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# PHYTOTOXINS. II. CHARACTERIZATION OF A PHYTOTOXIC FRACTION FROM *ALTERNARIA ALTERNATA* F. SP. *LYCOPERSICI*

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SUMMARY. - A host-specific phytotoxic fraction obtained from the cell-free culture filtrate of A. *alternata* f. sp. *lycopersici* is shown to consist of two esters of 1,2,3-propanetricarboxylic acid and 1-amino-11,15-dimethylheptadeca-2,4,5,13,14-pentol **1**. The sites of esterification are a terminal carboxyl of the acid and $C_{13}$ (major component **2a**) and $C_{14}$ (**2b**) of **1**.

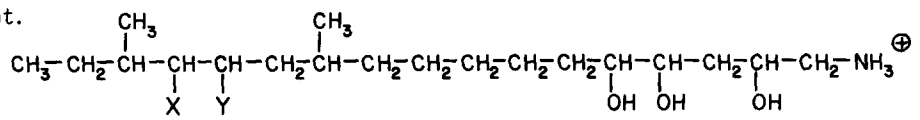
The stem canker disease of tomato, caused by A. *alternata* f. sp. *lycopersici*, is controlled in the host by a single genetic locus with two alleles.[1] Cell-free culture filtrates of the pathogen yield two ninhydrin-positive fractions, $T_A$ and $T_B$, which reproduce the macroscopic disease symptoms only on tomato and do so under quantitative control by the same genetic locus that regulates the disease reaction. $T_A$ and $T_B$ are separable by isoelectric focusing at pH 4-5, or by TLC on silica gel with 6:3:1 $CH_3CO_2C_2H_5$:$CH_3CO_2H$:$H_2O$, and are obtained most conveniently in >10 mg amounts by gel-permeation chromatography on polyacrylamide Bio-Gel P-2.[2] Based on current evidence $T_A$ and $T_B$, which exhibit necrotrophic biological activity at less than 10ng/ml, are produced only by this pathogenic *forma specialis*.

Hydrolysis of $T_A$ with aqueous NaOH yields 1,2,3-propanetricarboxylate and a base which is either 1-amino-11,15-dimethylheptadeca-2,4,5,13,14-pentol **1** or its 7,15-dimethyl isomer.[3] Methylation and deuteriomethylation[4] of $T_A$ gave products whose HRMS[5] has base peaks of 360.3126 ($C_{20}H_{42}NO_4^+$ = 360.3113) and 378.4260 ($C_{20}H_{24}D_{18}NO_4^+$ = 378.4244) and parent peaks of 633.4477 ($C_{33}H_{63}NO_{10}^+$ = 633.4452; 0.65% BP) and 657.5939 ($C_{33}H_{39}D_{24}NO_{10}^+$ = 657.5958; 1.31% BP). Also present in the HRMS of deuteriomethylated $T_A$ were peaks corresponding to loss of H, D, $CH_3$, $C_4H_9$, and one or more deuteriomethyls, deuteriomethoxyls and deuteriomethanols from M, peaks of m/e < 448.6 noted in the HRMS of the deuteriomethylated aminopentol,[3] and peaks of 130.0590 ($C_6H_4D_3O_3^+$ = 130.0584; 8.22% BP) and 193.0969 ($C_8H_5D_6O_5^+$ = 193.0983; 18.84% BP) which arise from the acid part. The HRMS data show that hydrolysis of $T_A$ is unaccompanied by skeletal rearrangement but they leave unanswered the questions as to location of the second methyl group ($C_7$ or $C_{11}$) and the site or sites of esterification in both the acid and the aminopentol parts. These questions are answered by consideration of the CMR spectrum of $T_A$.[6]

In addition to 4 bands in the carbonyl region from δ 174-182.5 ppm, with relative intensities of 1:1:2:2, the CMR spectrum of $T_A$ consists of 12 bands due to the major component, 12 bands due to a minor component, and 10 composite bands due to both components. The last set is made up of 3 bands whose line shapes are particularly sensitive to the presence of paramagnetic metal ions such as Cu(II) and 7 bands with the same chemical shifts to ± 0.05 ppm as bands in the spectrum of the aminopentol.[3] The 3 bands are assigned to the saturated carbons of the tricarboxylic acid part, and their chemical shifts and multiplicities - δ 34.73 (*t*), 40.35 (*t*), and 42.07 ppm (*d*) - confirm what can be inferred from the appearance

of the carbonyl bands; namely, the site of esterification is a terminal carboxylate (calc.[7] $\delta$ 37.4 [$C_1$,], 42.4 [$C_3$,] and 43.3 ppm [$C_2$,]) and not the $C_2$, carboxylate (calc.[7] $\delta$ 41.4[$t$ x 2] and 42.3 ppm [$d$]).[10] Of the 7 bands with chemical shifts in common with bands in the spectrum of the aminopentol, 4 have been assigned to $C_1$, $C_2$, $C_4$ and $C_5$. As these sites are not perturbed by the triacid part, the hydroxyls at $C_{13}$ and $C_{14}$ must be the sites of esterification. Significantly, the remaining 3 bands, all triplets, at $\delta$ 25.70, 31.49 and 35.74 ppm, have chemical shifts that agree well with values calculated for $C_7$ (25.0), $C_6$(33.0) and $C_3$ (38.4) of the 11,15-dimethyl structure **1a** when the uncertainties in correcting for the effect of nearby hydroxyl groups are taken into account.[7]

Each of the two sets of 12 bands is made up of 4 doublets, 5 triplets and 3 quartets. Consideration of the chemical shifts of the triplets with reference to those in the spectrum of the aminopentol leads to a clear choice between the 7,15- and 11,15-dimethyl structures. Of the calculated values for the chemical shifts of triplets in the spectrum of the 7,15-dimethyl isomer of **1a**, 5 are at $\delta$ > 30 ppm. The highest values - 36.9 ($C_8$), 38.4 ($C_3$) and 39.9 ($C_6$) - are for carbons that are 5 or more bonds removed from the sites of acylation, and these can be expected to show little change on acylation. In contrast, two of the 3 highest calculated values for chemical shifts of triplets in the spectrum of **1a** are for carbons much nearer to the sites of acylation - 36.9 ($C_{10}$) and 40.2 ($C_{12}$) - and the chemical shift of $C_{12}$, in particular, is expected to be affected by acylation at $C_{13}$ or $C_{14}$. Specifically, a 3-ppm upfield shift is estimated[7] as a result of acylation at $C_{13}$, and a 2-ppm downfield shift is estimated on acylation at $C_{14}$. The spectrum of $T_A$ has two triplets due to the major component at $\delta$ 33.82 and 37.02 ppm, two due to the minor component at $\delta$ 36.78 and 37.82 ppm, and one due to both components at $\delta$ 35.74 ppm; the spectrum of the aminopentol has triplets at $\delta$ 34.72, 35.73 and 37.59 ppm. From the foregoing, we conclude: the 35.74 band is due to $C_3$; the 34.72, 33.82 and 36.78 bands are due to $C_{12}$ of **1a**, **2a** and **2b**; and the 37.59, 37.02 and 37.82 bands are due to $C_{10}$ of **1a**, **2a** and **2b**. In short, the site of acylation in the major component is $C_{13}$, and $C_{14}$ is the site of acylation in the minor component.



**1**·$H^+$ = **1a**: X = Y = OH

**2a**: X = OH , Y = $^{\ominus}O_2C-CH_2-CH(CO_2^{\ominus})-CH_2-CO_2$

**2b**: X = $^{\ominus}O_2C-CH_2-CH(CO_2^{\ominus})-CH_2-CO_2$ , Y = OH

Observed and calculated CMR chemical shifts for **1a**, **2a** and **2b** are given in Table 1, and the proton-noise decoupled CMR spectrum of $T_A$ from $\delta$ 10-82 ppm is shown in Figure 1A. The 360-MHz PMR spectrum of $T_A$ is shown in Figure 1B.

Table 1. Calculated and Observed CMR Resonance Frequencies of **1a**, **2a**, and **2b** in $\delta$ ppm

| C | Calc.[b] | Observed | | | C | **1a** | | **2a**[c] | | **2b**[c] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **1a** | **2a** | **2b** | | Calc.[b] | Obs. | Calc.[b] | Obs. | Calc.[b] | Obs. |
| 1 | 47.9 | 45.28 | 45.28 | 45.28 | 11 | 27.5 | 28.59 | 29.5 | 28.62 | 27.5 | 28.37 |
| 2 | 66.6 | 64.94 | 64.97 | 64.97 | 12 | 40.2 | 34.72 | 37.2 | 33.82 | 42.2 | 36.78 |
| 3 | 38.4 | 35.73 | 35.74 | 35.74 | 13 | 73.6 | 69.98 | 77.6 | 74.46 | 70.6 | 69.16 |
| 4 | 73.7 | 70.49 | 70.46 | 70.46 | 14 | 85.9 | 79.18 | 82.9 | 76.30 | 89.9 | 81.72 |
| 5 | 79.0 | 74.72 | 74.67 | 74.70 | 15 | 37.6 | 36.05 | 39.6 | 36.35 | 34.6 | 34.89 |
| 6 | 33.0 | 31.44 | 31.49 | 31.49 | 16 | 24.6 | 23.96 | 24.6 | 24.27 | 26.6 | 24.70 |
| 7 | 25.0 | 25.72 | 25.70 | 25.70 | 15a | 14.1 | 14.85 | 14.1 | 14.48 | 16.1 | 14.80 |
| 8 | 30.2 | 29.10 | 28.92 | 29.15 | 1′ | - | - | 37.4 | 34.73 | 37.4 | 34.73 |
| 9 | 27.3 | 25.13 | 25.02 | 25.16 | 2′ | - | - | 43.3 | 42.07 | 43.3 | 42.07 |
| 10 | 36.9 | 37.59 | 37.02 | 37.82 | 3′ | - | - | 42.4 | 40.35 | 42.4 | 40.35 |
| 17 | 10.9 | 10.47 | 10.14 | 10.48 | | | | | | | |
| 11a | 19.6 | 20.36 | 20.16 | 20.28 | | | | | | | |

[a] Obtained at 50.3 MHz. [b] See Footnote 7. [c] Resonances at $\delta$ 174.51, 175.00, 180.45 and 182.15 ppm with relative intensities of 1:1:2:2 due to the carboxyl carbons were observed in the spectrum of $T_A$.

The upfield portion of the 360-MHz PMR spectrum of $T_A$ consists of incompletely resolved multiplets (18H) from 350-650 Hz and, in the methyl region, 4 doublets with $J$ = 7.3, 7.3, 6.6 and 6.6 Hz at $\delta$ 0.833 (**2b**), 0.853 (**2a**), 0.903 (**2a**) and 0.927 ppm (**2b**), and two triplets, $J$ = 7.3 Hz, at $\delta$ 0.857 (**2b**) and 0.878 ppm (**2a**) (9H). The remainder of the spectrum consists of: 5 well-defined multiplets (1H each) with the same or nearly the same parameters as those of bands assigned to protons at $C_1$, $C_2$, $C_4$ and $C_5$ of **1a**;[3] two coupled multiplets, $J$ = 2.8 Hz, at $\delta$ 3.458 and 5.135 ppm (0.57H each) due to protons at $C_{14}$ and $C_{13}$ of **2a**; two coupled multiplets, $J$ = 4.5 Hz, at $\delta$ 3.895 (0.43H) and 4.771 ppm (OH interference) due to protons

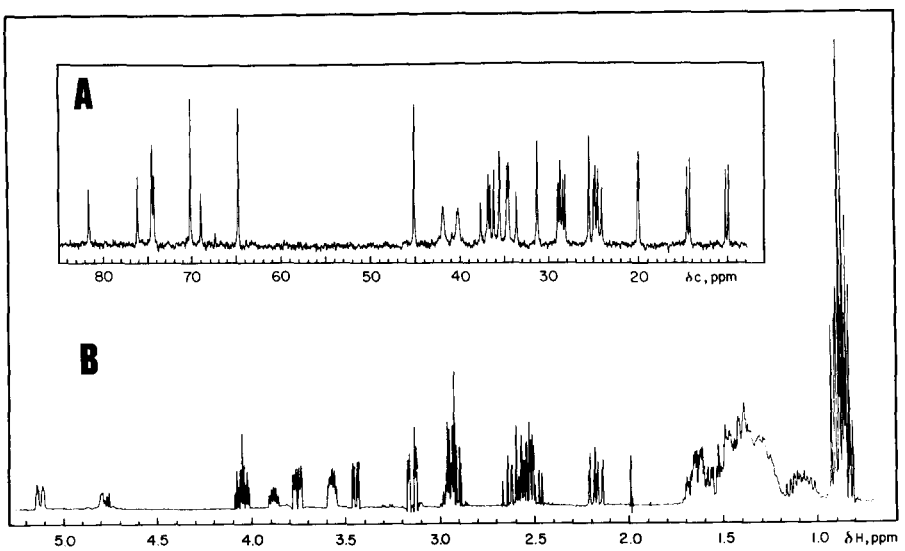



Figure 1: A, 50.3-MHz proton-noise decoupled CMR spectrum of $T_A$ from $\delta$ 10-82 ppm; B, 360-MHz PMR spectrum of $T_A$, resolution enhanced.

at $C_{13}$ and $C_{14}$ of **2a**; and 3 apparent multiplets with pH-dependent chemical shifts that are centered at *ca.* $\delta$ 2.2 (1H), 2.6 (3H), and 2.95 ppm (1H) in the spectrum of a $D_2O$ solution which gave a pH-meter reading of 8.2. These last bands are assigned to the protons at the saturated carbons of the acid part. It should be noted that the chiral centers at $C_2$, $C_4$ and $C_5$ of **1a**, and therefore **2a** and **2b**, have either the all-**R** or all-**S** configuration.[3]

Although $T_A$ obtained by different isolation techniques was a 4:3 mixture of **2a** and **2b**, it remains to be determined if this is the composition produced by the fungal pathogen rather than an equilibrated mixture. Also, the relative phytotoxicity of **2a** and **2b** remains to be determined. Evidence now available indicates that $T_B$ consists of two components with the same carbon skeletons as **2a** and **2b** but which lack the $C_5$ hydroxyl and differ in stereochemistry at one or more of the chiral centers from $C_{11}$ to $C_{15}$.

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## REFERENCES AND NOTES

1. D.G. Gilchrist and R.G. Grogan, *Phytopathology* **66**, 165 (1976).
2. The culture filtrate is concentrated to 10% of its original volume, treated with 0.43 part of 1.33 *M* barium acetate, centrifuged, and the centrifugate is extracted with butanol. The butanol is replaced with water on the rotovap, and the aqueous solution is concentrated to a yellow syrup, which is separated by gel-permeation chromatography using a pH 4.5 acetate buffer. The first ninhydrin-positive fraction contains $T_A$ and $T_B$ in a ratio of 12:1.
3. A.T. Bottini and D.G. Gilchrist, accompanying manuscript.
4. H. Morris, *FEBS (Fed. Eur. Biochem. Soc.) Letters* **22**, 257 (1972).
5. Electron impact mass spectra were obtained with a modified AEIMS-902 using a quartz-tip probe, a repetitive scanning rate of 8 sec./decade (m/z 70-900) at 10000 resolution (10% valley definition), accel. voltage 8 kv, and source temp 240-260°; the data were processed using the Sigma 7-LOGOS-II real-time data acquisition computer. The spectra were obtained at the Biomedical and Environmental Mass Spectrometry Resource, Space Sciences Laboratory, University of California, Berkeley; the resource is supported by NIH Division of Research Resources grant RR 00719.
6. CMR spectra were obtained with an NT-200 spectrometer; chemical shifts are reported relative to external TMS (capillary) at $\delta$ 0.00 ppm and are precise to $\pm$ 0.02 ppm. PMR spectra were obtained at 200-MHz and 360-MHz with NT-200 and NT-360 spectrometers; chemical shifts are reported relative to internal acetate at $\delta$ 1.903 ppm and are precise to $\pm$ 0.002 ppm. The spectrometers were made available through the UCD NMR Facility; the NT-200 spectrometer was purchased in part by NSF grant CHE 79-04832 to the Department of Chemistry. PMR and proton-noise decoupled CMR spectra of $T_A$, obtained by isoelectric focusing, were obtained at the Stanford Magnetic Resonance Laboratory (SMRL) with an HXS-360 spectrometer; the SMRL facility is supported by NSF grant GR 23633 and NIH grant RR 00711. Multiplicities of the bands in the CMR spectrum were determined by off-resonance decoupling of the PMR spectrum; assignments to bands in both spectra are consistent with the results of the off-resonance decoupling experiments.
7. CMR chemical shifts were calculated using the "Lindeman-Adams Rule"[8,9] and the empirical substituent parameters compiled by Wehrli and Wirthlin.[13] Values calculated for the carbons without $\alpha,\beta$ or $\delta$ substituents do not require use of substituent parameters and are expected to have a standard error of 0.8 ppm;[9] values for substituent parameters "are reliable only within a margin of $\pm 5 \div 10\%$."
8. L.P. Lindeman and J.Q. Adams, *Anal. Chem.* **43**, 1245 (1971).
9. F.W. Wehrli and T. Wirthlin, *Interpretation of Carbon-13 NMR Spectra*, Heydon and Son, Inc., Philadelphia, PA, 1978, pp. 36-42.

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