The cysteines and one tyrosine residue occur in almost identical positions, assuming one or two deletions, in both neurotoxins and this lytic factor. The carboxyl ends of the molecules, from residue 38 (lytic factor), are also very similar. The most apparent difference lies in the amino terminal half, where the lytic factor has predominantly hydrophobic amino acids and the typical neurotoxin hydrophilic. The invariant sequence found so far in all neurotoxins, Lys-X-Trp-X-Asp-X-Arg-Gly-, occurring between residues 24 and 32, is also missing in the lytic factor.

References

Aloof-Hirsch, S., De Vries, A., and Berger, A. (1968), *Biochim. Biophys. Acta 154*, 53.

Benson, J. V., Jr., Gordon, M. J., and Patterson, J. A. (1967), *Anal. Biochem. 18*, 228.

Brenner, M., Niederweiser, A., and Pataki, G. (1962), *in* Dünnschichtchromatographie, Stahl, E., Ed., West Berlin, Springer-Verlag, p 443.

Dayhoff, M. (1972), Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington, D. C., D-374.

Edman, P. (1970), *in* Protein Sequence Determination, Needleman, S. B., Ed., West Berlin, Springer-Verlag, p 211.

Edman, P., and Sjöquist, J. (1956), *Acta Chem. Scand. 10*, 1507.

Fryklund, L., Eaker, D., and Karlsson, E. (1972), *Biochemistry 11*, 4633.

Gross, E., and Witkop, B. (1962), *J. Biol. Chem. 237*, 1856.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Iwanaga, S., Wallén, P., Gröndahl, N. J., Henschen, A., and Blombäck, B. (1969), *Eur. J. Biochem. 8*, 189.

Karlsson, E., Arnberg, H., and Eaker, D. (1971), *Eur. J. Biochem. 21*, 1.

Karlsson, E., Eaker, D., Fryklund, L., and Kadin, S. (1972), *Biochemistry 11*, 4628.

Narita, K., and Lee, C. Y. (1970), *Biochem. Biophys. Res. Commun. 41*, 339.

Porath, J. (1956), *Biochim. Biophys. Acta 22*, 141.

Porath, J. (1964), *Sci. Tools 11*, 21.

Porath, J. (1966), *Mem. Inst. Butantan Sao Paulo 33*, 379.

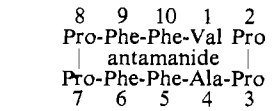
Strydom, A. J. C., and Botes, D. P. (1971), *J. Biol. Chem. 246*, 1341.

Antamanide Conformations in Nonaqueous Media. Dependence on Hydrogen-Bond Acceptor Properties of Solvent†,‡

Dinshaw J. Patel

ABSTRACT: Proton and carbon nmr studies and model building were combined to generate conformations for antamanide in nonaqueous solution. The conformation in weak hydrogen-bond acceptor nonaqueous solvent (designated I) contains all intramolecularly hydrogen-bonded peptide protons. This conformation occurs in rapid equilibrium with a conformation with all peptide protons exposed to solvent (designated II) in strong hydrogen-bond acceptor nonaqueous media. An analysis of the multiplicity of the C^{α} proline proton resonances and the chemical shifts of the C^{β} and C^{γ} proline carbon resonances for the nmr spectra of antamanide in solution suggests the presence of two cis and two trans X-Pro peptide bonds. Conformations were generated with cis peptide bonds either at Val_1-Pro_2 and Phe_6-Pro_7 or at Pro_2-Pro_3 and Pro_7-Pro_8 using Corey–Pauling–Koltun models which were in agreement with the nmr data and energy maps. The experimental evidence appears to be more consistent with the conformations 1,6-*cis*-I $\rightleftarrows$ 1,6-*cis*-II, containing cis peptide bonds at Val_1-Pro_2 and Phe_6-Pro_7, for antamanide in nonaqueous solution.

Nuclear magnetic resonance (nmr) spectroscopy and circular dichroism (CD) measurements were coupled with an approximate theoretical treatment to determine the conformational characteristics of the cyclic *all*-L-decapeptide antamanide (Tonelli *et al.*, 1971). Proton nmr decoupling and exchange studies for antamanide in $CDCl_3$ solution suggested that the observable peptide N protons were neither hydrogen bonded nor in solvent-shielded environments. The CD spectrum of antamanide (in methanol, dioxane, trimethyl phosphate, hexafluoroisopropyl alcohol) exhibited $[\theta] > -100{,}000°$ in the 190–205-nm region consistent with all X-Pro peptide bonds in the trans conformation. The solvent-dependent CD data exhibited no isosbestic point and were assigned to medium effects rather than conformational changes. The lowest energy cyclic conformation with all-trans peptide bonds generated in the theoretical portion of the study was not stabilized by intramolecular hydrogen bonding and possessed a pseudo-twofold center of symmetry (Tonelli *et al.*, 1971). The suggestion of all-trans X-Pro peptide bonds from the CD data needs reevaluation and is considered in detail later in the manuscript.



† From Bell Laboratories, Murray Hill, New Jersey 07974. *Received July 24, 1972.*

‡ This paper and the following paper both contain color plates. Plates 1 and 2 of this paper appear on p 685.

In contrast to the above results is the preliminary published report on the infrared (ir) and optical rotatory dispersion (ORD) of antamanide in solution (Ivanov *et al.*, 1971). The broad NH stretching band at ~3300 cm^{-1} in $CHCl_3$ and CH_3CN–CCl_4 suggested that all six N protons of antamanide participate in intramolecular hydrogen bonds. The ORD curves of antamanide in the range 210–250 nm exhibited strong solvent dependence (hexane–dioxane, methanol–water). The presence of an isosbestic point at 230 nm suggested only two forms of antamanide in equilibrium (Ivanov *et al.*, 1971).

From CD and uv studies a conformational change was observed for antamanide in nonpolar solvents on interaction with cations or polar solvents (Faulstich *et al.*, 1972). The conformation formed on addition of water was suggested to be similar to that of the Na complex. The unusually high negative dichroism at about 230 nm was ascribed to $n \rightarrow \pi^*$ transitions of transoid tertiary amide groups distorted out of plane. The CD data were suggested to support an antamanide conformation in nonpolar media, designated *trans*-M (Faulstich *et al.*, 1972). The merits of conformation *trans*-M will be evaluated at the end of this manuscript.

The investigations of antamanide in solution are thus in disagreement on the number of intramolecular hydrogen bonds and the number of solution conformations in equilibrium (Tonelli *et al.*, 1971; Ivanov *et al.*, 1971; Faulstich *et al.*, 1972). In order to shed further light on the subject, the nmr studies have been extended to an investigation of antamanide in several solvents. Model building coupled with theoretical calculations were used to generate structures defined in terms of the rotation angles φ, ψ, and ω (Edsall *et al.*, 1966) consistent with the additional nmr data. Recent nmr (Torchia *et al.*, 1972a,b; Deber *et al.*, 1971; Torchia, 1972; Wuthrich *et al.*, 1972) and X-ray (Sobell *et al.*, 1971) studies of proline-containing peptides show the presence of cis X-Pro peptide bonds. Thus, both cis and trans X-Pro peptide-containing antamanide structures have been investigated.

Experimental Section

Nmr spectra were run on a 220-MHz Varian nmr spectrometer equipped with a Varian variable-temperature unit. Ethylene glycol monitored temperatures to ±1°. A Fabri-Tek computer of average transients improved the signal-to-noise ratio. A Hewlett-Packard 651B test oscillator was used to carry out spin decoupling.

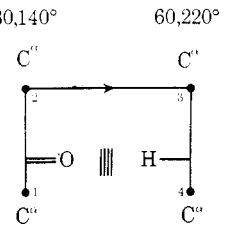
^{13}C nmr spectra were run on an XL-100 spectrometer interfaced with an F&H pulse programmer and a Fabri-Tek computer for operation in the Fourier transform mode (Sternlicht and Zuckerman, 1972). Proton decoupling was carried out under heteronoise conditions. Samples were dissolved in deuterated solvent and the instrument was locked on solvent deuterium.

Results and Discussion

The proton magnetic resonance parameters of interest are the proton–proton coupling constant $J_{H^NH^\alpha}$ in hertz, the temperature coefficients of peptide N resonances in parts per million per degrees centigrade and the H^α chemical shifts in parts per million. The coupling constant can be related to a range of values of the rotation angle φ from the relationship of Barfield and Karplus (1969). The temperature coefficient of the peptide NH resonance has provided a useful probe in the determination of the nature of the exchangeable proton (intramolecularly hydrogen bonded or exposed to solvent) (Kopple *et al.*, 1969a,b). Large temperature coefficients ($>6 \times 10^{-3}$ ppm/°C) are indicative of solvent exposure of peptide N proton while small temperature coefficients ($<2 \times 10^{-3}$ ppm/°C) are indicative of short coplanar intramolecular hydrogen bonds. Temperature coefficients in an intermediate range (~$2–6 \times 10^{-3}$ ppm/°C) may reflect either weak intramolecular hydrogen bonds, internal burial away from solvent of the exchangeable proton, or a conformational equilibrium represented by structures where the peptide proton experiences an average of intramolecularly hydrogen-bonded and solvent-exposed environments. Unlike the N proton chemical shifts, those of the H^α proton exhibit smaller solvent dependence and can be used as a qualitative measure of conformational change.

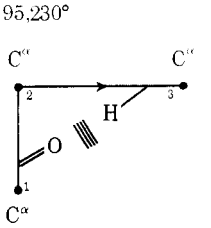
Conformational calculations (Ramachandran *et al.*, 1966; Venkatachalam, 1968) and the subsequent X-ray crystallographic investigations of proteins have provided information on the nature and stability of intramolecular hydrogen bonds. For a hydrogen bond between N—H and O=C, the N···O distance should be between 2.6 and 3.2 Å and the NH–NO angle < 30° (Ramachandran *et al.*, 1966).

For a tetrapeptide sequence containing L residues and trans peptide bonds only, the chain reverses its direction and forms a type I intramolecular 1 ← 4 hydrogen bond when the residue rotations at positions 2 and 3 have φ,ψ values of 130,140° and 60,220° respectively. Low-energy bends within ±20° of these values are predicted (Venkatachalam, 1968; Ramachandran *et al.*, 1970). Since the distance between C^α



residues at positions 1 and 4 is 4.8 Å, the chain can be extended in either direction to form an antiparallel pleated sheet held together by intramolecular hydrogen bonds (Venkatachalam, 1968).

For a tripeptide sequence of L residues and trans peptide bonds only, intramolecular type 1 ← 3 hydrogen bonds are generated when the residue rotations at position 2 have φ,ψ values in the region 95,230° (Ramachandran *et al.*, 1966).



In addition to intramolecular hydrogen bonds of type 1 ← 4, type 1 ← 3, and antiparallel β-sheet type discussed above, one must consider helical conformations held together by intramolecular hydrogen bonds. The 3_{10} and α helices (130, 120–150°) contain 1 ← 5 intramolecular hydrogen bonds.

The experimental results are outlined on the basis of the hydrogen-bond acceptor properties of the solvent system investigated.

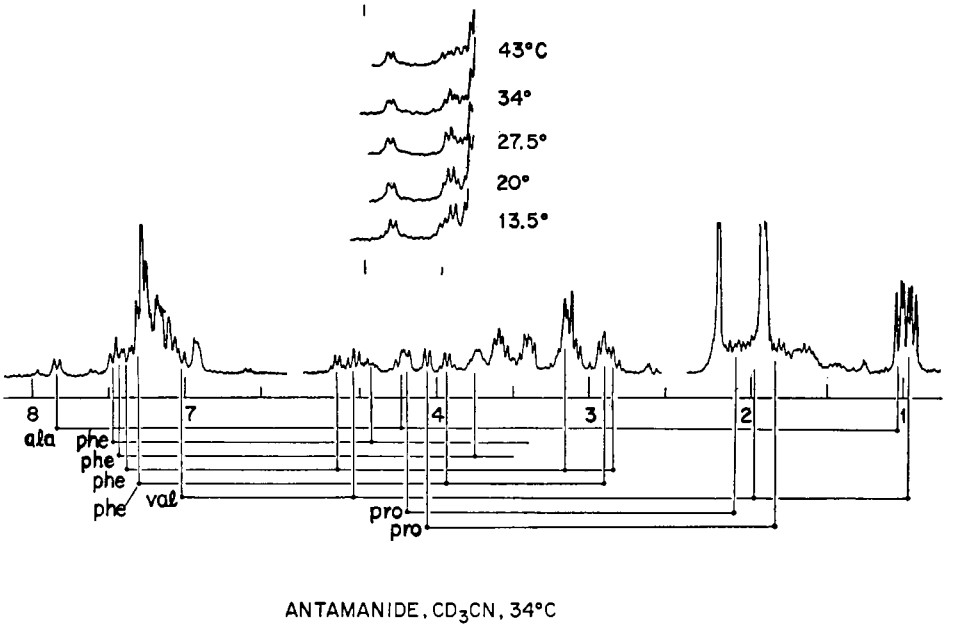


FIGURE 1: The proton nmr spectrum of antamanide in CD_3CN at 34°. The lines below the spectrum connect N, C^{α}, and C^{β} protons of individual residues derived from spin decoupling experiments

TABLE I: Proton Nmr Spectral Parameters (Chemical Shifts, Coupling Constants, and Temperature Coefficients) for Antamanide in Acetonitrile (34°).

	H^N	H^{α}	H^{β}	H^{γ}	$J_{H^NH^{\alpha}}$	$J_{H^{\alpha}H^{\beta}}$	Temp Coeff (ppm/°C) Low Temp	High Temp
Ala	7.83	4.23	1.04		8.5		-2.3×10^{-3}	0.45×10^{-3}
Val	7.02	4.54	1.99	0.96	8.5		2.5×10^{-3}	0.23×10^{-3}
Phe[a]	7.47	4.47			8.0		-2.7×10^{-3}	-1.7×10^{-3}
Phe	7.42	3.73			8.0		4.3×10^{-3}	2.0×10^{-3}
Phe	7.37	4.66	2.84, 3.16		8.0		4.1×10^{-3}	1.6×10^{-3}
Phe	7.30	3.93	2.86					
Pro		4.20	2.10			8.0, <1		
Pro		4.06	2.02			8.0, <1		

[a] Assigned to Phe_9 from Na ion binding studies outlined in the following manuscript (Patel, 1973).

I. Nonaqueous Weak Hydrogen-Bond Acceptor Solvents

Antamanide was investigated in polar CD_3CN and nonpolar CD_3CO_2H. Both are weak hydrogen-bond acceptor solvents.

The proton nmr spectrum of antamanide in CD_3CN at 34° is shown in Figure 1. Decoupling studies permit the assignments shown below the spectra. The chemical shifts and coupling constants are summarized in Table I. The peptide couplings, $J_{H^NH^{\alpha}}$, vary between 8.0 and 8.5 Hz for the five observable N protons. Two of the four proline H^{α} protons are doublets with $J_{H^{\alpha}H^{\beta}} = 8.0$ and <1 Hz. The temperature dependences of the peptide N protons of antamanide in CD_3CN are plotted in Figure 2 and the slopes tabulated in Table I. The data between 20 and 70° exhibit slopes of $\lesssim 2 \times 10^{-3}$ ppm/°C for the Ala_4, Val_1, and three of the four observable Phe peptide protons. The remaining Phe peptide proton is under the aromatic resonances and cannot be detected.

The proton nmr spectrum of antamanide in CD_3CO_2H at 32.5° has been investigated by decoupling studies and the data are summarized in Table II. The peptide couplings, $J_{H^NH^{\alpha}}$, vary between 6.5 and 8.5 Hz for all six peptide N protons. The temperature dependences of the peptide N protons of antamanide in CD_3CO_2H are plotted in Figure 2 and the slopes tabulated in Table II. The temperature coefficients for Val_1, Ala_4, and two Phe peptide protons are $\lesssim 0 \times 10^{-3}$ ppm/°C while two Phe peptide protons (similar C^{α} chemical shifts at 4.30 ppm) exhibit slopes of 3–4 $\times 10^{-3}$ ppm/°C.

The small or negative slopes ($\lesssim 2 \times 10^{-3}$ ppm/°C) as observed for Ala_4, Val_1, and two Phe residues (presumably Phe_9 and Phe_6 by symmetry) suggest the presence of short coplanar intramolecular hydrogen bonds while the remaining two Phe residues with similar C^{α} chemical shifts (presumably Phe_5 and Phe_{10} by symmetry) exhibit intermediate slopes (2–4 $\times 10^{-3}$ ppm/°C) suggesting the presence of weak intramolecular hydrogen bonds or solvent-shielded amide protons.

For an L-prolyl residue succeeded by another L-prolyl residue, *i.e.*, for Pro_2 and Pro_7, the pyrrolidine rings should have the poly-L-proline II (trans peptide bonds) or poly-L-proline

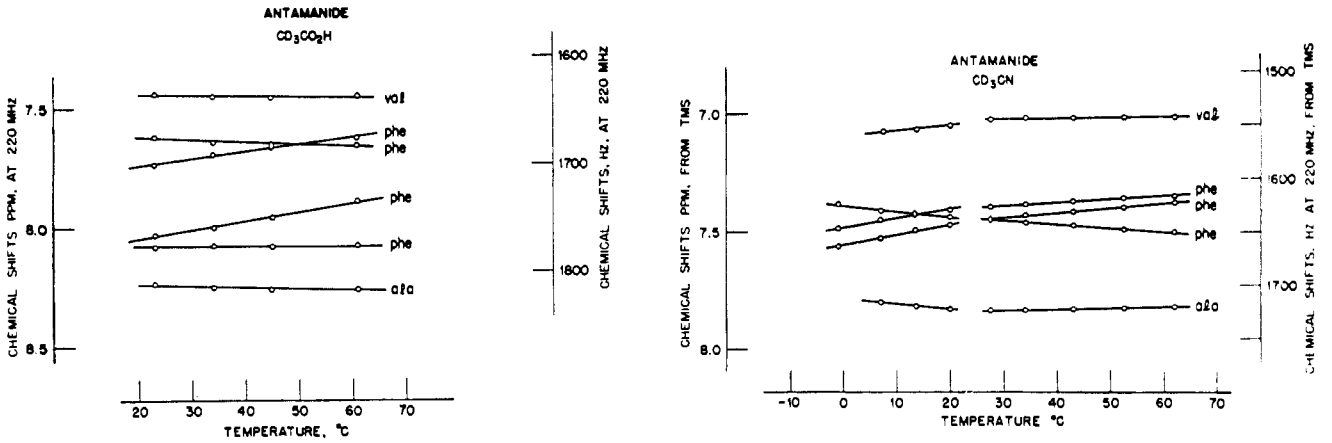


FIGURE 2: Temperature coefficient plots for antamanide in acetic acid and acetonitrile.

I (cis peptide bonds) geometry, namely ψ = 310–340° (Cowan and McGavin, 1955; Sasisekharan, 1959). For a residue preceding proline, conformational calculations predict ψ to be in the range 280–340° (Schimmel and Flory, 1968; Brant *et al.*, 1967).

The gramicidin S structure exhibits two type 1 ← 4 and two antiparallel β-sheet type short coplanar intramolecular hydrogen bonds (Hodgkin and Oughton, 1957; Schwyzer, 1959; Stern *et al.*, 1968; Schwyzer and Ludescher, 1968; Ovchinnikov *et al.*, 1970).

Starting from a gramicidin S like structure (Figure 3) a conformation was derived from model building in which strong intramolecular hydrogen bonds were formed by Ala_4, Phe_9 (type 1 ← 4) and Val_1, Phe_9 (antiparallel β-sheet type); the ψ values of Val_1, Pro_2, Phe_6, and Pro_7 are restricted to the range 280–340° and dihedral angles φ for nonproline residues are consistent with the experimentally observed coupling constants $J_{H^NH^\alpha}$ = 6.5–8.5 Hz. In this structural arrangement the bends necessary for cyclization are made at the Pro residues. There are cis peptide bonds between Pro_2-Pro_3 and Pro_7-Pro_8 in this conformation. The rotation angles derived from model building are summarized for the conformation, designated 2,7-*cis*-I in Table III and Corey–Pauling–Koltun (CPK) models presented in plate 1. For the peptide N protons of Phe_5 and Phe_{10} to participate in type 1 ← 3 intramolecular hydrogen bonds, the φ,ψ values of Ala_4 and Phe_9 have to be in the range (95,230°). For conformation 2,7-*cis*-I these values are in the range 90,270 and meet the condition for the hydrogen bond. Thus, the experimental requirements that Val_1, Phe_6, Ala_4, and Phe_9 participate in short coplanar hydrogen bonds and Phe_5 and Phe_{10} participate in weak intramolecular hydrogen bonds are met by conformation 2,7-*cis*-I.

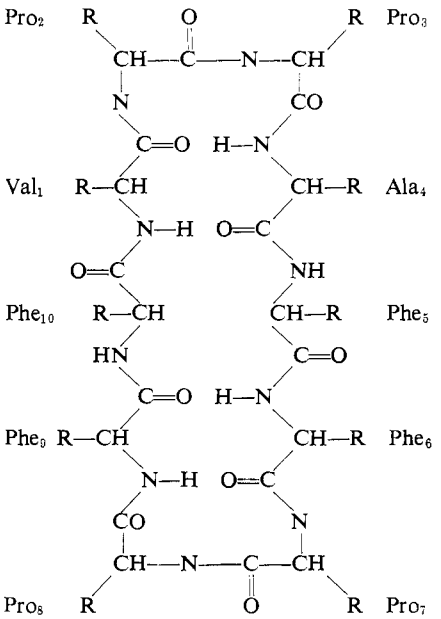


FIGURE 3: The gramicidin S backbone emphasizing four intramolecular hydrogen bonds; position of antamanide residues for conformation 2,7-*cis*-I.

A conformation was generated from CPK models that showed the following features. It exhibited cis peptide bonds between Val_1-Pro_2 and Phe_6-Pro_7, φ values consistent with experimental $J_{H^NH^\alpha}$ coupling constants, ψ values in the range 280–340° for Val_1, Phe_6, Pro_2, and Pro_7 in agreement with conformational maps of an X residue in an X-Pro sequence and short coplanar intramolecular hydrogen bonds for Val_1, Phe_6, Ala_4, and Phe_9 and weak intramolecular hydrogen bonds for Phe_5 and Phe_{10}. The backbone of this conformation, designated 1,6-*cis*-I, takes up a double helical-like geometry. The rotation angles defining this conformation are outlined in Table III and CPK models presented in plate 2.

The peptide N–H and O=C groups participating in the intramolecular hydrogen bonds in conformations 2,7-*cis*-I and 1,6-*cis*-I are summarized in Table IV.

TABLE II: Proton Nmr Spectral Parameters for Antamanide in CD_3CO_2H (32.5°).

	H^N	H^α	H^β	H^γ	$J_{H^NH^\alpha}$	Temp Coeff (ppm/°C)
Ala	8.25	4.59	1.18		8.0	-0.5×10^{-3}
Phe	8.08	4.94			8.5	~0
Phe	8.00	4.30			7.5	3.8×10^{-3}
Phe	7.70	4.30			7.5	3.2×10^{-3}
Phe	7.64	4.98			6.5	-0.8×10^{-3}
Val	7.45	4.61	2.18	1.01	8.0	-0.2×10^{-3}
Pro		4.52				
Pro		4.35				

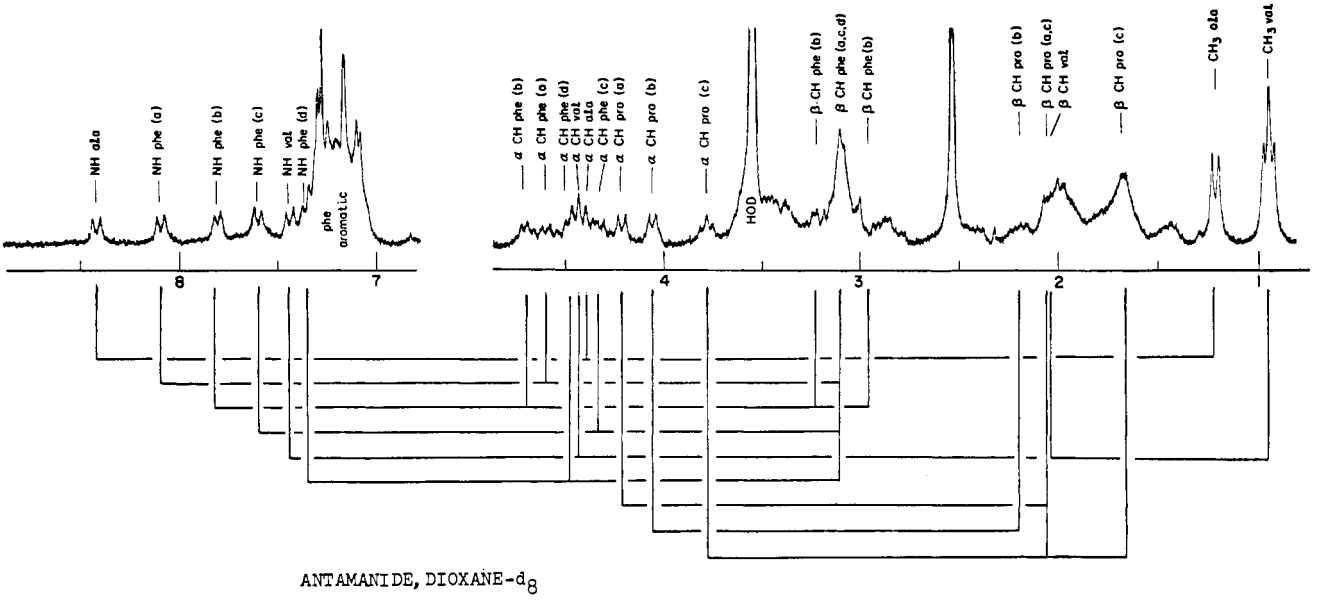


FIGURE 4: The proton nmr spectrum of antamanide in dioxane at 45°.

Attempts to generate an all-trans peptide bond conformation that met the intramolecular hydrogen bond and the rotation angle φ and ψ requirements outlined above were unsuccessful.

The above analysis predicts that antamanide conformation in nonaqueous weak hydrogen-bond acceptor solvents is represented by either 2,7-*cis*-I or 1,6-*cis*-I.

II. Nonaqueous Strong Hydrogen-Bond Acceptor Solvents

Antamanide was investigated in nonpolar dioxane and polar dimethylformamide. Both are good hydrogen-bond acceptor solvents.

The proton nmr spectrum of antamanide in dioxane-d_8 at 45° is shown in Figure 4. Decoupling studies permit the assignments shown below the spectra. The chemical shifts and coupling constants are summarized in Table V. The six peptide couplings, $J_{H^NH^\alpha}$, vary between 7.0 and 8.5 Hz. Two of the four proline H^α protons are doublets with $J_{H^\alpha H^\beta}$ = 8.0 and <1 Hz, while the remaining two C^α proline protons are multiplets. The temperature dependence of the peptide N protons of antamanide in dioxane-d_8 are plotted in Figure 5 and the slopes are tabulated in Table V. The slopes range from 2.4×10^{-3} (Val_1), 4.1×10^{-3} (Ala_4), and 3.6×10^{-3} (Phe, H^α at 4.61 and 4.71 ppm) to 5.6×10^{-3} (Phe, H^α at 4.35 and 4.48 ppm).

The temperature dependence of the peptide N protons of antamanide in dimethylformamide-d_7 are plotted in Figure 5. The slopes are in the range from 4.0 to 5.6×10^{-3} ppm/°C for the five observable residues.

The slopes in the strong hydrogen-bond acceptor solvents (dimethylformamide, dioxane) are much larger than in the weak hydrogen-bond acceptor solvents (CD_3CN, CD_3CO_2H). The temperature data in dimethylformamide and dioxane exhibit coefficients approaching $\gtrsim 6 \times 10^3$ ppm/°C, a condition for solvent exposure of peptide N protons. It is proposed that with increasing hydrogen-bond acceptor properties of solvent, conformation(s) with all peptide protons exposed to solvent are in equilibrium with the all-hydrogen-bonded antamanide conformation discussed in the last section.

A conformation containing cis peptide bonds between Pro_2-Pro_3 and Pro_7-Pro_8, and derived from 2,7-*cis*-I by rotation of the ψ angle at Pro_3 and Pro_8 from 150 to 300°, was investigated. The conformation, designated 2,7-*cis*-II, exhibits solvent-exposed protons and its rotation angles derived from molecular model building are summarized in Table VI.

A conformation containing cis peptide bonds between Val_1-Pro_2 and Phe_6-Pro_7, and derived from 1,6-*cis*-I by rotation of

TABLE III: Predominant Antamanide Conformation in Nonaqueous Weak Hydrogen-Bond Acceptor Solvents.[a]

	Val_1,Phe_6	Pro_2,Pro_7	Pro_3,Pro_8	Ala_4,Phe_9	Phe_5,Phe_{10}
2,7-*cis*-I φ	60	120	120	90	60
ψ	300	330	150	270	300
ω	0	180	0	0	0
1,6-*cis*-I φ	60	120	120	30	60
ψ	330	330	270	120	120
ω	180	0	0	0	0

[a] Rotation angles defined as in Edsall *et al.* (1966). The rotation angles φ, ψ, and ω are for conformations 2,7-*cis*-I and 1,6-*cis*-I, candidates for the antamanide conformation in poor hydrogen-bond acceptor solvents.

TABLE IV: Groups Involved in Intramolecular Hydrogen Bonds for Conformations 2,7-*cis*-I and 1,6-*cis*-I.

2,7-*cis*-I		1,6-*cis*-I	
N—H	O=C	N—H	O=C
Val_1	Ala_4	Val_1	Phe_6
Ala_4	Val_1	Ala_4	Phe_{10}
Phe_6	Phe_9	Phe_6	Val_1
Phe_9	Phe_6	Phe_9	Phe_5
Phe_5	Pro_3	Phe_5	Pro_2
Phe_{10}	Pro_8	Phe_{10}	Pro_7

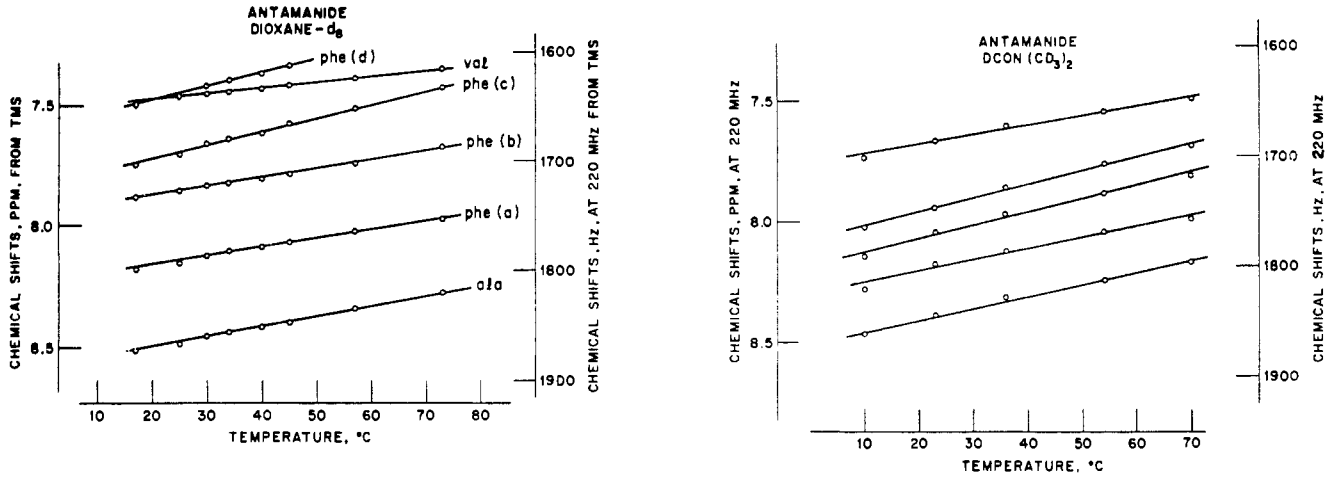


FIGURE 5: Temperature coefficient plots for antamanide in dioxane and dimethylformamide.

TABLE V: Proton Nmr Spectral Parameters for Antamanide in Dioxane (45°).

	H^N	H^α	H^β	H^γ	$J_{H^NH^\alpha}$	$J_{H^\alpha H^\beta}$	Temp Coeff (ppm/°C)
Ala	8.42	4.41	1.21		8.5		4.1×10^{-3}
Val	7.44	4.43	2.00	0.95	8.5		2.4×10^{-3}
Phe	8.10	4.61	3.08		8.3		3.6×10^{-3}
Phe	7.81	4.71	2.96, 3.26		7.0		3.7×10^{-3}
Phe	7.61	4.35	3.08		7.7		5.6×10^{-3}
Phe	7.36	4.48	2.82, 3.12		8.0		5.6×10^{-3}
Pro		4.21	2.10			8.0, <1	
Pro		4.06	2.23			8.0, <1	
Pro		3.78	2.06, 1.70			7.5, 7.5	
Pro		3.46	1.88, 1.66			9.0, 4.5	

the ψ angle at Pro_3 and Pro_8 from 270 to 150°, was investigated. The conformation, designated 1,6-*cis*-II, exhibits solvent-exposed protons and its rotation angles derived from molecular model building are summarized in Table VI.

A low-energy conformation with solvent-exposed peptide N protons and containing all-trans peptide bonds has been proposed previously by our group (Tonelli *et al.*, 1971).

Conformations 2,7-*cis*-II and 1,6-*cis*-II exhibit φ values consistent with $J_{H^NH^\alpha}$ = 7.0–8.4 Hz and ψ values for Val_1, Pro_2, Phe_6, and Phe_7 in the range 280–340° consistent with conformational maps of X residues in an X-Pro sequence. Since these conformations exhibit no intramolecular hydrogen bonds, they are flexible structures and the rotation angles in Table VI are representative of a conformation in a group of low-energy structures.

The above analysis predicts either conformation 2,7-*cis*-II or 1,6-*cis*-II to be the predominant antamanide conformation in nonaqueous strong hydrogen bond acceptor solvents.

TABLE VI: Predominant Antamanide Conformation in Nonaqueous Strong Hydrogen-Bond Acceptor Solvents.[a]

	Val_1,Phe_6	Pro_2,Pro_7	Pro_3,Pro_8	Ala_4,Phe_9	Phe_5,Phe_{10}
2,7-*cis*-II φ	30	120	120	60	90
ψ	300	300	330	300	270
ω	0	180	0	0	0
1,6-*cis*-II φ	30	120	120	90	30
ψ	300	330	120	300	330
ω	180	0	0	0	0

[a] Rotation angles defined as in Edsall *et al.* (1966). The rotation angles φ, ψ, and ω are for conformations 2,7-*cis*-II and 1,6-*cis*-II, candidates for the antamanide conformation in strong hydrogen-bond acceptor solvents.

III. Exchange Processes between Solvent-Dependent Antamanide Conformations

In the previous two sections it was suggested that the conformation of antamanide in nonaqueous solution was dependent on the hydrogen-bond acceptor properties of solvent. A rigid conformation (either 2,7-*cis*-I or 1,6-*cis*-I) held together by intramolecular hydrogen bonds of all six antamanide peptide N protons exists in weak hydrogen-bond acceptor nonaqueous solvents while a flexible conformation (either 2,7-*cis*-II or 1,6-*cis*-II) with all peptide N protons hydrogen bonded to solvent exists in strong hydrogen-bond acceptor nonaqueous solvents.

It is necessary to consider whether the solvent-dependent equilibrium I $\rightleftarrows$ II is in slow or fast exchange. Studies on the solvent dependence of the proton nmr spectra of *cyclo*(Pro-Gly-Ser)$_2$ (Torchia *et al.*, 1972b) and *cyclo*(Pro-Gly)$_3$ (Deber

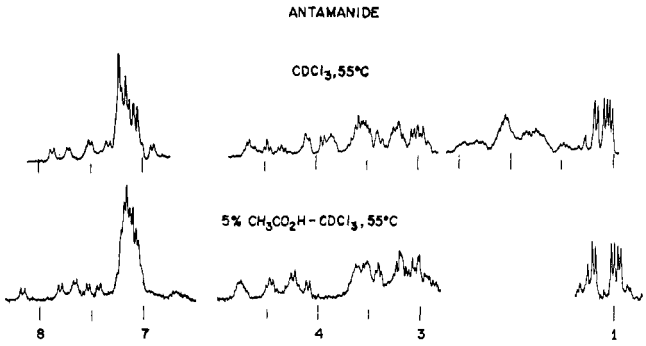


FIGURE 6: The proton nmr spectra of antamanide in chloroform and 5% acetic acid–chloroform.

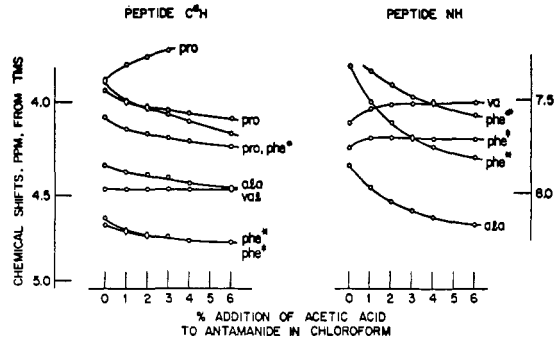


FIGURE 7: Plots of chemical-shift changes for N and C^{α} antamanide protons in chloroform on gradual addition of acetic acid.

et al., 1971) established that separate sharp nmr spectra are observed under conditions of slow exchange between conformations with cis peptide X-Pro bonds and those conformations with trans peptide X-Pro bonds. These features are consistent with the barrier to cis–trans peptide bond isomerization of ~20 kcal/mol. By contrast, average spectra consistent with rapid exchange among conformations are observed when rotation angle changes are located at φ,ψ values only (Torchia *et al.*, 1972a,b; Patel and Tonelli, 1973).

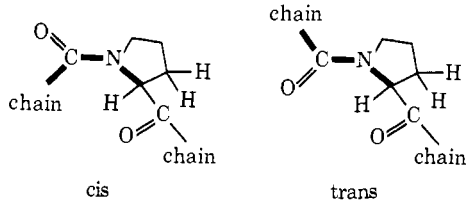
Addition of CD_3CO_2H to antamanide in $CDCl_3$ results in large changes in the H^{α} chemical shifts (Figures 6 and 7). (By contrast, addition of *i*-PrOH to antamanide in $CDCl_3$ had no effect on the H^{α} chemical shifts.) This suggests an acid-dependent variation in the populations of conformations I and II in the equilibrium I $\rightleftarrows$ II for antamanide in nonaqueous solution. The gradual addition of CD_3CO_2H from 0 to 6% to antamanide in $CDCl_3$ results in shifts of the individual resonances without doubling of their spectra (Figures 6 and 7).

The N, H^{α}, H^{β}, and CH_3 protons of the individual amino acids of antamanide in a particular solvent ($CDCl_3$, CD_3CN, CD_3CO_2H, dioxane, and dimethylformamide) do not show doubling of spectra.

The data are consistent with a rapid equilibrium for I $\rightleftarrows$ II without cis–trans peptide bond isomerization in nonaqueous solvents.

IV. The X-Pro Peptide Bond

Trans peptide bonds have been observed for linear peptides containing α-amino acids. For imino acids, like proline, the X-Pro peptide bond can be cis or trans.



Ramachandran *et al.* (1970) showed that cyclization of a peptide was facilitated by the presence of either D-amino acid(s) or glycine(s) in an L-amino acid sequence. The cyclic decapeptide antamanide contains neither D-amino acids nor glycines. However, it contains a high proline content and cyclization may be facilitated by the presence of cis peptide bonds.

In our earlier contribution (Tonelli *et al.*, 1971), *N*-acetyl-Pro-methyl ester was used as a model system for CD studies. For each trans X-Pro peptide bond, a contribution of $[\theta]_M = -20{,}000$ to $-30{,}000$ deg cm²/dmol for the π–π^* 190–205-nm band was deduced. Since antamanide in a series of solvents exhibited $[\theta]_M \sim -150{,}000$ deg cm²/dmol in the 190–205-nm region it was suggested that all four X-Pro peptide bonds were trans. Conformational analysis of antamanide in solution was therefore restricted to trans X-Pro bonds (Tonelli *et al.*, 1971). Faulstich *et al.* (1972) have reported on the synthesis and CD spectra of perhydroantamanide in solution. (The phenyl rings are reduced in perhydroantamanide.) For perhydroantamanide in methanol, $[\theta]_M = -70{,}000$ deg cm²/dmol in 195–210-nm region, suggesting that half the rotation observed for antamanide in solution in the $\pi \rightarrow \pi^*$ region originates in the four aromatic rings.

The CD data no longer support the premise that all four X-Pro peptide bonds in antamanide are trans (Tonelli *et al.*, 1971), and molecular model building and conformational calculations must consider cis X-Pro peptide bonds for antamanide conformations in solution.

The proline H^{α} proton chemical shift along with its splitting pattern due to coupling to H^{α} protons are characteristic for cis and trans X-Pro peptide bonds.

Cyclotriproline contains only cis peptide bonds and its spectrum in CD_2Cl_2 gave an H^{α} doublet at 5.05 ppm, $J_{H^{\alpha}H^{\beta}} = 7.0$ and 1.4 Hz (Deber *et al.*, 1971). The X-Pro peptide bond in evolidine has been assigned a cis configuration and exhibits an H^{α} doublet at 4.26 ppm, $J_{H^{\alpha}H^{\beta}} = 8.0$ and 0 Hz (Kopple, 1971). By contrast, polyproline II in D_2O which contains only trans peptide bonds gave an H^{α} multiplet at 4.7 ppm, $J_{H^{\alpha}H^{\beta}} = 8.5$ and 5.5 Hz (Torchia, 1971).

cyclo(Pro-Gly-Ser)$_2$ in Me_2SO and D_2O exhibits two sets of spectra for conformations containing cis peptide X-Pro and trans peptide X-Pro bonds in slow exchange. The cis X-Pro peptide bond conformation exhibits an H^{α} proline doublet at 4.75 ppm while the trans X-Pro peptide bond conformation exhibits an H^{α} proline multiplet at 4.35 ppm (Torchia *et al.*, 1972a). *cyclo*(Pro-Gly)$_3$ in CD_2Cl_2 exhibits cis X-Pro peptide bonds and the 4.75-ppm proline H^{α} resonance is a doublet. *cyclo*(Pro-Gly)$_3$–Na in Me_2SO exhibits trans X-Pro peptide bonds and the 4.35-ppm proline H^{α} resonance is a multiplet (Deber *et al.*, 1971). The chemical shift and multiplicity of proline H^{α} resonances are summarized in Table VII.

In summary, for a conformation containing cis and trans X-Pro peptide bonds, the proline H^{α} resonance is a doublet for the cis X-Pro peptide bond and a multiplet at higher field for the trans X-Pro peptide bond.

In the nmr study of antamanide in solution, the investigations were undertaken in the solvents CD_3CO_2H, CD_3CN,

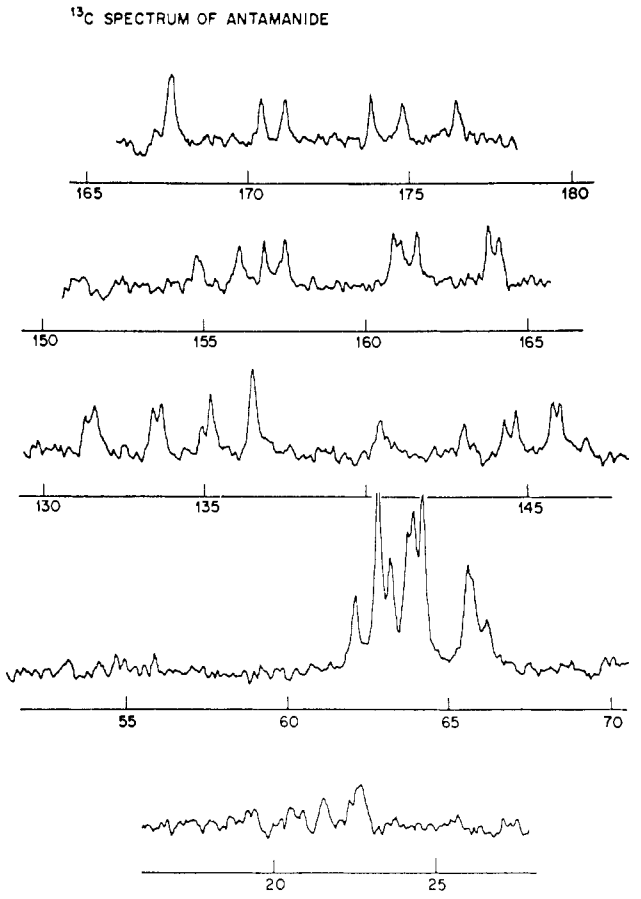


FIGURE 8: The heteronoise proton-decoupled ^{13}C nmr spectrum of antamanide in CD_3CN at 50°.

TABLE VII: Compilation of Proline H^{α} Chemical Shifts and Multiplicity for Proline-Containing Peptides.

Compound	Solvent	Proline H^{α} Chem Shift (ppm) Cis[a]	Trans[b]
cyclo(Pro-Gly-Ser)$_2$	D_2O		4.4
	Me_2SO		4.2
cyclo(Pro-Ser-Gly)$_2$	D_2O		4.4
	Me_2SO	4.7	4.25
cyclo(Pro-Pro-Pro)	CD_2Cl_2	5.05	
Poly(L-proline)-II	D_2O		4.7
t-Boc-Gly-Pro-OH	Me_2SO	4.5	4.25
(*S*-Bzl)-Cys-Pro-Leu-Gly-NH_2	Me_2SO	4.6	4.4
Evolidine	Me_2SO	4.26	
	Dimethyl-formamide	4.41	
Poly(Pro-Gly)	D_2O–CD_3CO_2D	4.6	4.35
	Me_2SO	4.5	4.4
Poly(Gly-Gly-Pro-Gly)	D_2O–CD_3CO_2D	4.6	4.4
	Me_2SO	4.5	4.3
Actinomycin D		3.9–4.3[c]	
cyclo(Gly-Pro)$_3$	CD_2Cl_2	4.75	
cyclo(Gly-Pro)$_3$-Na	Me_2SO		4.35

[a] H^{α} resonance is a doublet. [b] H^{α} resonance is a multiplet. [c] Fine structure of C^{α} resonance undetermined.

dioxane-d_8, $CDCl_3$, and C_6D_6. In all these solvents, two proline C^{α} resonances were doublets ($J_{H^{\alpha}H^{\beta}}$ = 8 and <1 Hz) and came downfield from the remaining two proline C^{α} resonances which were multiplets (see Table I and Table V for data in CD_3CN and dioxane-d_8). *The proline H^{α} splitting pattern in the proton nmr spectra strongly suggests that two X-Pro peptide bonds are cis and two are trans for antamanide in nonaqueous solution.*

The heteronoise proton-decoupled ^{13}C spectrum of antamanide in CD_3CN at 50° is outlined in Figure 8. Chemical shifts are referenced relative to CS_2 and assignments based on comparison with amino acid spectra. The assignments are summarized in Table VIII.

From a study of a series of proline-containing peptides with cis and trans X-Pro amide bonds, Dorman and Bovey (1972)[1] noted that the C^{β} and C^{γ} proline chemical shifts reflected the geometry of the peptide bond. The model systems used were *N*-acetylproline (cis and trans), *N*-acetylproline methyl ester (cis and trans), *N*-acetylproline amide (cis and trans), Gly-Pro (cis and trans), *cyclo*(Pro-Ser-Gly)$_2$ (trans), and polyproline II (trans). It should be noted that the geometry at the Pro ψ angle (*i.e.*, cis′ or trans′) may also have to be considered. In the above model systems Pro ψ is trans′.

For cis and trans X-Pro peptide bonds, the proline C^{γ} resonances have chemical shifts at 170.4 ± 0.3 and 168.5 ± 0.3 ppm, respectively, in the model systems. This permits assignment of antamanide proline C^{γ} resonances at 170.4 and 171.2 ppm to cis X-Pro peptide bonds while two proline C^{γ} resonances at 167.6 ppm are assigned to trans X-Pro peptide bonds (Table VIII).

For cis and trans X-Pro peptide bonds, the proline C^{β} resonances have chemical shifts at 161.5 ± 0.4 and 163.8 ± 1.1 ppm, respectively, in the model systems. This permits assignment of antamanide proline C^{β} resonances in the region 161.2 ± 0.4 ppm to cis X-Pro peptide bonds while the Pro C^{β} resonances at 163.7 and 164.0 ppm are assigned to trans X-Pro peptide bonds (Table VIII).

The ^{13}C nmr data strongly suggest that two X-Pro peptide bonds are cis and two are trans for antamanide in CD_3CN.

V. Discussion of Conformations

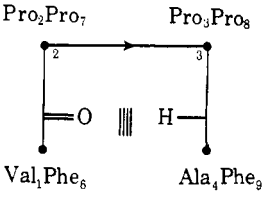
The solvent-dependent conformational behavior of antamanide in nonaqueous solution can be represented as one of two possibilities: 2,7-*cis*-I ⇄ 2,7-*cis*-II; 1,6-*cis*-I ⇄ 1,6-*cis*-II. The equilibrium shifts toward II in strong hydrogen-bond acceptor solvents. Some features of these conformations are discussed below.

The peptide NH resonance of Ala_4 in antamanide in nonaqueous solvents comes downfield from the remaining peptide NH resonances (Figures 1, 4, and 6). For conformation 2,7-*cis*-I, Ala_4 peptide NH forms an intramolecular type 1 ← 4 hydrogen bond. It has been observed that protons participating in type 1 ← 4 intramolecular hydrogen bonds come upfield from the remaining peptide NH protons (Urry and Ohnishi, 1970; Torchia *et al.*, 1972a,b). Under these conditions, the Ala_4 peptide NH in the 2,7-*cis*-I conformation would be expected to come at high fields in direct contradiction with experiment.

[1] Dorman, D., and Bovey, F. A. (1972), manuscript in preparation.

TABLE VIII: ^{13}C Chemical Shifts (Ppm Relative to CS_2) for Antamanide in CD_3CN at 50°.

CH_3, Ala,Val	176.5, 174.8, 173.8
C^{γ}, Pro	171.2, 170.4, 167.6, 167.6
C^{β}, Pro	164.0, 163.7
C^{β}, Pro,Val	161.6, 161.0, 160.8
C^{β}, Phe	157.5, 156.9, 156.1, 154.7
C^{δ}, Pro	145.9, 145.8, 144.7, 144.3
C^{α}, Ala,Phe	143.1, 140.5
C^{α}, Phe	136.5, 136.5
C^{α}, Phe,Val	135.2, 134.9
C^{α}, Pro	133.8, 133.5, 131.7, 131.5



Antamanide forms a complex with Na ion in nonaqueous solvents. The metal ion coordinates with the carbonyl groups of the cyclic peptide. Thus a cluster of carbonyls in the noncomplexed conformation may be a prerequisite for complexation. In conformation 2,7-*cis*-I there is no cluster of carbonyl groups (plate 1) while a set of four carbonyls in a cluster in 1,6-*cis*-I may serve as a recognition site for Na complexation (plate 2).

A definitive selection between these two possibilities can be approached by incorporation of proline-H^{α}-d_1 at either positions 2 and 7 or 3 and 8. Identification of Pro H^{α} proton doublets with particular proline residues would permit location of the cis peptide bonds.

VI. Conformation trans-M

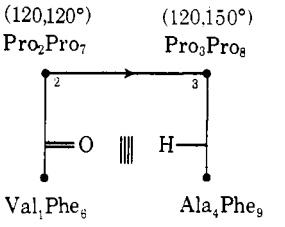
An Evaluation. Ivanov *et al.* (1971) have proposed an antamanide conformation in nonpolar media, designated *trans*-M. All peptide bonds are trans, and Faulstich *et al.* (1972) point out that the peptide bond between Val_1-Pro_2 and Phe_6-Pro_7 departs 30° from the planar trans configuration for this conformation. The rotation angles have not been reported but can be deduced from a CPK model of conformation *trans*-M built from a drawing reported by Faulstich *et al.* (1972). They are shown in Table IX.

A value of $\psi = 310°$ is predicted from energy calculations for an L-prolyl residue succeeded by another L-prolyl residue and observed experimentally for polyproline I, polyproline II, and model fragments containing proline sequences (Cowan and McGavin, 1955; Sasisekharan, 1959; Schimmel and Flory, 1968). Thus, the assignment $\psi_{Pro_2} = \psi_{Pro_7} = 120°$ for conformation *trans*-M suggests a very high conformational energy. By contrast Go and Scheraga (1970) have pointed out that the first Pro in a Pro-Pro sequence may exhibit $\psi = 120°$ if a flexible geometry is permitted for the Pro ring.

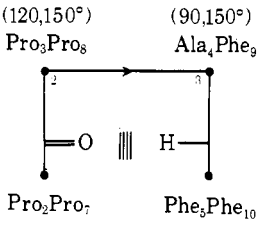
There are two sets of bends in conformation *trans*-M stabilized by type 1 ← 4 intramolecular hydrogen bonds. The first set involves two proline residues at positions 2 and 3 with a trans peptide bond between them. Venkatachalam (1968) has shown that a 1 ← 4 bend can be made with φ,ψ values of 120,150 and 120,150° for positions 2 and 3 respectively. However, this holds only if there are nonproline residue at positions 3 and 4 so that ψ values at positions 2 and 3 can assume values of ~150°. For conformation *trans*-M, Pro_3 and Pro_8 occupy position 3 of a type 1 ← 4 bend.

TABLE IX

	Pro_2,Pro_7	Pro_3,Pro_8	Ala_4,Phe_9	Phe_5,Phe_{10}	Val_1,Phe_6
φ, deg	120	120	90	250	240
ψ, deg	120	150	150	90	300



The second set of type 1 ← 4 bends in *trans*-M is shown below. For L-amino acids, trans peptide bonds and proline



(φ,ψ = 120,150°) at position 2, conformational calculations (Venkatachalam, 1968) predict a type I bend with φ,ψ = 60,240° at position 3. It is apparent that φ,ψ = 90,150° for Ala_4,Phe_9 at position 3 in conformation *trans*-M does not meet this requirement. Thus both bends stabilized by type 1 ← 4 intramolecular hydrogen bonds in conformation *trans*-M may be energetically unfavorable.

The peptide N protons of Ala_4, Phe_9, Phe_5, and Phe_{10} form short linear type 1 ← 4 intramolecular hydrogen bonds and the peptide N protons of Val_1 and Phe_6 form weak type 1 ← 3 intramolecular hydrogen bonds in conformation *trans*-M. Experimentally, Ala_4, Val_1, and two Phe residues (presumably Phe_9 and Phe_6 by symmetry) have slopes of $<2 \times 10^{-3}$ ppm/°C while the remaining two Phe residues (presumably Phe_5 and Phe_{10}) exhibit slopes of $\sim 4 \times 10^{-3}$ ppm/°C for antamanide in poor hydrogen-bond acceptor nonaqueous solvents. Thus antamanide conformation *trans*-M does not meet the requirements set by the temperature coefficient data.

The φ,ψ values for Phe_5 and Phe_{10} are 250,90° for conformation *trans*-M. Approximate intramolecular potential energy calculations (Brant and Flory, 1965; DeSantis *et al.*, 1965; Ramakrishnan and Ramachandran, 1965) do not predict an energy minimum in this region of the conformational energy map. By contrast, approximate quantum mechanical calculations (Pullman and Maigret, 1972) and calculations by Crippen and Scheraga (1969) suggest an energy minimum at φ,ψ (250,90°) for L-amino acid residues.

Finally, conformation *trans*-M exhibits two nonplanar trans peptide bonds. Winkler and Dunitz (1971) have evaluated the effect of small departures from the planarity of the

peptide bonds. A departure of 30° from planarity, however, only serves to raise the conformational energy of structure *trans*-M.

Summary of Conclusions

The solution conformations of antamanide in nonaqueous solution have been reinvestigated. The temperature coefficients of the peptide NH resonances in poor hydrogen-bond acceptor solvents suggested a conformation involving four strong and two weak intramolecular hydrogen bonds while the coefficients in strong hydrogen-bond acceptor solvents suggested a conformation with solvent-exposed peptide protons. The observation of average proton nmr spectra suggests rapid equilibrium among conformations and rules out cis–trans peptide bond isomerization. The multiplicity of the H^{α} proline protons and the carbon chemical shifts of the C^{γ} and C^{β} proline residues in antamanide are consistent with two cis and two trans X-Pro peptide bonds. Low-energy conformations constructed from model building were generated with cis peptide bonds between Phe_6-Pro_7 and Val_1-Pro_2 (conformations 1,6-*cis*-I $\rightleftarrows$ 1,6-*cis*-II) and also with cis peptide bonds between Pro_7-Pro_8 and Pro_2-Pro_7 (conformation 2,7-*cis*-I $\rightleftarrows$ 2,7-*cis*-II). These conformations exhibited φ values consistent with $J_{H^N H^{\alpha}}$ coupling constants, ψ values in the range 280–340 for the X residue in the X-Pro sequence, intramolecular hydrogen bonds suggested from temperature coefficient and exchange data, and rotation angles consistent with low-energy regions of conformational maps. These conformations are defined in terms of φ, ψ, and ω rotation angles and photographs of CPK models presented. The experimental evidence appears to be more consistent with the conformations 1,6-*cis*-I $\rightleftarrows$ 1,6-*cis*-II, containing cis peptide bonds at Val_1-Pro_2 and Phe_6-Pro_7, for antamanide in nonaqueous solution. The conformation for antamanide in nonpolar media suggested by Ivanov *et al.* (1971) and Faulstich *et al.* (1972) is reviewed.

Acknowledgments

Antamanide was generously provided by Professor Th. Wieland. Discussions with Professor Th. Wieland and Professor Yu. Ovchinnikov were most helpful.

References

Barfield, M., and Karplus, M. (1969), *J. Amer. Chem. Soc. 91*, 1.

Brant, D. A., and Flory, P. J. (1965), *J. Amer. Chem. Soc. 87*, 2791.

Brant, D. A., Miller, W. G., and Flory, P. J. (1967), *J. Mol. Biol. 23*, 47.

Cowan, P. M., and McGavin, J. (1955), *Nature (London) 716*, 501.

Crippen, G. M., and Scheraga, H. A. (1969), *Proc. Nat. Acad. Sci. 64*, 42.

Deber, C. M., Torchia, D. A., and Blout, E. R. (1971), *J. Amer. Chem. Soc. 93*, 4893.

DeSantis, P., Giglio, E., Liquori, A. M., and Ripamonti, A. (1965), *Nature (London) 206*, 456.

Edsall, J. T., Flory, P. J., Kendrew, J. C., Liquori, A. M., Nemethy, G., Ramachandran, G. N., and Scheraga, H. A. (1966), *Biopolymers 4*, 121.

Faulstich, H., Burgermeister, W., and Wieland, Th. (1972), *Biochem. Biophys. Res. Commun. 47*, 975.

Go, N., and Scheraga, H. A. (1970), *Macromolecules 3*, 188.

Hodgkin, D. C., and Oughton, B. M. (1957), *Biochem. J. 65*, 752.

Ivanov, V. T., Miroshnikov, A. I., Abdullaev, N. D., Senyavina, L. B., Arkhipova, S. F., Uvarova, N. N., Khalilulina, K. Kh., Bystrov, V. F., and Ovchinnikov, Yu. A. (1971), *Biochem. Biophys. Res. Commun. 42*, 652.

Kopple, K. D. (1971), *Biopolymers 10*, 1139.

Kopple, K. D., Ohnishi, M., and Go, A. (1969a), *J. Amer. Chem. Soc. 91*, 4264.

Kopple, K. D., Ohnishi, M., and Go, A. (1969b), *Biochemistry 8*, 4087.

Ovchinnikov, Yu. A., Ivanov, V. T., Bystrov, V. F., Miroshnikov, A. I., Shepel, E. N., Abdullaev, N. D., Efremov, E. S., and Senyavina, L. B. (1970), *Biochem. Biophys. Res. Commun. 39*, 217.

Patel, D. J. (1973), *Biochemistry 12*, 677.

Patel, D. J., and Tonelli, A. E. (1973), *Biochemistry 12*, 486.

Pullman, B., and Maigret, B. (1972), Conference on Conformation of Biological Molecules and Polymers, Jerusalem, Israel, April 3–9.

Ramachandran, G. N., Chandrasekaran, R., and Kopple, K. D. (1970), Second American Peptide Symposium, Cleveland, Ohio, August 17–19, paper No. 28.

Ramachandran, G. N., Vankatachalam, C. M., and Krimm, S. (1966), *Biophys. J. 6*, 849.

Ramakrishnan, C., and Ramachandran, G. N. (1965), *J. Biophys. 5*, 909.

Sasisekharan, V. (1959), *Acta Crystallogr. 12*, 897.

Schimmel, P. R., and Flory, P. J. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 52.

Schimmel, P. R., and Flory, P. J. (1968), *J. Mol. Biol. 34*, 105.

Schwyzer, R. (1959), *Rec. Chem. Progr. 20*, 146.

Schwyzer, R., and Ludescher, U. (1968), *Biochemistry 7*, 2519.

Sobell, H. M., Jain, S. C., Sakore, T. D., and Nordman, C. E. (1971), *Nature (London), New Biol. 231*, 200.

Stern, A., Gibbons, W. A., and Craig, L. C. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 735.

Sternlicht, H., and Zuckerman, D. (1972), *Rev. Sci. Instrum. 43*, 525.

Tonelli, A. E., Patel, D. J., Goodman, M., Naider, F., Faulstich, H., and Wieland, Th. (1971), *Biochemistry 10*, 3211.

Torchia, D. A. (1971), *Macromolecules 4*, 440.

Torchia, D. A. (1972), *Biochemistry 11*, 1462.

Torchia, D. A., diCorato, A., Wong, S. C. K., Deber, C. M., and Blout, E. R. (1972a), *J. Amer. Chem. Soc. 94*, 609.

Torchia, D. A., Wong, S. C. K., Deber, C. M., and Blout, E. R. (1972b), *J. Amer. Chem. Soc. 94*, 616.

Urry, D. W., and Ohnishi, M. (1972), Spectroscopic Approaches to Biomolecular Conformation, Urry, D. W., Ed., Chicago, Ill., American Medical Association Press, p 263.

Venkatachalam, C. M. (1968), *Biopolymers 6*, 1425.

Winkler, F. K., and Dunitz, J. D. (1971), *J. Mol. Biol. 59*, 169.

Wuthrich, K., Tun-Kyi, A., and Schwyzer, R. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 25*, 104.