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A NATURALLY-OCCURRING STRUCTURAL ANALOGUE OF THE PHYTOTOXIN CORONATINE

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Key Word Index—*Pseudomonas syringae* pv. *atropurpurea*; toxin; leaf chlorosis; coronatine; *N*-coronafacoylvaline; coronafacic acid.

Abstract—Chlorosis-inducing compounds in liquid cultures of the phytopathogenic bacterium *Pseudomonas syringae* pv. *atropurpurea* have been investigated. In addition to coronatine as previously reported, a new compound was discovered. This gave a mass spectral fragmentation pattern which indicated that it was, like coronatine, an amide of coronafacic acid. Acid-hydrolysis of the new toxin liberated the amino acid valine. This observation, together with mass spectral and NMR data, established the structure of the new toxin as *N*-coronafacoylvaline. Some implications to biosynthesis are discussed. Along with the two chlorosis-inducing compounds, the biologically inactive coronafacic acid was also isolated from the growth medium.

INTRODUCTION

Pseudomonas syringae pv. *atropurpurea* (Reddy & Godkin 1923) Young, Dye and Wilkie 1978, a pathogen of Italian ryegrass (*Lolium multiflorum* Lam.) often induces chlorotic haloes around necrotic bacterial infection-sites on leaves of the host plant. This leaf chlorosis has been ascribed to the action of the phytotoxin coronatine (**1**) [1]. Coronatine production in liquid cultures of *P.s. atropurpurea* is a feature common to most strains of *P.s. atropurpurea* [2] and may well be associated with the virulence of these organisms.

In an earlier account of coronatine produced by the pseudomonad *P. syringae* pv. *glycinea* [3], evidence was presented for the existence of minor chlorosis-inducing components, detected during the purification of coronatine. That observation has prompted detailed examinations of the chlorosis-inducing carboxylic acid complex of various *Pseudomonas* strains known [2] to be high-yielding producers of coronatine. I here report the isolation and structural elucidation of the first naturally occurring phytotoxic structural analogue of coronatine.

RESULTS AND DISCUSSION

Isolation and purification

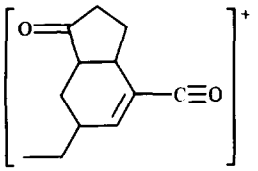
The carboxylic acid fraction extracted from the culture medium was subjected to the standard purification procedure (CC on LH20 Sephadex) for coronatine and those fractions with chlorosis-inducing activity were examined by TLC, revealing that biological activity occurred in two column fractions ahead of those that contained coronatine. These two earlier fractions had several UV-detectable components on TLC and were studied in detail. Thus, by use of preparative TLC, TLC and bioassay, the presence of a new chlorosis-inducing component (compound **2**) was established, with an R_f value on TLC lower than that of coronatine. Mixtures of compound **2** and coronatine were separated by column chromatography on silica gel, then the individual components were subjected to a final purification by preparative TLC.

The relative amount of coronatine compared with compound **2**, produced in liquid cultures of five separate single colony-forming units of *P.s. atropurpurea* 4328, was studied. Samples of the unpurified carboxylic acid fractions extracted from a concentrate of each culture supernatant were methylated and these derivatives were then subjected to GC analysis. The relative proportions of coronatine : compound **2** varied between 3 : 2 and 2 : 3 for the five cultures or for a repeat run of one culture.

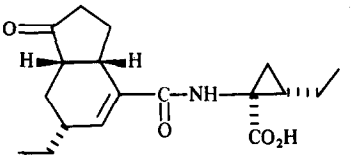
Structures

Coronatine and its methyl ester are most readily recognized from their characteristic mass spectra, which, in addition to the respective molecular ions each have a prominent fragment ion (ion a) at *m/z* 191, and a related ion at *m/z* 163 $[191 - CO]^+$ [4, 5]. The mass spectra of the new compound and its methyl ester also gave useful information about its structure. In each spectrum there was a prominent fragment ion at *m/z* 191 which was determined to have the molecular constitution $C_{12}H_{15}O_2$ by MS mass measurement. This result along with the presence of a fragment ion at *m/z* 163 indicated that the *m/z* 191 ion identified with ion a, and hence gave a strong indication that the new compound was a structural analogue of coronatine, differing in the amino acid portion of the amide structure of coronatine. Thus compound **2** was further investigated on this basis.

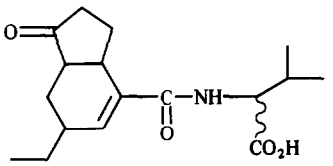
Compound **2** had MW 307 for $C_{17}H_{25}NO_4$ (by MS), and thus contained one less carbon atom than coronatine, and it formed a monomethyl ester, MW 321 for $C_{18}H_{27}NO_4$ (by MS). On acid hydrolysis, it gave two products one of which could be extracted from the aqueous, acidic hydrolysis mixture with ethyl acetate and was shown to be coronafacic acid by mass spectrometry. The other hydrolysis product, which remained in the



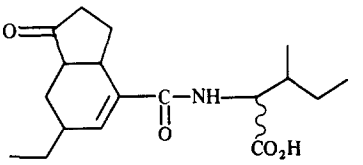
Ion a, *m/z* 191



1 Coronatine



2



3

aqueous phase after ethyl acetate extraction, behaved as valine on two dimensional (2D) thin layer electrophoresis (TLE)/TLC and visualization with ninhydrin. This was confirmed by co-chromatography with high sp. radioactivity ^{14}C-valine on 2D TLE/TLC: the ninhydrin colour, which arose solely from the hydrolysis product, was exactly coincident with the position of the radioactivity. In accord with the acidic properties of compound **2**, the component amino acid, valine, must be bonded to coronafacic acid by an amide linkage, which allows the deduction of structure **2** for this compound. The 200 MHz ^{1}H NMR spectrum was consistent with this structure; it showed the presence of an ethyl group (of the coronafacoyl moiety) and two non-equivalent methyl doublets for the isopropyl of the valyl moiety. The configuration of compound **2** has yet to be established. Nevertheless, since compound **2** co-occurs with coronatine, which is a product previously reported from *P.s. atropurpurea* with the established [5] stereochemistry **1**, a reasonable expectation is that compound **2** contains the *cis* ring-junction as in coronatine, and probably the same configuration at the α-amino carbon.

Coronafacic acid was also isolated from carboxylic acids produced by *P.s. atropurpurea* 4328, confirming the previous reported occurrence of this compound [1] from this organism. It was detected by TLC in LH20 column fractions immediately after the coronatine-containing fractions, and had no detectable biological activity. It was purified by preparative TLC and gave a mass spectral fragmentation the same as that previously reported for coronafacic acid isolated from *P. syringae* pv. *atropurpurea* [1].

Several coronatine analogues have previously been synthesized by the condensation of coronafacic acid with various amino-substituted compounds [5]. The compounds prepared were all reported to have biological activity to a lesser degree than the activity of coronatine. One such compound relevant to the present report is **3**, a homologue of compound **2**, which contains isoleucine in place of valine. The biological activity reported for **3** provides precedence for the biological activity that I have found with the related compound **2**.

The natural occurrence of the amide **2** may have relevance to biosynthetic studies. It points to a likelihood that a homologous amide such as **3** also might be a naturally occurring compound. A derivative of the coronafacoylisoleucylamide **3** may well be an intermediate on an enzyme-mediated oxidative-cyclization sequence to coronatine. Although the synthetic compound **3** is biologically active [5] it is noteworthy that its activity is lower than that of coronatine.

EXPERIMENTAL

CC used either Sephadex LH20 in MeOH–aq. 0.025 M ammonia (1:1) [2], or silica gel (Hopkin & Williams) eluted with $CHCl_3$. Fractions were monitored by TLC using silica gel PF_{254} and EtOAc–*i*PrOH–HOAc–H_2O (390:8:1:1). Components were detected by quenching of fluorescence under UV light. With preparative TLC, compounds were recovered from the gel by elution with MeOH, then the residues were partitioned between EtOAc and *ca* 0.005 M H_2SO_4 to give the EtOAc-soluble, free carboxylic acids. GLC was at 210° on 5% OV101 on 80/100 Chrom W-HP column (2 m × 2 mm i.d. steel) with N_2 at 25 ml/min and with FID. Amino acids were examined by 2D TLE/TLC using cellulose MN 300, as described in refs [6, 7]; detection utilized ninhydrin/Cd(OAc) spray [7]. Mass spectra were recorded at 20 eV with an AEI MS 30 instrument using a direct probe. Methyl esters were prepared using ethereal CH_2N_2.

Culture and bioassay. P.s. atropurpurea 4328 was obtained from the Plant Diseases Division Culture Collection. Single colony forming units were obtained directly by plating out from the reconstituted lyophilized culture. One 9 l. batch was grown for toxin isolation as described in [2, 3, 8] for *P.s. glycinea*. Toxin-containing solns were bioassayed on bean leaves by the prick assay as described in [9]. For GC survey of toxin produced by single colony forming units, 1.8 l. of each culture was grown.

Isolation and purification. Culture supernatant (9 l.) was concd (rotary evaporator, bath temp. = 40°) *ca* 40 × at neutral pH. The concentrate was cooled at 2° for 16 hr, centrifuged (27 000 *g*, 30 min), adjusted to pH 2, and extracted with EtOAc (3 × 200 ml). The EtOAc was reduced in vol. to *ca* 20 ml, and extracted with

1 M $KHCO_3$ (10 ml, 2 × 5 ml). The combined $KHCO_3$ extracts were washed with EtOAc (2 × 10 ml), acidified to pH 2, and extracted with EtOAc (3 × 20 ml), dried (Na_2SO_4) and the solvent evaporated. The residue, 164 mg, was chromatographed on a column of LH20 Sephadex, and the fractions collected were bioassayed and examined on TLC for content of coronafacic acid, coronatine, and compound **2**. Each of the three compounds was purified by silica gel CC, followed by prep. TLC (0.75 mm). The yields obtained were 8 mg coronafacic acid, 16 mg coronatine, and 20.5 mg compound **2**.

Compound **2**: TLC R_f 0.30 (cf. coronatine, 0.58). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.00 (3H, *t*, J = 6.6 Hz), 1.01 (3H, *d*, J = 6.6 Hz), 1.04 (3H, *d*, J = 6.7 Hz), 4.65 (1H, *dd*, J = 8.2, 4.4 Hz) and 6.2–6.5 (2H, *m*); MS *m/z* (rel. int.): 307.1772 $[M]^+$ ($C_{17}H_{25}NO_4$) (11), 289 $[M-H_2O]^+$ (7), 262 $[M-CO_2H]^+$ (7), 207 (coronafacamide ion) (16), 191.1071 (coronafacyl ion, $C_{12}H_{15}O_2$) (58), 190.0983 $[C_{12}H_{14}O_2]^+$ (100), 163 $[191-CO]^+$ (32). Acid hydrolysis afforded a single amino acid, valine, and coronafacic acid. Methyl ester MS *m/z* (rel. int.): 321.1967 $[M]^+$ ($C_{18}H_{27}NO_4$) (23), 289 $[M-MeOH]^+$ (4), 279 $[M-C_3H_6]^+$ (9), 207 (coronafacamide ion) (43), 191.1040 (coronafacyl ion, $C_{12}H_{15}O_2$) (100).

Coronafacic acid. Fractions containing this component, obtained during the purification of coronatine or compound **2**, were pooled and purified finally by prep. TLC (0.75 mm). MS *m/z* (rel. int.): 208 $[M]^+$ (100), 190 $[M-H_2O]^+$ (19), 179 $[M-Et]^+$ (15), 163 (16), 162 (16), 161 (17), 151 (22), 145 (15), 135 (15), 133 (22), 119 (34), 79 (14).

Acid hydrolysis. Compound **2**, 100 μg, in 50 μl 6 M HCl was sealed in a glass tube then heated in steam for 30 min. The sample was allowed to evaporate to dryness under vacuum over conc H_2SO_4 and NaOH pellets. The residue, which contained valine, was dissolved in 20 μl H_2O and 2 μl used for 2D TLE/TLC. In a second hydrolysis, 500 μg of compound **2** and 200 μl 6 M HCl were heated for 4 hr. The reaction mixture was cooled then diluted with H_2O to 1 ml and extracted with EtOAc (3 × 4 ml). These extracts were dried (Na_2SO_4) and evaporated; the product obtained co-chromatographed on TLC with coronafacic acid and had an identical mass spectrum to that of coronafacic acid.

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