and long-range selective proton decoupling spectra. In the last spectrum, the decoupling of the C-1′ carbon resonance to a singlet on irradiating the NH_2 protons finally confirmed the structure of compound **1** to be valoneic acid amide bilactone (epilobamide-A) (Fig. 6), which represents, to the best of our knowledge, the first reported natural occurrence of an ellagitannin amide.

Compound **2** gave chromatographic and UV spectral data (see Experimental section) identical with those of valoneic acid bilactone.[17,18] The negative ESI mass spectrum (Fig. 7) gave an $[M - H]^-$ ion at m/z 469 corresponding to an M_r of 470 and the dimer $[2M - 1]^-$ at m/z 939. Upon variation of the CID potential the same behaviour as with compound **1** was observed: fragment ions of m/z 425 indicating the carboxyl group (loss of CO_2) and the same fragments at m/z 301, 300 and 299 were recorded.

The FT-IR spectrum of **2** showed absorptions at ν_{max} 820.7 (C—O—C, sym. str. in lactone ring), 1043.3 (Ar—O—Ar, sym. str.), 1106.7 (C—O—C, asym. str.), 1245.8 (Ar—O—Ar, asym. str.), 1291.3 (C—O, str.), 1343.1 (O—H, deformation), 1441.3 (O—H, deformation in COOH group), 1726.3 (C=O, str.) and 3291.6 (O—H, str.) cm^{-1}. The ^{1}H NMR spectrum of **2** (400 MHz, DMSO-d_6, at room temperature) showed three sharp singlets at 7.49, 6.99 and 6.93 ppm which are characteristic for aromatic protons H-4, H-6′ and H-9, respectively (see formula, Fig. 8). The ^{13}C NMR spectrum of **2** (100 MHz, DMSO-d_6, at room temperature) exhibited 21 individual carbon resonances (Table 2), among which the three most downfield resonances at 166.09, 159.43 and 159.33 ppm were assigned to a carboxylic carbonyl carbon and the two non-equivalent carbonyl carbons in the lactone rings, respectively. From gated decoupling ^{1}H–^{13}C NMR experiments, the resonances at 110.63, 108.55 and 108.52 ppm were assigned to the protonated carbons, C-4, C-6′ and C-9 by large one-bond (direct) coupling constants ($J = 162$, 163 and 163 Hz, respectively). Assignment of the remaining carbon

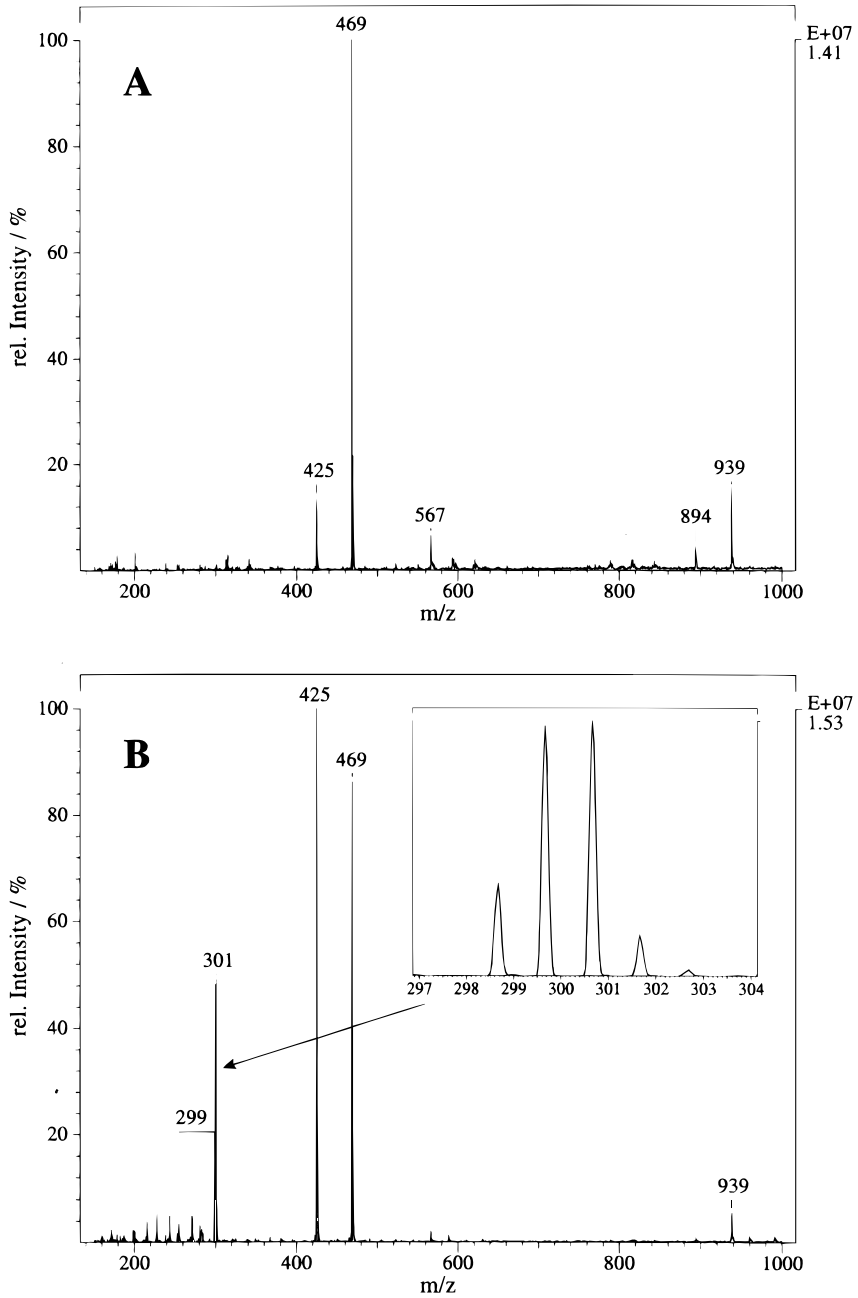

Figure 7. ESI-MS of valoneic acid bilactone. The spectrum at the top (A) was acquired with a moderate collision voltage, thus giving the $[M-H]^-$ and $[2M-H]^-$ ions and some smaller fragments; the bottom spectrum (B) was acquired with a high CID voltage and the fragments are now base signals. The three signals of the aglycone are shown in the inset. In contrast to the amide, loss of the carboxylic group is more pronounced.

signals was then done based on measurements of J values from this spectrum. These assignments were confirmed by 2D-HETCOR experiments.

ESI-MS with in-source CID

Direct flow injection ESI-MS was used at a sample flowrate of 1.25 µl min^{-1} to obtain more information about the structures of both **1** and **2** by variation of the CID voltages from −30 to −150 V to induce the fragmentation at different levels. Both compounds lose the carboxylic moiety or the carboxyl amid group, respectively. Surprisingly, upon increasing the CID voltage the fragment at m/z 299, which is the most abundant aglyconic fragment, at first decreases, whereas m/z 301, then 300 and 271 increase in intensity until they dominate this peak cluster. The behaviour is similar for both valoneic acid derivatives. A possible explanation is based on the unusual stabilization possibilities of that structure for radicals and negative charges due to the many tautomeric forms (Fig. 9). It seems that in a first step a rather stable, long-lived ion–molecule complex is

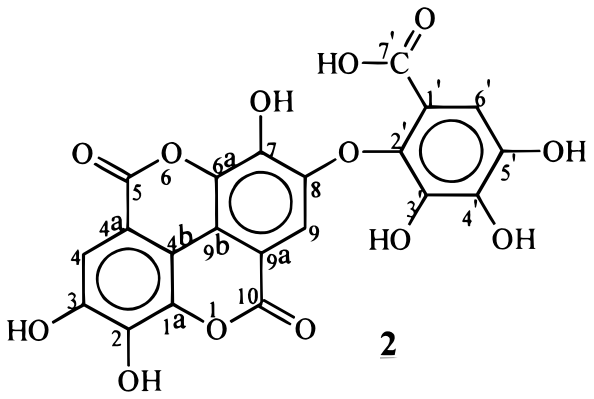

Figure 8. Structure of valoneic acid bilactone. The numbering refers to the NMR discussion in the text.

formed. It appears reasonable to suggest that the two aromatic ring systems provide the required stabilizing forces for such a complex.

With low collision energies, the ion–molecule complex exists long enough to allow oxidation of the valoneic moiety to a quinoidic system prior to dissociation. This means that two additional hydrogens have to be transferred to the neutral gallic acid moiety, which is subsequently lost. With higher energies the fragmentation time-scale becomes shorter and the collisions become more energetic. The result is that fragmentation with migration of only one hydrogen or even the simple bond fission leading to *m/z* 301 becomes favourable, since these are entropically less demanding. The driving force for the formation of such unusual distonic radical anions is their exceptional stability due to the complete distribution of both the radical and the negative charge in the extensive π-system of the molecule. The late appearance of *m/z* 271, which can be rationalized by a loss of C≡O from the oxidized aglycone, supports this explanation, since it reflects the next fragment generation already. Hence it may also contribute to the disappearance of the oxidized moiety.

CONCLUSION

The HPLC/ESI-MS approach described in this paper using detection of negative ions has been used successfully to screen complex mixtures rapidly in both, crude and refined extracts from plant material for known and unknown compounds. New target compounds were detected aided with the powerful 2D display, which allows one to detect unexpected compounds by simple and rapid inspection. The first structural information obtained led eventually to the fully characterized structures. This study confirmed that the response of negative ion LC/ESI-MS, particularly for assaying polyphenolic compounds, has great detection capabilities. Consequently, the detection and characterization of very small polyphenolic concentrations are possible. First experiments using micro-HPLC/MS and even capillary zone electrophoresis/ESI-MS have proved that the detection power of this technique can be extended to real micro-trace analysis, thus giving the prospect of a very powerful screening technique for new, potentially active compounds in natural sources based on small samples.[19] In combination with monitoring positive ions for basic compounds such as alkaloids in extracts of the same plants, a more comprehensive characterization of constituents in folk medicinal plants can be envisaged for the future.

In the course of this work, one new compound has been described and its structure has been completely elucidated based on all available spectroscopic data. The newly detected oxidation–fragmentation for the easily oxidized valoneic acid derivatives and the formation of stable distonic radical anions deserves further investigation and will be studied with similar compounds in the future using MS/MS techniques. It may have implications for structure elucidation and also for the discussion of the fragmentation processes of in-source collision-induced dissociations.

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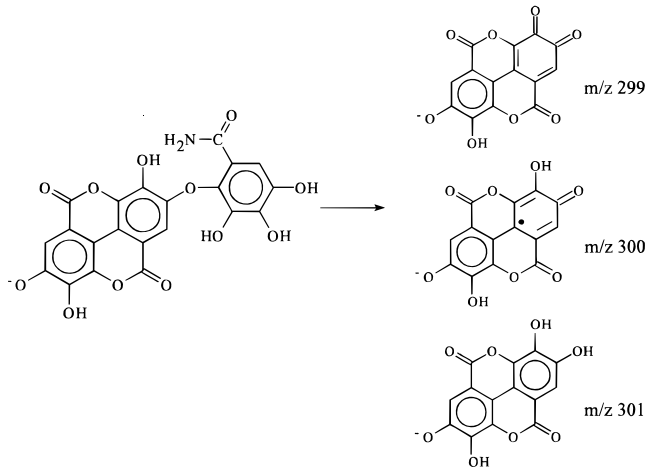

Figure 9. Fragments of both valoneic acid derivatives, shown here for the amide. The stability of the three main products, one of them is believed to be a distonic radical anion, is attributed to the many possible tautomeric forms; only one is indicated here in each case for clarity.

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