STRUCTURE ELUCIDATION OF A58365A AND A58365B, ANGIOTENSIN CONVERTING ENZYME INHIBITORS PRODUCED BY *STREPTOMYCES CHROMOFUSCUS*†

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A58365A and A58365B, angiotensin converting enzyme inhibitors isolated from the culture filtrate of *Streptomyces chromofuscus* NRRL 15098, are homologous compounds of molecular formulas $C_{12}H_{13}NO_6$ and $C_{13}H_{15}NO_6$. The molecular similarities of the two inhibitors were established by comparison of their ^1H NMR, ^{13}C NMR, and UV spectra. Catalytic hydrogenation of A58365A led to a tetrahydro-deoxy derivative, $C_{12}H_{17}NO_5$; extensive ^1H NMR decoupling studies at 360 MHz allowed all the non-exchangeable protons of the derivative to be connected in a continuous substructure. This fragment was combined with information from other spectroscopic methods to suggest the structures for A58365A (**1**) and A58365B (**2**); the conclusions were confirmed by an X-ray crystallographic analysis of A58365A-dimethyl ester.

Several inhibitors of angiotensin converting enzyme (ACE) have been discovered from fermentation sources[2], many of these inhibitors exert their effect primarily through chelation of the Zn^{2+} in the metallodipeptidase (aspergillomarasmines A and B, L-681,176, and phenacein). Previous papers from these laboratories have described the development of a high-volume, agar-based screen for the detection of ACE inhibitors produced by fermentations and the discovery of ACE inhibitory activity in the culture broth of *Streptomyces chromofuscus* NRRL 15098[3], the conditions for the biosynthesis of the ACE inhibitors A58365A and A58365B produced by this microorganism[4], and the isolation and characterization of the two ACE inhibitors from A58365[5]. A58365A and A58365B inhibition of ACE is not reversible by addition of divalent cations[3], hence strong chelation is not characteristic of these inhibitors. Furthermore, the structures of these novel fermentation products have provided a unique insight into the stereochemical requirements of captopril-related ACE inhibitors. A58365A has been determined to be 3-carboxy-1,2,3,5-tetrahydro-8-hydroxy-5-oxo-6-indolizinepropanoic acid (**1**); A58365B is the homologous 4-carboxy-1,3,4,6-tetrahydro-9-hydroxy-6-oxo-2*H*-quinolizine-7-propanoic acid (**2**). A58365A is thus a naturally occurring conformationally restricted analog of 2-methylglutaryl-L-proline (**3**), which was a part of the structure-activity relationship studies leading to captopril (**4**)[6].

Homology of A58365A and A58365B

The preliminary characterization of A58365A and A58365B indicated that the two compounds are homologs, having the molecular formulas $C_{12}H_{13}NO_6$ for A58365A (*m/z* 268.08189 by fast atom

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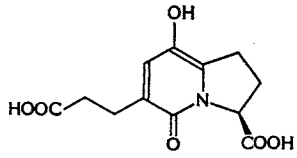
Fig. 1. Structures of angiotensin converting enzyme inhibitors A58365A (**1**), A58365B (**2**), 2-methyl-glutaryl-L-proline (**3**), and captopril (**4**).



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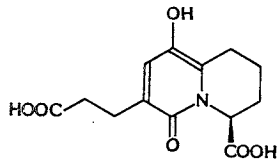

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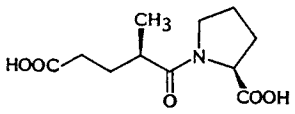

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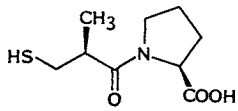

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Table 1. Comparison of ^{1}H (360 MHz) and ^{13}C (67.9 MHz) NMR parameters for A58365A and A58365B in D_2O solution.

A58365A substructures			A58365B substructures		
	^{1}H (δ)	^{13}C (δ)		^{1}H (δ)	^{13}C (δ)
-CH	5.01	63.80 d	-CH	5.10	58.10 d
CH_2	2.57, 2.30	26.76 t	CH_2	2.35, 2.08	26.05 t
$-CH_2$	3.15, 3.05	27.66 t	CH_2	1.86, 1.67	16.05 t
			$-CH_2$	2.93, 2.76	26.35 t
$-CH_2$	2.81, 2.74	25.66 t	$-CH_2$	2.78	23.40 t
$-CH_2$	2.64	33.19 t	$-CH_2$	2.66	33.44 t
-CH=	7.33	134.73 d	-CH=	7.35	132.99 d
Other carbon resonances		128.53 s 135.19 s 136.06 s 159.86 s 174.41 s 177.89 s			126.54 s 132.89 s 137.11 s 161.70 s 177.10 s 178.60 s

bombardment mass spectrometry (FAB-MS); calculated for M+H ($C_{12}H_{14}NO_6$) 268.08211) and $C_{13}H_{15}NO_6$ for A58365B (*m/z* 281.09035 by electron impact mass spectrometry (EI-MS); calculated for M^+ 281.08994)[5]. The homology of the two materials was characterized further by examining the ^{1}H and ^{13}C NMR spectra of each inhibitor. Homonuclear decoupling experiments on A58365A in D_2O solution accounted for 10 of the 13 protons, in three substructures:

$$\underset{(5.01)}{-CH}\!\!-\!\!\underset{\binom{2.57,}{2.30}}{CH_2}\!\!-\!\!\underset{\binom{3.15,}{3.05}}{CH_2}\!\!-, \qquad \underset{\binom{2.81,}{2.74}}{-CH_2}\!\!-\!\!\underset{(2.64)}{CH_2}\!\!-, \quad \underset{(7.33)}{-CH=}$$

The two-proton triplet near 2.64 ppm shifts with small changes in pH; the other resonances are less sensitive to such environmental changes. The same three substructures were found for A58365B,

except that the three-carbon fragment is lengthened by addition of a new methylene group to form $CHCH_2CH_2CH_2$. ^{13}C NMR spectra were obtained for each inhibitor in D_2O solution, and single-frequency irradiations were used to obtain the carbon-proton correlations given in Table 1.

The data in Table 1 indicate the close similarity between A58365 factors A and B. The equivalence or near equivalence of the protons of each methylene in the CH_2CH_2 units suggests that these fragments are not constrained in the overall structures, while the non-equivalence for each methylene in the three carbon (for A) or four-carbon (for B) substructures suggests that these fragments are in small rings. The carbon chemical shift of the CH of these substructures is similar to that for the α-carbon of an amino acid, such as proline (and indeed proline addition has been shown to stimulate production of ACE inhibitory activity by the NRRL 15098 fermentation[4]). If the two carbon resonances in the 170~180 ppm range for each inhibitor are assumed to be COOH groups, then the data in Table 1 account for $C_{12}H_{12}O_4$ for A58365A, and for $C_{13}H_{14}O_4$ for A58365B; each formula still lacks one N, two O's and one exchangeable H. Further structural studies were focused on A58365A only.

Chromophore Studies

Both A58365A and A58365B have a long-wavelength absorbance maximum near 330 nm which shifts to near 360 nm in the presence of base[5]. Several derivatives or model compounds were prepared and characterized by UV and NMR spectroscopies; the UV absorbance results for these products are collected in Table 2.

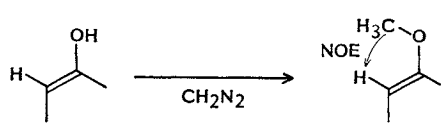
A58365A-dimethyl Ester (5)

Esterification of A58365A in acidic methanol produced a new material having an increase in mass of 28 daltons (MW 295, $C_{14}H_{17}NO_6$), supporting the earlier suggestion of two COOH groups in the parent compound. The ^1H NMR spectrum of **5** is similar to that for A58365A, with the addition of two new three-proton singlets; no nuclear Overhauser effects (NOE's) were observed in other parts of the spectrum when either of the CH_3 resonances was irradiated. The ^{13}C NMR spectrum of **5** also resembles that of A58365A, plus two new carbon resonances at 52.5 and 51.3 ppm. The UV spectrum of **5** in methanol indicates that the esterification causes no change in the chromophore; the long-wavelength absorbance maximum at 333 nm shifts to 362 nm in basic solution.

Trimethyl-A58365A (6)

Treatment of A58365A with excess CH_2N_2 in methanol for 16 hours generated a new trimethyl derivative (MW 309, $C_{15}H_{19}NO_6$); the UV spectrum of this material in both methanol and basic methanol has λ_{max} 330 nm. The ^1H NMR spectrum of **6** in $CDCl_3$ contains three CH_3 singlets at 3.79, 3.74, and 3.65 ppm; comparison with the spectrum of **5** suggests that the new CH_3 on the chromophore is represented by the peak at 3.74 ppm. The ^{13}C NMR spectrum of trimethyl-A58365A in $CDCl_3$ contains methyl resonances at 58.9, 52.5, and 51.3 ppm, confirming that the new CH_3 is attached to oxygen. Irradiation of the 3.74 ppm proton peak sharpens the 58.9 ppm carbon resonance in a selectively-decoupled ^{13}C spectrum; irradiation of the 3.74 ppm proton peak produced a strong NOE at the 7.3 ppm CH= resonance in the ^1H NMR spectrum of **6**. These results show that the titratable group on the A58365A chromo-

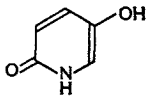
Scheme 1.



A58365A Trimethyl-A58365A

Table 2. UV spectroscopy of A58365A, A58365B, and related compounds.

Compound	λ_{max} (MeOH) nm (ε)	λ_{max} (basic MeOH) nm (ε)
A58365A (**1**)	325 (7,600), 232 (6,000)	353 (7,400), 243 (7,200)
A58365B (**2**)	332, 232	360, 243
Dimethyl-A58365A (**5**)	333, 235	362, 246
Trimethyl-A58365A (**6**)	330, 232	330, 230
5-Hydroxy-2-pyridone (**7**)	329 (5,120), 233 (6,340)	356 (5,120), 247
5-Hydroxy-6-methyl-2-pyridone (**8**)	333 (5,670), 231 (6,340)	362 (5,670), 245, 218



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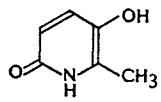

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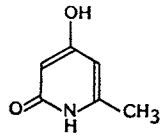

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phore is a phenol-like OH, adjacent to the single downfield proton site: (See Scheme 1).

When these conclusions are combined with the results in Table 1, only one oxygen and one nitrogen out of the $C_{12}H_{13}NO_6$ remain to be accounted for. The data collected thus far (five unsaturated non-carboxyl carbons, a phenol-like OH, plus nitrogen and another oxygen) suggest that the A58365A chromophore may be a hydroxypyridone.

5-Hydroxy-2-pyridone (**7**) and 5-Hydroxy-6-methyl-2-pyridone (**8**)

These model compounds were prepared according to published methods[7] and were fully characterized by ^1H and ^{13}C NMR spectroscopy (see Experimental section). Both display a long-wavelength absorbance maximum near 330 nm in methanol solution which shifts to near 360 nm in the presence of base, as listed in Table 2. The 5-hydroxy-2-pyridone chromophore thus provides a close match for the UV absorbance of A58365 factors A and B; in contrast, the long-wavelength λ_{max} for 4-hydroxy-6-methyl-2-pyridone (**9**), an isomer of **8**, is 282 nm[8].

Tetrahydro-deoxy-A58365A (**10**)

$$\underset{C_{12}H_{13}NO_6\ (MW\ 267)}{\text{A58365A (1)}} \xrightarrow[\text{Glacial acetic acid}]{H_2/PtO_2} \underset{C_{12}H_{17}NO_5\ (MW\ 255)}{\textbf{10}}$$

Catalytic hydrogenation of A58365A produced a white hygroscopic material which appeared to be a single compound by ^1H NMR, ^{13}C NMR, and HPLC analysis. The molecular weight of 255 was obtained by both field desorption (FD) and EI-MS, indicating that the reduction product had lost one oxygen and gained four hydrogens from A58365A; one non-carboxyl double bond remained unsaturated.

The 360 MHz ^1H NMR spectrum of **10** in D_2O solution is shown in Fig. 2; there are three CH-proton resonances near 4.3, 3.6, and 2.4 ppm, and there is one equivalent CH_2 group with a triplet resonance near 2.5 ppm. The remainder of the spectrum occurs between 2.3 and 1.5 ppm and contains five overlapped and non-equivalent methylenes. The connectivity of these peaks was traced

out using an extensive series of homonuclear decouplings at 360 MHz, as will be described below.

The ^{13}C NMR spectrum of **10** in D_2O contained twelve resonances; these are listed in Table 3. In addition to the two COOH groups previously identified for A58365A, **10** exhibits a third carbon resonance in the 175~180 ppm range. The carbons corresponding to the three CH's and the one equivalent CH_2 were assigned through the use of selective decoupling, as indicated in Table 3. The assigned CH_2 (^1H 2.49 ppm, ^{13}C 32.69 ppm) resembles the pH-dependent CH_2 in A58365A (^1H 2.64 ppm, ^{13}C 33.19 ppm; see Table 1), but few other correlations between the two molecules are clear.

The structure of **10** was established on the basis of a series of decoupling experiments on the spectrum in Fig. 2. The spectra were acquired at 0.244 Hz/point and plotted at 9 Hz/cm, allowing the detection of small changes in highly overlapped regions of the spectrum. Results of the decouplings are shown in Fig. 3, where resonances coupled to each other are connected by horizontal lines (only the peaks between ~2.6 ppm and ~1.5 ppm are shown in Fig. 3; the two downfield methines in Fig. 2 are at 4.34 and 3.62 ppm, as indicated). The five non-equivalent methylenes are the following resonance pairs in

Table 3. ^{13}C NMR parameters for **10** in D_2O solution (internal reference; dioxane at 67.4 ppm, 67.9 MHz).

Carbon-proton correlations assigned by single-frequency irradiations.

^{13}C Chemical shifts (ppm)	^1H Chemical shifts (ppm)
25.63 t	
26.09 t	
27.84 t	
28.71 t	
31.95 t	
32.69 t	2.49
39.24 d	2.40
59.44 d	4.34
60.80 d	3.62
175.49 s	
176.65 s	
178.93 s	

Fig. 2. 360 MHz ^1H NMR spectrum of **10**, the hydrogenation product obtained from A58365A. Solvent: D_2O (The sharp peak near 2 ppm is residual acetate).

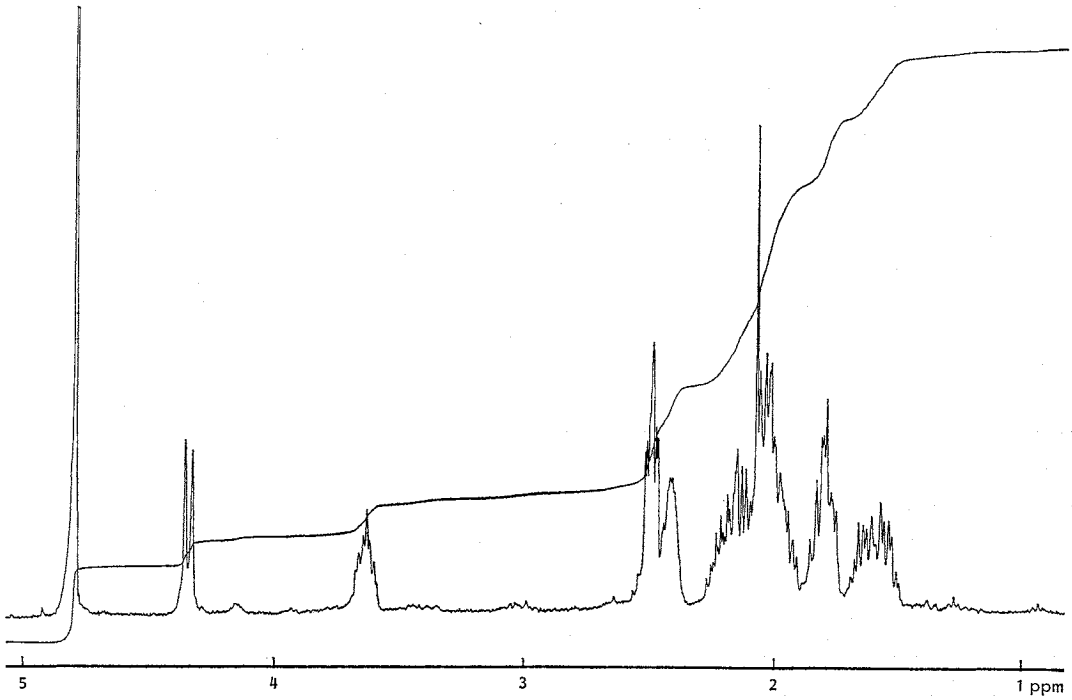


Fig. 3. *J*-Coupling connectivities for compound **10**, obtained by homonuclear decoupling experiments on the spectrum shown in Fig. 2 (360 MHz).

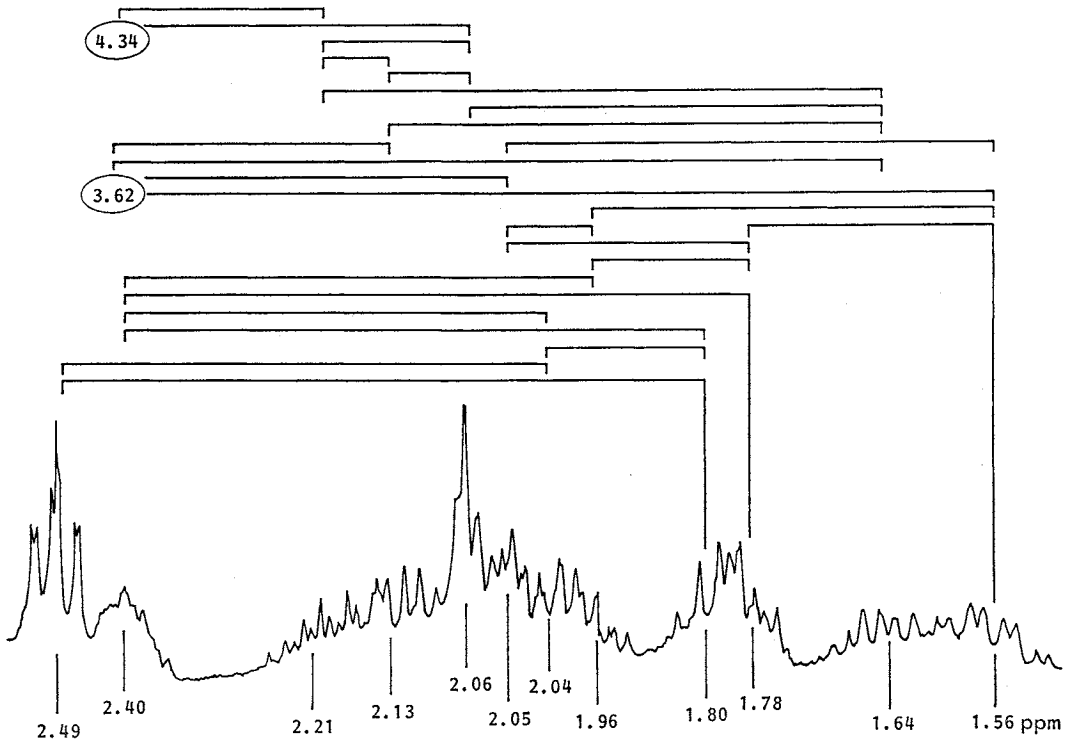


Fig. 4. A continuous substructure contained in compound **10**, based on the decoupling results shown in Fig. 3.

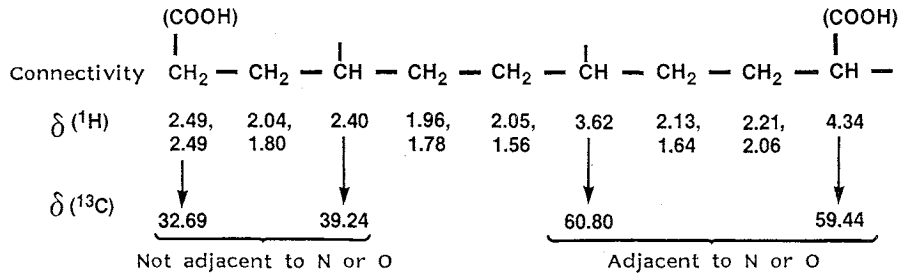
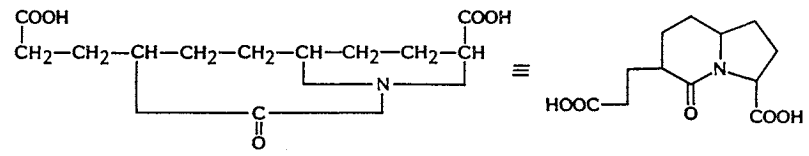


Fig. 3: (2.21, 2.06); (2.13, 1.64); (2.05, 1.56); (2.04, 1.80), and (1.96, 1.78). All fifteen non-exchangeable protons of **10** could be linked into a continuous substructure on the basis of the decoupling results in Fig. 3; this unit is shown in Fig. 4, along with the four known ^{13}C assignments. Two COOH groups have also been added to the substructure in Fig. 4 — one is connected to the equivalent CH_2 (^1H 2.49 ppm), since this appears to be derived from the pH-dependent CH_2 of A58365A, and the second is connected at the other end of the continuous fragment, to the CH at ^1H 4.34 ppm. This terminal CH of the substructure in Fig. 4 appears to be the same as the CH in the three-carbon fragment of A58365A (see Table 1).



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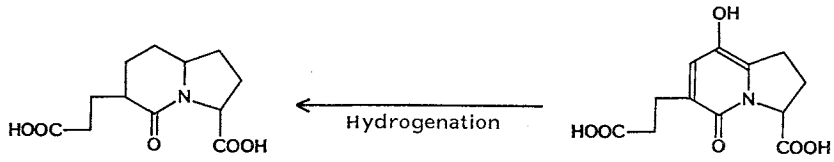
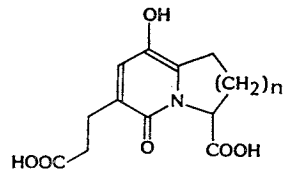
Scheme 2.



10
$C_{12}H_{17}NO_5$

1
$C_{12}H_{13}NO_6$

The addition of the two carboxyl groups to the fragment obtained from the ^1H NMR decoupling results leads to a partial molecular formula of $C_{11}H_{17}O_4$, which accounts for all of the atoms in **10** except $C_1N_1O_1$. The $C_1N_1O_1$ fragment must be added to the major substructure shown in Fig. 4 in a manner consistent with (1) the last carbon has a chemical shift of ~176 ppm, and (2) the methines at ^1H 3.62 ppm and 4.34 ppm are adjacent to a hetero atom. These constraints lead directly to the complete structure of **10**.



A58365A n=1
A58365B n=2

Structures of A58365A and A58365B

The UV spectra of the A58365 factors indicate that they are 5-hydroxypyridones (see Table 2). The structure of the tetrahydro-deoxy-A58365A (**10**) implies, therefore, that the parent A58365A has the structure **1** (see Scheme 2).

Since A58365B differs from A58365A only by having the substructure $CHCH_2CH_2CH_2$ rather than $CHCH_2CH_2$, the general structure for the two factors can be written as above.

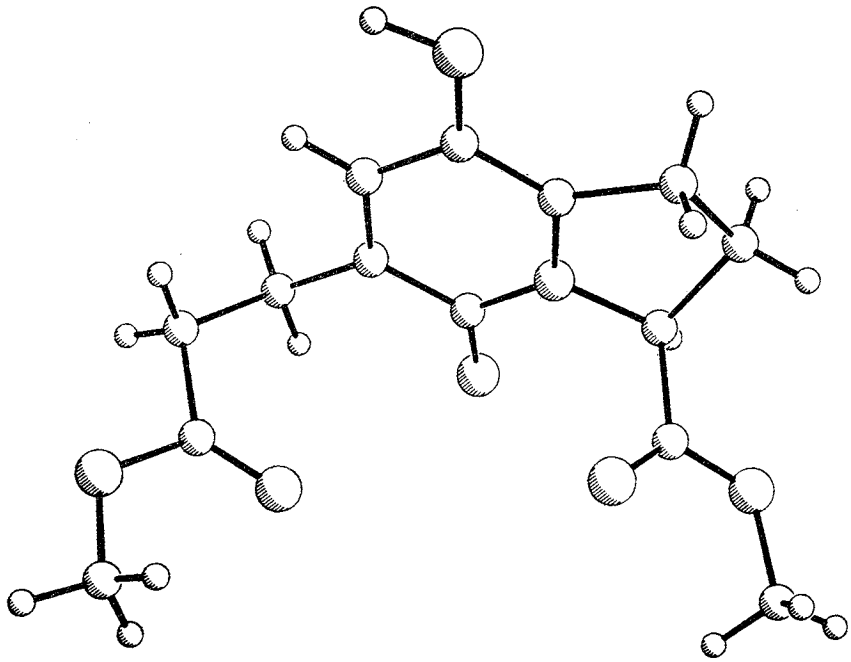
The structure of A58365A derived from the spectroscopic arguments presented here has been confirmed by solution of the X-ray crystal structure of the A58365A-dimethyl ester, **5**, as shown in Fig. 5. The absolute configuration was not determined, but the molecule is shown in Fig. 5 with the same configuration as L-proline.

Experimental

Physical Methods

UV spectra were run on a Cary model 118 spectrometer. NMR spectra were recorded on either a Bruker WH360 spectrometer (^1H spectra) or a Bruker WM270 spectrometer (^{13}C and some ^1H spectra). EI, FD, and FAB-MS were obtained on a Varian-MAT 731 spectrometer. X-Ray crystallography was accomplished on a Nicolet XRD P3/F automated four-circle diffractometer using monochromatic copper radiation.

Fig. 5. Structure of **5**, A58365A-dimethyl ester, as determined by X-ray crystallography.



A58365A-dimethyl Ester (**5**)

To a solution of A58365A in methanol (319 mg in 7.5 ml) was added 2.5 ml of methanol containing 4% by weight of hydrogen chloride; the solution was stirred at room temperature for 140 minutes. The esterification mixture was evaporated to dryness, then dissolved in approx 20 ml water. The aqueous solution, after adjustment to neutral pH, was extracted with methylene chloride (3× 100 ml). Following concentration under reduced pressure, the combined extracts yielded 151 mg A58365A-dimethyl ester. Crystallization from methylene chloride - diethyl ether afforded crystals suitable for X-ray analysis: ^{13}C NMR (67.9 MHz, $CDCl_3$) δ 173.45 (s), 170.55 (s), 158.86 (s), 135.25 (s), 133.83 (d), 132.16 (s), 128.54 (s), 62.10 (d), 52.52 (q), 51.34 (q), 32.54 (t), 27.11 (t), 26.67 (t), 25.84 (t).

X-Ray Analysis: Compound **5** crystallized as colorless prisms in the space group $P4_3$, Z=4, in a unit cell having dimensions a=8.314(2) Å, b=8.314(2) Å, c=20.463(8) Å. The calculated density was 1.386 gcm^{-3}. A total of 1168 unique reflections with 2θ less than 116.0° were measured. The structure was solved using the Random Tangent method (RANT) of the SHELXTL program library[9] and was refined by the least squares method with anisotropic temperature factors for all atoms except hydrogen. Hydrogen atoms were included with isotropic temperature factors at all calculated positions. The final R-factor was 0.0408 for 927 observed reflections.

Trimethyl-A58365A (**6**)

Approximately 10 mg of A58365A was dissolved in methanol and treated with excess CH_2N_2 for 18 hours; a single trimethyl product was obtained: ^{1}H NMR (270 MHz, $CDCl_3$) δ 7.27 (1H, s), 5.10 (1H, dd), 3.79 (3H, s), 3.74 (3H, s), 3.65 (3H, s), 3.11 (2H, t), 2.88 (1H, m), 2.80 (1H, m), 2.67 (2H, t), 2.49 (1H, m), 2.31 (1H, m); ^{13}C NMR (67.9 MHz, $CDCl_3$) δ 173.39 (s), 170.49 (s), 159.07 (s), 137.48 (s), 135.77 (s), 131.32 (d), 129.36 (s), 61.81 (d), 58.86 (q), 52.47 (q), 51.26 (q), 32.44 (t), 27.32 (t), 26.54 (t), 26.28 (t).

5-Hydroxy-2-pyridone (**7**)

^{1}H NMR (270 MHz, DMSO) δ 7.15 (dd, J=9.4 and 3.0 Hz, 4-H), 6.97 (d, J=3.0 Hz, 6-H), 6.29 (d, J=9.4 Hz, 3-H); ^{13}C NMR (67.9 MHz, DMSO) δ 159.07 (s, C-2), 141.31 (s, C-5), 133.02 (d, C-4), 121.64 (d, C-6), 117.10 (d, C-3).

5-Hydroxy-6-methyl-2-pyridone (**8**)

^{1}H NMR (270 MHz, DMSO) δ 7.12 (d, J=9.3 Hz, 4-H), 6.08 (d, J=9.3 Hz, 3-H), 2.08 (s, CH_3); ^{13}C NMR (67.9 MHz, DMSO) δ 159.62 (s, C-2), 137.53 (s, C-5), 133.15 (d, C-4), 131.63 (s, C-6), 114.12 (d, C-3), 14.25 (q, 6-CH_3).

Tetrahydro-deoxy-A58365A (**10**) (3-Carboxyoctahydro-5-oxo-6-indolizinepropanoic Acid)

Platinum oxide catalyst (600 mg) was added to a solution of 600 mg A58365A in 40 ml of glacial acetic acid. The suspension was stirred for 24 hours at 25°C under hydrogen (4.2 kg/cm^2). The catalyst was removed by filtration and the filtrate was lyophilized to yield the crude reaction product (583 mg). The crude product was purified by reverse phase HPLC on DuPont Zorbax ODS using a mobile phase of CH_3CN - HOAc - H_2O (8 : 0.2 : 91.8), yielding 182 mg of **10**.

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