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NORCORONATINE AND *N*-CORONAFACOYL-L-VALINE, PHYTOTOXIC ANALOGUES OF CORONATINE PRODUCED BY A STRAIN OF *PSEUDOMONAS SYRINGAE* PV. *GLYCINEA*

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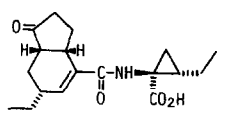
Key Word Index—*Pseudomonas syringae* pv. *glycinea*; toxin; leaf chlorosis; coronatine; norcoronatine; *N*-coronafacoyl-L-valine; biosynthesis; coronafacic acid; 1-amino-2-methylcyclopropane-1-carboxylic acid.

Abstract—Compounds in liquid cultures of the phytopathogenic bacterium *Pseudomonas syringae* pv. *glycinea* that cause chlorosis after application to young bean leaves have been investigated. Known compounds that were isolated and identified were coronatine, the major component, and *N*-coronafacoyl-L-valine, which are both biologically active, and coronafacic acid which is inactive. In addition a new minor component was isolated and purified. Mass spectrometry indicated that this was an amide of coronafacic acid, bearing one less methylene group than coronatine. Mass spectral and NMR data, together with a study of the products from acid hydrolysis of the new compound, established its structure to be norcoronatine (i.e. a methyl substituent in place of the 2-ethyl substituent on the cyclopropyl moiety of coronatine). The probable biosynthetic derivation of norcoronatine is discussed.

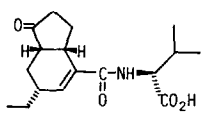
INTRODUCTION

Various strains of the bacterial phytopathogens *Pseudomonas syringae* pv. *atropurpurea* (Reddy & Godkin) Young, Dye & Wilkie and *Pseudomonas syringae* pv. *glycinea* (Coerper) Young, Dye & Wilkie produce the phytotoxic compound coronatine when grown in liquid cultures. Solutions of coronatine **1** cause chlorosis in young leaves 2–3 days after application to wounded sites, a symptom that often characteristically surrounds infection-sites on plants diseased by the pathogenic bacteria. Production of coronatine *in situ* is believed to be the cause of the disease symptoms [1].

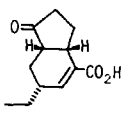
Earlier reports suggest [2] and establish [3] the occurrence of other minor chlorosis-inducing components present in liquid cultures along with coronatine; for a strain of *P. syringae* pv. *atropurpurea* one such compound has been isolated and purified, and characterized as *N*-coronafacoylvaline (**2**) [3]. I report here the characterization of norcoronatine, a new naturally occurring phytotoxic analogue of coronatine, produced by a strain of *P. syringae* pv. *glycinea* together with coronafacic acid (**3**), *N*-coronafacoyl-L-valine (**2**) and the major component coronatine (**1**).



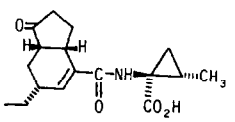
1 coronatine



2 N-coronafacoyl-L-valine



3 coronafacic acid



4 norcoronatine

RESULTS AND DISCUSSION

Coronatine has previously been reported [4] as a major product (4 mg/l.) in liquid cultures of *P. syringae* pv. *glycinea* 4182. The present study confirmed this earlier finding; GC analysis of the crude carboxylic acid fraction indicated the presence also of a similar quantity of coronafacic acid, and a smaller amount (*ca* 10% of the coronatine) of *N*-coronafacoylvaline, two compounds that previously have been reported to occur in liquid cultures of *P. syringae* pv. *atropurpurea*, [3, 5]. The identities of these two compounds in the present study were established by GC and MS following their isolation and purification. Other components in the crude mixture that were retained longer* during GC than coronafacic acid were minor in quantity, at most only 3% of the coronatine peak. Therefore, the isolation of these components to enable the evaluation of their chlorosis-inducing activity required fractionations in order to reduce and eventually remove the coronatine. This was achieved by subjecting the crude acids to chromatography on a long column of LH20 Sephadex and monitoring the composition of fractions by GC†; this resulted in the total separation

*Previous studies have demonstrated that biological activity is associated with amides of coronafacic acid, compounds which, when methylated, would be retained longer on GC than is methyl coronafacate

†The large size of the coronatine peak caused it to engulf most of the minor components (including *N*-coronafacoylvaline) that were later found to have chlorosis-inducing activity.

of some minor components, along with an enrichment of these minor components with respect to coronatine. Because chlorosis-inducing activity was found only in column fractions that contained coronatine (GC), or either of the fractions immediately before and after the coronatine peak, efforts were directed at purifying the three minor components contained in the various coronatine fractions, by column chromatography (silica gel) and preparative TLC. Each minor component thus obtained, had no coronatine detectable by GC analysis, yet each was biologically active. A mass spectrum of each showed the presence of a strong ion at m/z 191, typical of the coronafacoyl ion observed in the mass spectra of coronatine and *N*-coronafacoylvaline [3] indicating that each compound was an amide of coronafacic acid, and accordingly an analogue of coronatine. Of the three components obtained, two were established to be norcoronatine (**4**; discussed below), and *N*-coronafacoylvaline (**2**) as mentioned earlier. However, the third one was not homogeneous, giving an asymmetrical peak on GC, and the precise nature of its components has yet to be resolved.

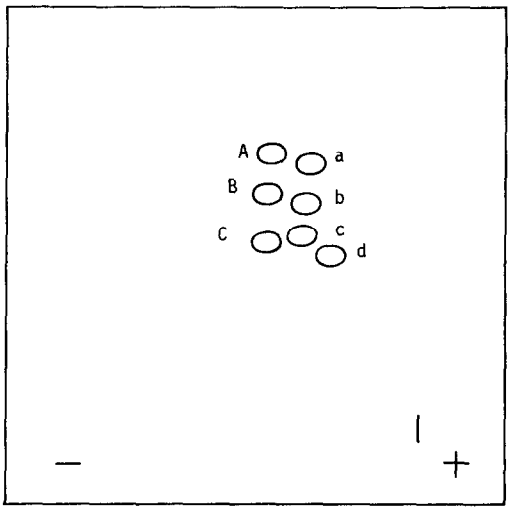


Fig 1. Migratory relationships between cyclopropyl amino acids and their corresponding saturated acyclic analogues on 2D TLE/TLC. This is a composite representation constructed from combinations of the individual components run with ^{14}C-alanine as a reference. Components are: A, ethyl-ACC (coronamic acid); B, product from norcoronatine; C, ACC (1-aminocyclopropane-1-carboxylic acid); a, isoleucine; b, valine, c, α-aminobutyric acid; d, α-aminobut-3-enoic acid. Ninhydrin colour reactions were A, B, C, each yellow → orange/brown; a, b, c, light magenta, d, brown

N-*Coronafacoyl*-L-*valine* (2)

The amino acid recovered from the acid hydrolysis of **2** was determined to be L-valine by GC of its *N*-trifluoroacetyl/methyl ester derivative. NMR spectra of *N*-coronafacoyl compounds with the *cis*-hydrindanone ring juncture display a broad signal at δ3.2–3.3 [6] due to the deshielded α-keto methine. A broad unresolved peak at δ3.24 in the NMR spectrum of **2** allows the deduction of the *cis*-configuration of the bicyclic ring system of *N*-coronafacoylvaline. The stereostructure of *N*-coronafacoyl-L-valine is therefore as depicted in structure **2**, where the configuration at each of the asymmetric centres is the same as found in coronatine [7]. This compound is identical with that previously reported [3] from the related pseudomonad *P. syringae* pv. *atropurpurea*.

Norcoronatine (4)

This component was found (by GC analysis) in fractions from an LH20 Sephadex column, and apparently eluted from this column as an unresolved shoulder on the leading side of the coronatine peak. The purified product, which was homogeneous by GC analysis, had M_r 305 for $C_{17}H_{23}NO_4$ (by MS) and thus differed from coronatine by one less methylene group. It formed a monomethyl ester, M_r 319 for $C_{18}H_{27}NO_4$ (by MS), and both this and the parent acid displayed a prominent ion at m/z 191 (base peak), typical of coronafacoyl compounds such as coronatine [3]. 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 TLC and mass spectrometry. The other hydrolysis product, which remained in the aqueous phase after ethyl acetate extraction, was found to differ on two dimensional thin layer electrophoresis (2D TLE)/TLC (Fig. 1) from common protein amino acids, and also from coronamic acid obtained from coronatine after the same hydrolysis conditions. However, after treatment with ninhydrin-cadmium acetate (on TLC) it developed a yellow–brown colour* which was similar to the colour given by coronamic acid and by 1-aminocyclopropane-1-carboxylic acid (ACC). On 2D TLE/TLC (Fig. 1), the hydrolysis product lay in a position intermediate between ACC and coronamic acid (= ethyl-ACC), consistent with the hydrolysis product being methyl-ACC and the three compounds forming an homologous series. An alternative possibility, that the hydrolysis product was the isomeric dehydroamino acid rather than a cyclopropyl-containing amino acid seemed to be less plausible based on the position of the dehydroamino acid α-aminobut-3-enoic acid (vinylglycine) on 2D TLE/TLC relative to α-aminobutyric acid, and the corresponding cyclopropyl analogue ACC (Fig. 1) which moved faster than the former two in the TLE dimension. By analogy, a dehydroamino acid from norcoronatine would be expected to be slower moving than valine in the TLE dimension, rather than faster moving as was observed. Furthermore, the two alternative structures possible for a dehydroamino acid moiety in norcoronatine would require the presence of a vinylic methyl group. The ^{1}H NMR spectrum clearly ruled out this possibility, but displayed a doublet at δ1.29 (J = 6.0 Hz) assigned to the cyclopropyl methyl substituent. A multiplet at δ3.18 is assigned to the α-keto methine and allows the deduction of a *cis*-configuration of the bicyclic ring system as in coronatine [6].

These observations, along with the MS and NMR data, establish the structure **4** for this product, except for the stereochemistry of the cyclopropyl substituents. Several lines of reasoning would suggest that norcoronatine has the same stereostructure as coronatine. Biosynthetically, coronatine is derived from L-isoleucine [8], and the

*The colour-reaction with 'basic' or 'neutral' amino acids is typically a light magenta.

configuration of the substituents at the α-carbon is unaltered in the conversion of isoleucine to the 1-amido-1-carboxy-2-ethylcyclopropyl moiety of coronatine. Similarly, L-valine was reported to be a precursor of coronafacoylvaline, and the data in the present report show that there has been a retention of the configuration of the L-valine moiety in *N*-coronafacoyl-L-valine. Furthermore, it is very probable that *N*-coronafacoyl-L-valine lies on the biosynthetic pathway to norcoronatine, paralleling the pathway proposed for coronatine biosynthesis [8] through *N*-coronafacoylisoleucine after coupling of coronafacic acid and L-isoleucine. The biosynthetic formation of the cyclopropane ring structure of coronatine is stereospecific, since previous work [5, 6] establishes that only one diastereoisomer (coronatine) is formed from the biosynthetic pathway. Since the biosynthetic derivation of the cyclopropane rings of norcoronatine and coronatine most likely utilize the same enzyme systems, then a reasonable expectation is that norcoronatine has the same configuration as coronatine. This was supported experimentally by studying the action of α-amino acid-L-acylase on the *N*-acetate derivatives of ethyl-ACC (from coronatine) and methyl-ACC (from norcoronatine). The acylase hydrolysed *N*-acetyl-L-alanine but not *N*-acetyl-D-alanine, confirming its specificity for the L-configuration. The *N*-acetates of ethyl-ACC and methyl-ACC were hydrolysed by the acylase, hence the two compounds are deduced to correlate with the L-α-amino acid configuration. The stereostructure **4** is therefore proposed for norcoronatine.

EXPERIMENTAL

Pseudomonas syringae pv. *glycinea* 4182 was obtained from the Plant Diseases Division Culture Collection. Toxin containing solutions were bioassayed by application to prick wound-sites on bean leaves as described in ref. [9]. Procedures of culture and general chemical procedures are described elsewhere [3]. Partition column chromatography on LH20 Sephadex (100 g, 97 × 2.4 cm) utilized MeOH–aq. 0.05 M NH_4OH (1·1) Yields of purified products from two 9 l batches were 78 mg of coronatine, and 2 6 mg of *N*-coronafacoylvaline. For GC, amino acids were derivatized to their methyl ester *N*-trifluoroacetyl derivatives; GC was at 110° on 5% SP-300 on 100/120 Supelcoport column (2 m × 2 mm i.d. steel) with N_2 at 25 ml/min and with FID [10] Retention times of the derivatives of D- and L-valine were 4.28 min and 4.78 min respectively. For GC of coronafacoyl derivs., see ref [3].

Norcoronatine (**4**). TLC R_f 0.42 (cf. coronatine, 0.58), 1 mg; ^{1}H NMR (200 MHz, $CDCl_3$): δ0.98 (3H, *t*, *J* = 7.5 Hz), 1 29 (3H, *d*, *J* = 6.0 Hz), 3.18 (1H, *mult.*) 6.35 (1H, *s*), 1.5–2.6 (several multiplets, unresolved); MS *m/z* (rel. int.): 305.1676 $[M]^+$ ($C_{17}H_{23}NO_4$ requires 305.1627) (12), 287 $[M-H_2O]^+$ (24), 191.1101 (coronafacoyl ion, $C_{12}H_{15}O_2$ requires 191.1072) (100), 163 $[191-CO]^+$ (83). Acid hydrolysis afforded a single non-protein amino acid and coronafacic acid. Methyl ester, MS *m/z* (rel. int.): 319 1778 $[M]^+$ ($C_{18}H_{25}NO_4$ requires 319.1784) (16), 287 $[M-MeOH]^+$ (20), 260 $[M-CO_2Me]^+$ (5), 191.1106 (coronafacoyl ion, $C_{12}H_{15}O_2$ requires 191.1072) (100), 163 $[191-CO]^+$ (53), 128.0721 (cyclopropyl compound from amide cleavage, $C_6H_{10}NO_2$ requires 128.0712) (51).

Acid hydrolysis. Norcoronatine (200 μg) was heated with 80 μl 6 M HCl for 1 hr in a sealed glass tube. The contents of the tube were then partitioned between 1 ml H_2O and 5 ml EtOAc, and the aq phase was extracted with EtOAc (2 × 5 ml). A single amino acid (2D TLE/TLC) was obtained from the aq. phase, and coronafacic acid (by GC and MS) from the EtOAc phase. Coronamic acid (1.5 mg) was similarly obtained from the acid hydrolysis of 5 mg of coronatine.

Acylase reactions. N-acetates of coronamic acid and norcoronamic acid were prepared at 18° using HOAc–Ac_2O *N*-Acetyl-D-alanine, *N*-acetyl-L-alanine and Acylase I were purchased from Sigma. Reactions of amino acid *N*-acetates (*ca* 150 μg) were at 38° in 100 μl 0.1 M NH_4HCO_3 solution containing 10 U Acylase I Hydrolyses were monitored by the appearance of a ninhydrin colour reaction and were complete within 1–2 hr.

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REFERENCES

1 Mitchell, R. E. (1984) *Ann Rev. Phytopathol* **22**, 215.
2 Mitchell, R. E., Young, H. (1978) *Phytochemistry* **17**, 2028.
3. Mitchell, R. E. (1984) *Phytochemistry* **23**, 791.
4 Mitchell, R. E. (1982) *Physiol. Plant Pathol.* **20**, 83.
5. Ichihara, A., Shiraishi, K., Sato, H. and Sakamura, S. (1977) *J. Am. Chem. Soc* **99**, 636.
6. Shiraishi, K., Konoma, K., Sato, H., Ichihara, A., Sakamura, S., Nishiyama, K. and Sakai, R. (1979) *Agric. Biol. Chem.* **43**, 1753.
7. Ichihara, A., Shiraishi, K. and Sakamura, S. (1979) *Tetrahedron Letters* 365.
8. Mitchell, R. E. (1985) *Phytochemistry* **24**, 247.
9. Mitchell, R E. and Bieleski, R. L. (1977) *Plant Physiol.* **60**, 723
10 Nakaparksin, S, Gil-Av, E. and Oro, J. (1970) *Analyt. Biochem.* **33**, 374