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Note

Gas chromatographic analysis of the *Alternaria* metabolite, tenuazonic acid

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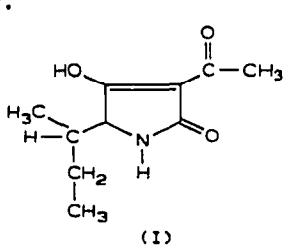
Tenuazonic acid (TA) (I) was first isolated from *Alternaria tenuis* in 1957[1] and its structure elucidated in 1959[2]. It has been identified as a metabolite of *Alternaria longipes* (Ell. and Ev.) Mason[3], *A. mali*, *A. kikuchiana*, *A. citri*, *A. japonica*, and *A. oryzae*[4], as well as *Pyricularia oryzae*[5] and some *Aspergillus* and *Sphaeropsidales* species[6].



(I)

Tenuazonic acid has been shown to possess antitumor activity[6,7]. It has been suggested that it is responsible for the lethality of crude *Alternaria* extracts to mice and rats[8,9]. It has also been associated with brown-spot disease of tobacco[10] and with black spot of apple and pear trees[4]. The potential hazards of this compound are implied in a recent review of *Alternaria* metabolites[11].

N-Nitrosotenuazonic acid is a new compound, which was prepared to evaluate potentially hazardous derivatives of TA. Since cigarettes are known to contain nitrites and nitrates, the possibility exists for the formation of this derivative during the smoking process.

The previously reported analytical method for TA is a spectrophotometric one, based on the absorbance at 277 nm (ref. 4). The method is limited by the tedious extraction procedure and the possibility of interference from materials absorbing in the same range.

EXPERIMENTAL

Analytical standards

Sodium tenuazonate and the N,N'-dibenzylethylenediamine salt were received

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gratis from the National Cancer Institute. They were also synthesized by the method of Harris *et al.*[12]. Purity was determined by its IR spectrum and thin-layer (TLC) and gas chromatographic (GC) behavior.

N-Nitrosotenuazonic acid was prepared by bubbling dinitrogen trioxide through a solution of TA in carbon tetrachloride. Removal of the solvent and recrystallization from hexane gave yellow needles, melting at 90–92° (mol. wt. = 226, mass spec.). The spectral data were consistent with N-nitrosation rather than with O-nitrosation. The amide absorption of TA (8.45 δ) was absent in the NMR spectrum (CCl_4) of the derivative, and the enolic hydroxyl absorption remained (13.2 δ). In the IR spectrum, the amide N–H absorption vanished upon nitrosation and the carbonyl peak shifted from 1720 to 1770 cm^{-1}.

Gas chromatography

A Model 2100 Varian Aerograph gas chromatograph with flame ionization detector and an accessory Model 480 Varian digital integrator were used for all analyses. Glass U-tubes (5 ft. × 2 mm I.D.) were packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh) and conditioned for 24 h at 250°. The detector and injection temperatures were 300°; the nitrogen carrier gas flow-rate was 25 ml/min; the electrometer range was 10^{-11} A/mV. The trimethylsilyl derivative of TA or its salts was prepared by dissolving in a mixture of N,O-bis(trimethylsilyl)acetamide–trimethylchlorosilane–pyridine (6:2:9) and warming for 10 min, at approximately 50°.

Method

The cigarettes employed were regular-sized (70 mm), non-filter, commercially available brands. They were spiked by injecting an ethyl acetate solution of TA into the cigarette with a microsyringe along the length of the cigarette. Controls were treated with ethyl acetate alone. The cigarettes were allowed to air-dry overnight at room temperature. The cigarettes were smoked to an average butt length of 23 mm, taking 30-ml, 2-sec puffs, one a minute, giving *ca.* twelve puffs per cigarette. The smoke was passed through a Cambridge filter into a methylene chloride trap at room temperature.

Fungi were grown on Richard's media or rice moistened with Czapeck's-Dox broth for two weeks at 28° in the dark. The media and mycelia were acidified with 1 *N* HCl to pH 2 and ground with ethyl acetate in a blendor. The mycelia were filtered off and the phases separated. The organic phase was concentrated *in vacuo*. An aliquot was silylated at a concentration of 100 μg/ml and a 100-μg aliquot was chromatographed in a temperature program of 100–250° at 8°/min. If TA was present, it occurred as a single sharp peak at *ca.* 11.5 min. The presence of TA was confirmed by co-chromatography with an authentic sample.

If interfering peaks are present due to background material, the organic phase can be liquid–liquid extracted with saturated sodium bicarbonate solution. The bicarbonate solution is then neutralized with 1 *N* HCl and re-extracted with ethyl acetate. This removes everything except the acidic materials, such as TA. A 75% recovery of TA is accomplished by this procedure.

The presence of TA is confirmed by TLC on silica gel plates. Using a solvent system consisting of toluene–ethyl acetate–formic acid (5:4:1), TA chromatographs as a broad tan spot. Due to the formic acid, any salts of TA are converted to the free

acid. The R_F value varies between 0.5 and 0.6, increasing with concentration. The minimum visual detection limit with ferric chloride spraying is *ca.* 2.5 μg.

N-nitrosotenuazonic acid chromatographs as a single yellow spot in the same solvent system with R_F 0.39. Spraying with a solution of diphenylamine in 85% sulfuric acid produces a deep blue color, indicating the presence of nitroso moiety[13]. N-nitrosotenuazonic acid could not be resolved by GC.

RESULTS AND DISCUSSION

The trimethylsilyl derivative of TA was well resolved on 3% OV-17, giving a linear response of integral counts to concentration from 1 to 20 μg. The lower detection limit was 0.1 μg under the conditions employed.

A variety of *Aspergilli*, *Helminthosporia* and *Alternaria* was screened for TA production. The *Aspergilli* and *Helminthosporia* were negative for TA, but the *Alternaria* were good producers, ranging from 0.1–12% of the crude extracts.

Since TA has been found in tobacco infected with *A. longipes*[10], it was of interest to study the extent to which TA was transported in cigarette smoke. Approximately 3% of the originally spiked TA (10 mg/cigarette) was detected in the Cambridge filters. None was detected in the methylene chloride trap and all controls were negative for TA. No evidence could be found for N-nitrosotenuazonic acid by TLC examination.

Since TA has been reported to be present in tobacco infected with *Alternaria*[10] and is believed to be one of the major toxins produced by the *Alternaria*[9], it is significant that it can be transported in cigarette smoke. The authors were unable to detect TA in a limited number of naturally moldy tobacco samples, but this may have been due to the age and amount of the samples available. Further work is encouraged to assess the environmental hazards implicated by the presence of TA in cigarettes.

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