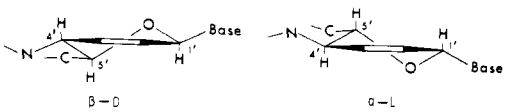
-Ac), and 1.98 ppm (s, -Ac). These data suggested that the serine moiety is bound to the 4′-amino function forming an amide bond.

Vigorous acid hydrolysis of **9** (3 N HCl, reflux, 15 h) gave a lactone dihydrochloride (**12**, $C_{11}H_{20}N_4O_4 \cdot 2HCl$, $[\alpha]^{23}_D$ ±0°) and **5**. **12** gave a monohydrochloride (**13**, $[\alpha]^{23}_D \pm 0°$) upon treatment with NH_4OH or IR-45, while a free base (**14**, $[\alpha]^{23}_D$ −7.9°, pK_a' = 2.8 (-COO^-), 8.6 (new 4′-NH_3^+), and >12 (guanidine)) was obtained by treatment with IRA-410. In the ^{13}C NMR spectrum of **14** the signals at $C_{3'}$ and $C_{5'}$ showed downfield shifts,[7] 4.4 and 6.8 ppm, when compared with those of **12**; therefore, the new primary amine should be located at 4′. The IR spectrum of **12** showed a strong absorption at 1770 cm^{-1} attributable to a five-membered lactone which disappeared in the spectra of **13** and **14**. The ^{13}C NMR signal of $C_{8'}$ shifted to 77.5 ppm in **12** from 68.9 ppm in **14**. In the ^1H NMR spectrum of **14** the signals at 3.75 (m, $H_{8'}$) and 1.95 ppm (m, $H_{7'}$) showed downfield shifts to 4.95 and 2.62 ppm (d like) in **12**, respectively. These data provided evidence for the structure of the lactone **12** as well as for the location of the carboxyl group in question.

Another ureido compound (**15**) was a key compound for establishing the location of the α-hydroxyl carboxylic acid. **14** was hydrolyzed (0.2 N NaOH, reflux, 2 h) to give ammonia and an ureido compound (**15**, mp 248 °C dec, $[\alpha]^{23}_D$ +5.9°, pK_a' = 7.75 (4′-NH_3^+) and 2.9 (-COO^-), δ 3.8 (d, $H_{5'}$) and 2.02 (m, $H_{7'}$)). On acetylation (Ac_2O/pyridine), **15** gave a triacetate (**16**, $[\alpha]^{25}_D$ +14.6°, pK_a' = 2.8 (-COO^-), ν 1700–1740 cm^{-1}, δ (Me_2SO-d_6) 1.98 (3 H, s) and 2.02 (3 H × 2, s)). On methylation (CH_2N_2/MeOH-Et_2O), **16** afforded a methyl ester (**17**, $[\alpha^{23}_D$ +14.1°, ν 1740 cm^{-1}, δ ($CDCl_3$) 3.80 (s, -$COOCH_3$).

Oxidation of **15** ($Pb(OAc)_4$/AcOH-water) yielded CO_2 and a ketone (**18**, $[\alpha]^{23}_D$ −1.2°, ν 1720 cm^{-1} (-CO-), δ 3.97 (d, $H_{5'}$) and 2.54 (m, $H_{7'}$)). In the ^{13}C NMR spectrum of **18** the signal of an isolated carbonyl group newly appeared at 210.9 ppm (s, $C_{6'}$) instead of the signal at 80.4 (s, $C_{6'}$) and 179.8 ppm (s, $C_{11'}$) in **14**. The 4′-*N*-acetate of **18** was reduced with $NaBH_4$/MeOH to give a diol (**19**, $[\alpha]^{27}_D$ +59.0°). Proton spin-decoupling studies of **19** confirmed the structure: when the 7′-methylene proton at 2.20 ppm (m) was irradiated, the methine signals at 4.25 (m, $H_{6'}$) and 4.03 ppm (m, $H_{8'}$) collapsed into a doublet (J = 7 Hz) and a double doublet, respectively. On irradiation of the methine proton at 3.78 ppm (q, $H_{5'}$) of **19**, the $H_{6'}$ methine signal at 4.25 ppm collapsed into a doublet (J = 4 Hz). From these data the presence of α-hydroxycarboxyl structure was established.

As for the absolute configuration of pyran-3-ene ring, the stereochemistry of $H_{4'}$ and $H_{5'}$ should be diaxial on the basis of the coupling constant of $J_{4',5'}$ = 10 Hz in **7**. Also the stereochemistry of $H_{1'}$ was assigned axial from $J_{1',2'}$ = 10 and 2 Hz in **3**. Thus, three bulky groups in the pyran-3-ene ring should reasonably be all equatorial. Only two sterically stable stereostructures of β-D or α-L could be permitted among all the possible isomers of pyran-3-ene as shown. Since these formula are mirror images, the Cotton effect of the CD spectrum in the B_{2u} band should be of opposite sign to each other.



β-D α-L

The CD spectra of the model and mildiomycin compounds follow: blasticidin S, $[\theta]_{270}$ −12 900; **1**, $[\theta]_{273}$ −8700; cytosinine, $[\theta]_{271}$ −9500; deseryl derivative of **1** (**20**, $[\alpha]^{24}_D$ +26.9°)), $[\theta]_{273}$ −9300; gougerotin, $[\theta]_{280}$ − 2700; **3**, $[\theta]_{285}$ −1800. The absolute configuration of blasticidin S and gougerotin has been determined as β-D.[8,9] These mildiomycin compounds showed negative Cotton effects quite similar to those of the model compounds, indicating that the pyranene ring should be β-D. The absolute configuration of **1** was thus assigned 1′*R*,4′*S*,5′*S*,2″*S*.

One of the interesting structural features of **1** is that the carboxyguanidino butyl group is bound to the unsaturated pyranoside with C-C bond. The aspects of the biosynthesis of this antibiotic provide another interesting problem—whether the quaternary carbon originates from an amino acid or sugar as a precursor.

Acknowledgments. We are grateful to Drs. E. Ohmura, K. Morita, M. Isono, and M. Yoneda in our Central Research Division for their encouragement throughout this work. We also thank the members of large-scale fermentation and physicochemical analysis sections for their support.

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(3) The IR spectra were measured in KBr pellet. The δ values in the ^1H and ^{13}C NMR spectra using XL-100 (Varian) were recorded in parts per million downfield from Me_4Si. All spectra herein were measured at the concentration of 20 mg/0.4 mL (^1H) and 200–300 mg/3 mL (^{13}C) in D_2O unless otherwise stated. In the ^{13}C NMR spectra dioxane was the internal standard (67.4 ppm).
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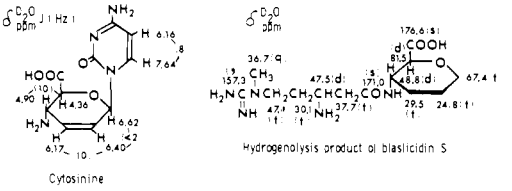

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Received February 27, 1978

^{13}C-Enriched *S*-Methylmethionyl Residues as a Probe of Protein Conformation[1]

Sir:

Specific ^{13}C labeling of proteins has enhanced the usefulness of ^{13}C NMR spectroscopy as a tool for the study of these macromolecules. One highly selective method for ^{13}C enrichment of proteins which permits their observation in an essentially native form is the methyl exchange reaction at methionyl residues.[2] This method has now been applied in our laboratories to the basic pancreatic trypsin inhibitor (BPTI).[3] In the course of this work we have had occasion to make spectroscopic observations on the ^{13}C labeled protein intermediate, which possesses an enriched *S*-methylmethionyl residue at position 52 ([ε-^{13}C-SMM-52]-BPTI). Detailed NMR spectroscopic studies of *S*-methylmethionine-containing

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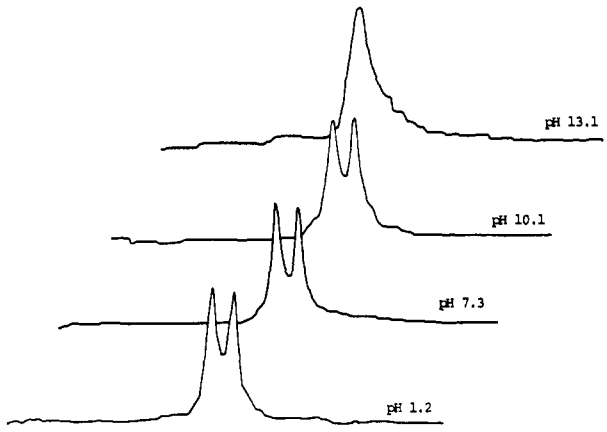

Figure 1. Four 100-Hz segments of the proton-decoupled 25.16-MHz ^{13}C FT NMR spectra of 1.8 mM [ϵ-^{13}C-SMM-52]-BPTI at indicated pH values (uncorrected). The solvent was a 1:2 D_2O–H_2O mixture. Each spectrum is the sum of 10 000 scans recorded at 32 °C. Successive spectra are offset by 10 Hz (0.40 ppm) for clarity of representation.

proteins have not been reported to date. We demonstrate here the potential of the ^{13}C labeled *S*-methylmethionyl residue as a probe of protein conformation.

The single[4] methionyl residue of BPTI (Met-52) lies in the center of the C-terminal helix[5] of the molecule. Studies on the renaturation of reduced BPTI[6] have indicated that this region of the molecule plays an important part in the refolding process. Previous modifications of the methionyl residue[7,8] have shown no detectable effect on the trypsin inhibiting properties of BPTI, and only slight effects on its capacity to renature.[9] Our experiments on the inhibitory and folding properties of [SMM-52]-BPTI have produced results fully consistent with these previous observations.[10]

Methylation of Met-52 of BPTI was carried out at room temperature in 70% formic acid, with a 100-fold excess of methyl iodide. These denaturing conditions differ from the general S-methylation procedure of Jones et al.[2] which, when applied to native BPTI, produced only minor modification. Kassell has shown that partial denaturation of BPTI is required if alkylation of the methionyl residue is to be achieved.[7] Under our denaturing conditions the extent of S-methylation in 18 h, as judged by analytical chromatography on (carboxymethyl) cellulose,[8] was 75–80%. Longer periods (40 h) or larger excesses of CH_3I (1000-fold) have not produced a significant increase in the degree of S-methylation. These results have been confirmed by amino acid analysis, which further shows no additional alterations of the protein during this reaction.

The ^{13}C NMR spectrum (Figure 1), recorded at pH 7, of [ϵ-^{13}C-SMM-52]-BPTI from alkylation with 90 at. % enriched $^{13}CH_3I$ shows two signals of equal intensity (±5%). These signals appear at chemical-shift positions upfield ($\Delta\delta = -0.22$ and -0.42 ppm) from the mean methyl signal of *S*-methylmethionine (δ 26.85 ppm, also recorded at pH 7). The appearance of the protein label as a pair of peaks is attributed to the two sterochemically distinct forms of the sulfonium group which arise upon methylation of the thioether moiety of the methionyl side chain. These singly ^{13}C-enriched methionine sulfonium residues are diastereomeric (the two methyl groups of an *S*-methylmethionyl residue are diastereotopic).[11] Separate methyl group signals (at δ 26.87 and 26.83 ppm) also appear in the NMR spectrum (Figure 2) of the amino acid, [*S*-$^{13}CH_3$]methionine, prepared from $^{13}CH_3I$ and methionine. Thus the nonequivalence of these methyl group signals is 0.04 ppm at pH 7.

Both the enhanced anisochromicity and the upfield chemical shifts of the diastereomeric resonances of the *S*-methyl groups of [ϵ-^{13}C-SMM-52]-BPTI, relative to those of *S*-methylmethionine, are believed to be a result of—*and sensitive to*—

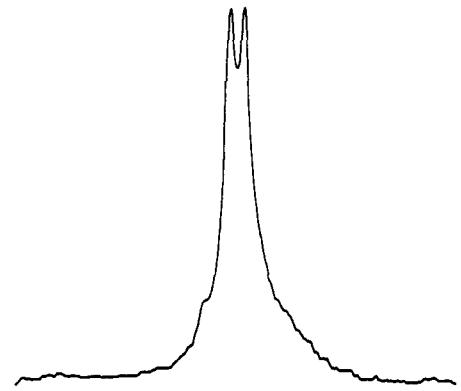

Figure 2. A 25-Hz segment of the proton-decoupled 25.16-MHz ^{13}C FT NMR spectrum of 0.1 M [*S*-^{13}C]methylmethionine at pH 6.9 (uncorrected) in 30 at. % deuterated H_2O. This trace is the sum of 16 scans at 32 °C.

the environmental effects of the protein. The chemical-shift separation of the resonances arising from the protein-borne *S*-methyl groups remains unchanged in spectra recorded over the pH range 1–10 (Figure 1). Their chemical-shift values are also unchanged in the range pH 7–10, with a slight ($\Delta\delta < 0.15$ ppm) downfield displacement at pH 1. These observations are consistent with the known conformational stability of unmodified BPTI over this same pH range. By pH 13 the *S*-methyl signals of [ϵ-^{13}C-SMM-52]-BPTI have coalesced and appear at lower field by 0.20 ppm and 0.39 ppm, respectively (Figure 1); the unmodified protein has been observed[12] to denature in the pH range 10–13. In addition, the spectrum of the ^{13}C-methylated protein which has been denatured by disulfide bond reduction and subsequent carboxymethylation of the thiol groups showed a substantial downfield movement (0.16 and 0.28 ppm) and a lessening of the chemical-shift nonequivalence (to 0.08 ppm) of the signals arising from the *S*-methyl label. These data support the conclusion that the *S*-methyl resonances in [ϵ-^{13}C-SMM-52]-BPTI are sensitive to the character of the protein environment.

The *S*-methylmethionyl residue differs from methionyl residues in possessing a positive charge. Its effects on protein structure may be considerably different from those of the residue it replaces. Our experience indicates, however, that this need not always occur, and that ^{13}C-enriched *S*-methylmethionyl residues can function as reporters of protein conformation. In the case of BPTI, the transformation has no effect on the activity of the molecule as an inhibitor of trypsin. Furthermore [SMM-52]-BPTI is capable of fully renaturing after disulfide bond reduction and at rates comparable with those of BPTI.[10] In addition, the similarity of the apparent pH denaturation profiles of [SMM-52]-BPTI and native BPTI indicate that the overall protein conformation and stability have not been significantly perturbed by this alteration. Detailed comparisons of the conformational properties of [SMM-52]-BPTI and native BPTI as well as the analogous use of [ϵ-^{13}C-Met-52]-BPTI chemical shifts as conformation probes are now underway. We believe that the twofold information available from ^{13}C-enriched *S*-methylmethionyl residues of proteins, in the form of both the chemical-shift changes and the enhancement of magnetic nonequivalence, may render this modification an especially useful probe in the observation of protein conformation and protein folding.

Acknowledgment is made to R. R. Inners for valuable assistance in the collection of initial spectral data on the modified protein. We also thank Bayer A. G., Wuppertal, for their gift of BPTI (Trasylol).

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(1) Supported by U.S. Public Health Service Grant GM-22927 and Robert A.

Welch Foundation Grant E-183. The Varian XL-100/Nicolet TT-100 NMR spectrometer was purchased through NSF Grant No. MPS 75-06162.

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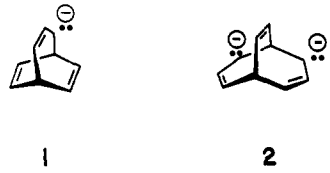
A $C_{11}H_{11}$ Limit of Longicyclic Stabilization

Sir:

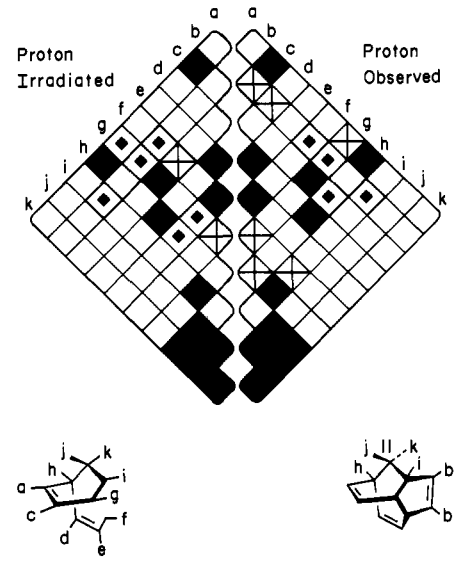
Synthetic applications of the Hückel MO model have largely been limited to those unsaturated hydrocarbons that are planar, or pericyclic, or both.[1] An alternative topology, the longicyclic, extends the range of the Hückel model from two dimensions into three.[2] The topologically required stabilization rule is then somewhat different.[3] Its limits, as yet, are less well defined. It rationalizes the stability of the norbornadienyl cation,[4] the inaccessibility of the norbornadienyl anion,[5] and the effect of charge on the direction of bicyclo[3.2.2]nonatrienyl ⇌ barbaralyl equilibration.[6]

The synthetic criterion is more demanding. One expects a fruitful stabilization rule to anticipate the successful preparation of easily isolable cations and anions, under reasonably standard conditions, and in the absence of empirical analogy. This criterion has now been satisfied in two longicyclic syntheses, those of the bicyclo[3.2.2]nonatrienyl anion (**1**)[7] and bicyclo[3.3.2]decatrienyl dianion (**2**).[7b]



1 **2**

The longicyclic rule[3] anticipates the next higher homologue, bicyclo[4.3.2]undecatetraenyl, to be stable *both* as the anion and as the cation.[8a] We here report our failure to obtain the anion under conditions closely similar to those that had earlier provided **1** and **2**. Satisfaction of the synthetic criterion is now frustrated by anionic rearrangement. Cationic rearrangement had similarly prevented isolation and characterization of the bicyclo[4.3.2]undecatetraenyl cation.[9] It is therefore the carbon skeleton, and not the charge, that facilitates rearrangement. The course of the anionic rearrangement and its absence from **1** and **2** both correspond to an explicitly predicted limit of longicyclic stabilization.[8b]



4a **4**

Figure 1. Complete spin decoupling results: solid block, elimination of 2–13-Hz coupling; ♦, elimination of 1.0–1.5-Hz coupling; +, inaccessible or ambiguous; open block, no discernible effect.

Table I. ^{1}H NMR Spectrum of **4**[a]

Assignment	δ	Area[b]	Appearance,[c] *J* in Hz
a	6.52	1.02	dd, J_{ac} = 7.5, J_{ah} = 8.5
b	6.09	1.95	d, J_{bf} = J_{bi} = 1.5
c	5.93	1.02	dd, J_{ac} = J_{cg} = 7.5
d	5.80	1.07	dd, J_{de} = 9.7, J_{dh} = 8.0
e	5.52	0.96	dd, J_{de} = 9.7, J_{ef} = 5.5
f	2.87	1.97	ddd, J_{ef} = 5.5, J_{fg} = 5.5, J_{bf} = 1.5
g	2.82		ddd, J_{cg} = 7.5, J_{fg} = 5.5, J_{gi} = 6.5
h	2.60	0.97	m
i	2.52	0.98	m
j	1.65	1.03	ddd, J_{jk} = 13.0, J_{ij} = 10.5, J_{hj} = 4.5
k	1.46	1.05	ddd, J_{jk} = 13.0, J_{ik} = 2.5, J_{hk} = 2.0

[a] 0.03 M in $CDCl_3$ at 300 MHz. [b] Normalized to 12 protons. [c] Apparent first-order splittings.

A persistent deeply purple solution was easily generated by treating *syn*-9-methoxybicyclo[4.3.2]undecatetraene (**3**)[10] with lithium in tetrahydrofuran. NMR spectra of this solution (and of its recrystallized solute) were unfortunately obscured by recalcitrant impurities. Structurally useful information could only be obtained by methanol protonation at −78 °C. This quenched the color and provided a new $C_{11}H_{12}$ hydrocarbon as the exclusive volatile product in 27% yield.[11,12]

The structure of this hydrocarbon was first defined to be an asymmetric tricycloundecatriene by its ^{13}C NMR (δ 142.1, 141.7, 139.7, 136.2, 135.8, 131.5, 48.0, 45.5, 42.1, 35.6, and 31.9 ppm) and ^{1}H NMR spectra (Table I). Next, a proton connectivity pattern (**4a**, Figure 1) was constructed by the sequential decoupling of all 2–13-Hz coupling constants. This pattern allows the residual *cis*-alkene to be joined in either one of two ways. Of these, only that represented by tricyclo[5.3.1.$0^{4,8}$]undeca-2,5,9-triene (**4**) is asymmetric. The complete list of possible isomers (48 tricyclics that lack quaternary carbons and exocyclic methylenes[13]) reveals no other viable candidate.

The ^{1}H NMR assignments of Figure 1 are generally con-

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