ANTIBIOTIC PRINCIPLE OF *EUPATORIUM CAPILLIFOLIUM*

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ABSTRACT.—Ethanolic extracts of *Eupatorium capillifolium* (Lam) Small showed activity against *Bacillus subtilis* grown in a chemically defined medium but not in a complex natural medium. The active principle was isolated as a colorless crystalline solid. A study of its properties and mass spectral fragmentation pattern showed that it was costic acid, a sesquiterpenic acid previously isolated from Costus root oil. The observed inactivity of costic acid against *B. subtilis* in a complex medium was shown to be due to the presence in the medium of glutamic acid, which is capable of reversing 90% of the activity of costic acid.

Eupatorium capillifolium (Lam) Small, is an annual weed which is found through-out the eastern United States and has been the subject of several investigations. Wagner et al., (1) reported the isolation of astragalin (kaempferol-3-β-glucoside) and hyperoside (quercetin-3-β-galactoside). In an extension of this work, Herz et al. (2) described the isolation of the flavonoid compounds (2R,3R)-3,4'-dihydroxy-5,7-dimethoxyflavanone and (2R,3R)-7-methoxy-3,5,4'-trihydroxyflavanone as the constituents of the chloroform extract of this plant. The lipophilic fraction was found by various investigators to contain the dimethyl ether of thymohydroquinone (3), phellandrene (3), borneol (3), limonene (3,4), and taraxasterol and its esters (5). Our interest in this plant started with the observation that the extracts exhibited antibacterial activity. This paper describes the isolation of the anti-bacterial principle and its characterization as the acidic sesquiterpene, costic acid.

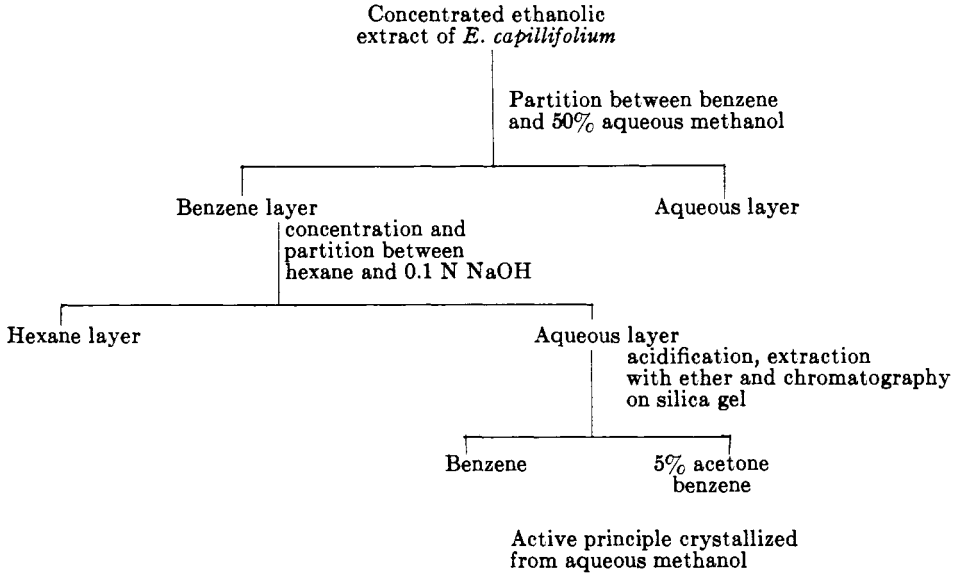
The ethanolic extract showed activity when tested against *Bacillus subtilis*, ATCC 6633, grown in a chemically defined medium. A disc-plate assay performed with this organism was used throughout the fractionation. The method of isolation is shown in scheme 1.

The antibiotic substance **1** was obtained as a colorless crystalline solid, mp 86–87°. Its purity was established by its behavior in thin layer chromatography (10% acetone-benzene: R_f 0.54; 20% acetone-chloroform: R_f 0.79; method of detection: yellow spot when sprayed with bromophenol blue and a brownish purple spot when sprayed with 1N H_2SO_4 followed by heat) and in gas chromatography, in which it appeared as a single peak with TR = 15.3 minutes.

The elemental analysis and the high resolution mass spectrum gave the molecular formula $C_{15}H_{22}O_2$ (M^+ observed: 234.1596; Calcd. 234.1619). The absorption spectral data (uv: 210 nm; log ϵ 3.78; ir: 1685, 1636, 1618 cm^{-1}) gave evidence for the presence of a carboxyl function and two double bonds, one of which was conjugated with the carboxyl group. The nmr spectrum showed two sets of olefinic protons: τ 3.56 and 4.24 ($CH_2=\overset{|}{C}-COOH$), τ 5.25 and 5.55 ($=CH_2$) and a methyl group on a tertiary carbon (τ 9.23). A methyl ester (**2**, M^+246) was prepared by a brief reaction with diazomethane (nmr: τ 3.80, 4.40, 2H; τ 5.26, 5.56, 2H; τ 6.30, 3H; and τ 9.25, 3H). When the reaction with diazomethane was prolonged, the methyl ester underwent further conversion to a compound **3**, with a lower R_f. Its nmr spectrum lacked the signals of the vinyl protons of the system $CH_2=\overset{|}{C}-COOCH_3$. This, coupled with its mass spectrum (M^+290, with a

SCHEME 1

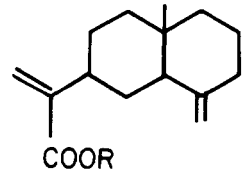
Isolation of the antibiotic principle from *Eupatorium capillifolium*.

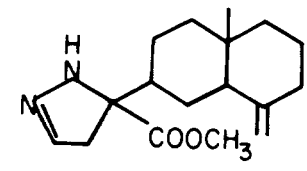


prominent peak at *m/e* 262 or M–28), showed that **3** was a pyrazoline derivative formed by the addition of diazomethane to the α,β-unsaturated carbonyl system. Similar behavior was described by Herz et al. (6) with the closely related illicic acid from *Ambrosia illicifolia*.

The above properties strongly suggested that the antibiotic principle was identical with the sesquiterpene costic acid (**1**) isolated earlier from Costus oil (7) and *Ambrosia illicifolia* (6). However, a direct comparison was not possible because a sample was unavailable from either of these investigators.

Mass spectral fragmentation patterns provided confirmation of the structure; since this information has not been published for costic acid, it is presented here. The molecular ion was present to the extent of 35%, followed by various fragmention peaks, of which three types are evident in the spectrum: a) those which retain the carboxyl function; b) those which result from decarboxylation; and c) those



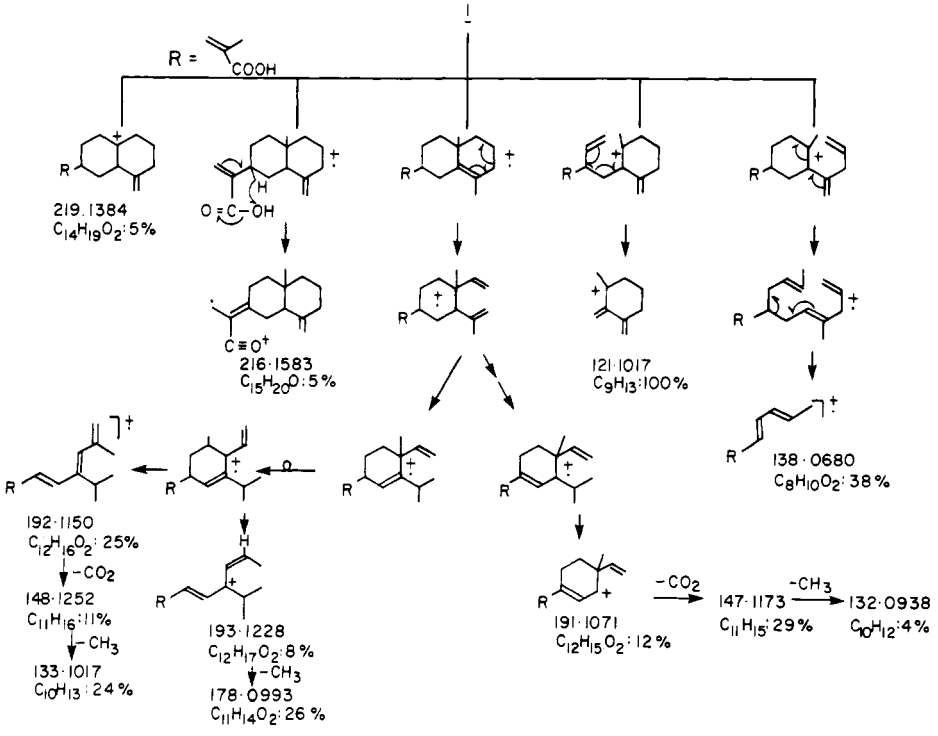


1, R = H

2, R = CH_3

3

which result from the loss of water. Of these, type b was the most numerous, and type c was the least numerous. The bulk of the high mass fragments contained the carboxyl group, thus showing that the α,β unsaturated carboxyl system acted as an effective charge-stabilizer. Each of these ions also had its counterpart in the decarboxylated fragments, which account for nearly all of the low mass fragments. Scheme 2 gives a possible fragmentation path for some of the significant peaks present in the high resolution mass spectrum.



SCHEME 2. Mass-spectral fragmentation of costic acid.

Antibacterial Activity

The organism *B. subtilis* (ATCC No. 6633) was grown in two different media: a chemically defined medium of composition: glucose 0.1%, sodium citrate 0.05%, dipotassium phosphate 0.7%, monopotassium phosphate 0.2%, ammonium sulfate 0.1% and magnesium sulfate 0.05%; and a complex natural medium: Difco Streptomycin agar.

For the "disc-plate" assay, filter paper discs (Schleicher and Schnell No. 740-E) were dipped in solutions of the compound in ethanol-water (1:1), dried for ten minutes to remove the ethanol, and placed on the appropriate agar plate. Because of the low solubility of costic acid in water, the plates were pre-incubated at 5° for 12 hours to permit maximum diffusion of the drug. After this, the plates were incubated at 37° for 10–16 hours, and the diameters of the clear zones were measured. The concentrations of the drug quoted refer to those of the solutions used in the assay. The activity of costic acid against *B. subtilis* in the synthetic medium is shown in table 1. The figures in table 1 were plotted and showed a linear relationship between the diameter of the zone and the logarithm of concentration with a minimum inhibitory concentration of 15 to 30 mcg/ml.

TABLE 1. Activity of costic acid against *B. Subtilis*

Concentration mcg/ml	Diameter of zone mm
750	26
500	24
250	22
120	19
60	16.5
30	14.5
15	0

The compound was also tested against *Escherichia coli* (ATCC 10536) in both natural and synthetic media and against *Staphylococcus aureus* (ATCC 6538P) in natural medium. It was inactive in both these tests.

Costic acid was inactive against *B. subtilis* when grown in a complex natural medium, thus suggesting that the drug acted as an antimetabolite. The identity of the target metabolite present in the complex medium was established by a stepwise elimination procedure. The effect of each of the components of the medium (peptone, yeast extract, etc.) on the activity of costic acid was determined first by the addition of each of these components (at three levels) to solutions of the drug (750 mcg/ml); the synthetic medium was used to test for activity. This showed that only yeast extract had the potential to reverse the activity of costic acid. Synthetic mixtures of B-vitamins, purine/pyrimidine bases and amino acids (in the form of soy-hydrolysate) were likewise tested for their effect on the activity of costic acid. Of these, only the amino acid mixture was found to be capable of reversing the activity of the drug. When the amino acid mixture (soy-hydrolysate) was passed through a weak base anion-exchanger, the effluent lost most of its ability to antagonize costic acid; this result suggested that the antagonist might be an acidic amino acid. A test of aspartic and glutamic acids showed that glutamic acid was highly effective in neutralizing the activity of costic acid; the results are shown in table 2. The percent inhibition values were obtained from the standard curve. Aspartic acid was inactive.

TABLE 2. Effect of glutamic acid on the activity of costic acid.

Costic acid mcg/ml	Glutamic acid mcg/ml	Zone of inhibition mm	% Inhibition
750	0	26.0	0
750	5,000	18.0	89
750	3,000	18.0	89
750	1,000	18.0	89
750	750	19.0	85
750	500	20.0	80
750	250	21.0	74
750	120	21.0	74
750	60	22.5	60
750	30	24.0	40
750	10	26.0	0

Among derivatives of glutamic acid, glutamine shared some reversal of activity in the range 5000–500 mcg/ml, but the reversal was not as intense as that with glutamic acid. Methyl ester of glutamic acid or glutathione showed no reversing activity. Even with glutamic acid, complete loss of activity was not observed as it was with yeast extract; thus the presence of other components in yeast extract was shown. Some evidence for this was obtained by the passage of yeast extract through a weak-base ion exchanger. The effluent did show activity, albeit at a higher concentration than was used before. No further investigation was undertaken to determine the identity of this metabolite.

EXPERIMENTAL[1]

EXTRACTION AND FRACTIONATION.—An air-dried, ground sample (1 Kg) of *Eupatorium capillifolium* was extracted with ethanol at 25° for two days. The extraction was repeated twice more with ethanol and the combined extracts concentrated to a thick syrup. It was partitioned between benzene (400 ml) and 50% aqueous methanol (400 ml). The solvent layer was concentrated to dryness and partitioned three times between hexane (400 ml) and 0.1 N aqueous sodium hydroxide. The combined aqueous layers were acidified and extracted with ether, and the concentrated extract was subjected to chromatography on silicic acid: cellulose (100 g) in benzene. After the column was washed with benzene, it was eluted with 5% acetone in benzene. The fractions containing the major band were combined and concentrated; the oily residue crystallized from methanol-water (2:1). The compound was obtained as colorless needles, mp 86–87°; yield, 0.8 g.

Anal calc. for $C_{16}H_{22}O_2$: C, 76.88; H, 9.46. Found: C, 76.98, H, 9.41.

ESTERIFICATION.—To a solution of **1** (0.2 g) in ether (20 ml) was added ethereal diazomethane drop by drop until there was a slight yellow color due to the excess reagent. When the solution was concentrated, a colorless oil was obtained which was shown to be homogeneous by thin-layer and gas-chromatograpy, *m/e*, 248.1788; Calc. for $C_{16}H_{24}O_2$: 248.1777.

When the reaction mixture was treated with an excess of the reagent, allowed to stand for two hours, and concentrated to dryness, a colorless crystalline solid was obtained, mp 38–40°; *m/e* 290.2010; Calc. for $C_{17}H_{26}N_2O_2$: 290.1996.

GAS CHROMATOGRAPHY.—The following conditions were used for the resolution of **1** and **2**. Column temperature, 150°; injector temperature, 250°; detector temperature, 230°.

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[1]Melting points were determined with a Fischer-Johns apparatus and were uncorrected. The spectra were determined on the following instruments: uv: Beckman 25; ir: Beckman Acculab 3; nmr: Varian T60 A with tetramethylsilane as internal stnadard and mass spectra: DuPont 490 chemical ionization spectrometer and Hitachi-Perkin-Elmer high resolution mass spectrometer, model RMU6E. Thin-layer chromatography (tlc) was performed with E. Merck silica gel HF 254+366. Column chromatography was carried out with a 1:1 mixture of silicic acid (Mallinckrodt 275–325 mesh) and cellulose (Brown & Co., tlc grade). Gas chromatography was performed with a Varian model 2100 instrument.