Biochemical and Biophysical Research Communications 452 (2014) 170–173

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The antitumor agent 3-bromopyruvate has a short half-life at physiological conditions

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ARTICLE INFO

Article history:
Received 12 August 2014
Available online 21 August 2014

Keywords:
Alkylating agent
3-Bromopyruvate
Cancer therapy
3-Hydroxypyruvate
Tumor
Ketone hydration

ABSTRACT

Clinical research is currently exploring the validity of the anti-tumor candidate 3-bromopyruvate (3-BP) as a novel treatment for several types of cancer. However, recent publications have overlooked rarely-cited earlier work about the instability of 3-BP and its decay to 3-hydroxypyruvate (3-HP) which have obvious implications for its mechanism of action against tumors, how it is administered, and for precautions when preparing solutions of 3-BP. This study found the first-order decay rate of 3-BP at physiological temperature and pH has a half-life of only 77 min. Lower buffer pH decreases the decay rate, while choice of buffer and concentration do not affect it. A method for preparing more stable solutions is also reported.

1. Introduction

From 1975 to 1995 3-BP was used extensively in research on enzyme structure–function relationships as an alkylating agent that often inactivated its targets. The alkylation occurs when a good nucleophilic group, typically a thiol, displaces the bromide leaving group in an S_N2 reaction [1]. After 2001 it has been frequently studied as a promising antitumor agent [2,3]. Initially conceived as a cancer treatment based on the suppression of glycolysis [2], 3-BP has also been shown to inhibit cell viability by increasing total reactive oxygen species [4,5] and inhibiting translation [6]. *In vivo* trials reveal that this relatively non-specific, highly reactive affinity label is surprisingly effective in discriminating between tumor and healthy tissues [3,7].

An important mechanism for this discrimination appears to be due to 3-BP gaining entrance to cells via monocarboxylate transporters (MCTs) which are overexpressed in cancer cells, as recently reviewed by Baltazar et al. [8]. Birsoy et al. performed a genome-wide haploid genetic screen that identified MCT-1 as the main determinant of 3-BP sensitivity. Furthermore, because the rate of 3-BP uptake is faster at lower pH, the increased acidity of extracellular fluid in tumor tissues constitutes a second factor that would increase the internal concentration of 3-BP to higher levels than in normal cells [9]. A key to the selective effectiveness of 3-BP may be tumor cells' preferential uptake of 3-BP, which is then broadly toxic inside the cell.

Some key properties of 3-BP, important for usage as well as understanding its mechanism of action, were published several decades ago in rarely-cited articles. These properties have not been mentioned in recent publications. The reaction properties are summarized in Fig. 1.

The electronegativity of the bromo group increases the instability of the neighboring carbonyl carbon, increasing its reactivity with water and nucleophiles [10]. In solution the rapid hydration equilibrium favors the gem-diol whether the carboxylic acid group is protonated or unprotonated. For the unprotonated form the equilibrium constant favoring the hydrate has been reported as 1.84 [10], while others found that the ratio of hydrate to keto forms was 4:1 [11]. In contrast, for pyruvate the equilibrium favors the keto form and only about 6% exists in solution as the hydrate [12]. That 3-BP is mostly in the hydrate form at neutral pH values apparently does not impact its transport by MCT-1, which has broad specificity for short-chain carboxylates substituted at the 2-position [13].

Most importantly, the prevalent gem-diol form of 3-BP base decays to 3-HP at neutral and, more rapidly, at basic pH values, producing HBr (aq) as a side product. The half-life of the decay at 20 °C and pH 8.20 measured by polarography was 87 min. For the greater destabilized ethyl 3-bromopyruvate ester the half-life was only 29 min at pH 7.45 [14].

Abbreviations: 3-BP, 3-bromopyruvic acid or 3-bromopyruvate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 3-HP, 3-hydroxypyruvate; TNB, 2-nitro-5-thiobenzoic acid.

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http://dx.doi.org/10.1016/j.bbrc.2014.08.066
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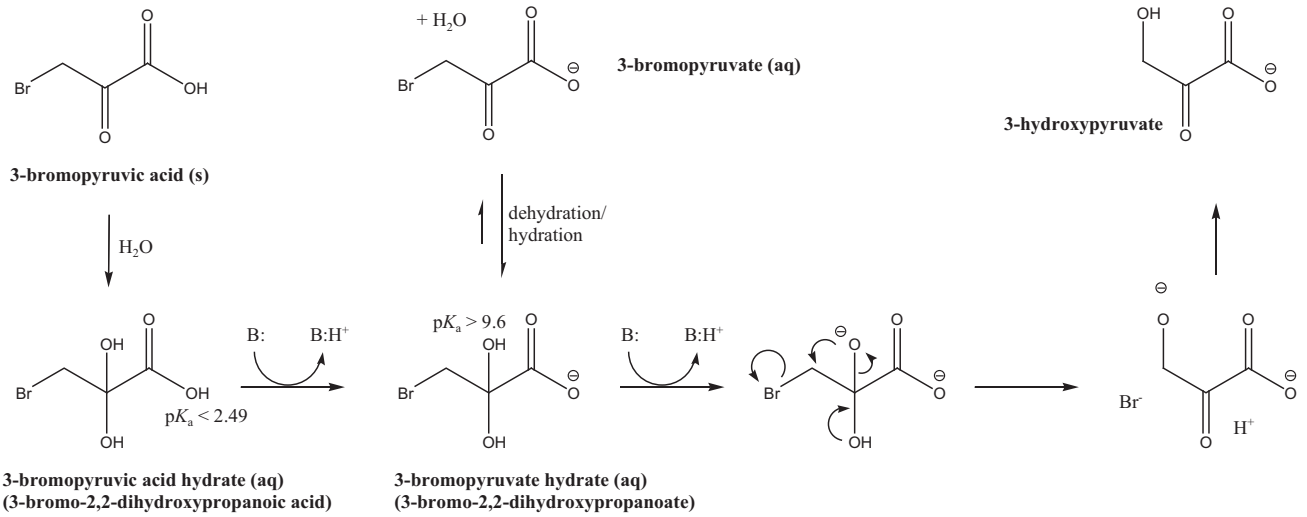

Fig. 1. Reactions and decay of 3-BP in solutions. Both 3-bromopyruvic acid and its conjugate base favor the gem-diol hydrate in solution, as reported by Fisher et al. [10]. The mechanism for the conversion of 3-bromopyruvatehydrate (aq) to 3-hydroxypyruvate (aq) is as proposed by Fleury et al. [14].

Yun and Suelter reported a 3-BP half-life of 3 h at pH 8.0 and 23 °C by employing a spectrophotometric method that was also utilized in the present study to determine the 3-BP half-life under physiological pH and temperature, which has not been previously reported. Their method determines solution concentrations of as low as 10 μM 3-BP by reaction with the yellow 2-nitro-5-thiobenzoic acid (TNB) anion, whose sulfhydryl displaces the 3-BP bromide, producing a colorless, alkylated TNB. The authors reported that the reaction is specific for ß-halopyruvate and demonstrated that 3-HP reacts several orders of magnitude slower than 3-BP, as expected due to the very poor leaving group ability of the hydroxide anion. No reaction with pyruvate was detected [15].

Abbreviations like "3-BP" have been used interchangeably for the acid and its carboxylate anion, which overlook two important distinctions. On one hand, the primary species in solution are actually 3-bromo-2,2-dihydroxypropanoic acid or its conjugate base. Second, the acid and conjugate base differ markedly in stability. Although this article will continue the convention of using the 3-BP abbreviation, careful attention must be paid to which structure actually predominates under given conditions.

3-BP is purchased in its acid form but converted to the carboxylate typically only at the point when added to reaction media. Some published reports adding millimolar 3-BP to weakly-buffered pH 7.2–7.4 solutions such as PBS apparently were unaware this would alter the solution pH, especially because each 3-BP acid releases two protons, the second slowly upon production HBr during decay to 3-HP. For example, adding 2 mM 3-BP to 10 mM pH 7.4 phosphate buffer immediately changes the pH to 7.0 and eventually to 6.6.

Stock solutions of the conjugate base, however, are typically unstable and cannot be stored, but must be prepared fresh and kept cold [15], although here we report a method for longer, stable storage. 3-BP solutions should never be neutralized with concentrated solutions of strong bases such as NaOH because the high concentration of hydroxide ion directly displaces the bromide, rapidly producing 3-HP [16]. It is not clear whether high concentrations of other nucleophiles in buffers, although weaker than hydroxide, might also be able to directly displace the bromide at appreciable rates. Likewise, the choice of buffer may impact the rate of the gem-diol anion internal rearrangement that also yields 3-HP. This was investigated in the present work.

Most proteins reported to have been modified by 3-BP were alkylated, typically at a cysteine side chain via direct displacement of the bromide. However, in at least one case where 3-BP was intended to covalently modify a protein it was not discovered until later that actually 3-HP had modified the protein via the 3-HP keto group forming a Schiff base with a lysine side chain [11,17]. In 3-HP the hydroxyl group has a slightly smaller effect than a bromo group at enhancing the reactivity of the keto group. For 3-HP in neutral solutions the keto form is slightly favored over the hydrate [11]. If the relative instability of the 3-HP keto group is sufficient to form a Schiff base with a lysine side chain, 3-BP, with a greater destabilized keto group, should be able to do the same with some proteins.

2. Materials and methods

2.1. Materials

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Life Technologies. 3-BP (acid form) and other reagents were purchased from Sigma–Aldrich.

2.2. Determination of 3-BP decay rates by reaction with TNB

The protocol for measuring the decay rates of 3-BP, adapted from that reported by Yun and Suelter, involved removing periodic aliquots from a 3-BP solution and reacting them with TNB. The decrease in concentration of TNB, determined at 412 nm (ε 12,800 M^{-1} cm^{-1}), equals the initial concentration of 3-BP in the cuvette [15].

TNB stock solutions were prepared by dissolving sufficient DTNB to 15 mM in pH 7.0 0.01 M Tris–MES, 1 mM EDTA. The DTNB was reduced with excess $NaBH_4$ (s), and TNB product was diluted with 0.1 M Tris-MES, 1 mM EDTA, pH 8.0 to 2 mM before being stored frozen.

Immediately prior to reaction with 3-BP, TNB stock solution was diluted to 72 μM in one of the indicated buffers. 3-BP was dissolved in the same buffer at 37 °C to a concentration of 2.0 mM. Multiple HP 8452A Diode-Array Spectrophotometers with thermostated cell holders all at the same temperature, either 37 or 40 °C, were used to monitor the reactions of 10 μL aliquots of the 3-BP solution removed at various times and added to 1.00 mL of the TNB solution in a cuvette. Absorbance values beginning at

0.6–0.9 were followed until the reaction stopped, typically after 10 min if performed at 40 °C.

2.3. Effect of pH on decay rates

Four 0.10 M potassium phosphate buffers were prepared from KH_2PO_4 and adjusted at 37 °C to pH 6.5, 7.0, 7.4, or 8.0 with KOH. A stock solution of the 3-BP conjugate base was prepared from the solid acid and $NaHCO_3$ (under vacuum to quickly degas CO_2) to be 200 mM 3-BP and 220 mM $NaHCO_3$. This stock solution was quickly diluted into each buffer (incubated at 37 °C) to yield 2.0 mM solutions of 3-BP whose concentrations were monitored as described above.

2.4. Effect of buffers on 3-BP decay rates

Decay rates for 3-BP were determined in one of three buffers: potassium phosphate, Tris–MES, or Tris–HCl. Each was adjusted to pH 8.0 at 37 °C using the appropriate conjugate acid or base for the first two buffers and either Tris or HCl for the third. Stock solutions of 200 mM 3-BP were made in 180 mM $NaHCO_3$ (aq) and diluted to 2.0 mM in one of the indicated buffers.

2.5. Storage of neutralized 3-BP stock solutions

A 2 mM 3-BP solution was adjusted to pH 3.0 with $NaHCO_3$ and aliquots stored at −10, 4, 21 or 37 °C. After time 3-BP concentrations were determined as described above.

2.6. ^{13}C NMR of the solvated, acid form of 3-BP

Twelve scans were performed on a concentrated solution of 1.1 g 3-BP (s) dissolved in 0.7 mL deionized water in an Anasazi 60 MHz FT-NMR. The instrument was zeroed with tetramethylsilane immediately beforehand. Chemical shift predictions were performed with Spartan '10.

3. Results and discussion

3.1. Effect of pH on decay rate

The 3-BP decay half-lives in 0.10 M potassium phosphate at pH 6.5, 7.0, 7.4, and 8.0 were found to be 430 ± 20 (3), 160 ± 30 (3), 77 ± 20 (3), and 37 ± 10 (3) min, respectively (Fig. 2). The increasing decay rate with increasing pH is congruent with the mechanism proposed in Fig. 1 and was observed by Fleury et al. [14], although they had not tested at physiological temperature and pH values.

In the physiological conditions of normal tissue the 77 min half-life of 3-BP is remarkably short, favoring minimal toxicity for healthy tissue. In the increased acidity of the extracellular fluid in most tumors, often with a pH of 6.5–7.0, the half-life is markedly longer. This would favor 3-BP toxicity for tumor tissues, in addition to the effect of MCTs, and may be one of the factors responsible for how this relatively non-specific, highly reactive affinity label discriminates between tumor and healthy tissues. The pH dependence of 3-BP decay also has implications for considering analogs for anti-tumor use, as well as *in vitro* investigations of 3-BP labeling of proteins during prolonged incubations at elevated pH or temperature.

3.2. Effect of buffer type and concentration on 3-BP decay rate

The first-order decay rates of 3-BP at pH 8.0, 37 °C in 0.10 M Tris–HCl, Tris–MES, or potassium phosphate were determined, giving half-lives with S.D. of 46 ± 1 (3), 46 ± 3 (3), or 42 ± 3 (3) min, respectively (Fig. 3). From the average half-lives no significant differences in decay rate are apparent. Thus, none of the buffer nucleophilic atoms or ions, including chloride, are sufficiently strong at normal buffer concentrations to displace the bromide from 3-BP. Neither do any of the buffers appreciably affect the rate of the internal rearrangement that displaces the bromide.

The effect of potassium phosphate buffer concentrations from 75 to 150 mM was determined and not found to affect the rate (results not shown). This was not surprising, given that the choice of buffer also appears not to affect the rate.

3.3. Long-term stable storage of 3-BP base

The pH-dependence of 3-BP decay rates suggests relatively stable solutions of the conjugate base should be possible at sufficiently low pH values that are 1–2 pH units above the pK_a of the carboxylic acid group. Its pK_a has been estimated at 1.60 [18] and <1.5 [10], and it would be expected to be lower than the pK_a of pyruvic acid, 2.49 [19].

By neutralizing solutions of 3-BP acid with $NaHCO_3$, as described in the Section 2, buffer-free solutions of sodium 3-bromopyruvate (<10% acid form) were made at pH 3.0. Solutions stored at −10 °C and 4 °C showed no detectable decay over two

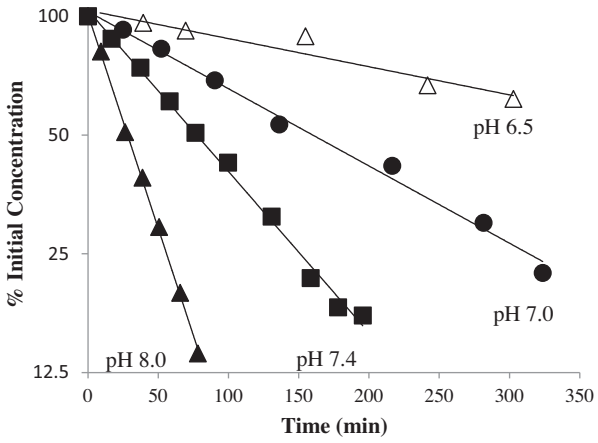

Fig. 2. Effect of pH on 3-BP decay rate. Aliquots of 2.0 mM solutions of 3-BP at 37 °C in 0.10 M KPi buffer at the indicated pH values were removed at various time points and the remaining concentration of 3-BP determined by reaction with TNB, as described in the Section 2. Note that the *y*-axis is logarithmic.

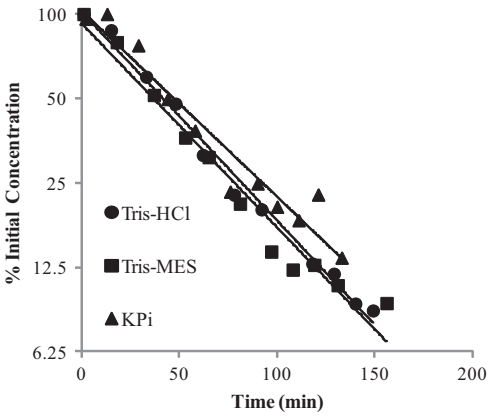

Fig. 3. Effect of buffer selection on 3-BP decay rate. Aliquots of 2.0 mM solutions of 3-BP in one of the indicated buffers, 0.10 M, pH 8.0, at 37 °C were removed at various time points and the remaining concentration of 3-BP determined by reaction with TNB, as described in the Section 2. Note that the *y*-axis is logarithmic.

Table 1
Calculated and experimental ^{13}C NMR of aqueous 3-BP, acid form.

Carbon	1	2	3
	δ (ppm)		
3-BP, keto (calc.)	157.9	185.5	34.3
3-BP, hydrate (calc.)	171.8	91.7	38.6
Experimental data	172.2	93.2	36.3

Values were determined as described in the Section 2.

months, while solutions at 18 and 36 °C had half-lives of 29 and 12 h, respectively (results not shown).

3.4. ^{13}C NMR of the solvated, acid form of 3-BP

The hydration status of 3-BP acid in solution was determined by ^{13}C NMR and compared to signals of those predicted for the keto and hydrate forms of the acid (Table 1). As shown, the measured shifts closely matched those of the hydrate, especially at the number two carbon. No other signals were apparent, including near 185 ppm, where a signal would be expected for the keto carbon. Apparently the keto-hydrate equilibrium favors the hydrate almost exclusively. This is consistent with the equilibrium being predicted to favor the hydrate more than in the conjugate base form due to the greater electron-withdrawing effect of the protonated carboxyl group. The hydrate form is also expected to be more favored than in pyruvic acid solutions, which are 60% hydrate [20].

3.5. Consideration of other reactions of 3-BP and its products

We observed that the same conditions that convert 3-BP to 3-HP also produce yellow solutions, with the color development being faster and continuing longer at higher pH values. 3-HP and HBr (aq), however, are colorless, meaning another species, presumably more conjugated, is being produced. While ketones also have enol forms that may need to be considered for their reactions, the bromo group does not appreciably affect the slow enolization of 3-BP, which only reaches 5% at equilibrium and would also not be expected to absorb visible light. 3-HP undergoes a slow aldol condensation, but above pH 10.5 [14]. The identity of the yellow side product remains unknown.

Funding

Funding was provided by Goshen College, including support from a Mininger Grant.

Acknowledgments

The authors would like to acknowledge Lynn Weaver, Ben Diedring, and Alan Leichty for helping with the initial implementations of the method for determining 3-BP decay rates. Doug Schirch would like to thank Roberto Contestabile, Martino Di Salvo, and Alessandro Paiardini at the Dipartimento di Scienze Biochimiche 'A. Rossi Fanelli', Sapienza Università di Roma, Italy for introducing him to 3-BP research.

References

[1] A. Galina, Mitochondria: 3-bromopyruvate vs. mitochondria? A small molecule that attacks tumors by targeting their bioenergetic diversity, Int. J. Biochem. Cell Biol. (2014), http://dx.doi.org/10.1016/j.biocel.2014.05.013.
[2] Y.H. Ko, P.L. Pedersen, J.F. Geschwind, Glucose catabolism in the rabbit VX2 tumor model for liver cancer: characterization and targeting hexokinase, Cancer Lett. 173 (2001) 83–91.
[3] J.-F.H. Geschwind, Y.H. Ko, M.S. Torbenson, C. Magee, P.L. Pedersen, Novel therapy for liver cancer: direct intraarterial injection of a potent inhibitor of ATP production, Cancer Res. 62 (2002) 3909–3913.
[4] L.S. Ihrlund, E. Hernlund, O. Khan, M.C. Shoshan, 3-Bromopyruvate as inhibitor of tumour cell energy metabolism and chemopotentiator of platinum drugs, Mol. Oncol. 2 (2008) 94–101, http://dx.doi.org/10.1016/j.molonc.2008.01.003.
[5] J.S. Kim, K.J. Ahn, J.-A. Kim, H.M. Kim, J.D. Lee, J.M. Lee, et al., Role of reactive oxygen species-mediated mitochondrial dysregulation in 3-bromopyruvate induced cell death in hepatoma cells: ROS-mediated cell death by 3-BrPA, J. Bioenerg. Biomembr. 40 (2008) 607–618, http://dx.doi.org/10.1007/s10863-008-9188-0.
[6] S. Ganapathy-Kanniappan, J.-F.H. Geschwind, R. Kunjithapatham, M. Buijs, L.H. Syed, P.P. Rao, et al., 3-Bromopyruvate induces endoplasmic reticulum stress, overcomes autophagy and causes apoptosis in human HCC cell lines, Anticancer Res. 30 (2010) 923–935.
[7] Y.H. Ko, B.L. Smith, Y. Wang, M.G. Pomper, D.A. Rini, M.S. Torbenson, et al., Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP, Biochem. Biophys. Res. Commun. 324 (2004) 269–275, http://dx.doi.org/10.1016/j.bbrc.2004.09.047.
[8] F. Baltazar, C. Pinheiro, F. Morais-Santos, J. Azevedo-Silva, O. Queirós, A. Preto, et al., Monocarboxylate transporters as targets and mediators in cancer therapy response, Histol. Histopathol. (2014) [Epub ahead of print].
[9] K. Birsoy, T. Wang, R. Possemato, O.H. Yilmaz, C.E. Koch, W.W. Chen, et al., MCT1-mediated transport of a toxic molecule is an effective strategy for targeting glycolytic tumors, Nat. Genet. 45 (2013) 104–108, http://dx.doi.org/10.1038/ng.2471.
[10] G. Fischer, M. Sieber, A. Schellenberger, The carbonyl reactivity of 3-bromopyruvate and related compounds, Bioorg. Chem. 11 (1982) 478–484, http://dx.doi.org/10.1016/0045-2068(82)90039-6.
[11] C.T. Bailey, M.G. Patch, C.J. Carrano, Affinity labels for the anion-binding site in ovotransferrin, Biochemistry (Moscow) 27 (1988) 6276–6282.
[12] A. Esposito, A. Lukas, J.E. Meany, Y. Pocker, The reversible enolization and hydration of pyruvate: possible roles of keto, enol, and hydrated pyruvate in lactate dehydrogenase catalysis, Can. J. Chem. 77 (1999) 1108–1117, http://dx.doi.org/10.1139/v99-071.
[13] A.P. Halestrap, Monocarboxylic acid transport, Compr. Physiol. 3 (2013) 1611–1643, http://dx.doi.org/10.1002/cphy.c130008.
[14] D. Fleury, M.B. Fleury, N. Platzer, Mechanistic study of the aldol condensation occurring in the alkaline solution of 3-hydroxy 2-oxo propanoate (β hydroxypyruvate), Tetrahedron 37 (1981) 493–501, http://dx.doi.org/10.1016/S0040-4020(01)92421-4.
[15] S.L. Yun, C.H. Suelter, Spectrophotometric determination of bromopyruvate by reaction with 2-nitro-5-thiobenzoic acid, Anal. Biochem. 85 (1978) 437–441.
[16] D.B. Sprinson, E. Chargaff, A study of beta-hydroxy-alpha-keto acids, J. Biol. Chem. 164 (1946) 417–432.
[17] M.G. Patch, C.J. Carrano, An affinity label for the anion binding site in ovotransferrin. Implications for iron release to reticulocytes, Biochim. Biophys. Acta 700 (1982) 217–220.
[18] W.L.F. Armarego, C. Chai, Purification of Laboratory Chemicals, seventh ed., Butterworth-Heinemann, Amsterdam, London, 2012.
[19] M.J. O'Neil, The Merck Index: An Encyclopedia of Chemicals, Drugs, and Biologicals, Merck, Whitehouse Station, NJ, 2001.
[20] Y. Pocker, J.E. Meany, B.J. Nist, C. Zadorojny, Reversible hydration of pyruvic acid. I. Equilibrium studies, J. Phys. Chem. 73 (1969) 2879–2882, http://dx.doi.org/10.1021/j100843a015.