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Study of the relative lactonization rates of pilocarpic acid and isopilocarpic acid in acidic media [1]

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Summary

The rate of specific acid catalyzed lactonization of pilocarpic acid and isopilocarpic acid at acidic pH has been studied by following the progress of the reaction by HPLC A 17 5-fold difference in the rate of lactonization to the corresponding parent lactone was observed in favor of isopilocarpic acid. The observed differences in the rates of lactonization were rationalized through application of molecular modelling and molecular orbital calculations Parameters studied were the calculated heats of formation, bond lengths and appropriate torsion angles for both pilocarpine and isopilocarpine. From these studies of the end products of the lactonization reactions, it appears that differences in the planarity of the lactone ring and its consequent effect on the formation of a reaction intermediate are possibly responsible for the different rates of lactonization

Introduction

Pilocarpine is a topical miotic commonly used in the treatment of glaucoma. The pH-dependent stability profile of pilocarpine in aqueous solution is well characterized and the mechanisms involved in the degradation of pilocarpine have been the subject of numerous investigations (Chung et al., 1970; Nunes and Brochmann-Hanssen, 1974; Bundgaard and Hansen, 1982). Aqueous solutions of pilocarpine are most stable in the pH range 4–6 and the pH-rate profile is characteristic of lactone ester hydrolysis (Chung et al., 1970; Lee, 1986).

There are two major first-order reactions which account for the instability of pilocarpine (P) solutions at alkaline pH. These are (i) hydrolysis of the lactone moiety to form pilocarpic acid (PA), and (ii) reversible epimerisation about the α-carbon to form isopilocarpine (IP) which can subsequently undergo hydrolysis to form isopilocarpic acid (IPA). At room temperature, the contribution of hydrolysis to the instability of P is of

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greater consequence than the epimerization reaction, although the relative rate of epimerization increases more rapidly with temperature than does the rate of hydrolysis (Nunes and Brochmann-Hansen, 1974; Bundgaard and Hansen, 1982).

In contrast to the degradative reactions of P, there is a scarcity of data regarding the relative lactonization kinetics of the two carpic acids. The acid catalyzed lactonization of PA has been studied using the change in optical rotation as a measure of reaction rate (Chung et al., 1970). These same workers suggested that the lactonization of IPA at pH 1 may occur at a more rapid rate than observed with the alternate isomer.

To facilitate development of an assay to provide baseline resolution and quantitation of P and IP and the two respective acids from different prototype formulations, we unsuccessfully attempted to prepare and isolate salts of the two carpic acids. It was apparent that once an alkaline solution of either carpic acid was neutralized to facilitate isolation of a salt, lactonization of the acid to the parent lactone was sufficiently rapid to significantly contaminate the isolated substance. Furthermore, it appeared that the rates of lactonization of PA and IPA were significantly different. Based on these observations, the present study was undertaken to characterize the relative lactonization kinetics of pilocarpic and isopilocarpic acid. Molecular modelling was also undertaken to rationalize the observed differences in the rate of the lactonization between the two isomeric carpic acids.

Materials and Methods

Chemicals

Pilocarpine hydrochloride and isopilocarpine nitrate were obtained from Sigma Chemical Co. (St Louis, MO). Sodium deuteroxide and deuterium oxide (both with isotopic purity of 99.9%) were obtained from Cambridge Isotope Laboratories (Woburn, MA). HPLC grade solvents were obtained from Mallinckrodt Australia (Clayton, Vic.). All other chemicals and solvents were at least reagent grade. Infrared spectra were recorded on a Hitachi 270-30 spectrophotometer from KBr discs.

Synthesis of carpic acids

PA was prepared by hydrolysis of a known quantity of P with 11% v/v of 10 M NaOD in D_2O. The reaction was followed using ^{1}H-NMR (300 MHz, Bruker) to confirm complete hydrolysis of the lactones to their respective acids. From the ^{1}H spectrum, hydrolysis of the lactone was accompanied by an upfield shift and change in the multiplicity of the C5 methylene proton adjacent to the ring oxygen atom. Samples of IPA were synthesized in an analogous manner using IP as the starting material.

Chromatography system

The chromatography system consisted of a Waters 510 pump (Milford, CT), an LDC Spectromonitor II (Riviera Beach, FL) variable wavelength detector (operated at 216 nm) and a Perkin Elmer (Norwalk, CT) 561 chart recorder. Sample introduction was via a Rheodyne (Berkeley, CA) 7125 loop injector.

Cation exchange (SCX) chromatography

Separation of carpic acids from the corresponding parent lactones was achieved using a Whatman (Clifton, NJ) Partisil 10 SCX (25 cm × 4.6 mm i.d., 10 μm) column and a mobile phase of 50:50 v/v methanol and 0.075 M phosphoric acid/ammonia buffer pH 3.5. The flow rate was 3 ml/min and the retention volume for either lactone (P/IP) was 27.6 ml and for either carpic acid (PA/IPA), 11.5 ml. The assay was linear over the concentration range 0–150 μg/ml and the within-day and between-day variability (coefficient of variation, CV) was 3.0% ($n = 3$) and 3.7% ($n = 4$), respectively.

Reverse phase chromatography

Quantitation and separation of IP and P was based upon the method of Wiesend (1988). The column was a Spherisorb (Queensbury, U.K.) S5 ODS2 column (25 cm × 4.6 mm i.d., 5 μm) and the mobile phase consisted of 990:10 v/v 0.084 M phosphoric acid/triethylamine buffer (pH 2.38) and methanol. The flow rate was 1.5 ml/min

and the retention volumes were 14.8 and 16.5 ml for IP and P, respectively. The assay was linear over the concentration range 0–150 μg/ml and the within-day and between-day variability (CV) was typically 2–4%.

Preparation of samples for kinetics

A known aliquot of the PA sample was diluted with HCl to give a final concentration in the range of 105–120 μg/ml. The ionic strength was maintained at $\mu = 0.25$ with NaCl and the temperature maintained at 25°C by a circulating water bath (Techne, Cambridge, U.K.). The pH of each sample was measured using an appropriately calibrated Metrohm pH meter (Herisau, Switzerland). IPA samples were prepared using similar methodology. In addition, the pH of each carpic acid sample was measured at the termination of the lactonization reaction and was found to differ from the original pH by no more than ± 0.05 pH unit. At solution pH values higher than 3.5, the carpic acid samples were buffered with three different concentrations (0.01, 0.02 and 0.04 M) of acetate buffer.

Sample analysis

The disappearance of either carpic acid and the appearance of the corresponding parent lactone was followed for at least four half lives with the cation exchange HPLC assay (which separated the lactones from the corresponding carpic acids). Peak height data and the corresponding time measurements were analyzed by regression and the observed first order rate constant (k_{obs}) was determined from the terminal portion of the linear profile. Appropriate mass balances were performed to verify that the loss of carpic acid corresponded only to the production of the lactone. The extent of epimerization which had occurred during the initial lactone hydrolysis (to prepare the carpic acids) was determined after completion of the lactonization reaction by the reverse phase assay.

Molecular orbital calculations

Semi-empirical molecular orbital calculations were performed on both P and IP using the AM1 Hamiltonian (Dewar et al., 1985) in MOPAC 5.0 (Stewart, 1989). This method was chosen as it provides, with reasonable computational costs, quite satisfactory information regarding the computational preferences of organic molecules (Fabian, 1988). By default, a Davidon-Fletcher-Powell geometry optimization was used together with the default convergers (a combination approach). The complex convergence and optimization criteria are described in the MOPAC manual and associated documentation (Stewart, 1989). All geometry optimizations reached SCF convergence and terminated normally. The level of convergence for each structure was specified using the keyword GNORM which was set to 1.0. These geometries were then further validated by additional calculations where a value of 0.1 was specified for GNORM (1018 SCF iterations for P and 1008 for IP). If this keyword is not used, calculations do not generally continue to adequate precision and the results may be unreliable. All calculations were performed on a Vax 11/750 computer. A complete geometry optimization of both P and IP was undertaken in this study. The initial molecular geometry for P was derived from the crystal structure (Codding and James, 1984). The chirality around C3 in P was inverted using the MORPHEUS graphics package (Andrews et al., 1989), of which the molecular modelling program CRYSX is a component, to obtain the starting geometry for the IP calculations. The input for the calculations referenced atoms in the imidazole ring of both structures to a central dummy atom. Such a geometry specification has been shown to decrease the convergence time for planar cyclic structures (Clark, 1985). Torsion angles were defined by clockwise rotations from the appropriate bonds according to the convention of Klyne and Prelog (1960).

Results and Discussion

The reaction scheme involving the possible lactonization and hydrolysis reactions for P and IP is shown in Fig. 1. The rate law describing the lactonization of IPA in acidic media is defined in Eqn 1 where k_1 is the second-order rate constant for the specific acid catalyzed lactonization of

IPA to form IP, and k_h represents the second-order rate constant for the specific acid catalyzed hydrolysis of IP to form IPA. Epimerization of IP to P does not occur under conditions of acidic pH.

$$-d[IPA]/dt = k_l[IPA][H^+] - k_h[IP][H^+] \quad (1)$$

The equilibrium between IPA and IP at acidic pH is strongly in favor of the lactone form with the equilibrium constant ($K = k_l/k_h$) being greater than 100 (less than 1% IPA present in an equilibrium mixture). Therefore, as $k_l \gg k_h$ and because the reactions were performed at constant pH, Eqn 1 can be simplified to yield Eqn 2 where k_{obs} is the observed pseudo first-order reaction rate constant for the lactonization of IPA at constant pH.

$$-d[IPA]/dt = k_{obs}[IPA] \quad (2)$$

This simple kinetic expression enabled the IPA peak height data from the SCX assay to be analyzed as a pseudo first-order reaction. The relationship between the observed rate constant (k_{obs}) and the second-order lactonization reaction rate constant is given by Eqn 3.

$$k_{obs} = k_l[H^+] \quad (3)$$

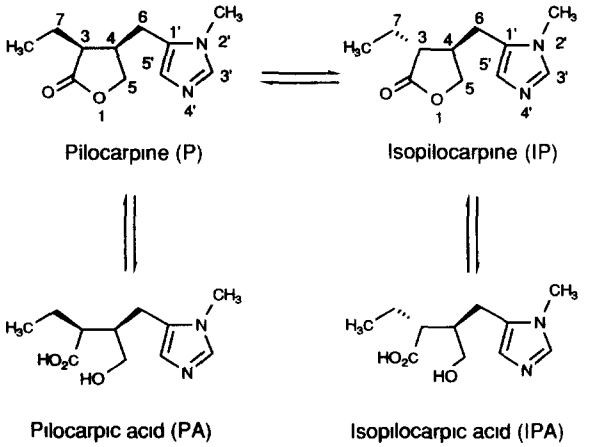

Fig. 1. Chemical structures of pilocarpine and related compounds and a reaction scheme depicting the associated equilibria

During the base catalyzed in-situ preparation of IPA from IP, some epimerization to P occurred which subsequently hydrolyzed to produce PA (see Fig. 1). The quantity of PA present in an in-situ prepared sample of IPA could not be directly quantitated with either the SCX assay (which did not separate the isomers) or reliably determined by the reverse phase assay (incomplete baseline resolution of IPA and PA). Therefore, at the conclusion of the lactonization reaction the quantity of IP (formed directly from IPA) and P (formed directly from PA) was determined by the reverse phase assay which readily resolved the two lactones. This approach was considered more accurate than utilizing chromatographic data from the peaks corresponding to the incompletely resolved IPA and PA under the conditions of the reverse phase assay. Using this procedure, the amount of PA present in a sample of IPA was up to 5% and this quantity reflected the ratio of the respective rate constants for the hydrolysis and epimerization of IP under basic conditions. This value is in good agreement with similar data reported by Bundgaard and Hansen (1982). When utilizing the SCX assay to follow the lactonization of IPA, the small quantity of PA present in the IPA sample did not significantly affect the determination of the lactonization rate constant for IPA as the difference in the reaction rate between the two carpic acids was 17.5-fold.

A similar kinetic treatment was utilized for the determination of the specific acid catalyzed lactonization of PA. Although the equilibrium between the hydrolysis and lactonization of PA and P was not as strongly in favor of the lactone form as that observed for IPA and IP ($K = k_l/k_h \approx 30$; Chung et al., 1970) it was still possible to utilize the above simplification for the treatment of the kinetic data. In an analogous manner to that described above, hydrolysis of P yielded PA and a small amount of IPA (resulting from the epimerization of P and subsequent hydrolysis) which was quantitated at the conclusion of the lactonization reaction by the reverse phase assay. The levels of IPA present were up to 20% and reflected the ratio of the respective rate constants for the epimerization and hydrolysis of P. As in the case of the lactonization of IPA, the quantity of the

isomeric carpic acid present in the sample of PA did not affect the determination of the lactonization kinetics due to the 17.5-fold difference in the rates.

Fig. 2 depicts the relationship between the logarithm of the normalized peak height as a function of time for the lactonization of IPA conducted at different solution pH values. The rate of lactonization increased with hydrogen ion concentration and was independent of the initial concentration of IPA indicative of a pseudo first-order reaction (data not shown).

Fig. 3 describes the kinetic data for the lactonization of PA as a function of solution pH. The values are presented as peak height data that were normalized to the initial peak height of the carpic acid peak from the SCX assay determined at the beginning of the reaction. The initial and rapid 20% decrease in the peak height data represents the more rapid lactonization of the IPA present in the in-situ prepared sample of the PA and the subsequent linear region represents the lactonization of PA. Buffer catalysis was not observed.

The pH-rate profile for the lactonization of IPA and PA at 25°C, derived from the data

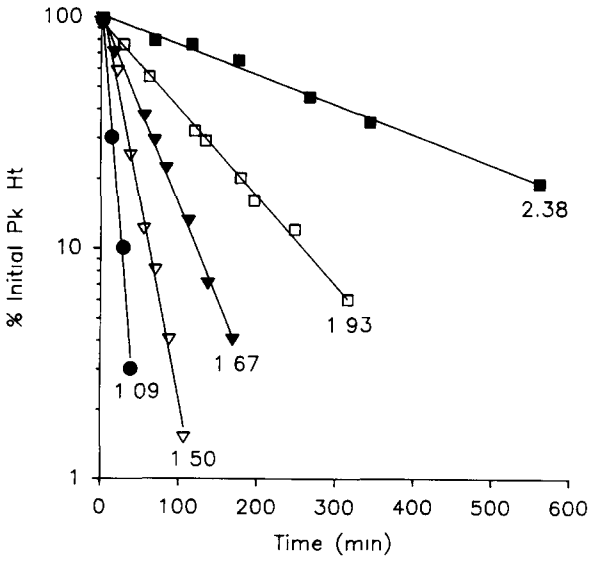

Fig. 2. Plot of the normalized peak height data of IPA as a function of time. The individual profiles refer to experiments performed at different pH values

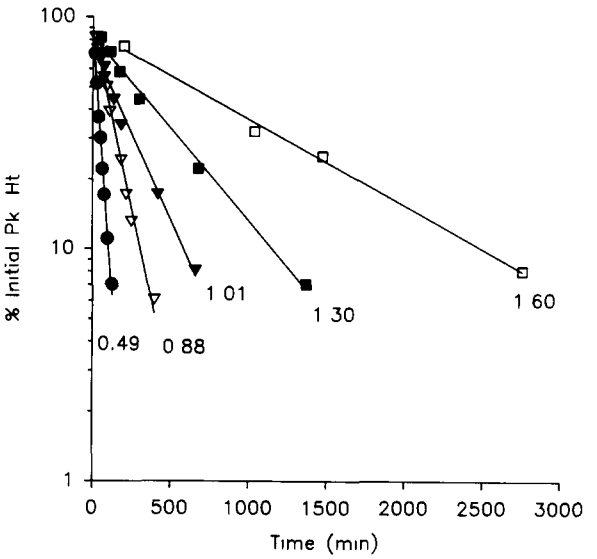

Fig. 3 Plot of the normalized peak height data of PA as a function of time. The individual profiles refer to experiments performed at different pH values

presented in Figs 2 and 3, is presented in Fig. 4. The data were fitted to Eqn 3 and the respective specific acid lactonization rate constants (mean ± SD) were: k_1(IPA) = 0.962 ± 0.032 l/mol per min and k_1(PA) = 0.055 ± 0.003 l/mol per min. The observed lactonization rate for PA is in good agreement with a reported value of 0.066 l/mol per min determined using polarimetry (Chung et al., 1970).

These data represent a 17.5-fold difference in the rates of lactonization of IPA relative to PA. From the above data, the reason for such a difference in lactonization rates between these two isomers was not obvious. Computer based molecular modelling and molecular orbital calculations were performed on both IP and P in an effort to more closely examine the ring geometries of these two molecules and to ascertain the reason for the significantly different lactonization rates of these two isomers.

AM1 is the most recently developed semi-empirical, molecular orbital method from the Dewar group, and is the only method capable of treating hydrogen bonding interactions or molecules with rotational freedom about single bonds between sp^2-hybridized atoms (Dewar et al., 1985; Boyd et

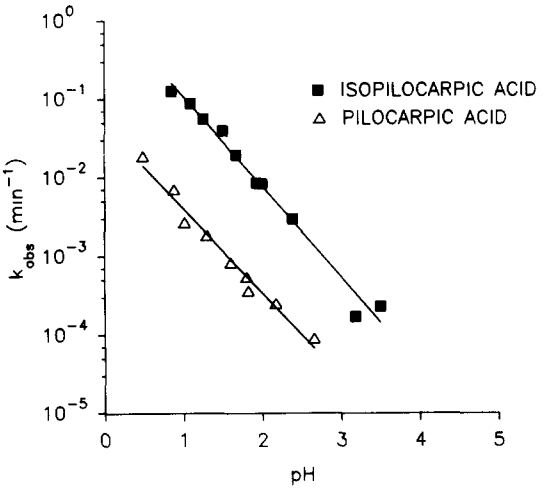

Fig. 4. Plot depicting the pseudo first-order rate constants for the lactonization of either IPA or PA as a function of solution pH

al., 1988). AM1 has been shown to be quite reliable in determining equilibrium torsion angles and preferred conformations over several conjugated and non-conjugated organic molecules when compared with experimental and ab initio data (Fabian, 1988; Masamura, 1988). Therefore, AM1 was used in preference to other methods (e.g., MNDO and MINDO/3) which are often compromised due to problems with geometry optimization where lone pairs of electrons and/or the potential for conjugation need to be considered (Clark, 1985).

Semi-empirical molecular orbital calculations on P and IP were performed to determine whether differences in steric strain around the lactone ring in these two isomers could account for the observed differences in the lactonization rates. Overall, the geometry of these two molecules was similar following complete energy minimization, as were the electronic energies, total energies and atomic charges. The difference in the calculated heat of formation for P and IP was 2.49 kcal/mol, indicating a preference for the trans orientation of the imidazole and ethyl substituents around the C3-C4 bond, as would be expected from the data describing lactonization rates of these two structures. Notable differences between the two geometries are shown in Table 1, where crystal structure values are also included for comparison. The atomic numbering scheme is given in Fig. 1. The torsion angle τ_1 shows the chiral inversion around C3 for these two isomers.

The calculated heat of formation values show that the AM1 conformation of P had been significantly optimized from the crystal structure geometry. Strain around the γ-lactone ring in the crystal structure has been eased considerably, with the bond lengths between C4-C3 and O1-C2 increasing and the bond angles C6-C4-C3 and C3-C4-C5 returning to more of a pyramidal geometry. Presumably, the steric interactions of the ethyl substituent at C3 and imidazole group at C4 in P have resulted in the bond angle C6-C4-C3 in the crystal structure becoming much larger than the expected pyramidal value of 109.5°. The strain from the steric interaction around C3-C4 can also be gauged by comparing the values of τ_4 and τ_5 for both IP and P in the AM1 generated structures (see Table 1). These values show that this interaction has considerably distorted the pyramidal geometry of C3 and C4 in P, whereas in IP the geometry is much closer to the expected value of 120°. The values for τ_4 and τ_5 in the crystal

TABLE 1

Computational data, derived from the AM1 program, describing the heat of formation, and relevant bond lengths and torsion angles present in pilocarpine and isopilocarpine

Parameter	Crystal structure	Isopilocarpine	Pilocarpine
Heat of formation (kcal/mol)	107.21	−47.28	−44.79
Bond length (Å)			
C4-C3	1.52	1 54	1 54
O1-C2	1 35	1 38	1.38
Bond angles (°)			
C6-C4-C3	118 6	113 0	116 8
C3-C4-C5	102.3	105.2	104 9
Torsion angles (°)			
τ_1: O1-C2-C3-C7	−104 7	122.9	−121.4
τ_2: C4-C5-O1-C2	−20.2	−0 9	−5 4
τ_3: C5-C4-C3-C2	−28 4	−1 6	−6 7
τ_4: C6-C4-C5-O1	156.9	124.0	134 1
τ_5 C6-C4-C3-C2	−148.7	−122.6	−129 1

structure of P are much more strained than in either of the optimized structures for P or IP.

Considerable skew of the lactone ring is experienced in the crystal structure which is reflected by the bond lengths, bond angles and torsion angles τ_1-τ_5, together with the relative calculated heats of formation for the crystal structure geometry compared to the optimized geometries. The planarity of the lactone ring is increased (relative to the crystal form) in pilocarpine following full geometry optimization, and even further increased in isopilocarpine. The strain, or non-planarity apparent in the lactone ring is a result of the steric interaction of the two substituents at C3 and C4 (see Table 1).

The formation of a cyclic lactone will involve a tetrahedral intermediate, where the two oxygen atoms each have a lone pair oriented antiperiplanar to the new bond (Deslongchamps, 1983). It also follows that the reverse process must follow for the formation of the respective acids from IP and P. It would be expected that it is the relative stabilities of these intermediates in IP and P which is being reflected in the 17.5-fold difference in lactonization rates measured in the current study. In the absence of more detailed calculations involving the characterisation of the transition states for the process of lactonization of both pilocarpine and isopilocarpine, it was postulated from these AM1 calculations, that the planarity of the lactone moiety in these two isomers was contributing significantly to the difference in the lactonization rates. However, a factor which cannot be discounted is the steric effect of the ethyl substituent in each of the isomers on the formation of the lactone moiety. As a tetrahedral intermediate would be anticipated to be involved in the lactone formation of both isomers, then steric interference would be expected to be quite similar whether the ethyl group was above or below the plane of the generated ring.

The influence of the non-planarity on the stability of the lactone moiety in these compounds, if as dramatic as inferred from the difference in the lactonization rates, should be reflected in the infrared vibration frequencies for the respective lactone functional groups. The carbonyl stretching vibration frequency for IP was measured at 1770 cm^{-1}, and 1752 cm^{-1} for P. When compared to standard values of 1760–1795 cm^{-1} for γ-lactones, and 1720–1750 cm^{-1} for saturated aliphatic esters (Cross and Jones, 1969), these values indicate that IP is more lactone-like than P. This observation is consistent with the hypothesis that the lack of planarity in the developing lactone based transition state contributes to the slower rate of lactonization observed with PA relative to IPA.

In summary, the specific-acid catalyzed lactonization rates for IPA and PA have been studied and a 17.5-fold difference was found in favor of IPA. The basis for this difference has been investigated using molecular modelling and molecular orbital calculations and the difference in the degree of planarity present in the postulated tetrahedral intermediate leading to lactonization has been implicated as a contributing factor to the different lactonization rates.

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References

Andrews, P.R, Quint, G., Winkler, D.A., Richardson, D., Sadek, M. and Spurling, T.H., MORPHEUS: A conformation-activity relationships and receptor modelling package *J. Mol. Graphics*, 7 (1989) 138–145

Boyd, D.B, Smith, D.W., Stewart, J.J P. and Wimmer, W., Numerical sensitivity of trajectories across conformational energy hypersurfaces from geometry optimized molecular orbital calculations: AM1, MNDO and MINDO/3 *J Comput Chem*, 9 (1988) 387–298

Bundgaard, H. and Hansen, S.H., Hydrolysis and epimerization kinetics of pilocarpine in basic solution as determined by HPLC. *Int J. Pharm.*, 10 (1982) 281–289.

Chung, P-H., Chin, T.-F. and Lach, J.L., Kinetics of the hydrolysis of pilocarpine in aqueous solution *J. Pharm Sci*, 59 (1970) 1300–1306.

Clark, T.A., *A Handbook of Computational Chemistry – A Practical Guide to Chemical Structure and Energy Calculations*, Wiley, New York, 1985.

Codding, P.W. and James, M N.G., The structure of pilocarpine hydrochloride, $C_{11}H_{17}N_2O_2.Cl$ A muscarinic alkaloid. *Acta Crystallogr*, B40 (1984) 429–434

Cross, A.D and Jones, R.A, *An Introduction to Practical Infrared Spectroscopy*, Butterworths, London, 1969, pp 86–87.

Deslongchamps, P., Stereoelectronic effects in organic chemistry In Baldwin, J.E (Ed.), *Organic Chemistry Series*, Vol 1, Pergamon, Oxford, 1983, pp 58–65

Dewar M.J.S, Zoebisch, E.G, Healy, E F and Stewart, J J P, AM1· A new general purpose quantum mechanical molecular model *J Am Chem Soc*, 107 (1985) 3902–3909

Fabian, W F, AM1 calculations of rotation around essential single bonds and preferred conformations in conjugated molecules. *J Comput Chem*, 9 (1988) 369–377

Klyne, W and Prelog, V, Description of steric relationships across single bonds. *Experientia*, 16 (1960) 521–523

Lee, V H.L., Pilocarpine – A stability monograph In Connors, K.A, Amidon, G L and Stella, V.J (Eds), *Chemical Stability of Pharmaceuticals*, Wiley, New York, 1986, pp 675–684

Masamura, M, Reliability of AM1 in determining the equilibrium structures of unionized amino acids. *J Mol Struct (Theorchem)*, 164 (1988) 299–311

Nunes, M.A. and Brochmann-Hansen, E., Hydrolysis and epimerization kinetics of pilocarpine in aqueous solution *J Pharm Sci*, 63 (1974) 716–721

Stewart, J P.P, MOPAC version 5 0, *QCPE Bull*, 9 (1988) 10

Wiesend, B, Die Bestimmung von Pilocarpin und seinen Zersetzungsprodukten mit einer neuen HPLC-methode *Pharm. Ztg Wiss*, 1 (1988) 44–47