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X-Ray Crystallographic and Proton Magnetic Resonance Spectroscopic Investigations of Nipecotic Acid, a Potent Inhibitor of γ-Aminobutyric Acid (GABA) Uptake

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The conformations of (±)-nipecotic acid in the solid state and in aqueous solution have been investigated by X-ray crystallographic methods and by ^{1}H NMR spectroscopy, respectively. Nipecotic acid exists in the crystalline state as well as in aqueous solution in a chair conformation with the carboxylate group in an equatorial position. The crystal structure is stabilized by hydrogen bonds. Nipecotic acid appears to be a better substrate than γ-aminobutyric acid (GABA) for the GABA transport system in rat brain slices. On the basis of the structure of nipecotic acid, it is suggested that GABA is transported by this system in a somewhat folded conformation with less than 5 Å between the zwitterionic centres.

A "high affinity" uptake system may play an important role in the function of GABA as an inhibitory transmitter in the brain.[1–5] Several structural analogues of GABA are potent inhibitors of this uptake system,[6–7] but these compounds have in addition "GABA-like" depressant actions. Specific inhibitors of the GABA uptake process *in vivo* have pharmacological interest and may furthermore provide information regarding the physiological role of the uptake process. Thus, considerable effort has been made to evaluate such compounds and recently (±)-nipecotic acid (piperidine-3-carboxylic acid) has been shown to be a powerful inhibitor of GABA uptake in rat brain slices.[8] Nipecotic acid administered electrophoretically to feline spinal neurones reversibly enhanced the depressant action of similarly administered GABA, but nipecotic acid did not significantly depress the firing of spinal neurones *per se*.[9] Nipecotic acid appears to be able to replace GABA in both the uptake system and the calcium-dependent, potassium-stimulated release system in rat brain slices.[10,11] This uptake system seems to be characterized by pronounced conformational specificity as revealed by investigations of a series of conformationally restricted analogues of GABA.[6,8,9,12]

EXPERIMENTAL

Nipecotic acid was prepared from nicotinic acid according to directions by Freifelder [13] and was identified by infrared and ^{1}H NMR spectroscopy supported by elemental analysis. The crystals used for the X-ray examination were crystallized from a water/*N*,*N*-dimethylformamide solution. Colourless prismatic crystals were formed.

The computations in connection with the X-ray structure determination were performed on an IBM 1130 and an IBM 370/165 computer, using *INDIFF*,[14] a local version of the *NRC 2A Picker Data Reduction Program*,[15] *MULTAN*,[16] The Program System X-Ray 72,[17] and *ORTEP*.[18] The X-ray atomic scattering factors used were those of Cromer and Mann [19] for oxygen, nitrogen, and carbon, and of Stewart, Davidson, and Simpson [20] for hydrogen.

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The ^{1}H NMR spectra were recorded with a Varian HA-100 spectrometer at 100 MHz on the δ-scale with tetramethylsilane as an external standard at 32 °C using a 0.1 M solution of nipecotic acid. The INDOR experiments [21] were performed using a modified HA-100 spectrometer with crystal locked digital frequency generation, and a Varian Spectro system 100 for accumulation of 20 scans.

X-Ray analysis

Crystal data. (±)-Nipecotic acid, $C_6H_{11}NO_2$, M = 129.16. Orthorhombic, $a = 11.177(3)$, $b = 6.123(1)$, $c = 9.539(4)$ Å, $U = 652.8$ Å^3, D_m (flotation) = 1.31 g cm^{-3}, $Z = 4$, $D_c = 1.314$ g cm^{-3}; $\mu(\lambda(\text{Mo}K\alpha) = 0.7107$ Å$) = 1.06$ cm^{-1}; $F(000) = 280$. Systematically absent reflections: $0kl$ when $k+l$ odd, $h0l$ when h odd; thus possible space groups are $Pna2_1$ and $Pnam$. The non-centrosymmetric $Pna2_1$ was indicated by intensity statistics and confirmed by successful refinement of the structure.

The unit-cell parameters were refined by least-squares techniques from the diffractometer measured 2θ angles observed for 35 reflections.

Data collection. Three-dimensional diffraction data were measured at room temperature on a Nonius three-circle automatic diffractometer using graphite monochromated Mo$K\alpha$ radiation. The ω scan technique with a scan speed of 1.2 ° min^{-1} was employed. Background counts were taken for half the scanning time at each of the scan range limits. One standard reflection was measured after every 25 reflections.

All the data were measured from a single crystal with approximate dimensions 0.20 × 0.30 × 0.22 mm. The crystal was mounted with [010] along the ϕ axis of the goniostat.

X-ray diffraction intensities within one quarter of the reciprocal sphere were measured in the range $2.5° \leq \theta \leq 28°$; averaging of equivalent reflections led to 832 unique reflections, 711 had $I_{net} \geq 3.0\sigma(I)$, where $\sigma(I)$ is the standard deviation from counting statistics. These were regarded as observed reflections, whereas the remaining reflections were regarded as unobserved and excluded from the refinement procedure. Lorentz and polarization corrections were applied but no absorption correction was made owing to the low absorption coefficient.

Structure determination. The structure was determined by direct methods with the multiple solution technique using the automatic phasing program MULTAN.[16] An E map computed from the set of phases with the highest combined figure of merit [22] revealed all the non-hydrogen atoms.

Individual atomic parameters of this model were refined, first with isotropic and then anisotropic thermal parameters using the full-matrix least-squares method. On convergence the R value was 0.073. The quantity minimized was $\sum w(|F_o| - |F_c|)^2$ where weights were taken as unity. A difference map at this point revealed the positions of all eleven hydrogen atoms (0.3 − 0.5 e Å^{-3}). These were included in all subsequent refinements with fixed isotropic temperature factors equal to the values of the attached non-hydrogen atoms. The least-squares refinement was completed with the introduction of a weighting scheme of the form $w = 1/\{1 + [(|F_o| - 5.75)/6.00]^2\}$.[17] On the last cycle of least-squares refinement the values of maximum and average shift/error were 0.03 and 0.009, respectively. The final R value is 0.033 ($R_w = 0.041$) for all observed independent reflections. A final difference synthesis

Table 1. Final positional and thermal (× 10^4 Å^2) parameters for non-hydrogen atoms. The standard deviations of positional and thermal parameters (× 10^4) are given in parentheses. The temperature expression is of the form:

$\exp[-2\pi^2(h^2a^{*2}U_{11} + k^2b^{*2}U_{22} + l^2c^{*2}U_{33} + 2hka^*b^*U_{12} + 2hla^*c^*U_{13} + 2klb^*c^*U_{23})]$

	x/a	y/b	z/c	U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
N(1)	0.4246(2)	0.2319(3)	0.9442 [a]	249(8)	314(9)	252(8)	47(7)	12(7)	22(8)
C(2)	0.3356(2)	0.2929(4)	0.8352(3)	283(9)	306(10)	235(10)	13(8)	−17(8)	0(9)
C(3)	0.2529(2)	0.4730(3)	0.8862(3)	243(8)	302(9)	231(9)	−2(7)	−6(8)	16(9)
C(4)	0.3264(2)	0.6687(4)	0.9365(3)	401(12)	306(11)	397(12)	37(9)	−111(12)	−58(11)
C(5)	0.4191(3)	0.6003(5)	1.0461(4)	435(14)	411(13)	416(13)	23(11)	−165(11)	−111(12)
C(6)	0.4989(2)	0.4215(4)	0.9902(4)	264(9)	438(12)	408(12)	−15(10)	−86(10)	−18(11)
C(7)	0.1698(2)	0.5446(3)	0.7677(3)	253(9)	238(9)	282(9)	−8(8)	−35(8)	24(8)
O(1)	0.0594(1)	0.5487(3)	0.7890(3)	241(7)	573(11)	451(10)	17(7)	16(7)	177(9)
O(2)	0.2184(2)	0.5977(3)	0.6548(3)	334(8)	622(11)	285(8)	11(8)	−1(7)	144(9)

[a] This parameter was held constant to define the origin.

Table 2. Final positional and thermal ($\times 10^3$ Å^2) parameters for hydrogen atoms. The estimated standard deviations ($\times 10^3$) of the coordinates are given in parentheses.

	x/a	*y/b*	*z/c*	U_{iso}
H(11)	0.387(2)	0.166(4)	1.021(3)	27
H(12)	0.475(2)	0.135(4)	0.909(3)	27
H(21)	0.375(2)	0.337(4)	0.759(3)	27
H(22)	0.292(2)	0.169(4)	0.810(3)	27
H(31)	0.205(2)	0.410(4)	0.973(3)	26
H(41)	0.368(2)	0.724(5)	0.860(3)	37
H(42)	0.269(2)	0.776(5)	0.973(3)	37
H(51)	0.377(2)	0.555(5)	1.126(3)	42
H(52)	0.473(2)	0.725(5)	1.073(4)	42
H(61)	0.550(2)	0.478(5)	0.904(3)	37
H(62)	0.553(2)	0.366(4)	1.062(3)	37

showed no peaks or depressions greater than 0.2 e Å^{-3}. Tables 1 and 2 list the final positional and thermal parameters for the non-hydrogen and hydrogen atoms, respectively. The terminal set of structure factors listed with the observed data are available by request.

Description of the structure. The X-ray diffraction analysis of (±)-nipecotic acid confirms the expected zwitterionic structure. The conformation of the molecule is shown in Fig. 1 in which the numbering of the atoms and major bond distances and angles are also indicated. The piperidine ring of nipecotic acid is in the chair form with the carboxylate group in an equatorial position. The least-squares plane through the carboxylate group and C(3) makes an angle of 82.6° with that of the puckered ring. The torsion angle O(1)−C(7)−C(3)−C(2) is ±127.6(2)°. The *intra*-molecular distances N(1)···O(1) and N(1)···O(2) are 4.756(3) and 4.237(3) Å, respectively.

The bond lengths and angles are in agreement with those of the equivalent bond lengths and angles found in several piperidinium derivatives, *e.g.* piperidinium *p*-hydroxybenzoate.[23] The mean values of the piperidine ring C−C and C−N bonds are 1.522 and 1.490 Å, respectively, and the internal bond angles in the piperidine ring are slightly larger than the regular tetrahedral angle. The geometry of the carboxylate group is quite normal. No significant differences are observed between the two C−O bond lengths, in accordance with the fact that each oxygen atom is an acceptor in a hydrogen bond.

The packing of the molecules in the crystals is illustrated in Fig. 2. The crystal structure is stabilized by hydrogen bonds, one for each hydrogen atom covalently bonded to nitrogen. Table 3 lists hydrogen bond distances and angles and van der Waals contacts between non-hydrogen atoms less than 3.4 Å.

Analysis of proton magnetic resonance spectrum

The ^{1}H NMR spectrum of nipecotic acid in deuterium oxide solution is shown in Fig. 3. The spectrum can be divided into 3 sections: the four protons on C(2) and C(6) are assigned to section I, the proton on C(3) to section II, and the four protons on C(4) and C(5) to section III. Section I contains the AB part of an ABX system, the AB coupling constant being characteristic of a pair of geminal protons. The AX and BX coupling constants should describe the interaction between the geminal protons and a single vicinal proton. Section II appears as a symmetrical multiplet of 7 bands. In section I of the spectrum the signals due to the H(6) protons are expected to be present as a very complex many line pattern, each line having only a small intensity. The most prominent lines would therefore intuitively

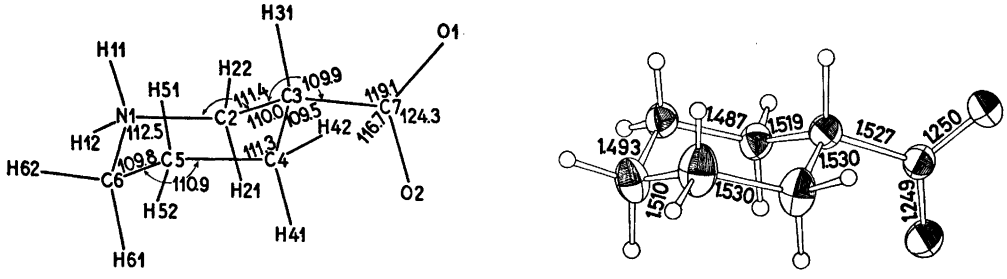

Fig. 1. Perspective drawings of nipecotic acid. (a) The numbering of the atoms and bond angles involving only non-hydrogen atoms. All e.s.d.'s are 0.2°. Bond angles involving hydrogen atoms range from 106 to 112°. E.s.d.'s are 1−3°. (b) The thermal ellipsoids for the non-hydrogen atoms, scaled to 50 % probability; hydrogen atoms are represented as spheres of arbitrary radius. Bond lengths between non-hydrogen atoms. E.s.d.'s are 0.003−0.004 Å. Bond lengths involving hydrogen atoms range from 0.89 to 1.06 Å. All e.s.d.'s are 0.03 Å.

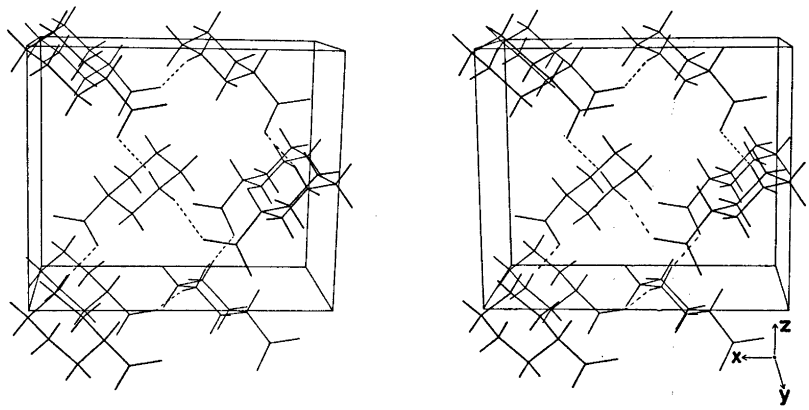

Fig. 2. Stereo diagram illustrating the molecular packing of nipecotic acid. Hydrogen bonds are drawn with broken lines.

Table 3. Hydrogen bond lengths and angles and van der Waals contacts between non-hydrogen atoms less than 3.4 Å.

A———H	B	B equipoint	A–H (Å)	A···B (Å)	H···B (Å)	∠AHB (°)
N(1)–H(12)···O(1)		$(\frac{1}{2}+x,\frac{1}{2}-y,z)$	0.89(3)	2.723(3)	1.86(3)	164(3)
N(1)–H(11)···O(2)		$(\frac{1}{2}-x,-\frac{1}{2}+y,\frac{1}{2}+z)$	0.93(3)	2.695(3)	1.79(3)	163(2)
C(2)	O(1)	$(\frac{1}{2}+x,\frac{1}{2}-y,z)$		3.290(3)		
C(2)	O(2)	$(\frac{1}{2}-x,-\frac{1}{2}+y,\frac{1}{2}+z)$		3.330(4)		
C(4)	O(2)	$(\frac{1}{2}-x,\frac{1}{2}+y,\frac{1}{2}+z)$		3.389(4)		

be ascribed to the two protons at C(2). Based on this a first assignment is made as shown in Fig. 3. The approximate values of the coupling constants would be in agreement with a geminal coupling constant of 12.4 Hz and vicinal coupling constants of 4.0 Hz and 8.6 Hz for the equatorial-axial and axial-axial configurations, respectively. No virtual coupling is expected to contribute to the line separation in the AB part of the ABX system since δ(H-3) $-$ δ(H-4) is of the order of ten times the vicinal couplings between H(3) and H(4).

To verify the assumption above Homo INDOR experiments were undertaken. One of the small lines marked with an asterisk was irradiated, and the remaining part of section I was scanned with the H_2 field. Under appropriate conditions the INDOR spectra corresponding to an AB case were observed. This permitted the accurate location of the lines belonging to each of the AB subspectra in section I. No analysis was attempted of section II in the spectrum, but the approximate position of the chemical shift can be taken as δ 3.1. The available data were used in the iterative program Laocoon III,[24] which yielded chemical shifts and coupling constants. The actual values of the coupling constants δ_A and δ_B depend on the chosen value of δ_X The error limits indicated for the coupling constants result from varying δ_X within ± 3 Hz, which is considered a conservative measure. The computed coupling constants were: $J_{AB} = -12.78 \pm 0.07$ Hz, $J_{AX} = 3.94 \pm 0.05$ Hz (equatorial-axial), and $J_{BX} = 9.32 \pm 0.02$ Hz (axial-axial).

In simple piperidine derivatives the equatorial proton on C(2) is found downfield from its axial counterpart.[25] Estimating the vicinal coupling constants for the protons on C(4) to be $J_{a,e} = 4.4$ Hz and $J_{a,a} = 8.6$ Hz, the multiplet at δ 3.09 assigned to the proton on C(3) could be analyzed as illustrated in Fig. 3, suggesting that this proton is axial and the carboxylate group is equatorial. The vicinal coupling constants used in this assignment are in general agreement with those found in most 6-membered rings [26] as is the geminal coupling constant in piperidines.[27] The spectrum as assigned is consistent with the piperidine ring of nipecotic acid in aqueous solution being predominantly in the chair form with the carboxylate group in an equatorial position.

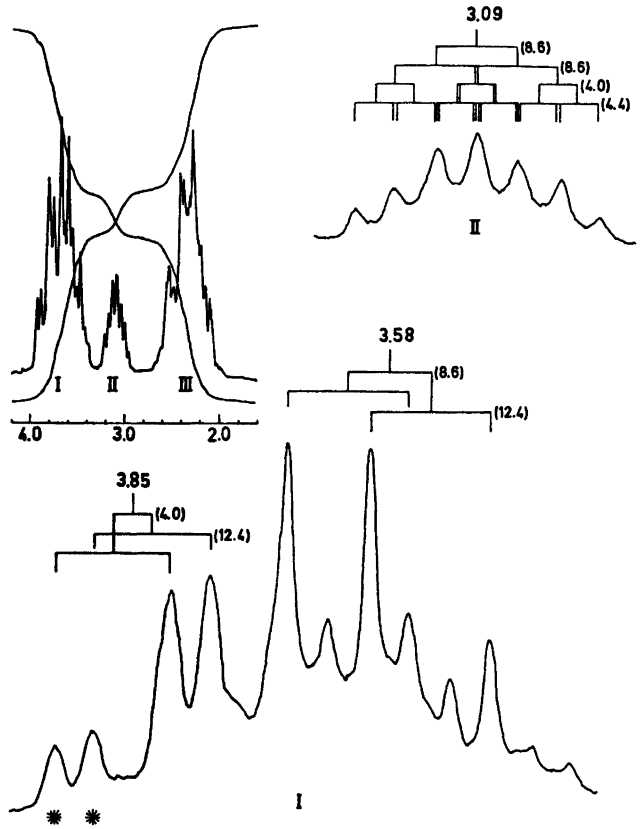

Fig. 3. Proton magnetic resonance spectrum of nipecotic acid. The spectrum is measured at 100 MHz on the δ-scale. Sections I and II of the spectrum are expanded and have been subjected to an approximate first order partial analysis in terms of the chemical shifts of the protons on C(2), δ 3.85 and 3.58, and C(3), δ 3.09, and their respective coupling constants (Hz) as indicated. The asterisks indicate the lines monitored during INDOR experiments.

DISCUSSION

The GABA molecule has considerable flexibility and the ability to adopt different conformational modes may well be essential to its physiological activity.[9] Different conformations of GABA may be required for the initial attraction to postsynaptic receptors and for the subsequent modification of the conductance properties of the postsynaptic membrane.[28] Furthermore, GABA may be transported across membranes in a conformation different from that required for initial binding to the uptake system.[6] Problems concerning the "active conformations" of GABA are approached indirectly by examination of structure-activity correlations of conformationally restricted GABA analogues.

Compared to the GABA molecule, nipecotic acid is a relatively bulky and inflexible molecule. Nonetheless, studies with radioactive nipecotic acid indicate that GABA and nipecotic acid can be counter-transported in brain slices *via* the same mobile carrier and that nipecotic acid appears to have a higher affinity than GABA for this carrier.[11] The X-ray study of nipecotic acid revealed a chair conformation with the carboxylate group in an equatorial position. The torsion angle O(1)−C(7)−C(3) −C(2) is ±127.6° and the *intra*-molecular distances between the nitrogen atom and the two oxygen atoms are 4.756 and 4.237 Å,

respectively. The proton magnetic resonance study indicates that in aqueous solution nipecotic acid exists predominantly in a chair conformation with the carboxylate group equatorial. In solution the carboxylate group can rotate so that the distance between the nitrogen atom and one oxygen atom ranges from 4.3 to 4.9 Å (Dreiding stereomodel). From a study of some conformationally restricted GABA analogues which were competitive inhibitors of GABA uptake it was concluded that the zwitterionic centres should be between approximately 5 and 6 Å apart in order to bind to the transport carrier.[6] The results obtained with nipecotic acid indicate that GABA is transported in a somewhat folded conformation with less than 5 Å between the zwitterionic centres. Analogues of restricted conformation with a greater minimum charge separation than 5 Å may inhibit the transport system by binding to the carrier to form a complex which does not penetrate the membrane.

The β-alanine molecule can assume the same distance between the zwitterionic centres as does nipecotic acid, but it does not inhibit GABA uptake in rat brain slices.[29] Factors other than charge separation may be important for interaction with the GABA transport carrier. The pronounced difference between β-alanine, which has two methylene groups, and nipecotic acid, which has four methylene groups, with respect to GABA uptake may reflect the importance of hydrophobic bonding in interactions with the transport carrier. It has been argued previously that conformational adaptability may be important in interactions with the carrier.[6] Data pertinent to the conformational adaptability of nipecotic acid might be obtained from magnetic resonance relaxation studies and from molecular orbital calculations.

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