of dimethyluracil dimers, consistent with the occurrence of a chain reaction.

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Structure of Efrapeptins from the Fungus *Tolypocladium niveum*: Peptide Inhibitors of Mitochondrial ATPase[1]

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Efrapeptins, a group of peptide toxins, were isolated from the culture filtrates of the fungus *Tolypocladium niveum* and complete stereostructures of five efrapeptins C–G were established. The peptides are mitochondrial ATPase inhibitors and have insecticidal properties.

Efrapeptins are a complex mixture of peptide antibiotics produced by the fungus *Tolypocladium niveum* (syn. *Tolypocladium inflatum, Beauveria nivea*), a soil hyphomycete. The peptides are inhibitors of mitochondrial oxidative phosphorylation and ATPase activity[2] and inhibit photophosphorylation in chloroplasts.[3] Efrapeptins are catalytic site competitive inhibitors that bind to the soluble (F_1) part of the mitochondrial ATPase,[4] thus blocking accessibility of an essential arginine residue on the enzyme at the adenine nucleotide binding site.[5] Some ATPases and mutant cell lines are resistant to efrapeptins.[6] There are several reports of the use of efrapeptins as a tool for studying energy-transfer reactions in biological systems.[7] In spite of their unique biological activity, the complete structures of these peptides were not known, although a partial structure with the amino acid sequence of one of the peptides was published before.[8] In a recent communication, we reported the structure of the unusual C-terminal blocking group in efrapeptins.[1] In this paper we present the amino acid sequence of five peptides, efrapeptins C–G (1–5), along with evidence corroborating the structure of the C-terminal blocking group, a bicyclic amine that apparently plays an important role in the biological activity of efrapeptins. These peptides are rich in α-aminoisobutyric acid (Aib) and are composed of 15 amino acid residues with an acetylated N-terminus. Efrapeptins are distinct from fungal peptaibols which are α-Aib rich peptides with an acetylated N-terminus and an amino alcohol at the C-terminus.[9]

1 Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Aib-X
2 Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Iva-X
3 Ac-Pip-Aib-Pip-Iva-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Gly-Leu-Iva-X
4 Ac-Pip-Aib-Pip-Aib-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Ala-Leu-Iva-X
5 Ac-Pip-Aib-Pip-Iva-Aib-Leu-β-Ala-Gly-Aib-Aib-Pip-Aib-Ala-Leu-Iva-X
6 H-Aib-Gly-Leu-Iva-X
7 H-Pip-Aib-Gly-Leu-Iva-X
8 Ac-Aib-Gly-Leu-Iva-X
10 H-Pip-Aib-Ala-Leu-Iva-X

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As part of our work on bioactive metabolites from entomopathogenic fungi, we discovered that the lipid soluble extract from the broth of an isolate of *T. niveum* displayed insecticidal activity. This fungus is known to produce cyclosporins, cyclic peptides that are important immunosuppressive drugs, and elvapeptins, peptides that are probably closely related to efrapeptins.[10] Bioassay guided fractionation resulted in concentration of the activity in a peptidal fraction that was identified as a mixture of efrapeptins. The mixture was resolved in HPLC into five peaks (C–G) labeled according to the order of elution from a reverse phase column.[11] Our data suggest that peptide D is identical to efrapeptin D as reported by Jackson et al.[11] For separation of individual components, a methodology was developed that involved the use of flash chromatography on silica gel[12] followed by Sephadex LH-20 chromatography which furnished a clean mixture of peptides. By the use of preparative TLC on silica gel,[13] two major peptides (D and F) could be enriched into different fractions which, upon extensive reverse-phase HPLC, furnished pure compounds.

Amino acid analysis of **4** by a combination of techniques (TLC, HPLC, GC–MS analysis of the trimethylsilyl (TMS) derivative[14] of the complete hydrolyzate) showed the presence of Aib, alanine (Ala), β-alanine (β-Ala), glycine (Gly), pipecolic acid (Pip), leucine (Leu), and isovaline (Iva). Also, FAB mass spectrometric analysis of the complete hydrolyzate of the peptide mixture showed the presence of M + H$^+$ ions of Ala/β-Ala (m/z 90), Aib (m/z 104), Iva (m/z 118), Pip (m/z 130) and Leu (m/z 132).[15] The presence of *N*-acetyl-Pip was confirmed by GC–MS analysis of the partial hydrolyzate (TMS, M + H$^+$ at m/z 244, chemical ionization, reagent gas isobutane). **2** was found to be devoid of Ala. All the chiral amino acids in efrapeptins were reported to have the *S* configuration.[8] We have confirmed the *S* configuration of Ala and Leu by GC analysis of their *N*-TFA, *O*-Me esters on a chiral column packed with *N*-*n*-lauroyl-*N*-L-valine-*tert*-butylamide. The absolute configuration of Pip as *S* was confirmed by its direct isolation and the measurement of the optical rotation. The absolute configuration of Iva as *S* was confirmed by X-ray analysis of a C-terminal dipeptide crystal by its relative relationship to a Leu residue of known *S* configuration.[1] This is unusual as Iva has been shown to have the *R* configuration in fungal peptaibols.[16]

FAB mass spectra of peptides **4** and **2** showed molecular ions at m/z 1634 and 1620, respectively, notwithstanding the reported (M + H$^+$) of 1619 for **2**.[8] Because of the presence of a quaternized nitrogen in the structure, these peptides produce unusually intense molecular ions instead of protonated pseudomolecular ions under FAB mass spectrometric conditions (as confirmed for **12** below). Such a phenomenon has been demonstrated in secondary ion mass spectrometry[17] and in FABMS of peptides.[18] A

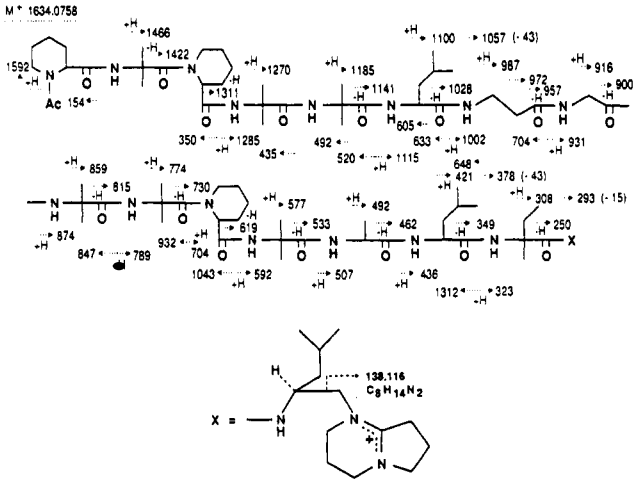

Figure 1. FAB mass spectral fragmentation of **4**.

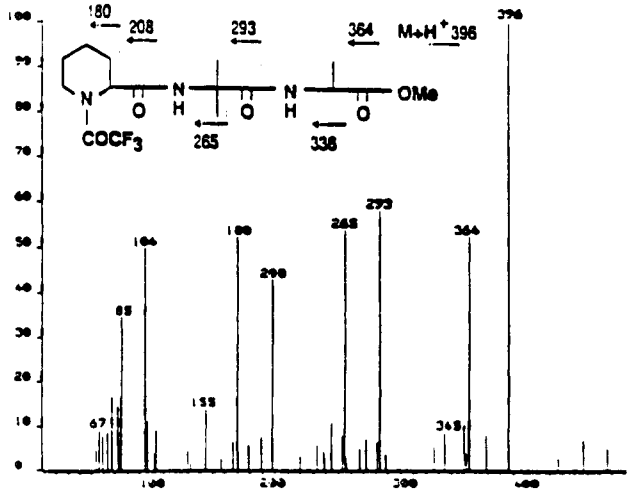

Figure 2. GC–CIMS of TFA-Pip-Aib-Ala-OMe.

high-resolution FAB mass spectrum of **4** showed an M$^+$ ion at m/z 1634.0758 which is consistent with an elemental composition of $C_{82}H_{141}N_{18}O_{16}$ (calculated value, 1634.0773). An intense peak in both the spectra at m/z 154 indicated the presence of *N*-acetyl-Pip as the N-terminal residue. The spectra showed fragment ions corresponding to C-terminal alkyl (Z), amino (Y′) and acyl ('X), and N-terminal acylium ions (B).[19] The most intense peaks in the clusters of C-terminal acyl ions appear to be 1 mass unit lower than the corresponding fragment mass probably because of a proton loss during fragmentation. A possible explanation for this is the formation of a double bond rearrangement product (formation of C8′–C8′a double bond with concomitant proton loss) under mass spectrometric conditions. Figure 1 shows the representative ions as observed from **4**. Complete amino acid sequence of both the peptides could be derived from analyses of the FAB mass spectra.[20] Except for an Aib residue, the amino acid sequence of **2** was in agreement with that proposed by

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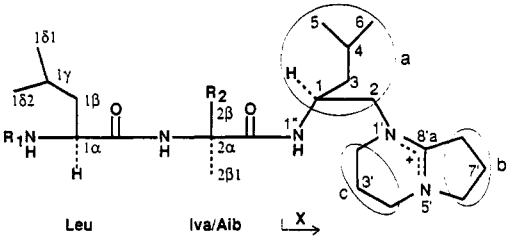
Bullough et al.[8] The Gly residue which is closer to the C-terminal in **2** is replaced by an Ala in **4** which was suggested by the presence of fragment ions at 507 (Y'_3) and 533 ($'X_3$). These sequence assignments were further substantiated by GC–MS (electron impact and CI, isobutane) analysis of the partial hydrolyzates of the efrapeptins.[21] GC–MS of the *O*-Me, *N*-TFA derivative[22] showed the fragments Aib-Aib, Aib-Ala, Aib-Gly, Pip-Aib, and Pip-Aib-Ala. Identical analysis of the TMS derivative of the partial hydrolyzate[23] showed the fragments Pip-Aib, Aib-Aib, Aib-Ala, Aib-Pip, Pip-Aib-Ala, Aib-Aib-Leu, and Gly-Aib-Aib. A representative Cl mass spectrum of the fragment TFA-Pip-Aib-Ala-OMe is shown in Figure 2. This sequence also confirmed the position of Ala in **4**. The above data, in conjunction with the published sequence of **4**, firmly established the amino acid sequence of the two peptides. The mass spectral and the amino acid analysis data suggested that the microheterogeneity of the peptides was due to the differences in amino acid residues and that the peptides had a common blocking group at the C-terminus. The ^1H and the ^{13}C NMR spectra of **4** were too complex to give any significant information about the structure of the C-terminal residue. Low yields of the complex peptide mixture from fungal cultures combined with difficult resolution of the individual components further complicated the issue. Attempts were therefore made to do partial hydrolysis and isolate and identify smaller fragments.

Efrapeptins can be neither acetylated (C_5H_5N–Ac_2O) nor reacted with CH_2N_2, suggesting the absence of any free amino, hydroxyl, or carboxyl functionalities. The peptides are also resistant to base treatment (1 M NaOH/MeOH or MeOH–K_2CO_3), Edman degradation, and dansylation. The NMR and UV data of the peptides did not indicate the presence of any olefinic double bonds and aromatic rings. Partial hydrolysis of the peptides followed by HPLC analysis showed the presence of several fragments from which two major components (**6** and **7**) were isolated. On the basis of amino acid and FAB mass spectrometric analysis, **6** and **7** were assigned the structures H-Aib-Gly-Leu-Iva-X (M^+, m/z 578) and H-Pip-Aib-Gly-Leu-Iva-X (M^+, m/z 689), respectively (X = blocking group). Neither of the peptides reacted with CH_2N_2, suggesting the presence of a blocked carboxy terminal. Acetylation of **6** furnished two products that were identified as a major product Ac-Aib-Gly-Leu-Iva-X (**8**) (FABMS, M^+, m/z 620), and a minor product (hydrolysis followed by acetylation) Ac-Leu-Iva-X (**9**) (FABMS, M^+, m/z 478). Another fragment was identified as H-Pip-Aib-Ala-Leu-Iva-X (**10**) (FABMS, M^+, m/z 703). These observations, in addition to furnishing support to the amino acid sequence assignments, indicated that the C-terminal blocking group was rather difficult to hydrolyze.

The FAB mass spectrum of a hydrolyzate of the efrapeptins, along with the parent ions of the free amino acids, showed the presence of a molecular ion of a peptide at m/z 436 (**11**), which was isolated as follows. The hydrolyzate was chromatographed on cellulose, and the material eluted with $CHCl_3$–CH_3OH (90:10) was subjected to HPLC to afford **11** and **13**. Amino acid analysis of **11** confirmed the presence of Leu and Iva. The FAB mass spectrum showed an M^+ at m/z 436 and characteristic ions at 421 (Z_2), 349 ($'X_1$), 323 (Y'_1), 308 (Z_1), and 250 ($'X_0$), indicating the sequential loss of Leu and Iva. High-resolution analysis of the molecular ion gave an exact mass of 436.3647 which was consistent with an elemental composition of $C_{24}H_{46}N_5O_2$ (calculated value, 436.3652). The molecular formula of the peptide requires five degrees of unsaturation with an ionized species. **11** did not react with CH_2N_2, and acetylation furnished a monoacetylated product (FABMS, M^+, m/z 478) which was identical to **9**.



9 R_1=$COCH_3$, R_2=CH_2-CH_3
11 R_1=H, R_2=CH_2-CH_3
12 R_1=$COCF_3$, R_2=CH_2-CH_3
13 R_1=H, R_2=CH_3
14 R_1=$COCH_3$, R_2=CH_3

^1H NMR of **11** in CD_3OD showed the presence of all characteristic Leu and Iva signals. Detailed analyses of the ^1H HOM2DJ resolved,[24] COSYPS,[25] and COSY-45 spectra of the peptide established the presence of, in addition to Leu and Iva moieties, three spin systems: $(CH_3)_2CHCH_2CH(CH_2)$– (a), $-CH_2CH_2CH_2-$ (b), and $-CH_2CH_2CH_2-$ (c) as in **11**. The methyl doublets at 0.9 and 0.94 ppm (part of a) showed connectivity with the proton at 1.66 ppm (m). The multiplet at 1.69 ppm showed connectivity to the signal at 1.22 ppm, which was between the geminally coupled methylene protons (3-CH_2) which were also connected to the downfield multiplet at 4.37 ppm (1 H). The proton at 4.37 ppm showed connectivity to the geminally coupled methylene protons centered at 3.39 and 3.58 ppm (2-CH_2). The multiplet at 1.61–1.77 ppm which integrates for 5 protons also represents three Leu (β-methylene and γ-methine) protons. The Leu α-proton resonated at 3.92 ppm (m) and showed a cross peak against the multiplet at 1.69 ppm. Leu methyls resonated as partially overlapping doublets at 1.0 and 1.02 ppm, which was confirmed by a HOM2DJ resolved spectrum.

The other spin system (b), clearly discernible from the COSY-45, was the $-CH_2CH_2CH_2-$ system where the methylene multiplet at 2.15 ppm (7′-CH_2) showed connectivity to the two pairs of geminally coupled methylene multiplets centered at 3.73 and 3.81 ppm (6′-CH_2), and 3.03 and 3.18 ppm (8′-CH_2). These methylenes did not show any other direct connectivities. The Iva methyl triplet at 0.85 ppm showed cross peaks with the geminally coupled methylene multiplets (β) at 1.9 and 2.15 ppm. The β1 methyl of Iva resonated at 1.37 ppm as a singlet. The only remaining proton signals in the spectrum were the three methylene multiplets (part of spin system c), two of which at 2.06 (3′-CH_2) and, 3.43 and 3.64 ppm (2′-CH_2), showed connectivities. The third methylene resonances (4′-CH_2) were overlapped by the multiplet centered at 3.41 ppm. As supported by the other analysis (vide infra), this methylene could only be a part of the $-CH_2CH_2CH_2-$ system whose COSY connectivity with the methylene at

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Table I. NMR Chemical Shifts of 11–13[a]

	11 in CD_3OD		11 in pyridine-d_5		12 in CD_3OD,	13 in CD_3OD	
carbon	proton	carbon	proton	carbon	carbon	proton	carbon
1α	3.92 (m, 1 H)	53.15 (CH)	4.49 (m, 1 H)	53.41 (CH)	54.95	3.83 (m, 1 H)	53.02 (CH)
1β	161–1.77 (m, 2 H)	41.71 (CH_2)	1.96–2.18 (m, 2 H)	41.55 (CH_2)	41.4	1.63–1.72 (m, 2 H)	41.66 (CH_2)
1γ	1.61–1.77 (m, 1 H)	25.52 (CH)	1.96–2.18 (m, 1 H)	24.81 (CH)	25.69	1.63–1.72 (m, 1 H)	25.54 (CH)
1δ1	1.00* (d, 6.14 Hz, 3 H)	22.11* (CH_3)	0.8* (d, 5.6 Hz, 3 H)	22.82* (CH_3)	21.92*	0.99* (d, 6.4 Hz, 3 H)	22.37* (CH_3)
1δ2	1.02* (d, 6.14 Hz, 3 H)	23.07* (CH_3)	0.91* (d, 5.6 Hz, 3 H)	23.13* (CH_3)	23.17*	1.01* (d, 6.4 Hz, 3 H)	22.9* (CH_3)
1C=O		169.94 (q)		170.73	172.61		170.12
2α		61.76 (q)		61.0	61.65		58.11
2β	1.9, 2.15 (each m, 1 H each)	27.86 (CH_2)	2.23, 2.6 (each m, 1 H each)	28.29 (CH_2)	29.1	1.42† (s, 3 H)	27.42† (CH_3)
2γ	0.85 (t, 7.32 Hz, 3 H)	8.27 (CH_3)	0.99 (t, 7.5 Hz, 3 H)	8.15 (CH_3)	8.2		
2β1	1.37 (s, 3 H)	23.64 (CH_3)	1.54 (s, 3 H)	23.46*	23.3	1.55† (s, 3 H)	23.75† (CH_3)
2C=O		176.83 (q)		175.26	176.75		176.78
1	4.37 (m, 1 H)	46.88 (CH)	4.49 (m, 1 H)	45.63 (CH)	46.43	4.4 (m, 1 H)	46.72 (CH)
2	3.39, 3.58 (each m, 1 H each)	57.91 (CH_2)	3.29 (dd, 3.36, 14 Hz, 1 H) 4.1 (br dd, 10.88, 14 Hz, 1 H)	56.45 (CH_2)	57.79	3.4, 3.47 (each m, 1 H each)	58.08 (CH_2)
3	1.22, 1.69 (each m, 1 H each)	41.1 (CH_2)	1.16, 2.08 (each m, 1 H each)	39.95 (CH_2)	41.06	1.23, 1.6 (each m, 1 H each)	41.04 (CH_2)
4	1.66 (m, 1 H)	25.84 (CH)	1.92 (m, 1 H)	24.81 (CH)	25.99	1.63–1.72 (m, 1 H)	25.95 (CH)
5	0.9† (d, 6.34 Hz, 3 H)	21.75† (CH_3)	0.84† (d, 6.5 Hz, 3 H)	21.69 (CH_3)	21.64‡	0.9‡ (d, 7.2 Hz, 3 H)	21.62‡ (CH_3)
6	0.94† (d, 6.34 Hz, 3 H)	23.79† (CH_3)	0.93† (d, 6.5 Hz, 3 H)	21.69 (CH_3)	23.68‡	0.95‡ (d, 7.2 Hz, 3 H)	23.65‡ (CH_3)
2′	3.43, 3.64‡ (each m, 1 H each)	45.91‡ (CH_2)	3.2, 3.88‡ (each m, 1 H each)	44.3† (CH_2)	45.71	3.41, 3.62+ (each m, 1 H each)	45.93+ (CH_2)
3′	2.06 (m, 2 H)	19.94 (CH_2)	1.81, 2.04 (each m, 1 H each)	19.05 (CH_2)	19.91	2.05 (m, 2 H)	19.86 (CH_2)
4′	3.41‡ (m, 2 H)	43.56‡ (CH_2)	3.2, 3.45‡ (each m, 1 H each)	42.49† (CH_2)	43.49	3.4+ (m, 2 H)	43.49+ (CH_2)
6′	3.73, 3.81 (each m, 1 H each)	55.57 (CH_2)	3.45, 3.77‡ (each m, 1 H each)	54.5 (CH_2)	55.55	3.72, 3.85 (each m, 1 H each)	55.55 (CH_2)
7′	2.15 (m, 2 H)	19.15 (CH_2)	1.81, 2.04 (each m, 1 H each)	18.19 (CH_2)	19.15	2.16 (m, 2 H)	19.08 (CH_2)
8′	3.03, 3.18 (each m, 1 H each)	32.18 (CH_2)	2.93, 3.66† (each m, 1 H each)	31.24 (CH_2)	31.9	3.06 (m, 2 H)	31.98 (CH_2)
8′a		166.12 (q)		165.33	166.39		165.97

[a] s = singlet, d = doublet, m = multiplet, dd = double doublet, br = broad; *, †, ‡, + assignments may be interchanged within the column; q = quaternary carbons confirmed by "quaternary only" sequence.

2.06 ppm is superimposed on the connectivity of the signal at 2.06 ppm with that at 3.43 ppm. These results were substantiated by the ^1H NMR and COSYPS spectra of the peptide in C_5D_5N which showed better resolution of the signals in the 2.8–4.2 ppm range. The interpretations were further corroborated by a RELAYH 2D NMR experiment[26] in CD_3OD which showed long-range connectivities. These connectivities are shown in Figure 3.

^{13}C NMR of 11 in CD_3OD showed the presence of 24 carbons. DEPT analysis[27] confirmed the presence of 6 methyls, 10 methylenes, and 4 methines. The presence of 4 unprotonated carbons was also confirmed by a quaternary only sequence[28] which showed the resonances of four carbons at 176.83, 169.94, 166.11, and 61.76 ppm. Considering the two downfield signals as the amide carbonyls of Leu (169.94 ppm) and Iva (176.83 ppm), the molecule must have a C=N moiety to account for the third carbon signal at 166.11 ppm and to satisfy the molecular formula as deduced by the HRFABMS (vide supra). This also suggested the presence of a bicyclic system in the molecule which must be the unknown C-terminal blocking group because the whole fragment is a linear peptide as indicated by the acetylation reaction and the FAB mass spectral fragmentation. The signal at 61.76 ppm was assigned to the α-carbon of Iva. The ^{13}C–^1H connectivities were determined by a HETCOR[29] experiment. All the individual assignments, which were supported by the observation of direct connectivities ($^1J_{CH}$), have been listed in Table I which also lists the carbon chemical shifts in C_5D_5N. The presence of 1-pyrroline ring system in the blocking group which accounts for the C=N moiety as well as the COSY fragment b was inferred by COLOC experiments.[30] The combined results of several COLOC experiments run under

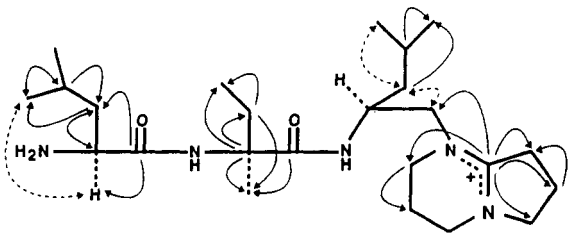

Figure 3. COLOC (—) and RELAYH (- - -) connectivities for 11.

(26) Bax, A.; Drobny, G. *J. Magn. Reson.* **1985**, *61*, 306. For the present experiment following values were used: RELAY = 2, TAU = 0.02.
(27) Doddrell, D. M.; Pegg, D. T.; Bendall, M. R. *J. Magn. Reson.* **1982**, *48*, 323. Levitt, M. H. *J. Magn. Reson.* **1982**, *48*, 234.
(28) Bendall, M. R.; Pegg, D. T. *J. Magn. Reson.* **1983**, *53*, 272.
(29) Bax, A.; Morris, G. A. *J. Magn. Reson.* **1981**, *42*, 501. Freeman, R. *Proc. Roy. Soc. London A* **1980**, *373*, 149. Rutar, V. *J. Am. Chem. Soc.* **1983**, *105*, 4095.
(30) Kessler, H.; Griesinger, C.; Zarbock, J.; Loosli, H. R. *J. Magn. Reson.* **1984**, *57*, 331. Krishnamurthy, V. V.; Casida, J. E. *Magn. Reson. Chem.* **1987**, *25*, 837.

different sets of parameters[31] are summarized in Figure 3. The sp^2 carbon of the heterocyclic system shows long range ($^2J_{CH}$ and $^3J_{CH}$) connectivities to all the three carbons of the 1-pyrroline ring.

Based on the above information, the rest of the molecular structure could be constructed into an *N*-peptidoisobutyl[2,3,4,6,7,8-hexahydro-1-pyrrolo[1,2-α]pyrimidiniumyl]ethylamine system as in **11**. Such an arrangement would satisfy the molecular weight requirement of having a bicyclic system, and would be consistent with the presence of COSY fragments a and c. The only remaining question was the position of the isobutyl side chain which could either be on the carbon next to the pyrimidinium nitrogen or on the carbon next to the amide nitrogen. This was unambiguously confirmed by a COLOC experiment where a $^3J_{CH}$ connectivity was observed between the methylene resonances at 3.39 and 3.58 ppm (2-CH_2) and the sp^2 carbon of the pyrrolopyrimidine moiety at 166.11 ppm. This clearly established the position of the isobutyl chain as being next to the nitrogen bearing the peptide chain. On the basis of the combined evidence, the final structure of the fragment was assigned as in **11**. The structure also explains the observation that efrapeptins and the smaller fragments which have an intact C-terminal blocking group show a prominent peak at m/z 138 in their FAB mass spectra. This peak can be generated by a homolytic cleavage of the C1–C2 bond only if the isobutyl chain is on the carbon next to the amide nitrogen. High-resolution analysis[32] of the peak derived from the FAB of **4** gave an exact mass of 138.116 which was consistent with the elemental composition of $C_8H_{14}N_2$ (calculated value, 138.1157). That this is the structure of the blocking group in the parent efrapeptins was substantiated by the presence of an m/z 138 peak in the FAB mass spectra of **1–5**, and by the presence of a signal at 166.1 ppm (C-8′a) and the absence of any olefinic carbon signals in the ^{13}C NMR of **4**. DEPT of **4** in CD_3OD showed 10 methines, thus eliminating the possibility of the presence of an alternative C-terminal structure with a C8′–C8′a double bond which would instead have 11 methines. An examination of the geminally coupled 2-methylene proton resonances in the ^1H NMR of **11** in C_5D_5N revealed that one of the protons at position 2 resonated at 3.29 ppm as a double doublet with coupling constants of 3.36 and 14 Hz. The other proton resonated at 4.1 ppm as a broad double doublet with coupling constants at 10.88 and 14 Hz. The observation suggests the existence of restricted rotation around C1–C2 bond.[33]

The unambiguous determination of the structure of the blocking group came from a single-crystal X-ray analysis of the trifluoroacetyl derivative **12** (^{13}C NMR, Table I). The FAB mass spectrum of **12**, acquired with thioglycerol, showed an intense peak at m/z 532. That this peak is due to the molecular ion was confirmed by acquiring the spectrum with NaI where no sodiated molecular ion peak ($M + Na^+$) was observed. Also, as expected for a molecular ion peak, the FAB mass spectrum of the sample with increasing amounts of NaI showed a decrease in the intensity of the peak at m/z 532 along with an increase in the intensity of $Na(NaI)_n^+$ cluster ions.

12 afforded crystals from ethyl acetate–hexane at 5 °C. A crystal with dimensions 0.1 × 0.3 × 0.7 mm was chosen for all subsequent X-ray measurements. Preliminary X-ray photographs displayed monoclinic symmetry and accurate lattice constants of a = 11.101 (3), b = 14.844 (2), and c = 11.660 (2) Å, and β = 116.23 (1)° were determined from a least-squares analysis of 30 diffractometer-measured 2θ values. Subsequently, all diffraction maxima with $2\theta \leq 115°$ were collected at −35 °C with a computer-controlled four-circle diffractometer using θ:2θ scans and graphite monochromated Cu Kα (1.547 18 Å) radiation. A total of 2454 unique reflections were measured, and after correction for Lorentz, polarization, and background effects, 2292 (93%) were judged observed at $|F_o| \geq 4\sigma|F_o|$.

Direct methods were used to find a phasing model for **12** in the space group $P2_1$. One peptide-fragment molecule, one molecule of water, and one trifluoroacetate ion were found to comprise the asymmetric unit. While the initial solution of the structure of this molecule was not uniquely difficult, the refinement process was not at all routine. Significant disorder in the trifluoro groups of both the peptide fragment and the trifluoroacetate ion could not be alleviated by the use of low-temperature data collection conditions. As a result, it was necessary to model the trifluoro groups as being composed of six partial site-occupancy fluorine atoms instead of three unit-occupancy fluorine atoms, with a total occupancy of 3.0 for each of the trifluoro groups. In addition, the lengths of all the C–F bonds in the two molecules were constrained to 1.38 (±0.02) Å. Subsequently, full matrix least-squares refinement was done. All heavy atoms of the peptide fragment, excluding the fluorines, were refined anisotropically with isotropic riding hydrogen atoms. The atoms of the trifluoroacetate ion and water molecule were allowed to refine isotropically and in this way a final crystallographic residual of 6.92% has been achieved.

The other three minor efrapeptins were identified as **1**, **3**, and **5**. The FAB mass spectrum of **1** (efrapeptin C) showed a molecular ion at m/z 1606, which indicated that **1** had a difference of 14 mass units from **2**. Amino acid analysis of **1** confirmed the presence of Leu, Aib, Pip, Gly, and β-Ala. In the FAB mass spectrum of **1**, along with the characteristic corresponding fragment ions as observed for **2**, the presence of C-terminal ions at m/z 294 (Z_1), 309 (Y'_1), and 335 ($'X_1$) suggested the presence of an Aib residue instead of an Iva next to the C-terminal blocking group which also accounts for the difference of 14 mass units between **1** and **2**. This was confirmed by direct isolation of **13** as described above.

Amino acid analysis of **13** confirmed the presence of Leu and Aib in an equimolar ratio. The FAB mass spectrum of **13** showed a molecular ion at m/z 422. The presence of fragment ions at m/z 250 ($'X_0$), 294 (Z_1), 309 (Y'_1), and 407 (Z_2) suggested the amino acid sequence as -Leu-Aib-. **13** did not react with CH_2N_2 and acetylation furnished a monoacetylated derivative **14** (FABMS, M^+, m/z 464).

The ^1H NMR of **13** in CD_3OD resembled that of **11**. The replacement of Iva in **11** with an Aib in **13** was indicated by the appearance of two methyl singlets at 1.42 and 1.55 ppm and the disappearance of the characteristic Iva methyl triplet and methylene multiplets. ^1H–^1H connectivities were determined by a DQFCOSY[34] experiment where Leu methyl doublets at 0.99 and 1.01 ppm showed connectivity to the multiplet at 1.63–1.72 ppm (Leu γ-C*H*

(31) COLOC of **11** was recorded with Varian supplied pulse sequence (COLOCS = *n*, COUPLD = *n*). Three different experiments were performed using fixed evolution time d3 of 30, 40, and 50 ms and a refocusing delay d4 of 32 ms. COLOCS experiment was performed using d3 = 40 ms and d4 = 32 ms.

(32) The FAB mass spectrum of **4** was acquired with thioglycerol and the exact mass of the peak at m/z 138.116 was measured with thioglycerol reference peaks at 126.0589 ($C_3H_{12}O_2SN$, thioglycerol + NH_4^+) and 181.0357 [$C_6H_{13}O_2S_2$, (thioglycerol − H_2O)$_2$ + H^+].

(33) Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512. Jackman, L. M.; Sternhell, S. *Application of NMR Spectroscopy in Organic Chemistry*, 2nd ed.; Pergamon Press: New York, 1969; p 281.

(34) Piantini, U.; Sorensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800.

and β-CH_2) which, in turn, showed connectivity to the one proton multiplet centered at 3.83 ppm (Leu α-CH). The rest of the connectivity pattern in the spectrum lends support to the structure of the blocking group. The 1-H of the blocking group (4.4 ppm, m) showed cross peaks against the methylene resonances centered at 3.4 and 3.47 (2-CH_2), and 1.23 and 1.6 (3-CH_2) ppm. The proton resonance at 1.23 ppm (3-HC*H*) showed connectivity to the multiplet centered at 1.63–1.72 ppm (4-CH) which showed cross peaks against the methyl doublets centered at 0.9 and 0.95 ppm (5 and 6-CH_3). Two $-CH_2CH_2CH_2-$ spin systems were also clearly discernible. The methylene multiplet centered at 2.16 ppm (7′-CH_2) was directly coupled with the methylene resonances at 3.06 (8′-CH_2), and 3.72 and 3.85 ppm (6′-CH_2). The methylene multiplet at 2.05 ppm (3′-CH_2) showed cross peaks with the methylene multiplets centered at 3.41 and 3.62 ppm (2′-CH_2). The connectivity of the 3′-CH_2 signal with the methylene multiplet at 3.4 ppm (4′-CH_2) was superimposed on the connectivity between the 3′-CH_2 and one of the protons of 2′-CH_2. The ^{13}C NMR spectrum of **13** in CD_3OD showed the presence of 23 carbons which accounted for 6 methyls, 9 methylenes, 4 methines, and 4 quaternary carbons (DEPT). The *C*=N^+ of the heterocycle showed a characteristic resonance at 165.97 ppm.

On amino acid analysis **3** (efrapeptin E) showed the presence of Pip, Aib, Leu, β-Ala, Iva, and Gly. The FAB mass spectrum of **3** showed a molecular ion at m/z 1634. The sequence-specific fragment ions at 1270 (Z_{12}), 1285 (Y'_{12}) and 1311 ($'X_{12}$) clearly indicated the presence of an Iva unit in **3** in place of the Aib residue in **2**. That this Iva residue is flanked by Pip and Aib units in accordance with the amino acid sequence of **2**, was confirmed by the presence of fragment ions at 1171 (Z_{11}), 1186 (Y'_{11}), 1212 ($'X_{11}$), and 1396 (Y'_{13}). The complete structure of the peptide was assigned as in **3**.

Amino acid analysis of **5** (efrapeptin G) confirmed Pip, Aib, Leu, β-Ala, Gly, Iva, and Ala as the constituent amino acids. The FAB mass spectrum of the peptide showed a molecular ion at m/z 1648 which indicated a difference of 14 mass units from **4** (M^+, m/z 1634). A detailed analysis of the fragment ions of **5** suggested an amino acid sequence identical to that of **4** except for the substitution of Iva at residue 12 which also accounts for the difference of 14 mass units in the molecular weights of the two peptides. This was suggested by the presence of fragment ions at m/z 1185 (Z_{11}), 1284 (Z_{12}), 1299 (Y'_{12}), and 1410 (Y'_{13}). The complete structure of the peptide is as shown in **5**. Indirect support for the amino acid compositions came from our studies on directed biosynthesis where supplementing culture media with different amino acids resulted in enhanced production of appropriate efrapeptins.[35]

On the basis of biosynthetic considerations and structural analogy, it seems plausible that part of the blocking group with the isobutyl side chain is derived from *S*-Leu (amide formation with Iva followed by modification and reduction of the carboxyl) where the chiral center of Leu remains undisturbed during the bioconversion. To the best of our knowledge this is the first report of a peptide linked to hexahydropyrrolo[1,2-α]pyrimidine (also known as 1,5-diazabicyclo[4.3.0]non-5-ene, DBN) which is particularly interesting because of the unique biological activity associated with these peptides. The blocking group is an alkylation product of DBN with *S*-leucinol.

Both **2** and **4** showed insect toxicity against *Leptinotarsa decemlineata* (Coleoptera) (foliar spray assay, LC_{50} at 18.9 and 8.4 ppm, respectively). All the peptides showed mitochondrial ATPase inhibitory activity when tested against preparations from entomopathogenic fungi (e.g. *Metarhizium anisopliae* and *T. niveum*) and insects (e.g. flight muscles from *Musca domestica*). No ATPase inhibitory activity was observed with **9**. **8**, however, showed a clear dose response when bioassayed although the specific activity was lower than the parent peptides. This observation suggests a possible role for the blocking group in the biological activity of efrapeptins. Details of these studies have been published elsewhere.[36]

Experimental Section

Fungal Culture. The fungal isolate of *T. niveum* was kindly provided by the USDA-ARS Entomopathogenic Fungi Collection (ARSEF no. 616) located at Ithaca, NY. The fungus was maintained on nutrient agar slants and cultured in six 1-L Fernbach flasks in Czapek-Dox medium enriched with 0.5% bactopeptone. The fungus was grown at ambient temperature on a rotary shaker (120 rpm) and was harvested 10 days after inoculation.

Isolation of Toxins. The fungal culture (6 × 1-L) was filtered through several layers of cheese cloth and the filtrate was extracted with CH_2Cl_2. The solvent was removed in vacuo to afford a residue (472 mg) which was flash chromatographed on silica gel (40 μm). The elution was monitored by TLC (silica gel, CH_2Cl_2–MeOH, 86:14, visualization; I_2 vapors, Dragendorff's reagent or UV 254 nm) and identical fractions were combined. Bioassay of the resultant fractions revealed that insect toxicity was associated with the fractions eluted with CH_2Cl_2–MeOH (92:8) (58 mg). TLC of this fraction showed a major spot which was Dragendorff's reagent positive but ninhydrin negative. The mixture was further purified by chromatography over Sephadex LH-20 and the toxin was eluted with $CHCl_3$–MeOH (3:1). Removal of the solvent furnished a white powder (36 mg) which showed the presence of at least five peaks (Efrapeptins C–G, labeled after comparison of chromatographic properties as described in ref 11, 1–5) when examined by analytical HPLC: column RPC_8 (0.4 × 15 cm), particle size, 5 μm; solvent MeCN–12.5 mM $(NH_4)_2SO_4$ (63:37) or MeCN–20 mM $MeCOONH_4$ (60:40); flow 1 mL/min; detection UV 225 nm.

For further purification of individual components, the mixture was separated into two bands by preparative TLC on silica gel (plates, 20 × 20 cm; solvent, toluene–2-butanone–EtOAc–HCOOH–H_2O, 3:12:25:5:5; visualization, I_2 vapors or Dragendorff's reagent; R_fs, 0.5 and 0.45). The two bands were scraped off and the toxin eluted with $CHCl_3$–MeOH (88:12) (upper band, 12 mg; lower band, 12 mg). HPLC examination revealed that the upper band was composed of efrapeptins E, F, and G (**3**–**5**) and the lower band was a mixture of efrapeptins C, D, and E (**1**–**3**). Final purification of individual components was achieved by repetitive semipreparative HPLC; column RPC_8 (0.9 × 50 cm); solvent MeCN–12.5 mM $(NH_4)_2SO_4$ (75:25); flow 7 mL/min; detection UV, 225 nm. Individual peaks were pooled and further purified by recycling and peak shaving. The solvent was removed in vacuo, and the residue suspended in H_2O. The toxin was extracted with CH_2Cl_2. **1**: $[\alpha]_D^{22}$ −2.5° (*c* 0.08, $CHCl_3$); UV (λ_{max}, MeOH) 209, 230 (sh) nm; FABMS, M^+, m/z 1606. **2**: $[\alpha]_D^{22}$ −3.1° (*c* 0.32, $CHCl_3$); UV (λ_{max}, MeOH) 208, 230 (sh) nm; FABMS, M^+, m/z 1620. **3**: $[\alpha]_D^{22}$ −2.2° (*c* 0.37, $CHCl_3$); UV (λ_{max}, MeOH) 208, 230 (sh) nm; FABMS, M^+, m/z 1634. **4**: $[\alpha]_D^{22}$ −5° (*c* 0.4, $CHCl_3$); UV (λ_{max}, MeOH) 208, 230 (sh) nm; HRFABMS, M^+ at m/z 1634.0758 was peak matched against the peaks of $Cs^+(CsI)_4(^{85}RbI)_2$ (1595.7776) and $Cs^+(CsI)_5{}^{85}RbI$ (1643.7713). Glycerol was used as the matrix. **5**: $[\alpha]_D^{22}$ −5.3° (*c* 0.42, $CHCl_3$); UV (λ_{max}, MeOH) 208, 230 (sh) nm; FABMS, M^+, m/z 1648.

Complete Hydrolysis of the Efrapeptins. The upper band from preparative TLC (R_f 0.5; 2.5 mg) was treated with 0.5 mL of 6 N HCl, degassed (freeze–thaw cycle), and sealed in vacuo. The solution was heated at 115° for 24 h and then diluted with deionized water (2 mL). The solution was extracted with ether (2 × 2 mL), and the water layer was dried in vacuo followed by

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(36) Krasnoff, S. B.; Gupta, S.; St. Leger, R. J.; Renwick, J. A. A.; Roberts, D. W. *J. Invertebrate Pathol.* **1991**, *58*, 180.

desiccation over NaOH. The residue was dissolved in water and examined by TLC for amino acids (cellulose, 1-butanol-H_2O-AcOH, 4:1:2; silica gel, $CHCl_3$-MeOH-NH_4OH, 75:25:4; visualization, ninhydrin). The lower band from preparative TLC was also subjected to similar analysis. Amino acid analyses were also carried out on an amino acid analyzer where amino acids in the hydrolyzate were identified as the phenylthiohydantoin derivatives by comparing their HPLC retention times with those of the standards.

Chiral GC of the *N*-TFA, *O*-Me Derivative of the Complete Hydrolyzate. The complete hydrolyzate (1 mg) was dissolved in 1 mL of dry MeOH, and the solution was chilled in ice bath. To this chilled solution was added 100 μL of $SOCl_2$, and the vial was sealed under dry N_2. After standing at 40 °C for 2 h, the solution was evaporated to dryness by a stream of dry N_2 followed by vacuum desiccation. The residue was dissolved in 500 μL of trifluoroacetic anhydride (TFAA) and allowed to stand at room temperature for 2 h. The solvent was then removed under a stream of N_2, and the residue was dissolved in 500 μL of CH_2Cl_2 for injection on a gas chromatograph: column Supelco SP300, glass 3 m (1-mm i.d.), temperature 110 °C; Carrier gas He; flame ionization detector (FID) 150 °C; injector 150 °C; retention time (t_R, min) Aib 3.23, Iva 4.5, *S*-Ala 5.01, Gly 7.75, β-Ala 9.78, Pip 15.98, *S*-Leu 19.07. The retention times were consistent with those of the standards.

GC of the TMS Derivative of the Complete Hydrolyzate. The complete hydrolyzate (0.5 mg) was treated with 100 μL of TriSil BSA/P (solvent pyridine, Pierce) and sealed under dry N_2. The solution was heated on a heating block at 60 °C for 12 h and then diluted with dry pyridine (500 μL). This solution was used for injection on GC: column capillary 5% methyl silicone, 12 m; 100 °C (4 min) to 270 °C (10 min), rate 6 °C/min; carrier gas He; flow 48 mL/min; FID 250 °C; injector 250 °C; t_R (min) Ala 5.57, Aib 6.58, Iva 8.5, Leu 9.55, Gly 10.37, Pip 11.57, β-Ala 13.5. Derivatives from the standards had identical retention times.

GC-MS of Partial Hydrolyzate: *N*-TFA, *O*-Me Derivative. The Sephadex LH-20 purified peptide mixture (5.4 mg) was mixed with concd HCl (500 μL) and sealed under N_2. The solution was allowed to stand at 37 °C for 12 h, and the solvent was then removed by a stream of dry N_2 followed by vacuum desiccation over NaOH. The residue (1.53 mg) was dissolved in MeOH (1 mL), and an excess of ethereal solution of CH_2N_2 (4 mL) was added to it. The sealed solution was allowed to stand overnight at 5 °C, and then the solvent was removed. The vacuum dried residue was treated with TFAA (200 μL) at room temperature for 2 h, and then the solvent was removed by a stream of dry N_2. The residue was dissolved in 250 μL of CH_2Cl_2 and subjected to GC-MS analysis resulting in the identification of the fragments as described in the text. Column capillary 5% methyl silicone, 12 m; 40 °C (4 min) to 270 °C (10 min), rate 10 °C/min; carrier gas He; flow 42 mL/min; FID 250 °C; injector 250 °C.

GC-MS of Partial Hydrolyzate: TMS Derivative. Sephadex-purified material (2.61 mg) was mixed with 6 N HCl (250 μL), degassed (freeze-thaw cycle), and vacuum sealed. The mixture was heated at 110 °C for 25 min and then HCl was removed by a stream of N_2 followed by vacuum desiccation over NaOH. The residue (1.25 mg) was dissolved in dry DMF (100 μL), and the solution was treated with BSTFA/TMSCl (500 μL). The sealed mixture was then heated at 65 °C for 2.5 h and then directly injected on the GC-mass spectrometer resulting in separation of the fragments as described in the text. Column capillary 5% methyl silicone, 12 m; 130 °C (4 min) to 270 °C (10 min), rate 8 °C/min; carrier gas He; flow 33 mL/min; FID 250 °C; injector 250 °C.

Treatment of Efrapeptins with 1 M NaOH. The efrapeptin mixture (upper band from preparative TLC, 1 mg) was mixed with 1 M NaOH (100 μL) and MeOH (100 μL). The mixture was stirred at room temperature for 3 h, and the solution was then adjusted to pH 6 with dilute HCl. The solvent was removed in vacuo, and the residue was resuspended in H_2O (1 mL). Extraction with $CHCl_3$ (3 × 2 mL) furnished a residue which was found to be identical to the starting material upon TLC and HPLC analysis.

Treatment of Efrapeptins with MeOH/K_2CO_3. The efrapeptin mixture (upper band from preparative TLC, 1 mg) was dissolved in MeOH (200 μL), and a few crystals of K_2CO_3 were added to it. The above solution was stirred overnight at room temperature. Solvent was removed in vacuo and the residue was suspended in water (1 mL). Extraction with CH_2Cl_2 (3 × 2 mL) afforded a product which was found to contain major efrapeptins by HPLC analysis.

Isolation of 6 and 7. Preparative TLC purified material (lower band, 40 mg) was treated with 6 N HCl (1 mL), degassed (freeze-thaw cycle), and sealed. The solution was heated at 110 °C for 5 min, and the solvent was removed in vacuo followed by vacuum drying over NaOH. The residue was dissolved in MeOH (4 mL) and subjected to HPLC: column, RPC_4 (0.9 × 30 cm); inject, 100 μL; solvent, 0.1% TFA (linear gradient, 30 min) to 0.1% TFA-0.1% TFA in MeCN (50:50); flow, 7 mL/min; detection, UV 225 nm. Two major peaks were collected and solvent removed to afford, according to the order of elution, **6** (3.5 mg) [FABMS, m/z 578 (M^+); AA: Gly, Leu, Aib, Iva] and **7** (2.7 mg) [FABMS, m/z 689 (M^+); AA: Gly, Leu, Aib, Pip, Iva].

Isolation of 8 and 9. **6** (3 mg) was treated with dry C_5H_5N (200 μL) and Ac_2O (200 μL) and sealed under N_2. The sample was heated at 50 °C for 4 h, and the solvent was removed in vacuo. The residue was dissolved in MeOH and subjected to HPLC: column, RPC_4 (0.9 × 30 cm); solvent, 0.1% TFA-MeCN (72:28, isocratic); flow, 7 mL/min; detection, UV 225 nm. Two peaks were collected (in the order of elution): **9** (minor); FABMS, m/z 478 (M^+) and **8** (major); FABMS, m/z 620 (M^+).

Isolation of 10. The Sephadex LH-20 purified peptide mixture (50 mg) was treated with 6 N HCl (1 mL) and sealed after degassing. The solution was heated at 110 °C for 15 min and the solvent removed in vacuo. The residue was dissolved in MeOH (500 μL) and subjected to HPLC: column, RPC_{18} (0.9 × 50 cm); solvent, 0.5% TFA-0.5% TFA in MeCN (70:30) to (linear gradient, 30 min) 0.5% TFA-0.5% TFA in MeCN (30:70); flow, 3.3 mL/min; detection, UV 220 nm. The well-resolved peak was pooled and solvent removed in vacuo to afford **10**. AA: Pip, Aib, Ala, Iva, Leu; FABMS, m/z 703 (M^+).

Isolation of 11 and 13. Sephadex LH-20 purified peptide mixture (280 mg) was treated with 6 N HCl (2 mL) and sealed in vacuo after degassing. The solution was heated at 110 °C for 12 h, and the solvent was removed. The hydrolyzate (230 mg) was loaded on a column of microgranular cellulose packed in $CHCl_3$. The column was eluted with $CHCl_3$ followed by increasing proportions of MeOH in $CHCl_3$. The elution was monitored by TLC (cellulose, butanol-acetic acid-water, 4:1:2; visualization, ninhydrin) and identical fractions were combined. Fractions eluted with $CHCl_3$-MeOH (90:10), upon removal of the solvent, afforded a residue (30.2 mg) which was dissolved in MeOH (3 mL) and subjected to HPLC: column, RPC_4 (0.9 × 30 cm); solvent, 0.1% TFA in MeCN-0.1% TFA (15:85) to (linear gradient, 30 min) 0.1% TFA in MeCN-0.1% TFA (30:70); flow 7, mL/min; detection, UV 225 nm. Two peaks were pooled and the solvent was removed to afford **11** (12.4 mg) [$[\alpha]^{22}_D$ + 2.2° (c 0.7, MeOH); AA: Iva, Leu; HRFABMS, M^+, m/z 436.3647 (the peak match was done against PEG reference peaks at 415.2543 and 459.2805)] and **13** (2.7 mg) [$[\alpha]^{22}_D$ −0.5° (c 0.4, MeOH); AA: Leu, Aib; FABMS, m/z 422 (M^+)].

Acetylation of 11. **11** (2.5 mg) was dissolved in dry MeOH (1 mL), and Ac_2O (1 mL) was added under dry N_2. The solution was allowed to stand at ambient temperature overnight, and the solvent was removed in vacuo. The residue (2.5 mg) was dissolved in MeOH (1 mL) and subjected to HPLC: column RPC_8 (0.4 × 15 cm); solvent, MeCN-H_2O-12.5 mM $(NH_4)_2SO_4$, 40:40:20; flow, 1.2 mL/min (isocratic); detection, UV 225 nm. The major peak was collected and the solvent evaporated. The residue was extracted with dry MeOH and filtered and the solvent removed to afford **9** (1.2 mg): TLC, single spot (silica gel; solvent, $CHCl_3$-MeOH, 80:20; visualization, Dragendorff's reagent; ninhydrin negative); FABMS, m/z 478 (M^+).

Preparation of 12. **11** (11.6 mg, TFA salt) was mixed with TFAA (1 mL) under ice cooling and sealed under N_2. The sample was allowed to stand at ambient temperature for 2 h, and then the solvent was removed with a stream of N_2. The vacuum-dried residue was dissolved in MeOH (1 mL) and subjected to HPLC: column RPC_4 (0.9 × 30 cm); solvent, 0.1% TFA in MeCN-0.1% TFA (10:90) to (linear gradient, 30 min) 0.1% TFA in MeCN-0.1% TFA (80:20); flow, 5 mL/min; detection, UV 225 nm. The single major peak was collected and solvent removed to afford **12** (6.1 mg). Slow evaporation of solvent from a solution of **12**

in ethyl acetate–hexane at 5 °C afforded clear crystalline needles in 3 days.

Preparation of 14. 13 (0.6 mg) was reacted with dry C_5H_5N/Ac_2O (100 μL each) overnight at ambient temperature. 14 was purified by HPLC: column, RP C_4 (0.9 × 30 cm); solvent, 0.1% TFA in H_2O–0.1% TFA in MeCN (85:15) to (linear gradient, 30 min) 0.1% TFA in H_2O–0.1% TFA in MeCN (30:70); flow, 5 mL/min; detection, UV (225 nm); FABMS, m/z 464 (M^+).

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Supplementary Material Available: FAB mass spectra of 1–14, ^1H, ^{13}C, and DEPT NMR spectra of 4, NMR spectra of 11 in CD_3OD (HOM2DJ resolved, COSYPS, HETCOR, DEPT, "quaternary only", COLOC, RELAYH) and in C_5D_5N (^1H, ^{13}C, COSYPS), and the X-ray structure of 12 (30 pages). Ordering information is given on any current masthead page.

Isolation and Identification of Two New Metabolites from Silver Leaf Fungus *Stereum purpureum*

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Two new sterpurene sesquiterpenes have been isolated from *Stereum purpureum* and identified as 4,12-dihydroxysterpurene (1) and 5,12-dihydroxysterpurene (3) mainly on the basis of mass spectra and extensive nuclear magnetic resonance spectrometry of compounds 1 and 3 and their diacetate derivatives 2 and 4.

The fungus *Stereum purpureum*, which causes the so-called silver leaf disease of a variety of fruit trees and scrubs, grows slowly on a malt extract broth to produce a complex mixture of sesquiterpene metabolites belonging to a new structural type among the sesquiterpenoids. *S. purpureum* was grown in malt extract–dextrose–peptone liquid culture. Extraction of the culture broth with dichloromethane provided crude metabolites which caused "silvering" in mountain ash seedings.[1,2] The crude metabolites were first separated by TLC and further purified by column chromatography over silica gel to give a viscous, yellow product, followed by preparation of their diacetate derivatives and final purification by HPLC using two different solvent systems.

Compound 1 has the same skeleton as 4,12-dihydroxysterpurene. The IR spectrum of 1 shows a characteristic hydroxyl absorbance at 3586 cm^{-1} ($CHCl_3$). Two proton signals disappear from the ^1H-NMR spectrum of 1 upon addition of D_2O, indicating the presence of two hydroxyl groups. The remaining functionality of the 4,12-dihydroxysterpurene skeleton (three quaternary methyls, a fully substituted double bond) was established from examination of ^1H- and ^{13}C-NMR, NOE, and COSY spectra of 1 and/or 2. For example, the 400-MHz ^1H-NMR spectrum of 1, analyzed in detail in the Experimental Section, includes an AB quartet at 4.50 ppm (allylic primary alcohol) and a multiplet at 4.33 ppm (secondary alcohol); these signals serve to confirm the locations of the two hydroxyl groups. The ^1H-NMR and ^1H–^1H COSY spectra of 1 show the presence of three methyl groups (1.03, 1.07, and 1.25 ppm), an isolated allylic methylene group (2.25 ppm, C-11), an allylic methine proton (2.75 ppm, C-3) coupled to two otherwise isolated methylene groups (C-7 and C-9), an isolated 4-spin system corresponding to two methine protons (C-3 and C-4), and a methylene group (C-5) on a cyclobutane ring. These data thus establish the fundamental 4,12-dihydroxysterpurene skeleton.

Compound 1 readily forms a diacetate 2 when treated with acetic anhydride–pyridine. 2 was first separated by TLC and then repeatedly purified through HPLC. In the ^1H-NMR of 2, the hydrogens on carbons bonded to oxygen are moved downfield to 4.58 (2 H, s, H-12) and 4.43 (1 H, m, H-4) ppm. Singlets at 141.9 and 127.5 ppm in the APT-^{13}C NMR spectrum of 2 correspond to the fully substituted double bond of the allylic alcohol. The 400-MHz ^1H-NMR spectrum and COSY of 2, analyzed in the Experimental Section, include an isolated allylic methylene group (C-11) and an allylic methine proton (C-8) coupled to two methylene groups (four protons on C-7 and C-9). This produces the multiplet at 2.75 ppm. In addition, an isolated 4-spin system on the cyclobutane ring appears. These data further confirm the fundamental carbon skeleton of 1.

The mass spectrum of 1 shows a very weak molecular ion at m/z 236 (0.6); its exact mass measurement m/z 236.1772 corresponds to the molecular formula $C_{15}H_{24}O_2$ (required 236.1776), indicating four double-bond equivalents. Its striking characteristic peaks appear at M – 18, M – 44, and 159. The peak m/z 192 (M – 44), shown by high-resolution measurements to be due to reaction i and then cleavage of the cyclobutane ring with loss of C_2H_4O

(1) Westcott, C. *Plant Disease Handbook*, 3rd ed.; Van Nostran Reinhold, Toronto, 1971; p 354.
(2) Hepting, G. H. *Agriculture Handbook*; Agriculture Forest Service: Washington, DC, 1981; No. 386, pp 61, 84, 90, 236.

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