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Chemical characterization of a new carbohydrate metabolite in the vitreous of Black Moor goldfish by liquid chromatography-electrospray mass spectrometry and nuclear magnetic resonance

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Abstract. Our recent report has described the occurrence of a remarkably high amount of an unknown compound with light absorption maximum at 290 nm in the aqueous humor of Black Moor goldfish. The absorption maximum is shifted to 270 nm in 0.01% trifluroacetic acid. The concentration of this compound was very low in the eye of common goldfish and was absent in ocular fluids or serum of mammalian species. This compound was isolated by chromatography on a carbohydrate column eluted by 10 mM ammonium phosphate followed by chromatography on a Delta PAK C_{18} column eluted by acetonitrile–water–trifluoroacetic acid. The ion size of the unknown compound determined by electrospray for protonated and deprotonated ions are m/z 205 and 203, respectively. The protonated ion was fragmented to m/z 187, 169, 157 and 141 as the cone voltage increased. The data from mass spectrometry and nuclear magnetic resonance spectroscopy indicate the chemical structure of a carbohydrate with 7 carbons containing a lactone ring.

Keywords: Goldfish, aqueous humor, megalophthalmia, high pressure liquid chromatography, mass spectrometry, nuclear magnetic resonance

1. Introduction

Aqueous humor is secreted from the ciliary epithelium into the posterior chamber. It moves to the anterior chamber through the pupil and exits through the trabecular meshwork. The normal circulation of the aqueous humor supplies nutrients to tissues surrounding the posterior and anterior chamber, and removes toxic catabolic products. Therefore, abnormal metabolic activities in intraocular tissues can be assessed from the biochemical contents in the aqueous humor.

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In our previous study, we compared the biochemical contents in the aqueous humor of Black Moor goldfish with that of common goldfish in order to identify metabolic changes associated with megalophthalmia in Black Moor goldfish [1,2]. Black Moor goldfish is a mutant of the common goldfish, *Carassius auratus* [3]. A special feature of Black Moor goldfish is their remarkably large eye size [4,5]. Like many fish and mammalian species, the eyes of common goldfish grow very slowly after birth. The eye volume of common goldfish falls within a narrow range among fishes with body weights between 2 to 100 g [2]. The genetic capability in restricting eye growth after birth is lost among Black Moor goldfish. Their eye volumes increase proportional to their body sizes [2]. Our previous reports [1,2] have described a marked elevation of an unknown compound in the aqueous humor of Black Moor goldfish having a light absorption maximum at 290 nm, and a molecular weight of 204. We designate this unknown as compound 204. We now have characterized the unknown compound using a mass detector to monitor the ion size of compounds eluted from the column, and collected a large amount of the unknown through repeated chromatography for nuclear magnetic resonance spectroscopy (NMR).

2. Experimental

2.1. Extraction of compound 204 from aqueous humor

Black Moor goldfish was obtained from Hoipei Aquarium, Hong Kong. They were anaesthetized by being immersed in 0.05% 3-aminobenzoic acid ethyl ester (Sigma Chemical, St. Louis, MO, USA). The aqueous humor was aspirated by puncturing through the cornea with a 25 gauge needle connected to a tuberculin syringe.

About 6 ml of aqueous humor from megalophthalmic eyes and evaporated to dryness under vacuum in a Freezone 4.5 free dry system (Labconco, Kanasas City, MO, USA). The residue was extracted by vigorous mixing in a vortex mixer with 100 μl of acetonitrile. The residue was extracted 4 times in the same manner. There was negligible compound 204 in the acetonitrile extraction in the 4th extraction. The extract was evaporated to dryness and redissolved in 0.5 ml of 10 mM ammonium phosphate. We have also extracted the unknown compound from the vitreous in the same manner. The result was very similar to that from aqueous humor.

2.2. Chromatography on carbohydrate column

In our previous study, μ-Bondapax NH_2 column was used to demonstrate the occurrence of compound 204 in aqueous humor. We now use a Carbohydrate column (4.6 $\times$ 250 mm, supplied by Waters Associates, Milford, MA, USA). The chromatographic result of the present study is identical to that reported previously. The chromatograms of aqueous humor and vitreous are very similar to each other. An aliquot of 0.02 ml was injected to the column each time. The column was eluted by 10 mM ammonium phosphate. The fractions containing the major peak from each chromatographic purification step were combined, evaporated to dryness in a Freezone 4.5 freeze dry system.

2.3. Effect of solvent on light absorption spectrum

The residue obtained from the above step was dissolved in 0.5 ml of acetonitrile–water–trifluoroacetic acid (2 : 97.99 : 0.01). An aliquot of 0.01 ml obtained from the Carbohydrate column chromatography (see Section 2.2) was diluted with 0.1 ml of 10 mM ammonium phosphate. Another aliquot of 0.01 ml

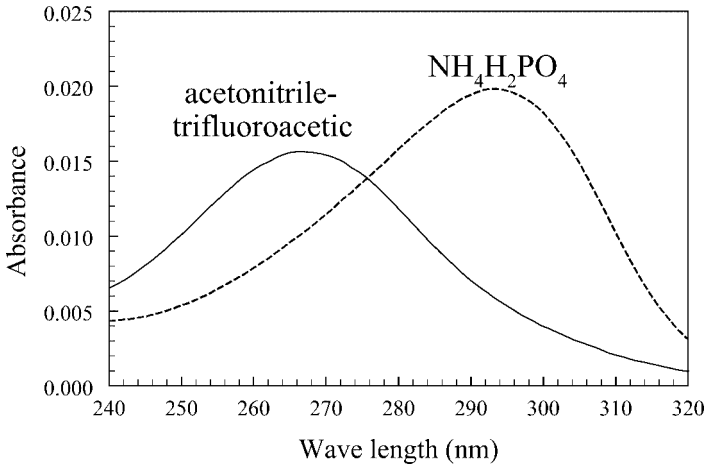

Fig. 1. Purification of the unknown on a Delta PAK C_{18} column. The major compound eluted from a μ-Bondapak-NH_2 column was applied to a Delta PAK C_{18} column and eluted by trifluoroacetic acid–acetonitrile as described in the text. Absorption spectrum after dilution by ammonium phosphate (solid line) and by trifluoroacetic acid–acetonitrile (broken line) is shown in figure.

was diluted with acetonitrile–water–trifluoroacetic acid (2 : 97.99 : 0.01) in order to compare the light absorption spectrum of the sample in two different solvents. A diluted sample was injected directly into the photo diode array detector to record the light absorption spectrum. The results are shown in Fig. 1.

2.4. Chromatography on Delta PAK C_{18} column and mass spectroscopy

For mass spectrometric analysis, an aliquot of 1 μl of the sample described in Section 2.2. was injected into a Delta PAK C_{18} column (15-μm particle size, 3.9 $\times$ 300 mm, supplied by Waters Associates) and eluted by acetonitrile–water–trifluoroacetic acid (2 : 97.99 : 0.01) at 0.4 ml/min. The eluant was delivered through a fine tubing (inside diameter of 0.005 inch) to a photo diode array detector, and a mass detector (Micromass Platform LCZ supplied by Waters Associates, Medford, MA, USA). The optimized settings in the mass detector were: nitrogen gas flow – 500 l/hr; capillary voltage – 3.5 kV, cone voltages – 10 V, source temperature – 140°C, desolvation temperature – 350°C. All mass spectra were recorded under a full scan operation for both positive and negative ions, with a scan range from m/z 20 to 600. In order to observe fragmentation at different cone voltage, the same amount of sample was analyzed in the same manner at different cone voltages specified in the legend of Fig. 3.

2.5. NMR spectroscopy

A large amount of sample was needed for nuclear NMR analysis. The unknown collected from the carbohydrate column chromatographic preparation (see Section 2.2) was further purified on a Delta PAK C_{18} column as described above (see Section 2.4). An aliquot of 0.05 ml was injected into the column each time. The fractions containing the major peak from many chromatographic preparations were combined, lyophilized to dryness and sent to “NMR Analysis and Solution”, Decatur, IL for NMR analysis.

The ^{1}H spectrum was run at 400 MHz, on a Varian VXR 4000 Spectrometer. A matched susceptibility tube (Shigemi, Inc) was used in order to maximize the sensitivity, since so little sample was available. The conditions were the following: 4000 Hz spectral width, 39^0 RF pulse, 16 sec relaxation delay, 4.1 sec

acquisition time, 32 transients. The water signal was not suppressed. Homonuclear decoupling experiments were run to establish connectivity.

The ^{13}C spectrum was run at 100 MHz on the same instrument, again using the Shigemi tube. The conditions were the following: 22,000 Hz spectral width, 39^0 RF pulse, no relaxation delay, 1.2 sec acquisition time, Waltz-16 broadband decoupling at 2.4 kHz power, 27,000 transients. 2 Hz line broadening was used to manipulate the free induction decay spectrum. The total run time required to obtain the spectrum was 9 hr. In spite of this the resulting ^{13}C spectrum did not have a sufficient signal to noise ration to detect any carbonyl groups.

3. Results and discussion

3.1. Effect of solvent on light absorption spectrum

Our previous data showed the presence of a remarkably high amount of an unknown compound in the aqueous humor and vitreous of Black Moor goldfish [1]. This compound has a light absorption maximum at 290 nm. Ammonium phosphate was used to elute the compound from the column in previous studies. Since ammonium phosphate interferes with mass spectroscopic analysis, we removed ammonium phosphate by a Delta PAK C_{18} column, eluted by acetonitrile–water–trifluoroacetic acid in this study. However, the compound eluted from Delta PAK C_{18} column had a light absorption maximum at 270 nm. The difference in absorption maximum must be due to different elution solvents used in the two chromatographic procedures. In order to document the influence of elution buffer on the light absorption maximum, an aliquot of the unknown recovered from the carbohydrate column step was diluted 10 times with each elution solvent and injected into the detector to measure the light absorption spectrum. The results (Fig. 1) confirmed the effect of solvent on light absorption maximum of the unknown.

3.2. Sensitivity of the unknown to hydrolysis in HCl

A typical chromatogram of the unknown on a Delta PAK C_{18} column is shown in Fig. 2A (upper tracing). A single light absorption peak was observed at 8.7 min in the 270 nm-chromatogram. This peak was completely lost after incubation with HCl at 90°C for 2 hours (Fig. 2A, lower tracing). The chromatogram detected by electrospray for the deprotonated ion, m/z 203, showed one peak (Fig. 2B, upper tracing) coinciding with the light absorption peak (Fig. 2A, upper tracing). The ion, m/z 203, was also completely destroyed by HCl (Fig. 2B lower tracing). A weak signal for a deprotonated ion, m/z 221, appeared at the retention time of 9 min after hydrolysis (Fig. 2C, lower tracing). The deprotonated ion, m/z 221 was not observed before hydrolysis (Fig. 2C, upper tracing). Carbohydrates have very low signal in electrospray analysis. The weak signal of the unknown after opening the lactone ring by HCl was expected. There was no other compound detectable by light absorption, nor electrospray for protonated or deprotonated ion after hydrolysis.

3.3. Fragmentation of the unknown at different cone voltage

Three chromatographic analyses of the same sample monitored at different cone voltage are shown in Fig. 3. When the eluant from the column was analyzed by electrospray for protonated ions, the major ion was m/z 205 (Fig. 3A). Electrospray for deprotonated ion showed the presence of m/z 203 (see Fig. 2B). Therefore, the molecular weight of the unknown must be 204. The ion, m/z 227, must be the

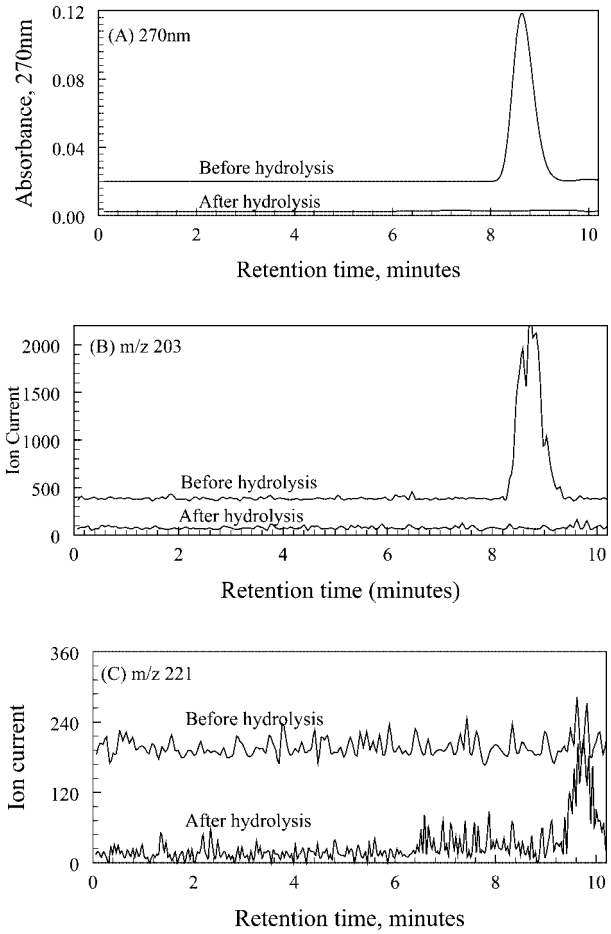

Fig. 2. Effect of HCl on light absorption and ion size: Purified compound 204 was incubated in 6 N HCl at 95°C for 60 minutes, the compound was analyzed before and after hydrolysis as described in the text: The eluant was monitored (A) at 270 nm, (B) by electrospray for deproteinized ion, m/z 203, and (C) deproteinized ion, m/z 221.

sodium adduct of the ion, m/z 205. A small fragment, m/z 187, was noticed in the spectrum obtained at 10 volt. When the cone voltage was increased to 20 volt, the ion, m/z 205, was reduced, and m/z 187 increased. In addition, the ions, m/z 169, 157, 141 increased (Fig. 3B). As cone voltage increased to 30 volts, ions m/z 227, 205 were lost. The major ions were m/z 157, and 141 (Fig. 3C). When the eluant was monitored for deprotonated compound at 10 volt, only m/z 203 was observed. Increased voltage destroyed m/z 203. The degradation products were not detectable by electrospray for deprotonated ions.

3.4. Nuclear magnetic resonance spectroscopy

The purified sample was analyzed for ^{1}H spectrum (Fig. 4) and ^{13}C spectrum. There was no signal at higher frequency than the water peak at 4.8 ppm, which rules out aromatic and sp2 hybridized materials.

The strongest set of signals in the ^{1}H spectrum are the methyl doublet 1.44 ppm and the quartet 4.40 ppm at an integrated ratio of 3 : 1. This set of signals indicated lactic acid and was corroborated by the ^{13}C spectrum (19.0 ppm, 66.2 ppm). The integral intensities for compound 204 are all 1 : 1 : 1 : 1 : 1

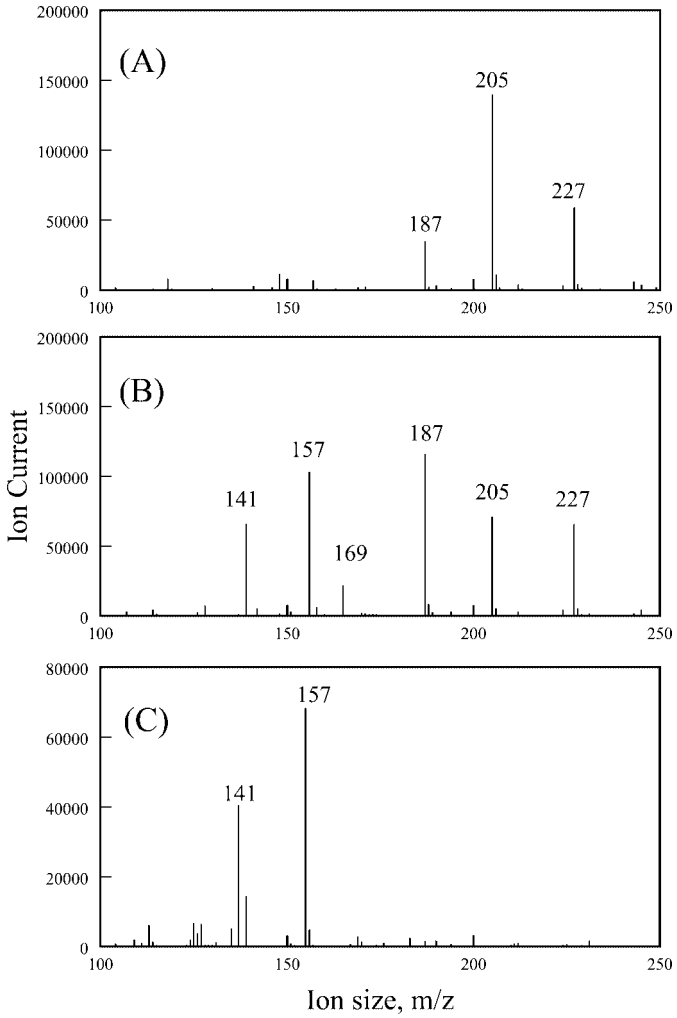

Fig. 3. Mass spectrum at retention time 8.7 min detected by different cone voltage: (A) 10 volt, (B) 20 volt and (C) 30 volt.

for the five signals involved. There was also a strong singlet at 3.62 ppm, however it was not observed in the spectrum of a second sample of material. The 3.62 singlet integrates to 3.45 times the peak from compound 204. Therefore it was concluded to arise from still another contaminating material.

There was another compound present indicated by another set of signals. This second compound was present at 0.30 moles to 1.0 moles of lactic acid. It's ^{1}H signals consisted of the following: an ABX system, 2.89 ppm, 2.58 ppm, 4.31 ppm respectively, $J_{AB} = 17.6$ Hz, $J_{AX} = 0$, $J_{BX} = 0.70$ Hz; and an AB system 3.75 ppm, 3.60 ppm, $J_{AB} = 12.1$ Hz.

The 17.6 Hz coupling in the ABX system is large and unusual and must result from a geminal coupling, i.e., hydrogens A and B reside on the same carbon. The second coupling (J_{BX}), 0.70 Hz is also unusual. Coupling constants are indicative of H–H bond angles. The small J_{AX} is more likely due to the bond angle between A and X. If the bond angle between A and X is zero degree, the coupling constant is minimized and may be undetectable. This data can be rationalized by a six or possibly seven membered ring. The carbon bearing AB hydrogens are bonded to a carbonyl group, and a methine group with its

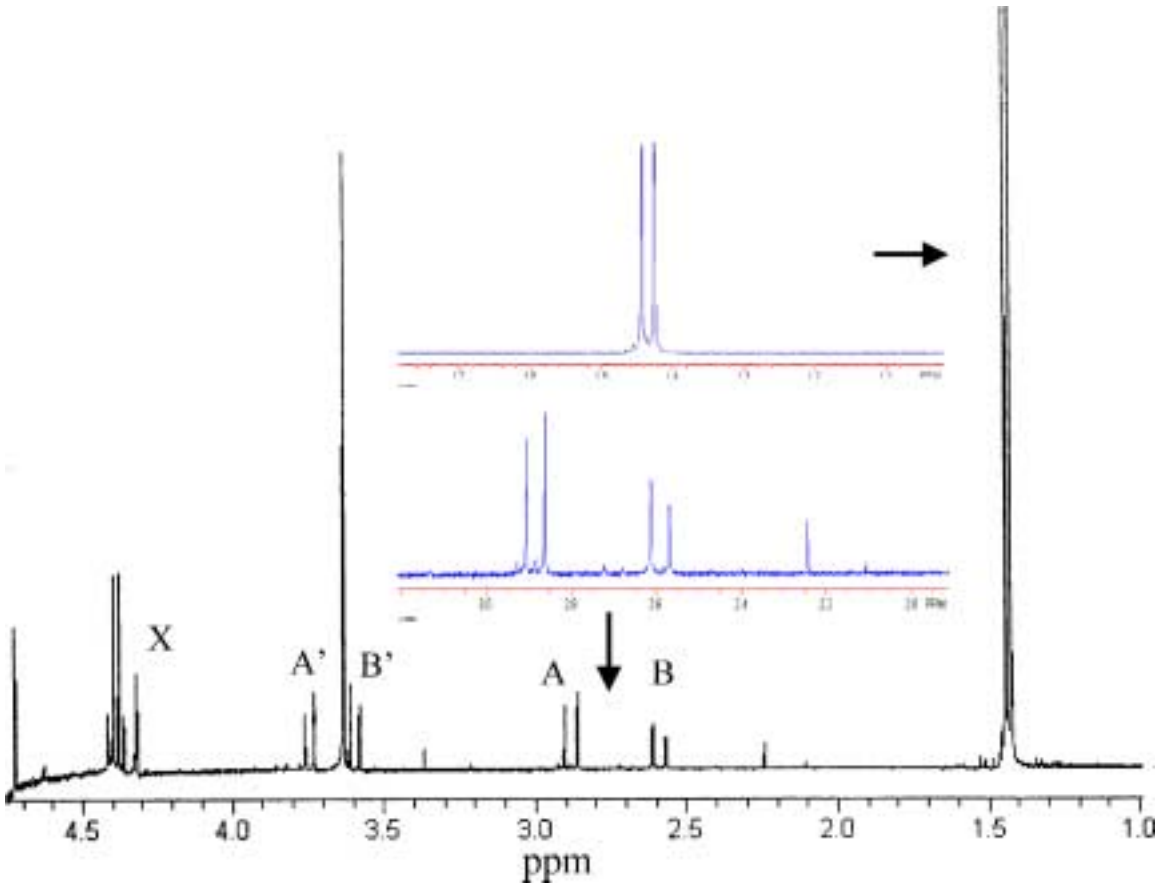

Fig. 4. ^{1}H Nuclear magnetic resonance spectrum.

hydrogen in an axial position. This methine must have two electron withdrawing groups attached to explain the 4.31 ppm chemical shift. The chemical shifts and coupling of the AB spectrum, is similar to those seen in carbohydrates, when there are two adjacent hydrogens in an equatorial relationship.

4. Conclusion

Mass spectrometry data reveals the molecular size of 204. Although NMR spectroscopy indicates the presence of lactic acid in our preparation of compound 204, lactic acid has a molecular weight of 90 and is optically inactive. When the eluant from the column was monitored by photo diode array detector at 270 nm, the light absorption peak coincides with the deprotonated ion, m/z 203, and protonated ion m/z 205. Lactic acid was detected by electrospray for the deprotonized ion, m/z 89. The m/z 89 peak was observed immediately behind the 270 nm peak (Fig. 5). The data indicate that compound 204 has the light absorption characteristic of the second compound observed in the NMR spectrum.

The light absorption characteristic of compound 204 at 270 nm and mass signal for the ion, m/z 203, was lost upon hydrolysis. The appearance of m/z 221 after hydrolysis supports the structure of a lactone. Opening the lactone ring of the ion m/z 203 by water increased the molecular size by 18 to m/z 221.

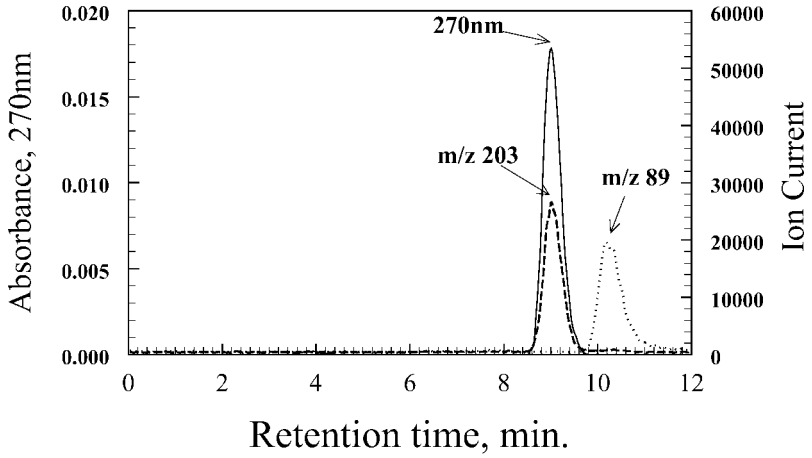

Fig. 5. Chromatogram of aqueous humor from a Black Moor goldfish: Comparison of retention time of compound 204, lactic acid and the unknown with 270 nm absorption: An aliquot of 2 μl of aqueous humor was injected to a Delta PAK C_{18} column, and eluted by acetonitrile-trifluoroacetic acid at 0.4 ml/min as described in the text. The eluant was recorded at 270 nm by a photo diode array detector, followed by a mass detector.

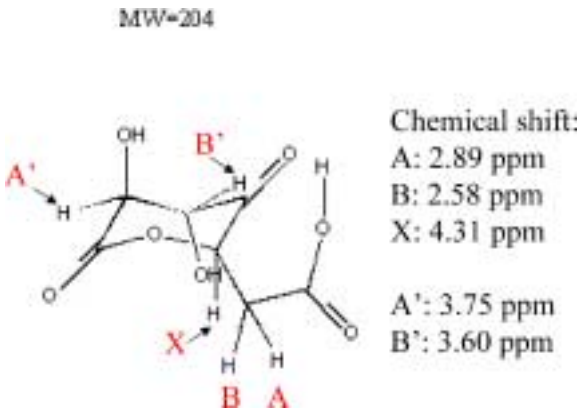

Fig. 6. Proposed structure for compound 204.

The structure proposed for compound 204 is shown in Fig. 6. This structure has been rationalized from the NMR spectrum for a compound with a molecular size of 204. The six membered ring in which the ABX system resides can result from the strong hydrogen bonding of the acid H to the keto group. Another concern is the chemical shifts of the AB protons, which can be rationalized by spatial relationships of them to the ketone and lactone carbonyl groups. The proposed structure can be fragmented to ions m/z 187, 169, 157 and 141 as shown in Fig. 7.

We have observed compound 204 in the serum of 5 different species of goldfish. Its occurrence in the eye was observed only in Black Moor goldfish. It has not been observed in the serum of rat, rabbit, chicken, bovine, and human. This compound is a carbohydrate metabolite of fishes, absent in mammalian tissues.

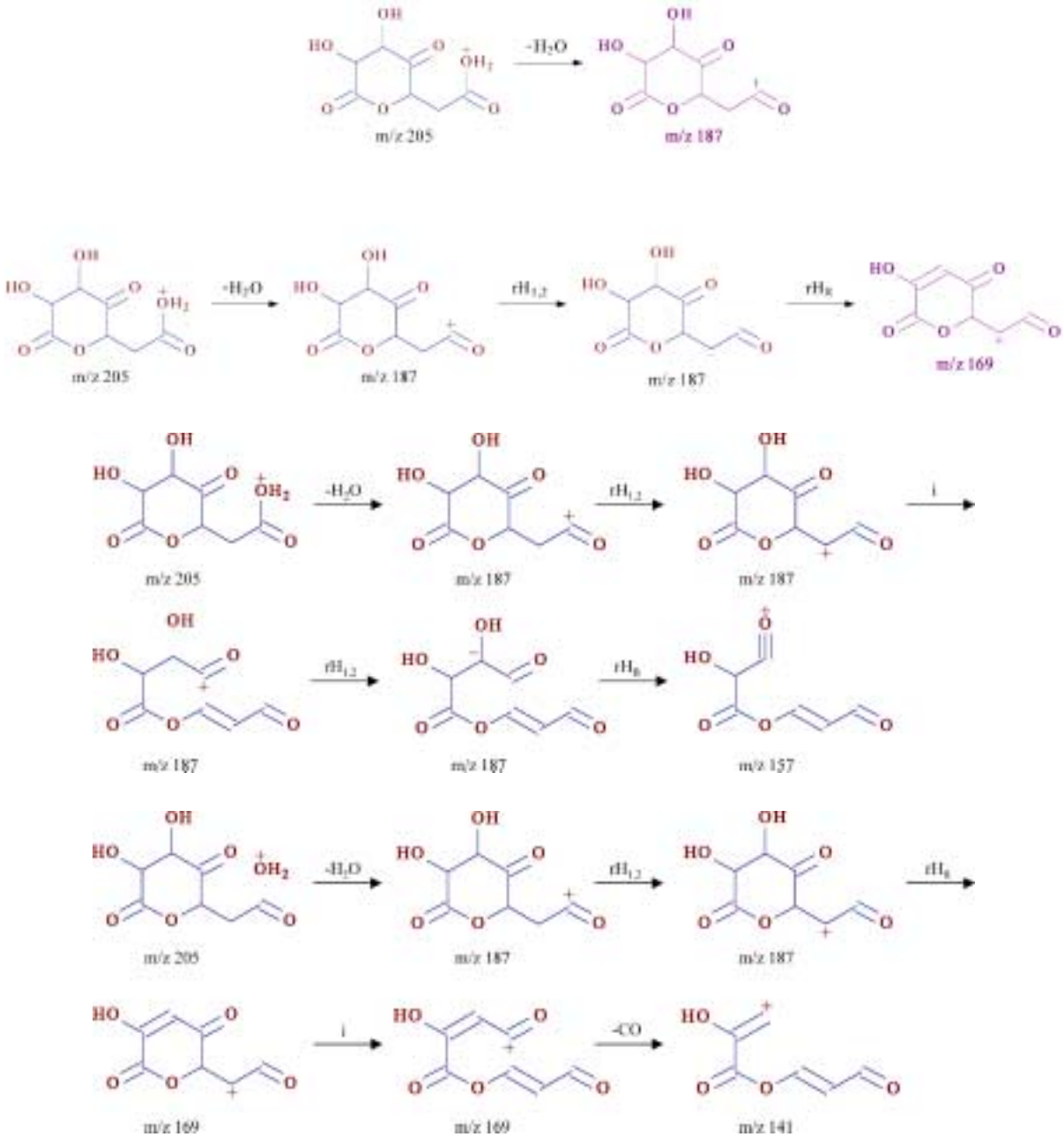

Fig. 7. The possible degradation fragments (m/z 187, 169, 157 and 141) and fragmentation mechanism from compound 204.

Acknowledgement

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