and if the reaction of the neutral purine with e^-_{aq} occurs within its lifetime, one should be able to observe an increase in the intensity of the band at 320 nm as the e^-_{aq} signal decreases, i.e., approximately 2 μs after the laser pulse. This increase in absorption was not observed. A possible explanation is that, once formed, the radical anion reacts instantaneously to produce the species absorbing at 320 nm.

In the pulse radiolysis of 2'-deoxyadenosine Hissung et al.[42] observed that the radical anion presented a rather weak and featureless absorption above 300 nm which, in 0.2 μs, decayed into a neutral radical. At pH 5.2, Moorthy and Hayon[26] assigned the absorption maxima at 315 and 430 nm to the species H_3P^+, which resulted from the one-electron reduction of purine, HP, and subsequent protonation to form a dihydro radical cation.

Another possible species contributing to the absorption at 320 mn is the purine radical cation. During continuous UV irradiation of frozen purine solutions in 8 M $NaClO_4$, a permanent, faint blue coloration on the sample was observed as well as a decrease in absorbance of the purine absorption band at 264 nm. The appearance of a new band with a maximum near 320 nm was also observed.[17] The blue coloration was explained in terms of the presence of trapped electrons detected by EPR and their visible absorption band. Warming the irradiated solution to room temperature produced a partial regeneration of the purine absorption, while the absorption at 320 nm was no longer observed. This band was assigned to a purine radical cation. The radical cation was identified from its EPR spectra consisting of a singlet with line width of 22 G and g value of 2.004.[19] On the other hand, in the laser pulse experiments, irradiated purine solutions containing N_2O did not present an increase in the absorbance at 320 nm as should be expected if it were to correspond to the absorption of a radical cation.

In summary, simple models for one of the nucleic acid components, purine free base and 6-methylpurine, undergo monophotonic ionization as one of their primary photochemical processes. This is evidenced by the presence of a visible band assigned to the hydrated electron absorption band. This band is quenched by well-known electron scavengers, confirming the presence of the electron. Similarly, the effect of triplet quenchers such as O_2 and paramagnetic ions on the 390-nm band, as well as the sensitization of the crocetin triplet, provided evidence for the presence of a purine triplet state. The contribution of this state to the photoionization process is minor inasmuch as the electron initial yield is not affected by the presence of triplet quenchers. Furthermore, the triplet's appearance occurs 15 ns after that of the electron. The photoejected electron adds to purine at almost diffusion-controlled rates, implying the formation of radical anions and products resulting from their reaction with water.

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Registry No. Purine, 120-73-0; 6-methylpurine, 2004-03-7.

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Characterization of the *Fusarium* Toxin Equisetin: The Use of Phenylboronates in Structure Assignment

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Abstract: *Fusarium equiseti* has repeatedly been identified in environments where several genetically unrelated individuals each developed leukemia. The screens on cultures of this fungus revealed interesting biological activities and turned up equisetin, a previously identified yet uncharacterized metabolite. The molecule has now been shown to contain two domains, a bicyclic hydrocarbon and an *N*-methyltetramic acid, connected by a bridging carbonyl. The steric requirements of the two bridging carbon–carbon bonds dictated much of the physical and chemical behavior of the toxin. A phenylboronic ester derivative proved to be essential in defining the enol form of the tetramic acid and in making possible the assignment of the scalar and dipolar spin exchange interactions in the complete characterization of the molecule.

Genetic and environmental factors are both known to contribute to the development of leukemia.[1] However, individual cases usually cannot be clearly attributed to either genetic predisposition or specific etiologic agents and probably result from several interacting factors.[2] In instances when "clusters" of genetically unrelated individuals living in the same environment all develop the same form of leukemia,[3,4] environmental leukemogens are more strongly implicated and can potentially be identified.

A striking medical report appearing in 1969 associated four cases of leukemia with a single home in Georgia.[4] Existing evidence suggesting that mycotoxins may be causative factors in environmentally induced leukemia[5] led Wray and O'Steen[6] to screen the house and surroundings for fungal species. Of 11 fungal isolates tested, an extract of a *Fusarium sp.* was shown to have the greatest toxicity to animals.[6] In a subsequent study, *Fusarium equiseti* was found in the home of a husband and wife who both

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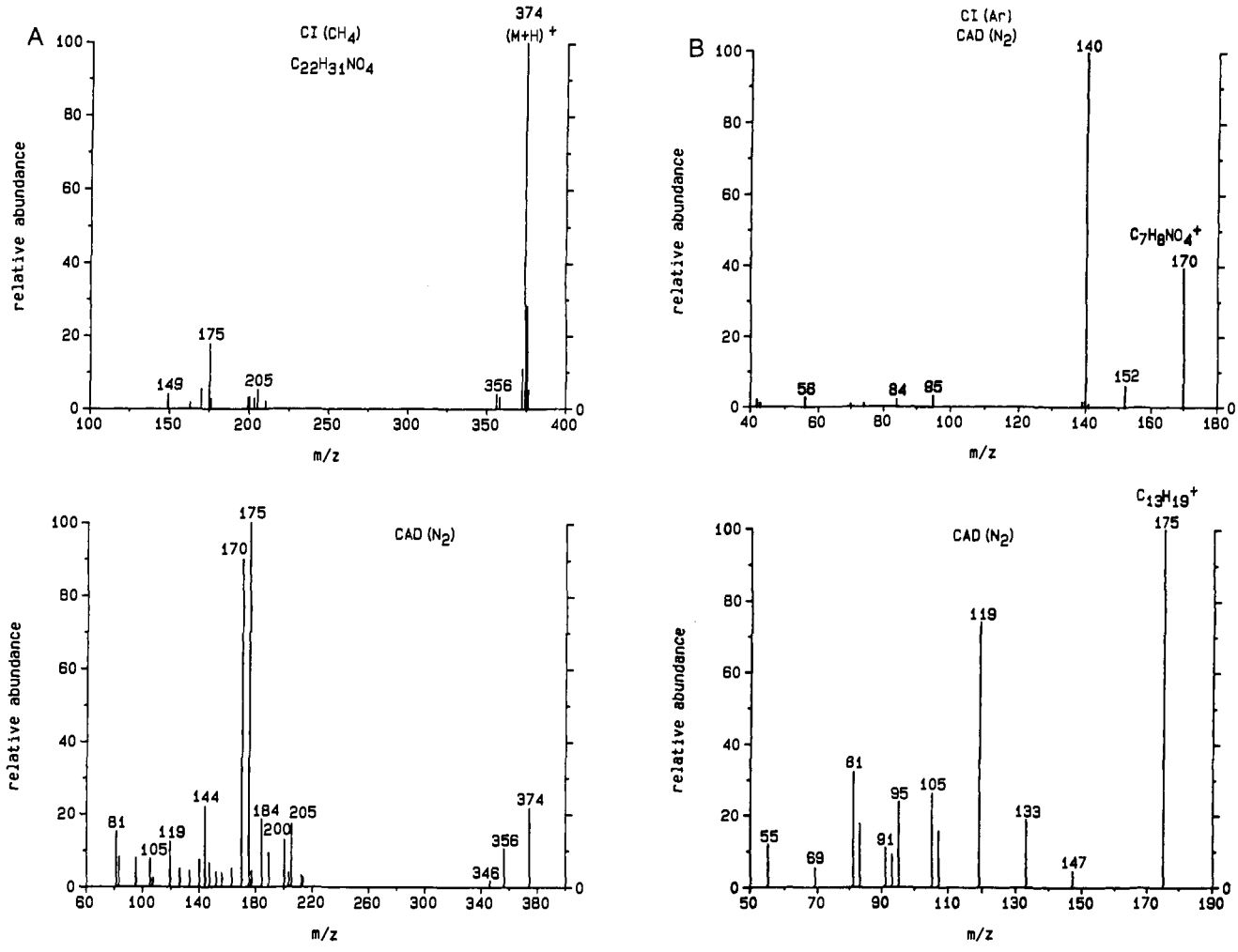

Figure 1. (A) Methane chemical ionization spectrum of **1** (top). Daughter ion spectrum of the N_2 collision-activated decomposition (CAD) of **1** (bottom). (B) CAD (N_2) daughter ion spectra of the m/z 170 (top) and 175 (bottom) ions from the Ar chemical ionization spectrum of **1**.

developed acute myelomonocytic leukemia within 6 months of each other.[7] An extract of this sample was shown to suppress the immune response to intradermal phytohemagglutinin in 4-month-old guinea pigs,[7] whereas fungal isolates from nearby control homes had no effect. The toxicity and immunosuppressive activity of these fungal extracts implicated the *Fusarium* metabolites as possible leukemogens. Our recent investigations of environmentally induced chronic human diseases[8] stimulated our interest in this possible correlation, and we now report the complete chemical characterization of the principal toxic metabolite produced by *F. equiseti*.

Results

The white mold was found to grow best in liquid culture[9] and was produced under 2-week growth cycles at ambient temperature in Fernbach flasks. The samples were screened for general cytotoxic activity[9] in the hope of picking up all the biologically active components. Chromatographic separation of a $CHCl_3$ extract yielded a clear oil, which accounted for all the detectable activity. The organic-soluble material exhibited intense electronic transitions in MeOH at λ^1_{max} 292 nm (ϵ 10000) and λ^2_{max} 248 nm (ϵ 7500). The ^1H NMR spectrum, independent of solvent and field strength, gave abnormally broad lines, making it impossible to define individual resonances. Only a few basic features of the molecule could be immediately identified (500 MHz, $CDCl_3$); four vinyl protons (δ 5.1–5.4), three protons on heteroatom-bearing carbons (δ 3.6–4.1), an *N*-methyl group (δ 3.07), two methyl doublets (δ 1.56 and 0.92), and one low-field exchangeable proton (δ 17.3). The three protons on the heteroatom-bearing carbons were assigned as an isolated AMX spin system, but interpretation of the few cross peaks present in the high-field region of the COSY spectrum was not possible. Only coupling between one of the vinyl protons and the methyl group at δ 1.56 could be assigned with certainty.

The EI(+) mass spectrum (MS) of the compound identified an apparent molecular ion at m/z 373 (30%). The corresponding $(M + H)^+$ ion, m/z 374, was observed by FAB (glycerol) and positive ion CI (CH_4) MS. The negative ion CI spectrum (N_2O) showed a major M^- ion, m/z 373 (100%), along with a weak $(M - H)^-$ ion, m/z 372 (13%). Exact mass measurement of the EI(+) m/z 373 ion suggested a composition of $C_{22}H_{31}NO_4$ (373.2289, calcd 373.2253). The nitrogen and oxygen heteroatoms and the eight degrees of unsaturation were supported by the intense electronic transitions and carbonyl and hydroxyl stretching frequencies in the vibrational spectrum.

While the positive ion CI (CH_4) spectrum showed an abundant parent ion, the fragment ion intensity was very low (Figure 1A, upper). Collision-activated decomposition (CAD, N_2) of the m/z 374 ion in a triple-quadrapole mass spectrometer,[10,11] however, gave two major daughter ions at m/z 170 and 175 (Figure 1A, lower). The complete absence of ions at higher masses suggested that the molecule was fragmenting into two separate domains,

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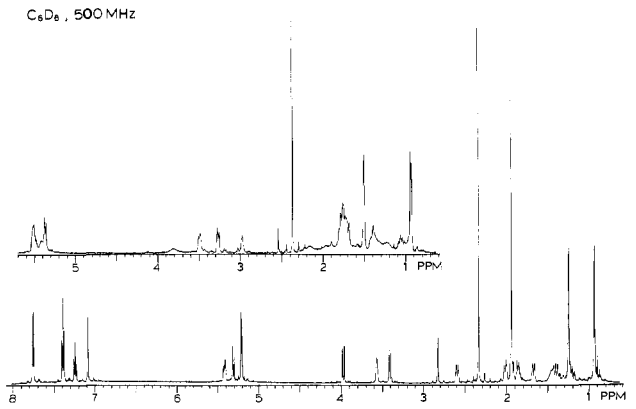


Figure 2. ^{1}H NMR (C_6D_6, 500-MHz) spectra of **1** (inset) and the phenylborate derivative **2**.

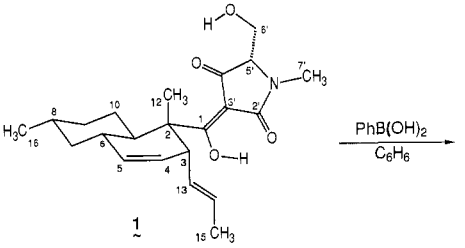
and CAD (N_2) analysis of the two ions supported this assignment. Figure 1B (lower) shows the daughter ion spectrum of the m/z 175 ion. This ion sequentially lost units of 14, suggesting a predominantly carbocyclic fragment as further confirmed by an exact mass measurement (175.1493, calcd 175.1486, $C_{13}H_{19}$). The m/z 170 ion showed very little fragmentation on collisional decomposition other than a loss of H_2O to m/z 152 and formaldehyde to a stable m/z 140 ion (Figure 1B, upper). Exact mass measurement of the m/z 170 ion established its composition as $C_7H_8NO_4$ (170.0460, calcd 170.0452), a fragment containing all the heteroatoms of the *Fusarium* metabolite.

Gas-phase deuterium exchange (CI^+, MeOD)[12] gave a parent ion at m/z 377 $(Md_2 + D)^+$, indicating two exchanges. However, only a monosilylated derivative of the toxin could be prepared, which under CI^+ conditions gave major fragment ions at m/z 175 and 242 $(m/z\ 170 + TMS)^+$. Deuterium exchange of this derivative gave a molecular ion at m/z 448 $(Md + D)^+$, and an intense ion at m/z 243 relegated the other exchangeable proton to the heteroatom-containing fragment as well.

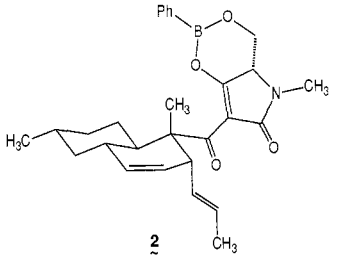
The MS/MS data established the *Fusarium* metabolite as a molecule containing two distinct domains; a hydrocarbon moiety and a heteroatom-rich system with two exchangeable protons and one silylation site. These two distinct domains were also supported by the EI(+) spectrum, which showed two ions, m/z 203 (39%) and m/z 170 (100%), whose combined mass equals the molecular weight of the toxin.

The Heterocyclic Domain. The mass spectral studies established the presence of a heterocyclic fragment of composition $C_7H_8NO_4$ containing four degrees of unsaturation. Correlation of this information with existing ^{1}H NMR data suggested that all eight of the protons on the system could be accounted for in four isolated spin systems: an NCH_3, an AMX system bounded by heteroatoms, and two exchangeable protons. Four carbons and three oxygens remained to be assigned, arguing strongly for at least two carbonyls in the heterocycle. An *N*-methyltetramic acid, **1**, accounted for all of the atoms in the system and avoided an N–O bond, for which there was no chemical evidence. The proposed system contained the requisite proton spin systems, would be expected to give an intense ion in the mass spectrum as a result of α cleavage between the carbonyl and the hydrocarbon, and was consistent with a partial structure assigned for equisetin, a metabolite of *F. equiseti*, previously identified by Burmeister et al.[13] The β,β'-triketone would also be expected to undergo deuterium exchange without silylation.

The low-field-shifted exchangeable proton (δ 17.3, $CDCl_3$) in the ^{1}H NMR spectrum was assigned as the enol proton of the β,β'-triketone but a range of pH's and various organic solvents were unsuccessful at altering the exchange rate so as to improve the NMR spectral resolution. Base-catalyzed acetylation led to decomposition, and attempts to direct the enolization through acetal formation with the primary alcohol gave low yields of an epimerized product. Simple exposure of the sample to pyridine also led to epimerization. However, the mild reaction with phenylboronic acid in benzene[14] gave a 1:1 adduct, **2**, which could be chromatographically purified and easily hydrolyzed back to the natural product on exposure to mild acid.



1



2

The electronic spectrum of **2** was very similar to the natural product, suggesting that the chromophore was little affected. The ^{1}H NMR spectrum of the phenylborate derivative, however, was markedly different (Figure 2). Throughout the spectral region, sharp and well-resolved lines appeared, the most marked of which was the "new" methyl singlet at δ 1.92. At 500 MHz, all 29 nonexchangeable protons of the adduct were identifiable.

The Hydrocarbon Domain. The structural identity of **1** as equisetin was established by comparisons with a small sample of the original isolate.[13] While the hydrocarbon domain of the compound had not been structurally assigned almost certainly because of the poor spectral resolution,[13] the NMR spectrum of the phenylborate derivative, **2**, was well resolved and could be mapped out by 2D spectroscopy. Two sets of olefinic protons were both coupled to a multiplet at δ 3.57, a signal that appeared only as a broad hump at δ 3.82 in the spectrum of the natural product. The relatively small coupling constant between the resolved olefinic protons (J = 10 Hz) suggested a cis geometry for one double bond. The other olefin signals, overlapping in C_6D_6 but well resolved in $CDCl_3$, showed a large trans 15 Hz coupling, with one proton also coupled to the low-field-shifted methyl doublet. These assignments characterized an isolated homoallylic diene system accounting for two of the four degrees of unsaturation indicated by the composition of the hydrocarbon domain, $C_{15}H_{23}$.

The use of a relatively small data set and an 8 ppm sweep width made the COSY spectrum of **2** (Figure 3) highly selective, digitizing only J values greater than approximately 7 Hz (^2J and $^3J_{ax-ax}$ couplings). As a result, the equatorially disposed protons H_{10e}, H_{9e}, and H_{7e} showed only one cross peak to their respective geminal partners, whereas the axial protons in the system had more than one coupling connection. Acquisition of the data with different delay times, Δ, following the excitation pulses[15,16] selectively

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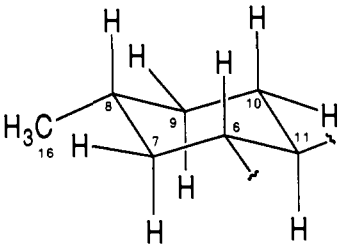

$$(T_w - 90°\Delta - t_1 - 90°\Delta - t_2)n$$

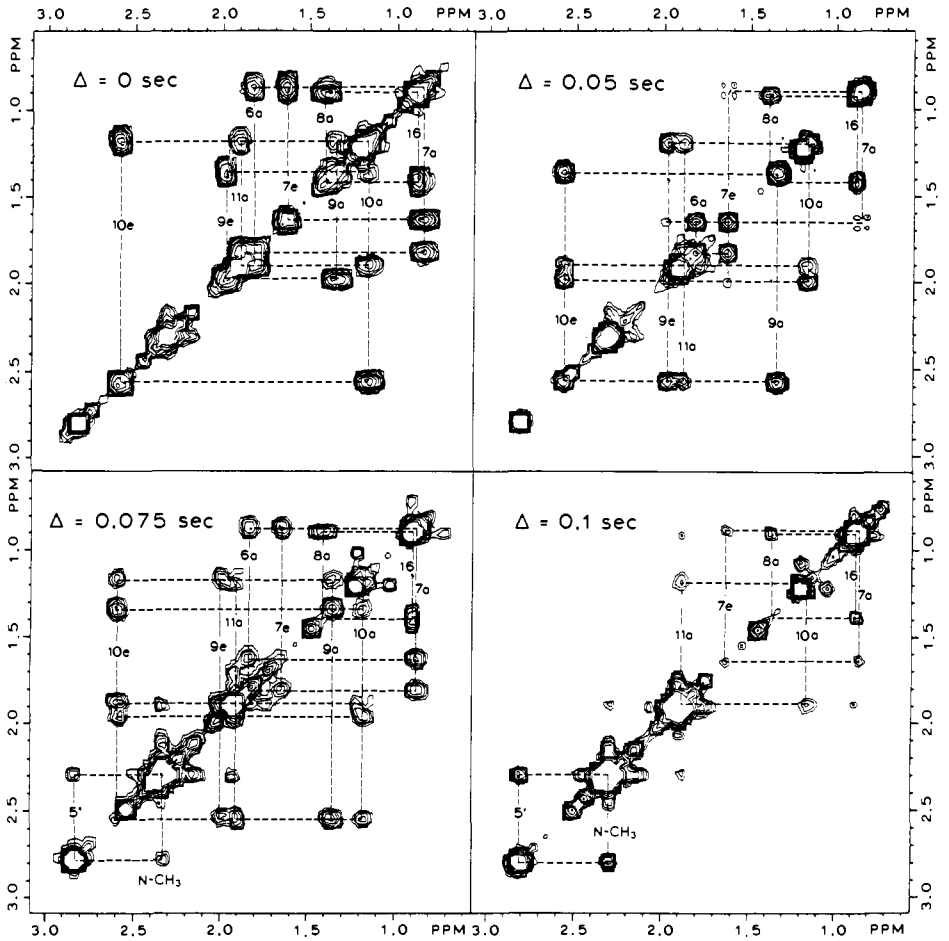

Figure 3. Effects of various delay times (Δ) on the high-field region of the delayed COSY spectrum (C_6D_6, 500 MHz) of **2**. A total of 256 (t_1) by 512 (t_2) data-point spectra were acquired to produce 256 × 256 reals. The time domain data were apodized with a sine bell function and the data sets were symmetrized.[42]

amplified different spin interactions. With Δ = 0.05 s, the couplings between H_{10e} and three additional protons, H_{9e}, H_{9a}, and H_{11a}, were revealed and the larger J coupling cross peaks were absent or of reduced intensity. Cross peaks representing longer range interactions, such as the coupling between H_{6a} and the olefinic protons H_4 and H_5 establishing the connection between the two spin systems and the ^4J coupling between the NCH_3 and $H_{5'}$, are clearly seen with longer delay times. While there are other methods, including spin locking experiments, which can identify small or long-range coupling interactions, the degree of simplification of the 2D spectra afforded by these delay times is evident in Figure 3.

The two carbons and three hydrogens still unassigned, when considered with the 3 H singlet at δ 1.92 and the additional degree of unsaturation, were assigned as in **1**. The relative stereochemistry of C_2 and C_3 could not be established by evaluating coupling connectivities. Several experiments were attempted to assign the configuration through long-range coupling interactions. Coupling between H_3 and H_5 observed at Δ = 0.1 s suggested that H_3 was equatorial. The absence of observed coupling between H_3 and Me_{12} provided negative evidence for the H_3 and axial Me_{12} assignment.[16] Unfortunately, the resonance overlap between Me_{12} and H_{11a} precluded the detection of ^4J trans-diaxial coupling between those two resonances that would have verified the assignment.

Dipolar exchange experiments[17] were investigated in an attempt to assign the stereochemistry, and of the several mixing times tested, τ_{mix} = 0.75 s provided the most information (Figure 4). An exchange cross peak occurring between H_{11a} and the isochronous olefinic signals, H_{13} and H_{14}, suggested that the propenyl branch at C_3 was axial. The presence of an exchange cross peak between Me_{12} and H_{10a} supported an axial orientation for the methyl group, and the additional cross peak between Me_{12} and H_3 supported an assignment in which Me_{12} and the propenyl side chain were on opposite faces of the decalin system. NOE difference experiments[18] further confirmed these assignments. Irradiation of the $H_{13/14}$ resonance gave enhancements of H_{11a} (2.5%) as well as H_4 (5.7%), H_3 (6.9%), and Me_{15} (3.5%).

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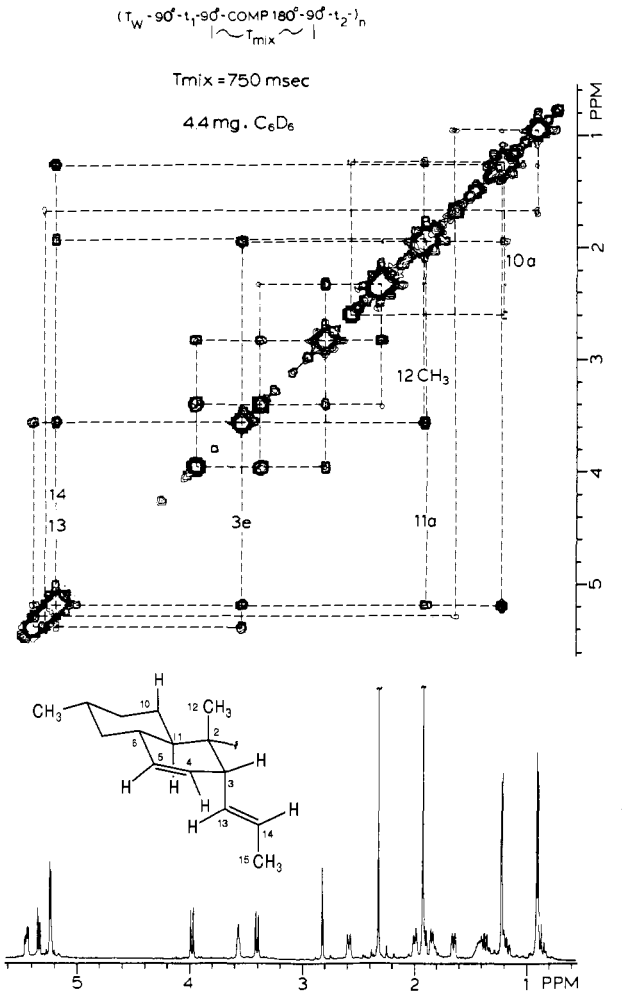


Figure 4. High-field expansion of the 2D cross-relaxation spectrum (τ_{mix} 0.75s) of **2**.

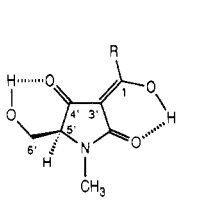
However, the resonance overlap between H_{11a} and Me_{12} still prevented further NOE difference experiments to definitively establish the axial orientation of Me_{12}.

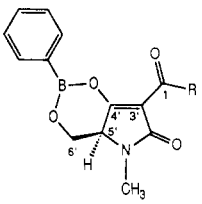
Variable-Temperature ^{1}H NMR Studies. The broad lines in the room-temperature ^{1}H NMR spectrum of the natural product were attributed to dynamic enolization. Heating to 60 °C in C_6D_6 had very little effect on the rate of tautomerization; however, on cooling to −20 °C in $CDCl_3$, the spectrum was as well resolved as that of the phenylborate derivative. Rather than trapping out many isomeric forms, the low-temperature spectrum appeared to represent a single major conformer.

The correlation experiments, which allowed for complete assignment of the proton spectrum of the borate derivative, worked in an analogous fashion at −20 °C. The same selectivity of the cross peak detection was possible with delayed correlation experiments. Furthermore, the low-temperature spectrum of the natural product did not have the resonance overlap problems present in the spectrum of the borate derivative. H_{11a} was seen as a well-resolved broad triplet at δ 1.66, 110 Hz downfield from Me_{12}, and delayed COSY spectra with Δ = 0.05, 0.075 and even more strongly at 0.15 s established the long-range coupling between these two signals. Such ^4J trans-diaxial couplings are routinely observed in terpenoid systems and are diagnostic for axial methyl substituents.[16]

Further support for the configurational assignment of Me_{12} came from 1D NOE difference data. Irradiation of H_{11a} produced a 6.9% enhancement of H_{13} along with enhancements of H_{14} (2.0%), H_{9a} (3.1%), and H_{7a} (2.8%). The enhancement of H_{13} seen upon irradiation of H_{11a} was complementary to the enhancement of H_{11a} (2.5%) seen upon irradiation of the $H_{13/14}$ resonance in the phenylborate derivative. Both observations confirmed the axial orientation of the propenyl branch. No enhancement of Me_{12} was observed when H_{11a} was irradiated and the irradiation of Me_{12} produced enhancements of H_{3e} (6.8%) and H_{6a} (3.0%). A reciprocal, although less selective, irradiation of H_{6a} produced a 1.7% enhancement of Me_{12}, in addition to enhancements of $H_{4/5}$ (3.1%) and H_{10a} (3.3%). The dipolar couplings between H_{6a}, H_{10a}, and Me_{12} support their 1,3-diaxial orientation and established the relative stereochemistry of the substituents as in **1**.

Table I. ^{13}C NMR Assignments





carbon	δ (−20 °C)	carbon	δ (25 °C)
2′	176.7	2′	173.9
3′	99.8	3′	101.2
4′	198.9	4′	189.6
5′	66.4	5′	69.6
6′	60.0	6′	60.8
7′	27.2	7′	28.3
1	190.6	1	194.2

Enol Form of the Tetramic Acid. Low-temperature ^{13}C NMR experiments together with the use of the borate adduct of defined enol position made it possible to assign the enol structure of the tetramic acid. Polarization transfer (DEPT) spectral editing experiments[19] carried out at −20 °C assigned the proton multiplicities of every carbon.[20] The difficult assignment of the methylenes in the decalin system was made by empirical chemical shift calculations and by comparison to the structurally and probably biosynthetically related system in ilicicolin H.[21] Comparison of the low-temperature spectrum with that taken at 25 °C showed that at room temperature all the carbons proximal to the enolizable C_1 carbon (2, 3, 11, 12, 2′, 3′, 4′, and 5′) were broadened significantly, some to the point of being undetected ($C_{3'}$). Therefore, all but the olefinic carbons were unequivocally assigned.

The chemical shift values of the tetramic acid carbons of the borate adduct were different from those observed in the natural product at −20 °C (Table I). The C_1 carbonyl in the borate is shifted downfield by almost 4 ppm while the $C_{4'}$ enol carbon of the borate is shifted upfield by more than 9 ppm. These chemical shift assignments were consistent with other simple tetramic acid systems, which undergo rapid tautomerizations to multiple enol forms.[22-25] The hydroxymethyl at $C_{5'}$ could add a degree of stabilization by hydrogen bonding to the $C_{4'}$ carbonyl oxygen, making possible an additional intramolecular H bond[22] from the C_1 enol OH to the electron-rich amide carbonyl.[23] This assignment is supported by the downfield shift of the $C_{2'}$ carbonyl in the low-temperature spectrum.[22] Such a bis-hydrogen-bonding structure should greatly stabilize one exocyclic enol over the others and suggests that a major contributing factor to the broad NMR lines would be hindered bond rotation between the quaternary C_2 carbon and the C_1 enol carbon.[25]

Mass Spectrometric Assignments. Since the electron impact fragmentation of the original isolate of equisetin led to the assignment of a two-carbon straight-chain bridge between the two domains of the molecule,[13] an assignment inconsistent with structure **1**, it was important that the fragmentation pathways

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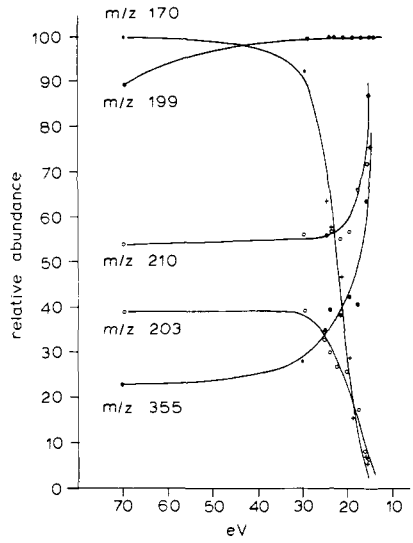


Figure 5. Plot of relative ion intensity vs ionization potential for the five selected ions.

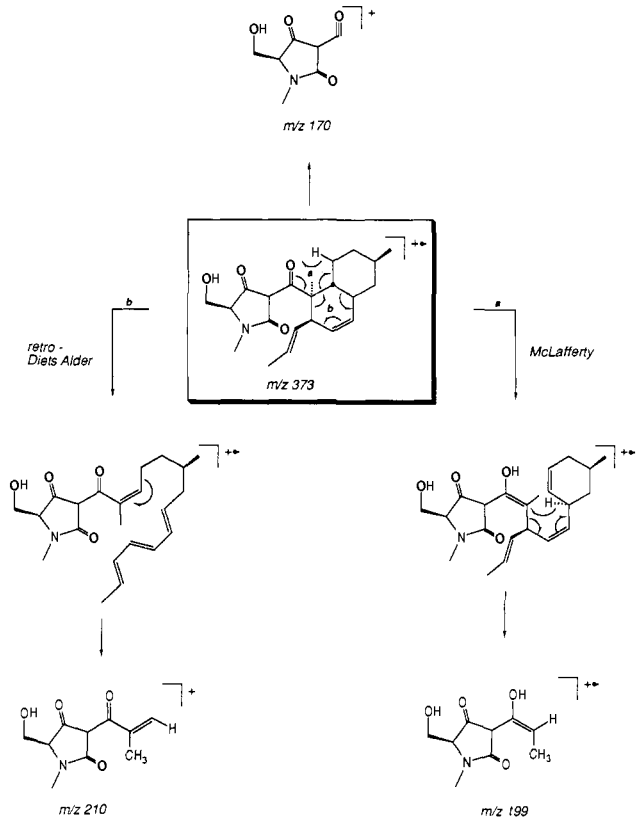
be assigned. Collision-activated fragmentation of the CI^+ (CH_4) generated parent ion of **1** gave two abundant fragment ions at m/z 170 and 175 (Figure 1A, lower). The ion at m/z 170 would be expected to arise from α cleavage between the bridging carbonyl and the hydrocarbon domain. This ion occurred as the base peak in the EI (70 eV) spectrum and an exact mass measurement supported the α cleavage origin. The m/z 175 ion, which was much weaker in the EI spectrum, had a composition of $C_{13}H_{19}$, losing the equivalent of C_2H_4 from the carbocyclic domain. The mechanism for this loss was not apparent from structure **1**. The major fragment ions at m/z 199 ($C_9H_{13}NO_4$) and m/z 210 ($C_{10}H_{12}NO_4$) in the EI spectrum were complementary ions corresponding to the tetramic acid containing C_2H_5 and C_3H_4 alkyl fragments, respectively. Likewise, a straightforward mechanism for their generation from **1** was not obvious.

Parent ion scans established that both m/z 199 and 210 arose directly from the molecular ion and supported two independent fragmentation pathways. Variation of the acceleration potential in the EBQQ hybrid spectrometer produced clear differences in the fragment ion intensities of the m/z 170 ion relative to the ions at m/z 199 and 210. This observation suggested that a study of the fragment ion intensity as a function of ionization potential could clarify the fragmentation mechanism.[26]

At low ionization energy, most of the low mass fragment ions disappeared from the spectrum and the ions at m/z 170 and 140 were very weak, whereas the ions at m/z 199, 210, and 355 dominated the spectrum. A plot of this change in relative intensity for the five selected ions is shown in Figure 5. The m/z 210 and 355 $(M - H_2O)^+$ ions clearly dominated under low-energy conditions, the m/z 203 and 170 ions increased at high energy, and the intensity of the m/z 199 ion was not altered within the energy range tested. The higher energy generation of the m/z 170 and 203 ions was again consistent with the α cleavage between the bridging carbonyl and the carbocyclic domain, charge retention being possible on either fragment. The lower energy processes that gave rise to the 210 and 199 ions could be assigned as two independent rearrangements initiated by either McLafferty or retro-Diels–Alder fragmentations (Scheme I). These mechanisms were consistent with the observed ion composition measurements and added further support to the assigned connection between the two molecular domains.

Absolute Stereochemical Assignment. The $C_{5'}$ chiral center dominated the CD spectrum of **1**, giving three negative Cotton effects at 340 ($\Delta\epsilon$ −0.328), 288 ($\Delta\epsilon$ −10.2), and 240 nm ($\Delta\epsilon$ −5.60). This spectrum was very similar to that seen for a simple tetramic acid, tenuazonic acid, [lit.[27] (MeOH) 275 ($\Delta\epsilon$ −6.9), 235 nm ($\Delta\epsilon$ −3.7)], whose absolute stereochemistry was rigorously proven by degradation studies.[28] Tenuazonic acid was shown to be biosynthesized from two molecules of acetate and one of L-isoleucine, maintaining the chirality of the amino acid in the final product.[29] Exposure of tenuazonic acid to base at elevated temperatures racemized the center[28] and exposure of **1** to pyridine at room temperature for a period of 2 days gave two clear sets of proton signals for $H_{5'}$, $H_{6'}$ and $H_{6''}$. Therefore, the tetramic acid ring of the *Fusarium* metabolite was assigned as having the absolute configuration of **1** derived from the direct biosynthetic incorporation of the amino acid L-serine.[30]

Scheme I[a]



[a] Proposed mechanisms for the mass spectral fragmentations.

The ability to freeze out a single conformation across the sterically hindered bridging carbonyl suggested the possibility of using dipolar exchange to relate the absolute stereochemistry of the tetramic acid to the C_2 center of the decalin system. While no Overhauser enhancements were detected between the C_2 methyl group and the tetramic acid ring protons in either the low-temperature spectrum of **1** or the spectrum of **2**, irradiation of both H_{9a} and H_{10e} on the bicyclic hydrocarbon produced enhancements of the ortho protons of the phenyl ring of the borate (1.3% and 1.5%, respectively). These enhancements required the phenyl ring to be close to one side of the decalin system and, if substantiated, this preferred orientation would permit the relay of the assignment of absolute stereochemistry from one molecular domain to the other.[17c]

The small coupling constants observed between $H_{5'}$ and $H_{6'}$ (J = 1.5 Hz) and $H_{5'}$ and $H_{6''}$ (J = 2.0 Hz) suggested that the ester ring existed in a pseudoboat with $H_{5'}$ bisecting the angle between the two methylene protons. This assignment was further supported by Overhauser enhancements of both $H_{6'}$ (3.1%) and $H_{6''}$ (2.0%) on irradiation of $H_{5'}$ as well as the complementary irradiations

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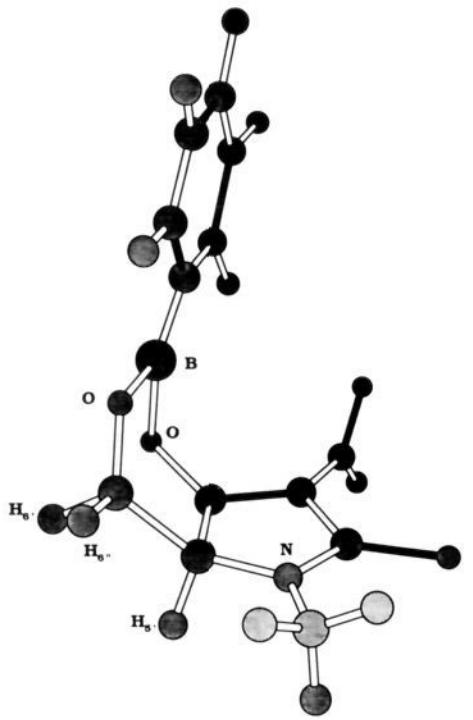
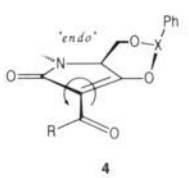
of $H_{6'}$ and $H_{6''}$, which both produced enhancements of $H_{5'}$ (6.1% and 5.3%, respectively). However, only irradiation of $H_{6''}$ produced an enhancement of the NCH_3 resonance (1.6%). When the NCH_3 signal was irradiated, enhancements of $H_{5'}$ (5.1%), $H_{6''}$ (3.0%), and the ortho protons on the phenyl ring (1.7%) were observed. Small enhancements of the ortho protons (1.2%) were also observed upon irradiation of $H_{6'}$ and $H_{6''}$. These NOE measurements were consistent with the ring conformation shown in **3** and highlighted the significant cross relaxation occurring to the phenyl ring protons.



3

The establishment of the solution conformation of the two molecular domains of **2** made it possible to model the conformational preferences of the two bridging carbon–carbon bonds. Molecular mechanics calculations[31] were used to minimize the individual domains (X = C^+ or sp^2 carbon), and rigid rotor approximations were used to establish the minimum energy positions around each of the bonds in question. Increasing steric bulk of the substituent R in **4** forced the exocyclic carbonyl from con-



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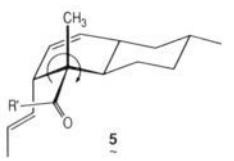
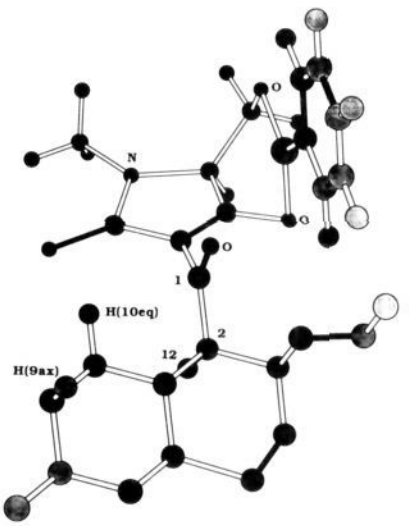


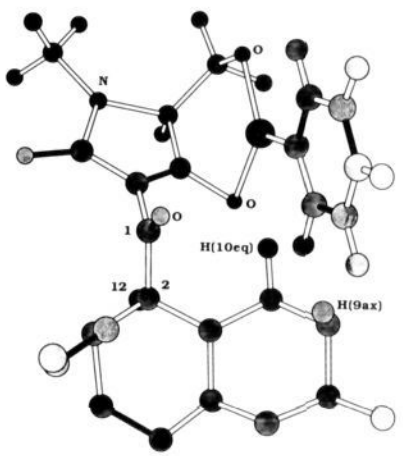
5

jugation to the convex or endo face of the fused ring system. The difference in strain energies for the endo vs exo conformations was approximately 7 kcal with R = *tert*-butyl. Likewise, the C_1–C_2 bond in **5** was lowest in energy with the carbonyl oxygen anti to the C_2 methyl group. For R′ = isopropyl, the difference in energy for anti vs syn was 5 kcal; for R′ = phenyl the difference was 2.1 kcal and the other conformations were of considerably higher energy.

This endo–anti conformation was significantly stabilized in the models and this same general trend held for the calculations on **1**. In both decalin enantiomers, **6** and **7**, the endo–anti conformations were the most stable. **6**, however, orients the phenyl ring away, whereas in **7**, the phenyl ring is positioned over the decalin ring. Furthermore, the calculations on **7** suggest a significant 4.8 kcal potential well, whereas in **6** the conformations differ by only ~0.5 kcal and variable-temperature NMR experiments on the borate could detect no conformer interconversions. The endo–anti conformation of **7** places the ortho protons of the phenyl ring directly over H_{9a} and H_{10e}, supporting the absolute stereochemistry shown in **1**.



6



7

Discussion

The principal toxic metabolite isolated from *F. equiseti* proved to be a dynamic structure that was not amenable to ^1H NMR analysis. Electronic spectra and mass spectrometry defined the molecule as one containing two separate domains: a tetramic acid heterocycle and a bicyclic hydrocarbon. Preparation of a phenylboronic ester locked the equilibrating heterocycle into one enol form and permitted the complete characterization of the molecule by 1D and 2D ^1H NMR methods.

While boron esters have found utility in regiochemical control of polyols and carbohydrates,[14,32] in aldol chemistry,[33] and even

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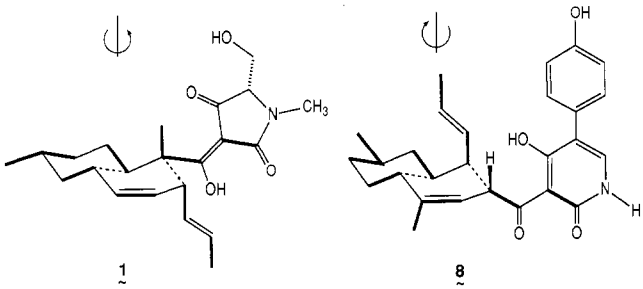
in the chromatographic separation of diols,[34] they have seen little use in organic spectroscopy. Much of the chemistry of these boron esters is dictated by the favorable overlap of the empty orbital on boron and the oxygen lone-pair electrons.[32-35] The large spatial area over which cross relaxation was detected in **2** may arise from increased flexibility in the B–C_{phenyl} bond due to favorable oxygen back-bonding.[36] The phenylboronate esters are easily formed and removed and have greatly extended the spectroscopic information available for the assignment of **1**.

We have demonstrated the relay of absolute chirality from the tetramic acid across acylic bonds to the C_2 center of the decalin ring. Attempts to prove the assignment by hydrolytic and oxidative removal of the tetramic acid[37] or spectral analysis of the C_5, epimerized product were unsuccessful at least in part because of the limited amount of material available, but total synthesis has now provided the conclusive proof.[38] Based on an acetate/propionate biosynthetic pathway, which has ample precedent in several related natural products,[23] the absolute sense of the biosynthetic cyclization defining the stereochemistry at C_2 can be assigned. This cyclization is opposite[39] to that found for the most closely related material, ilicicolin H, **8**, elaborated by both *Cylindrocladinin ilicicola*[21] and *Beauveria sp.*[40]



1 8

A number of antibiotic tetramic acids have been previously identified as fungal metabolites;[23] however, the mechanism of their toxicity has never been established. The identification of a possible environmental role associated with the development of chronic human disease, the proof of structure, and the synthesis of structural analogues of **1** have provided new approaches for these biological investigations. The compound has now been shown to be toxic to a selective range of mammalian cell types and to bind directly to DNA. The further characterization of this activity is currently in progress[41] and may help to clarify the possible role of this mycotoxin in the pathogenesis of leukemia.

Experimental Section

Spectroscopy. A Perkin-Elmer Lambda 5 UV/Vis spectrophotometer, a Cary 60 CD/ORD spectrometer modified with a Cary 1115 repetitive scan accessory, and a Perkin-Elmer 1430 ratio recording spectrophotometer or a Nicolet 20SXB FTIR instrument were used routinely.

Mass spectra were acquired on a VG 70-250E or a VG 7070 EBQQ. CAD (N_2) spectra [$CI^+(CH_4)$ and $CI^-(N_2O)$] were obtained with a Finnigan 3200 triple-quadrapole mass spectrometer or the VG 7070 EBQQ system. GC–MS runs were conducted with an HP 5790A series GC with an H/P capillary column interfaced with the VG 7070.

NMR spectra were recorded on a home-built 500-MHz console with a 1280/293C data system and NMCFT software or a Varian XL-400 spectrometer. Further details of the spectroscopy experiments as well as fully assigned correlation spectra, variable-temperature ^1H and ^{13}C NMR spectra, mass spectra at different ionization potentials, and parent ion MS/MS data are available as supplementary material.

Purification of 1. Fungal cultures were grown in 46 Fernbach flasks for 2 weeks at room temperature prior to extraction with cold $CHCl_3$. The dry residue obtained after evaporation of the organic solvent was applied to a silica gel column and sequentially eluted with toluene, ether, EtOAc, and acetone. By use of the bioassay procedures previously described,[9] the ether, EtOAc, and acetone fractions were all found to be toxic to day-old cockerels. The EtOAc and acetone fractions were pooled together and applied to a second SiO_2 column and eluted with toluene and ether. Toxicity was associated with a band of fractions that eluted with ether and contained a common spot, which charred purplish-red on SiO_2 TLC plates sprayed with benzaldehyde/HCl. Active fractions were pooled together and applied to a Florisil column eluted with a gradient from toluene to EtOAc, followed by a gradient from EtOAc to acetone. The desired spot eluted during the EtOAc to acetone gradient and during a final column flush with acetone. When combined and concentrated, these fractions produced a white precipitate. Final purification could be accomplished in either of two ways.

(i) A SiO_2 pTLC plate (0.25 mm) was dipped twice in 0.1 M oxalic acid in MeOH and allowed to air dry. Following application of crude toxin (15–80 mg), the plate was developed in benzene/10% CH_3OH. The UV-active toxin band (R_f = 0.38) was extracted 2× with $CHCl_3$/10% CH_3OH. Generally, the compound thus obtained was suitable for NMR spectral studies. If highly pure sample was required, a second pTLC plate (not oxalic acid treated) was occasionally run (benzene/15% CH_3OH).

(ii) A 15 cm × 1.5 cm column of reversed-phase gel (octadecylsilane bonded to SiO_2) was flushed with CH_3OH before equilibration with CH_3OH/20% H_2O and application of toxin (15–100 mg). The column was eluted with a step gradient from CH_3OH/20% H_2O to CH_3OH, and the early eluting fractions contained pure compound: UV (MeOH) λ^1_{max} 292 nm (ϵ 10 000), λ^2_{max} 248 nm (ϵ 7500); CD (MeOH) 340 ($\Delta\epsilon$ −0.328), 288 ($\Delta\epsilon$ −10.2), 240 nm ($\Delta\epsilon$ −5.60); IR (film, NaCl plate) ν_{max} (intensity), 3300 (s), 3020 (m), 2940 (s), 2910 (s), 2840 (s), 1690 (s), 1660 (s), 1560 (s), 1500 (s), 1450 (s), 1380 (m), 1250 (m), 1210 (m), 1070 (m), 1035 (m), 970 (m), 920 (m); ^1H NMR (500 MHz, $CDCl_3$, −20 °C) δ H_{3e} (3.32 br d, J = 9.0 Hz, 1 H), H_4/H_5 (5.40, br s,‡ 2 H), H_{6a} (1.84, br t*, J = 12 Hz, 1 H), H_{7a} (0.90, q,* J = 12 Hz, 1 H), H_{7e} (1.81, br d,* J = 12 Hz, 1 H), H_{8a} (1.48, m, 1 H), H_{9a} (1.11, q,* J = 12 Hz, 1 H), H_{9e} (1.76, br d,* J = 12 Hz, 1 H), H_{10a} (1.04, q,* J = 12 Hz, 1 H), H_{10e} (1.96, br d, J = 12 Hz, 1 H), H_{11a} (1.66, br t, J = 11 Hz, 1 H), H_{12} (1.46, s, 3 H), H_{13} (5.17, dd, J = 15, 9.0 Hz, 1 H), H_{14} (5.25, dq, J = 15, 6.5 Hz, 1 H), H_{15} (1.56, d, J = 6.5 Hz, 3 H), H_{16} (0.92, d, J = 6.5 Hz, 3 H), $H_{5'}$ (3.67, dd, J = 5.0, 3.5 Hz, 1 H), $H_{6'}$ (4.07, dd, J = 12, 3.5 Hz, 1 H), $H_{6''}$ (3.90 dd, J = 12, 5.0 Hz, 1 H), $H_{7'}$ (3.07, s, 3 H), enol H (17.3, s, 1 H) (*, partially obscured; ‡, overlapping); ^{13}C NMR (50 MHz, $CDCl_3$, −20 °C) δ 198.9 ($C_{4'}$), 190.6 (C_1), 176.7 ($C_{2'}$), 130.4 (C_5 or C_{13}), 129.8 (C_5 or C_{13}), 127.1 (C_4 or C_{14}), 126.2 (C_4 or C_{14}), 99.8 ($C_{3'}$), 66.4 ($C_{5'}$), 60.0 ($C_{6'}$), 48.4 (C_2), 44.6 (C_3), 41.9 (C_7), 39.6 (C_{11}), 38.4 (C_6), 35.5 (C_9), 33.3 (C_8), 28.1 (C_{10}), 27.2 ($C_{7'}$), 22.5 (C_{16}), 18.2 (C_{15}), 13.7 (C_{12}); EI(+) MS (70 eV, 250 °C) m/z 373 (31, $C_{22}H_{31}NO_4$), 355 (23, $C_{22}H_{29}NO_3$), 210 (54, $C_{10}H_{12}NO_4$), 203 (39, $C_{15}H_{23}$), 200 (50, $C_9H_{14}NO_4$), 199 (89, $C_9H_{13}NO_4$), 183 (26, $C_9H_{13}NO_3$), 175 (38, $C_{13}H_{19}$), 170 (100, $C_7H_8NO_4$), 143 (40, $C_6H_9NO_3$), 140 (52, $C_6H_6NO_3$).

Epimerization of 1. Exposure of **1** to pyridine for 3 days produced a 50/50 mixture of two compounds. The ^1H NMR spectrum of the mixture ($CDCl_3$, 500 MHz, 25 °C) showed two sets of signals for the following resonances:

	1	epi-1
$H_{5'}$	3.63	3.66
$H_{6'}$	4.03	4.06
$H_{6''}$	3.88	3.85
$H_{7'}$	3.07	3.05

Phenylboronic Acid (PBA) Esterification. A solution of **1** (43.3 mg, 0.116 mmol) and PBA (10.6 mg, 0.0869 mmol) in 50 mL of dry, distilled benzene was allowed to reflux for 22 h in a Dean–Stark trap. After being cooled to room temperature, the reaction mixture was evaporated to dryness in vacuo. TLC showed a single product (SiO_2, toluene/20%

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CH_3OH, R_f = 0.80), in addition to some unreacted starting material (R_f = 0.28). The product was purified on a small SiO_2 flash column (17 cm × 1.5 cm) eluted with CH_2Cl_2. Yield of clear glass: 9.5 mg, 0.021 mmol, 24%. [Unreacted starting materials (33 mg) were eluted with CH_2Cl_2/10 to 50% CH_3OH]: UV (hexanes) λ^1_{max} 296 nm, λ^2_{max} 243 nm; ^1H NMR (500 MHz, C_6D_6) δ H_{3e} (3.57, m, 1 H), H_4 (5.44, ddd, J = 10, 5.5, 3.5 Hz, 1 H), H_5 (5.33, br d, J = 10 Hz, 1 H), H_{6a} (1.83, br t, J = 12 Hz, 1 H), H_{7a} (0.87, q,* J = 12 Hz, 1 H), H_{7e} (1.64, br d, J = 12 Hz, 1 H), H_{8a} (1.41, br m, 1 H), H_{9a} (1.36, dddd, J = 12, 12, 12, 3.5 Hz, 1 H), H_{9e} (1.99, br d, J = 12 Hz, 1 H), H_{10a} (1.18, br q,* J = 12 Hz, 1 H), H_{10e} (2.58, br d, J = 12 Hz, 1 H), H_{11a} (1.91, t,* J = 12 Hz, 1 H), H_{12} (1.92, s, 3 H), H_{13}/H_{14} (5.23, m,‡ 2 H), H_{15} (1.21, d, J = 4.5 Hz, 3 H), H_{16} (0.90, d, J = 6.5 Hz, 3 H), $H_{5'}$ (2.82, s, 1 H), $H_{6'}$ (3.97, dd, J = 12, 1.5 Hz, 1 H), $H_{6''}$ (3.40, dd, J = 12, 2.0 Hz, 1 H), $H_{7'}$ (2.32, s, 3 H), H_{ortho} (7.81, d, J = 8.0 Hz, 2 H), H_{meta} (7.43, t, J = 8.0 Hz, 2 H), H_{para} (7.28, t, J = 8.0 Hz, 1 H). (*, partially obscured; ‡, overlapping); ^1H NMR (500 MHz, $CDCl_3$) δ H_{3e} (3.16, m, 1 H), H_4 (5.32, ddd, J = 10, 5.0, 2.5 Hz, 1 H), H_5 (5.37, br d, J = 10 Hz, 1 H), H_{6a} (1.87, br t,* J = 12 Hz, 1 H), H_{7a} (0.88, q, J = 12 Hz, 1 H), H_{7e} (1.81, br d, J = 12 Hz, 1 H), H_{8a} (1.54, br m, 1 H), H_{9a} (1.15, dddd, J =12, 12, 12, 3.5 Hz, 1 H), H_{9e} (1.90, br d, J = 12, 1 H), H_{10a} (0.96, br q,* 1 H), H_{10e} (2.20, br d, J = 12 Hz, 1 H), H_{11a} (1.63, t, J = 12 Hz, 1 H), H_{12} (1.51, s, 3 H), H_{13} (4.93, ddd, J = 15, 8.5, 1.5 Hz, 1 H), H_{14} (5.08, dq, J = 15, 6.5 Hz, 1 H), H_{15} (1.23, d, J = 6.5 Hz, 3 H), H_{16} (0.96, d, J = 6.5 Hz, 3 H), $H_{5'}$ (3.50, br s, 1 H), $H_{6'}$ (3.97, dd, J = 12, 1.5 Hz, 1 H), $H_{6''}$ (3.71, dd, J = 12, 3.0 Hz, 1 H), $H_{7'}$ (3.25, s, 3 H), H_{ortho} (7.54, dd, J = 8.0, 1.5 Hz, 2 H), H_{meta} (7.36, br t, J = 8.0 Hz, 2 H), H_{para} (7.31, br t, J = 8.0 Hz, 1 H). (*, partially obscured); ^{13}C NMR (100 MHz, CD_2Cl_2) δ 194.2 (C_1), 189.6 ($C_{4'}$), 173.9 ($C_{2'}$), 132.1 ($2C_{ortho}$), 131.6 (C_5), 130.6 (C_{para}), 127.8 ($2C_{meta}$), 127.6 (C_4 or C_{13} or C_{14}), 126.8 (C_4 or C_{13} or C_{14}), 126.6 (C_4 or C_{13} or C_{14}), 101.2 ($C_{3'}$), 69.6 ($C_{5'}$), 60.8 ($C_{6'}$), 48.9 (C_2), 46.0 (C_3), 42.7 (C_7), 40.9 (C_{11}), 39.1 (C_6), 36.4 (C_9), 34.1 (C_8), 28.8 (C_{10}), 28.3 ($C_{7'}$), 22.7 (C_{16}), 17.6 (C_{15}), 14.0 (C_{12}); CI(-) MS (NH_3) m/z 459 (16), 357 (54), 355 (100), 343 (38), 341 (37), 339 (31), 337 (23), 327 (22), 195 (32), 181 (41), 167 (31), 153 (20); CI(-) MS (NH_3), CAD (N_2) m/z 459 (M^-, 100), 355 (28), 337 (51).

Acetonide Formation. One crystal of pTSA was added to a solution of the *Fusarium* toxin (4.0 mg, 10.7 μmol) in dimethoxypropane (2 mL). After being stirred at room temperature for 2 h, the solution was diluted with 10 mL of $CHCl_3$ and extracted 1× with 2% $NaHCO_3$ and 1× with brine and the organic layer was dried over Na_2SO_4. TLC indicated a mixture of product (SiO_2, benzene/30% CH_3OH, R_f = 0.81) and unreacted starting material (R_f = 0.57). The product was purified on two small SiO_2 columns (pasteur pipets) eluted with benzene/10% CH_3OH and benzene/5% CH_3OH: yield after purification 0.5 mg, 1.21 μmol, 11% (not optimized); GC-MS m/z 413 (19), 395 (8), 355 (18), 337 (17), 336 (18), 250 (38), 239 (44), 203 (37), 192 (30), 182 (39), 181 (57), 180 (46), 175 (42), 152 (100), 119 (29), 105 (65).

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Supplementary Material Available: Experimental details of the spectroscopy experiments and seven figures of fully assigned correlation spectra, variable-temperature ^1H and ^{13}C NMR spectra, mass spectra at different ionization potentials, and parent ion MS/MS data (10 pages). Ordering information is given on any current masthead page.

Total Synthesis of the *Fusarium* Toxin Equisetin: Proof of the Stereochemical Relationship of the Tetramate and Terpenoid Sectors

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Abstract: A total synthesis of the *Fusarium* mycotoxin equisetin, in a manner that establishes its stereochemistry, is described. The key steps involve a lactonic variation of the ester enolate Claisen rearrangement and a novel construction of a 1-acyltetramic acid from an L-*N*-methylserine derivative and a β-keto ester.

Background and General Synthetic Strategy

The fungal metabolite equisetin, isolated from the white mold *Fusarium equiseti*, is suspected to be a promoter of chronic environmental diseases including certain leukemias.[1] Early investigations indicated equisetin to also be a potent antibiotic.[2] Evidence has already been gathered to the effect that equisetin shows selective toxicity to mammalian cells, and that it binds strongly to DNA.[3] Following the isolation and partial characterization of the *Fusarium* toxin by Burmeister and co-workers, a complete structure assignment has been advanced by Lynn and collaborators.[2,3] The identification of an acyltetramic acid moiety, derivable from L-*N*-methylserine, involved a combination of ultraviolet, magnetic resonance, and polarimetric (Cotton effect) measurements. Extensive NMR experiments allowed the Chicago workers to formulate an octalinoid substructure, with the relative configurations shown in system **1**. Left undefined in a rigorous sense was the stereochemical relationship of this octalinoid ("terpenoid") moiety to the stereogenic center at $C_{5'}$[4] in the tetramate sector. Recently the Chicago workers addressed this issue through the novel use of a phenylboronate ester. Analysis of low-temperature NMR data of the boronate derivative of equisetin revealed several important intramolecular dipolar coupling interactions. On the basis of molecular modeling computations (particularly MM2), it was argued that these interactions would be better rationalized if the overall stereochemistry is that shown in expression **1** rather than that arising from an "ent-terpenoid" diastereomer (see compound **18**). Since the validity of these arguments had not been extensively tested in practice,

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