Exploring 2-acetyl-1-pyrroline loss by high resolution mass spectrometry and nuclear magnetic resonance

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Abstract

2-Acetyl-1-pyrroline (2AP) is the characterizing odorant in foods such as aromatic rice and popcorn. 2AP is a potent odorant with a detection threshold of 0.1 ppb and contributes a roasted, cracker-like or popcorn-like aroma character. As such, 2AP would be appealing as an added flavoring but this poses a challenge due to the molecule's unstable nature. When 2AP is neat or in a concentrated aqueous solution, the color will rapidly change from colorless to red as 2AP reacts. Mass spectral data from our lab show a decline in a 25 mg/mL aqueous solution of 2AP in only 5 minutes. Buttery et al. postulated in 1983 that this molecule undergoes a polymerization process. Yet, little information is available in the literature to support this hypothesis. Our research has probed 2AP loss in water by high resolution mass spectrometry (HR-MS) and NMR (1D and 2D) and confirmed that a polymerization process does occur. We have observed that 2AP polymerization is a complex process, generating many unstable intermediates. The intermediates are highly unsaturated molecules which contain an increasing number of 2AP moieties, accompanied by the loss of water. Increasing dehydration occurs as molecular weight increases. NMR shows the increase of other small molecules in 2-6 hours of reaction. We have assembled a list of structural features of the polymeric species via 2D NMR and MS^2. The research presented will focus on the insights gained about 2AP's reactions products. Stabilization strategies for 2AP will be briefly mentioned.

Introduction

2-Acetyl-1-pyrroline (2AP) is the characterizing aroma compound in aromatic rice, popcorn and the Pandan leaf with a low odor threshold (0.1 ppb) [1]. 2AP can be formed biochemically, such as in rice, the Pandan leaf and the bread flower. Additionally, it is a Maillard reaction compound; and therefore, 2AP is present in a huge variety of cooked foods. Unfortunately, 2AP is very unstable when isolated in its neat form. 2AP rapidly undergoes a color change from a pale yellow oil to a viscous red material in minutes after being concentrated.

2AP was discovered in 1982 by Ron Buttery's group [2]. In the following year, this research group offered the hypothesis that 2AP undergoes a polymerization process between the carbonyl group and the five position of other molecules [1]. During the 35 years since 2AP's discovery, there has been a wealth of research done on 2AP that has included the development of new synthetic routes, the identification and quantification of 2AP in various foods, and a variety of chemical and physical methods developed to increase the compound's storage stability. Figure 1 shows some highlights from 2AP's history. Yet, 2AP's fate has been accepted as polymerization without analytical characterization.

The current work provides strong data to explain the fate of 2AP. This research is motivated by the clear interest of the scientific community in this molecule and the potential of 2AP as a flavoring molecule in the food industry. A deeper understanding of

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2AP's reaction fate can offer insight into stabilization attempts and provide knowledge about the chemistry of the structurally similar family of cracker-like aroma compounds.

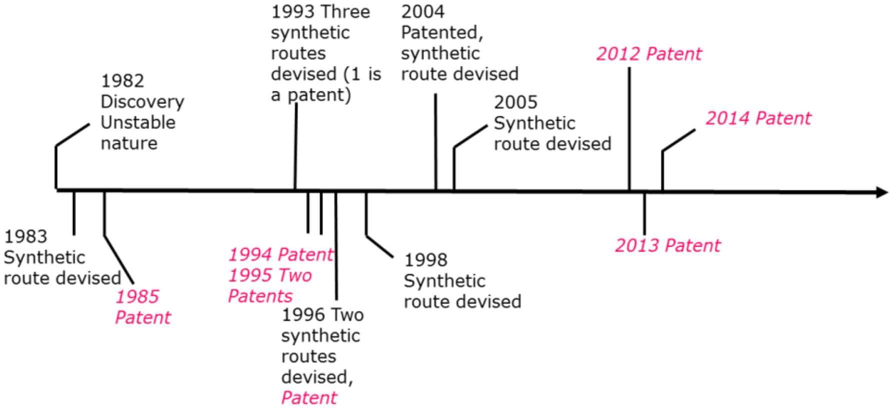

Figure 1: Timeline of 2AP's discovery and synthesis and patent history. The italicized work is focused on 2AP stabilization. Compiled from references 1-13.

Experimental

2AP was synthesized according to known methods [5]. The product was stored as a dilute solution in dry ether (sodium sulfate present) in the freezer. Immediately prior to an experiment, neat 2AP was obtained by filtering away the sodium sulfate and removing the ether under a stream of nitrogen gas. Initial experiments explored 2AP degradation in methanol. However, 2AP was relatively stable (days) and deuterium exchange proved to be a problem when working with deuterated solvents. The acetyl group can undergo keto-enol tautomerization. This is observed in NMR by the loss of a singlet corresponding to 2AP's methyl group, accompanied by the formation a triplet and pentet just upfield of the methyl signal. This is consistent with the incorporation of one or two deuterium atoms, respectively, based on the deuterium 2n+1 rule.

Further studies used water as the dilution solvent for 2AP. This is an appropriate solvent choice since water is a major component of many foods. Normal (protonated) water was used for NMR studies as well to overcome problems with exchange. Time course data was collected with high resolution mass spectrometry (HR-MS) and NMR for times within a few minutes of the reaction start and up to seven hours. MS2 data was used to obtain additional structural insights. 2D NMR experiments (COSY, TOCSY, HSQC, HMBC) were also conducted to gain connectivity data. Isotopically labeled d3-2AP was synthesized from d3-iodomethane. HR-MS studies in deuterium oxide were conducted on the labeled molecule.

Results and discussion

From the time course data collected with HR-MS (not shown) it is notable that 2AP's peak area declines in only 5 minutes [14]. Table 1 demonstrates the formation of a series of products with increasing molecular weight [14]. These masses are a multiple of 2AP's mass plus a proton. Alternatively, the masses correspond to a multiple of 2AP's mass, accompanied by the loss of water. For example, the mass 205.1338 is the equivalent mass of 2 molecules of 2AP, the loss of 18 Daltons from water and the addition of a proton to create a positive ion, caused by the ionization process. Another feature observed from the data is that the abundance of the dimer (223.1444) increases at early time points and then declines (data not shown) [14]. This would also be consistent with a polymerization process, where products continue to react and increase in size.

From Table 1, the mass accuracies are all within 3 ppm, which gives confidence that the proposed chemical formulas are correct. The same time course experiment was repeated with d3-2AP. In order to get useful data, the experiment must be conducted in deuterium oxide, since the deuteriums on the acetyl group exchange with the deuteriums of the solvent. Table 2 shows some very interesting results, which indicate that exchange must occur between the solvent and a proton on the pyrroline ring, indicating that a ring opening mechanism is involved [14]. The rationale for this conclusion is based on the fact that the observed masses have one more deuterium that the predicted formulas for the isotopic species. For example, the dimer has six deuterium atoms from the two molecules coming together and one deuterium from the ionization process, giving a total of seven deuterium atoms. Since eight deuterium atoms are actually present on the dimer, this strongly points to a ring opening mechanism. Further, this observation gives further confidence that a chemical reaction is occurring, as opposed to aggregates forming in the mass spectrometer.

Table 1: 2AP product masses with chemical formula assignments and qualitative descriptions

General name	*Chemical formula (protonated)*	*Exact mass*	*Mass accuracy, ppm*
2AP	$C_6H_{10}NO$	112.0760	2.762
dehydrated dimer	$C_{12}H_{17}ON_2$	205.1341	2.731
dimer	$C_{12}H_{19}O_2N_2$	223.1447	2.669
dehydrated trimer	$C_{18}H_{26}O_2N_3$	316.2026	2.044
trimer	$C_{18}H_{28}O_3N_3$	334.2131	1.741
doubly dehydrated tetramer	$C_{24}H_{33}O_2N_4$	409.2605	1.704
dehydrated tetramer	$C_{24}H_{35}O_3N_4$	427.2710	1.480
triply dehydrated pentamer	$C_{30}H_{40}O_2N_5$	502.3179	0.494
doubly dehydrated pentamer	$C_{30}H_{42}O_3N_5$	520.3287	0.929
triply dehydrated hexamer	$C_{36}H_{49}O_3N_6$	613.3874	2.175
doubly dehydrated hexamer	$C_{36}H_{51}O_4N_6$	631.3980	2.169

Table 2: Products observed from the loss of d_3-(Me)-2-acetyl-1-pyrroline in deuterium oxide

General name	*Chemical formula (protonated)*	*Predicted d_3-analogue (in D_2O)*	*Actual d_3- analogue (in D_2O)*	*Exact mass*	*Mass accuracy, ppm*	*Complications*
2AP	$C_6H_{10}ON$	$C_6H_6{}^2H_4NO$	$C_6H_6{}^2H_4NO$	116.10081	0.108	none
dehydrated dimer	$C_{12}H_{17}ON_2$	$C_{12}H_{12}{}^2H_5ON_2$	$C_{12}H_{11}{}^2H_6ON_2$	211.17116	-0.190	1 D exchanged for H
dimer	$C_{12}H_{19}O_2N_2$	$C_{12}H_{12}{}^2H_7O_2N_2$	$C_{12}H_{11}{}^2H_8O_2N_2$	231.1942	-0.512	1 D exchanged for H
dehydrated trimer	$C_{18}H_{26}O_2N_3$	$C_{18}H_{18}{}^2H_8O_2N_3$	$C_{18}H_{15}{}^2H_{10}O_2N_3Na$	348.24626	-1.165	2 D exchanged for 2 H
trimer	$C_{18}H_{28}O_3N_3$	$C_{18}H_{18}{}^2H_{10}O_3N_3$	$C_{18}H_{16}{}^2H_{12}O_3N_3$	346.28784	-1.413	2 D exchanged for 2 H
trimer			$C_{18}H_{16}{}^2H_{11}O_3N_3Na$	367.26306	-1.218	2 D exchanged for 2 H
dehydrated tetramer	$C_{24}H_{35}O_3N_4$	$C_{24}H_{24}{}^2H_{11}O_3N_4$	$C_{24}H_{21}{}^2H_{14}O_3N_4$	441.35799	-0.571	3 D exchanged for 3 H

The NMR data was collected in an array. There was no delay between each step in the array, allowing the maximum sensitivity by continuous acquisition of spectra. The major signals in the time zero data are all associated with 2AP and it structural dimer (data not shown) [14], the latter of which is formed during 2AP's chemical synthesis and is difficult to separate from 2AP using silica gel chromatography. After 2 hours of reaction, it is clear that much of the 2AP has been lost and new species have formed. The challenge of NMR in general is that milligram quantities of product are needed. In this reaction system, numerous products are being generated in trace quantities. Another particular challenge is the system's dynamic nature. NMR sensitivity doubles as the number of scans is quadrupled; however, this system does not give the option of long acquisition times. The problem is being overcome through the use of a quick sample preparation procedure using solid phase extraction. We have successfully isolated the dimer in excess of 90% purity. With the low quantity of 2AP, the dimer was stable for at least a 24-hour time period, quite adequate for acquiring quality 2D data. NMR spectra was collected using protonated solvents (regular water and methanol) due to the exchange problem. Presaturation of the methyl signal from methanol interferes with dimer cross peaks in the 2D data. Studies are currently underway with d3-methanol (CD3OH) to overcome this problem.

The data given here provide strong evidence that 2AP undergoes a polymerization process. Dilution is a simple strategy to extend 2AP's shelf life, also suggested by Buttery when he hypothesized that 2AP undergoes polymerization [1]. Various stabilization strategies have been developed for 2AP. Physical methods have included entrapment in cyclodextrin [9] and spray drying [15]. Unfortunately, these methods have suffered from poor loading and a limited increase in shelf life. The most promising strategy to date has been 2AP complexation with zinc halides [13]. The zinc forms a covalent bond to both the nitrogen and oxygen groups of 2AP. This complex is stable for months if stored under moisture free conditions. Further, since zinc, chloride and 2AP are all GRAS molecules, the complex should not pose a problem for obtaining GRAS status. This concept of holding the reactive groups on 2AP captive is a key idea from both the current work and the zinc halide complexation. Future stabilization work should continue to utilize this concept.

Summary and Conclusions

From this research we have provided mass spectral data to show that 2AP undergoes polymerization. We see that 2AP forms up to at least a hexamer in dehydrated form. MS^2 data indicates that the 2AP ring opens during formation of the dimer. Further, NMR suggests that the dimer contains carbonyl and carbon-carbon and/or carbon-nitrogen double bonds. We are continuing efforts to identify the dimer structure. Holding one or more of the reactive groups on 2AP captive is a promising approach to stabilizing this potent flavor molecule.

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