A Cartilage Derived Novel Compound DDP (2,6-dimethyldifuro-8-pyrone): Isolation, Purification, and Identification

HARPAL K. GAHUNIA, ALAN LOUGH, REINHOLD VIETH, and KENNETH P.H. PRITZKER

ABSTRACT. ***Objective.*** Fluorescent biomolecules within cartilage matrix can be used as specific markers of cartilage metabolism. While establishing the protocol to evaluate mature collagen crosslinks in articular cartilage (AC) associated with maturation, aging, and osteoarthritis, chromatographic analysis of the crosslinks also revealed an apparently novel fluorescent peak. Preliminary investigation of this compound (now abbreviated DDP) in various tissues from rabbits, calves, chickens, and humans showed that this compound is AC-specific. We aimed to isolate, purify, and identify this fluorescent compound.

Methods. Fully encapsulated, bovine metacarpophalangeal joints (n = 350, age < 2 years) were used as the source for AC. DDP was isolated and purified by reverse phase high pressure liquid chromatography, and its elution was monitored using a fluorescence detector at excitation λ = 306 nm, and emission λ = 395 nm. The liquid phase of DDP was characterized by mass spectrometry and nuclear magnetic resonance spectroscopy. DDP solution (5.7 μg/μl) was crystallized in 100% deuterated methanol and the DDP crystal was characterized by single crystal x-ray diffraction.

Results. From bulk preparations, 12 μg (58 nmol) per gram dried AC of the novel compound was isolated and purified. Analytical techniques to identify this AC-specific compound, 2,6-dimethyldifuro-8-pyrone, corroborate and confirm its molecular structure and atomic connectivity in both liquid and solid phase. DDP is a symmetrical aromatic compound with molecular weight 204, molecular formula $C_{11}H_8O_4$, and a molar extinction coefficient 4700 M^{-1} at maximal UV absorption (λ = 306 nm).

Conclusion. 2,6-dimethyldifuro-8-pyrone (DDP) is a novel cartilage-specific compound that could have potential application as a unique biochemical marker in joint diseases involving articular cartilage degradation. (J Rheumatol 2002;29:147–53)

Key Indexing Terms:
ARTICULAR CARTILAGE BIOCHEMICAL MARKER CRYSTALLOGRAPHY
MAGNETIC RESONANCE SPECTROSCOPY MASS SPECTROMETRY DDP

During normal metabolic processes and in disease, tissues release various compounds into the blood and body fluids. Some of these compounds are specific to particular tissues and can, therefore, be used as diagnostic markers to monitor its status. Articular cartilage (AC) is a specialized connective tissue that functions in joints and at other skeletal sites to resist compressive forces associated with locomotion. The ability of AC to perform this function can be compromised by changes in its properties that occur with age or manifestation of diseases such as osteoarthritis (OA) and rheumatoid arthritis (RA). These changes reflect metabolism that can be monitored by analysis of appropriate biological markers[1]. Hence, treatments designed to slow or reverse the effects of abnormal metabolism can benefit enormously from early detection of the disease state.

While fluorescence emission has been used extensively to study protein and carbohydrate structure and dynamics, few studies have utilized fluorescence emission for studies of AC. Fluorescent molecules in AC are produced either by enzymatic reactions of proteins (e.g., pyridinium collagen crosslinks) or by non-enzymatic reaction products of sugars or oxidized lipids (e.g., pentosidine)[2,3]. Pyridinoline (Pyd) was first isolated and characterized from rat tail tendon by Fujimoto, *et al*[4,5]. Pyd is present in collagen-containing tissues such as cartilage, synovial membrane, meniscus, bone, and ligament. It is far more abundant in cartilage than in bone[6]. Pentosidine was iso-

From the Department of Pathology and Laboratory Medicine, Samuel Lunenfeld Research Institute, Mount Sinai Hospital; and Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada.

H.K. Gahunia, PhD, Senior Scientist, Department of Orthobiologic Technologies, DePuy Orthopaedics, formerly Graduate Student, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, and Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto; A. Lough, PhD, Service Manager, X-Ray Crystallography Laboratory, Department of Chemistry, University of Toronto; R. Vieth, PhD, Clinical Biochemist, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Associate Professor, Department of Laboratory Medicine; K.P.H. Pritzker, MD, FRCPC, Pathologist-in-Chief, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Professor, Department of Laboratory Medicine and Pathobiology and Department of Surgery, Faculty of Medicine, University of Toronto.

Address reprint requests to Dr. K.P.H. Pritzker, Department of Pathology and Laboratory Medicine, Suite 600-1, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, Canada M5G 1X5.
E-mail: kpritzker@mtsinai.on.ca

Submitted October 27, 2000; revision accepted August 16, 2001.

lated from human dura mater and characterized by Sell and Monnier[7]. Pentosidine has also been detected in skin, ocular lens, and cartilage. Its level increases exponentially with age[8]. Pyd and its deoxy form (Dpyd) and pentosidine are used as biochemical markers of cartilage and joint tissue degradation[9–12].

While establishing the conventional procedure[6] to quantify collagen crosslinks using reversed phase high pressure liquid chromatography (HPLC) in our laboratory, we detected an additional fluorescent peak that eluted roughly 12 to 14 minutes after Pyd and Dpyd (elution time at 8 to 10 min). Preliminary investigation of this compound (now abbreviated DDP) in various human and animal tissues (ligament, articular cartilage, bone, tendon, meniscus, skin, etc.) indicated its articular cartilage-specific nature. An extensive literature search did not reveal any documentation of this peak. We aimed to isolate and purify this fluorescent compound from articular cartilage and to elucidate its molecular structure for further biological and chemical characterization.

MATERIALS AND METHODS

Tissue samples. Fully encapsulated calf metacarpophalangeal joints (age < 2 yrs) were obtained from the abattoir (Ryding Regency, Toronto, ON, Canada) and dissected within 24 h of death. AC was excised, freeze-dried, and stored at –70°C until the day of experiment.

Reagents. The Hewlett-Packard HPLC system consisted of 2 HPLC pumps (model 1050), an automatic injector (series 1100), and a diode array absorbance detector (model 1050). The peak fluorescence was monitored using a Perkin-Elmer fluorescent detector (model LC240). The following solvents were used: hydrochloric acid (Sigma), HPLC grade water (Omnisolve, Fisher Scientific), HPLC grade methanol (Caledon Laboratories), and HPLC grade acetonitrile (Caledon Laboratories).

Cartilage hydrolysis and preparation. A total of 116 g of dried cartilage was used to isolate and purify DDP for chemical analysis. Eight to 10 g of lyophilized AC flakes were hydrolyzed with 6 M HCl (20 mg/ml) for 24 h at 110°C. The hydrolyzed AC was kept at ambient temperature for 6 h, and the acid was evaporated in a round Pyrex flask using a rotoevaporator (Fisher Scientific). The dried hydrolyzate was reconstituted in 25 ml (total volume) of 50% methanol and 50% deionized (DI) water. After rigorous vortexing, the black slurry was filtered through a sterilized 0.22 μm Gelman filter and aliquoted into 2 ml HPLC injection vials for automatic injection.

HPLC separation and purification of DDP. Reverse phase HPLC and a total of 3 cycles (steps) of sample injection, peak collection, lyophilization, and resuspension stages were used to separate and purify DDP from hydrolyzed AC. The 3 steps differed either in the constitution or concentration of the mobile phase and the dimensions of the HPLC column used. The fluorescence detector was set at excitation λ = 306 nm and emission λ = 395 nm. Step 1 involved injection of 1 ml of AC hydrolyzate onto a Phenomenex Sphereclone semipreparatory C18 HPLC column [dimension 250 × 10 mm, particle size 5 μm, octadecyl silicate (ODS) 2]. The mobile phase comprised 30% acetonitrile in 70% DI water, flow rate was 4 ml/min, and back pressure was about 170–190 bars. The eluant fractions corresponding to DDP peak were manually collected (tails of the peak were discarded) from all the sample injections and the fractions were pooled, evaporated, and resuspended in 5 ml of 50% methanol and 50% DI water. Step 2 involved injection of 1 ml of semipure DDP onto a new semipreparatory HPLC column with dimensions as in step 1. The mobile phase comprised 20% acetonitrile to separate any compound coeluting with DDP. The DDP peak eluant was manually collected from all the sample injections, and the fractions were pooled, evaporated, and resuspended in 4 ml of 50% methanol and 50% DI water. Step 3 involved injection of 2 ml DDP solution onto a Phenomenex Sphereclone analytical C18 HPLC column (dimensions 250 × 4.6 mm, pore size 5 μm, OD 2), and mobile phase comprised 50% methanol and 50% distilled water. The flow rate was 1 ml/min and pressure 190 bar. For each step, DDP was manually collected and care was taken to discard the tails of DDP peak to minimize any possible coeluting and contaminating compound. DDP was transferred onto a preweighed test tube and dried under nitrogen. A control sample (700 ml of 6 M HCl) was prepared under similar experimental conditions as DDP.

Preparation of purified DDP for nuclear magnetic resonance and mass spectroscopy. The purified DDP (1.4 mg) was resuspended in 80 μl of 100% deuterated methanol, briefly vortexed, and placed in a 3 mm inner diameter nuclear magnetic resonance (NMR) tube (Nerelac Glass Ware). The above procedure was repeated twice, making a total volume of 240 μl. Mass spectrometry and NMR spectroscopy were used to characterize DDP-deuterated methanol solution and also the control sample in similar solution.

Mass spectrometry. The DDP was analyzed by electrospray ionization mass spectrometry (ESIMS) and by tandem mass spectrometry (MS/MS). The ESIMS experiment, used to detect the parent ion in solution, was performed using Perkin-Elmer/Sciex (Concord, ON, Canada) API-III triple quadrupole mass spectrometer. The mobile phase consisted of a solution of 50% acetonitrile and 50% water, 1 mmol ammonium acetate, and 0.1% acetic acid. The mobile phase was pumped at a flow rate of 0.020 ml/min using LKB Bromma (Sweden) HPLC pump. The voltage applied to the tip of the ion spray needle was 5 kvolts, and the voltage applied to the orifice was 80 volts. One to 20 μl of the sample solution was injected into the mass spectrometer. On the other hand, MS/MS experiment gave information on the structure of selected parent ion and its fragmented daughter ions. An aliqout of DDP solution was injected and ionized on a needle probe, i.e., where ions were formed. MS/MS scans were obtained by mass selecting a parent ion that was observed in the normal mass spectrometry scan into the second quadrupole. The pressure of the collision gas (argon) in the second quadrupole determined the degree of fragmentation and was set such that the collision gas target had a value of 200 to 250 bars. To further confirm the structural analysis, high resolution experiments were conducted on 2 separate preparations of DDP and at 2 different times. The instrument was operated at 10,000 resolution (10% valley) 2• = 5.7 PPM based on 27 measurements of the molecular ion of cholesterol.

NMR spectroscopy. One-dimensional (1-D) proton (^{1}H) and carbon (^{13}C) NMR spectroscopic experiments were acquired using a 500 MHz Varian Unity NMR spectrometer. A 3 mm closed NMR tube containing DDP deuterated methanol solution (5.7 mg/ml) was placed in the 3 mm Nalorac direct microprobe for analysis. The experiments were run at ambient temperature and recorded using a single pulse protocol. The ^{1}H NMR spectrum was obtained with 16 scans using a 60° pulse and a 0.98 s recycle delay. The ^{13}C NMR spectrum was obtained with 4711 scans, using a 90° pulse and 50 s recycle delay.

Single crystal x-ray diffraction. The NMR tube containing DDP solution (5.7 mg/ml, pH 7) was placed at –9°C, and pale yellow crystals were formed by day 10. The molecular structure and atomic connectivity of DDP in crystal phase was investigated using single crystal x-ray diffraction analysis. A crystal (volume 13.72×10^{-3} mm^3) was selected, mounted on a glass fiber, and stabilized by embedding in epoxy. Data were collected at room temperature on a Nonius KappaCCD diffractometer using graphite monochromated MoK• radiation (ß = 0.71073 Å). Three hundred sixty frames of 1° rotation in phi were exposed for 60 s each. Indexing, integration, and scaling were carried out using the Denzo package. Lorentz and polarization corrections were carried out, but no absorption correction was applied. The structures were solved and refined using the SHELXTL\PC package[13]. Refinement was by full-matrix least squares on F^2 using all data (negative intensities included).

RESULTS

HPLC separation and purification of DDP. A total of 1.4 mg of DDP was purified from 116 g of dried calf metacarpophalangeal AC (12 μg or 58 nmol of DDP/g dried AC). During

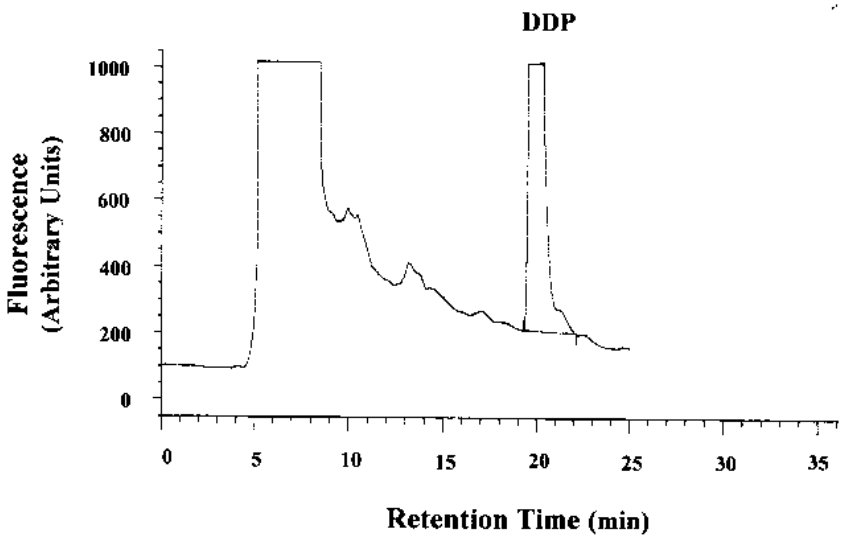

Figure 1A. Reverse phase HPLC chromatograph of DDP in acid hydrolyzed bovine metacarpophalangeal articular cartilage during purification step 1. The sample was eluted with mobile phase consisting of acetonitrile/deionized water (30:70). The eluant peak was monitored using fluorescence detector with excitation and emission wavelength 306/395 nm. Typical elution time of DDP was 19 min with the elution time range 19–21 min.

purification steps 1 and 2 (acetonitrile solution), DDP fluorescent peak eluted at retention times of 19 min (Figure 1A) and 41 min, respectively. At the purification step 3 (50% methanol), DDP peak eluted at 15.5 min with the elution time range from 15.5 to 22 min (Figure 1B). The control sample showed no peak corresponding to the retention time of DDP collection in the various purification steps. The measured UV absorbance of DDP at maximum wavelength (306 nm) was 0.69 AU. The molar extinction coefficient of DDP in 50% methanol was $4700.M^{-1}.cm^{-1}$.

Mass spectrometry. The data obtained from ESIMS experiment clearly showed a striking difference between the 2 collected fractions, i.e., DDP solution (Figure 2A, upper panel) versus control solution (Figure 2A, lower panel). The $[(H_2O)_4$ MeOH]H^+ fragment originates from the methanol:water solvent (50:50) in which the sample (DDP or control) was dissolved. The species of MW 204 appeared over a relatively narrow time range as the probe was heated and could be "separated" from the bulk of the impurity in the spectrum.

High resolution measurement of DDP yielded a value of 204.04 amu for the molecular ion (Figure 2B). When this value was submitted for possible "fits" for C, H, O (0–4), N

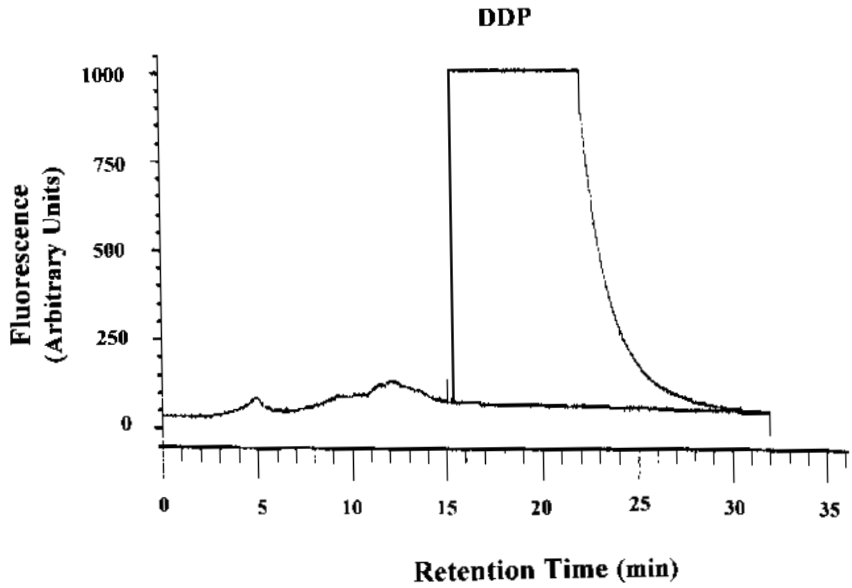

Figure 1B. Reverse phase HPLC chromatograph of DDP observed during purification step 3, eluted with mobile phase consisting of methanol/deionized water (50:50). Typical elution time of DDP was 15.5 min with elution time 15.5–22 min.

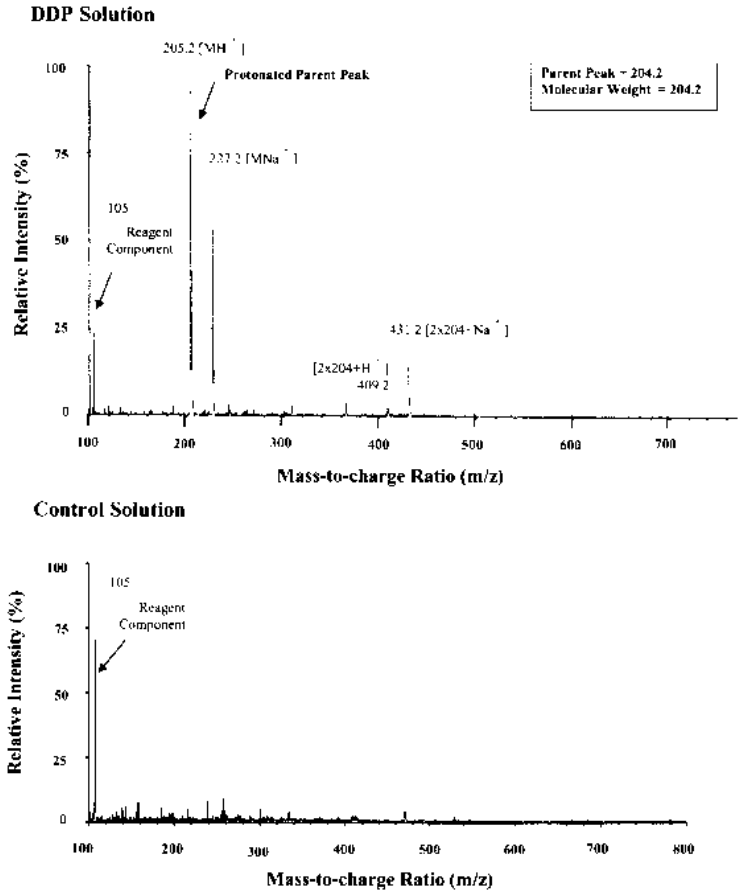

Figure 2A. Electrospray ionization mass spectrometric analysis of DDP versus a control sample. Upper panel shows the mass spectrum of DDP solution. The peak at 205.2 m/z depicts protonated DDP. Lower panel shows the mass spectrum of control sample solution. The peak at 105 corresponds to the reagent peak. Note the absence of a peak corresponding to 205.2 m/z.

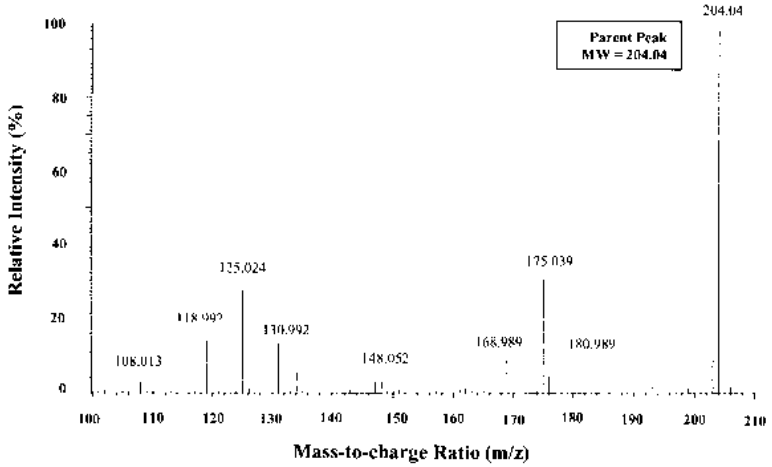

Figure 2B. Tandem mass spectrometric analysis shows the mass spectrum of DDP. The peak at 204.04 m/z depicts DDP. The molecular ion (m/z 204) decomposed through loss of m/z 29 (CHO) to afford m/z 175 ($C_{10}H_7O_3$) and a loss of m/z 79 (C_5H_3O) to yield m/z 125 ($C_6H_5O_3$).

(0–2), and P (0–2), the possibility that emerged was $C_{11}H_8O_4$. The fragment at 175.04 amu was identified as $C_{10}H_7O_3$ arising via CHO loss from the appropriate 204. The fragment at 125.02 amu was identified as $C_6H_5O_3$ arising from the loss of C_5H_3O (C_5H_4O when protonated) from the respective 204 and was thought to have a trihydroxy benzene structure. The frag-

ment at m/z 80.03 amu was identified as C_5H_4O. From examination of the isotopic distribution of the molecular ion, the correct formula for DDP appeared to be $C_{11}H_8O_4$.

Nuclear magnetic resonance spectroscopy. The ^1H NMR spectrum showed 2 peaks of interest, one at 2.5 ppm (methyl singlet) and another at 6.6 ppm (olefinic singlet). All ^1H chemical shifts were recorded with respect to trimethylsulfonyl. The singlet peaks indicate that these functional groups neighbor no other protons. The methanol and water proton peaks were the prominent peaks in the spectrum. The other weak resonances observed in Figure 3A originated from impurities.

A typical ^{13}C NMR spectrum of DDP showed 6 singlets, showing a high degree of symmetry in its molecule (Figure 3B). Peak assignment was performed in standard ^{13}C chemical shifts[14]. There were 3 resonances apparent in the aromatic region (aromatic carbons) of the spectrum, 156 ppm, 158 ppm, and 161 ppm, with a relative ratio of 2:1:2. The ^{13}C singlets at 156 ppm and 161 ppm correspond to fully conjugated aromatic/olefinic carbons. The ^{13}C singlet at 161 ppm is assigned to carbon in the aromatic ring directly connected to the methyl group. The 158 ppm peak belongs to a carbonyl carbon in the aromatic ring with no attached protons. The other singlets were observed at 14.4 ppm (methyl carbon), 100.5 ppm (olefinic methine carbon), and 139 ppm. The ^{13}C singlet at 100.5 ppm is an olefinic carbon with an attached proton.

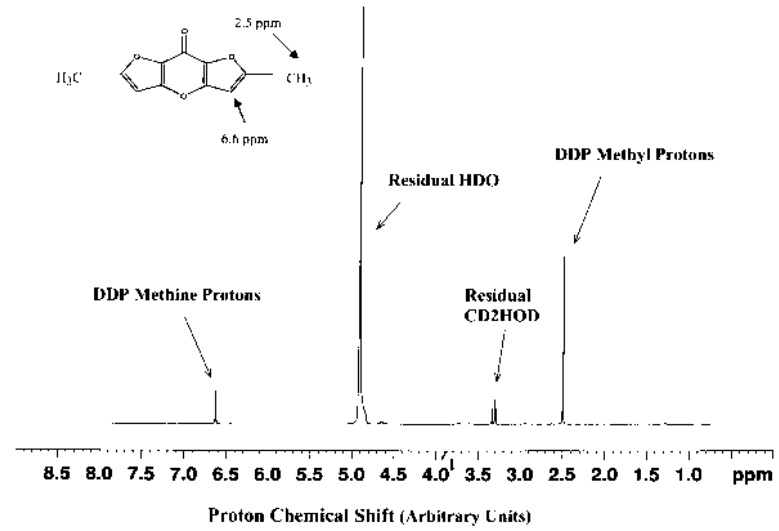

Figure 3A. One dimensional proton NMR spectra of DDP in deuterated methanol. Two proton environments were noted at 2.5 ppm and 6.6 ppm, corresponding to DDP methyl and DDP methine protons, respectively.

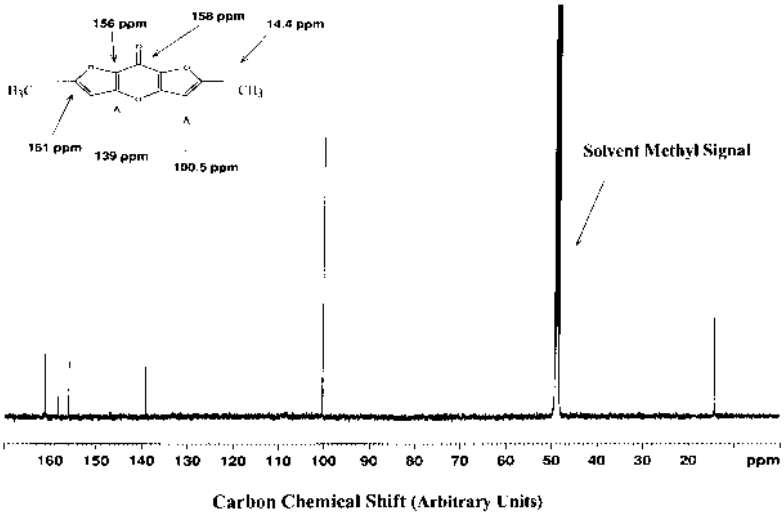

Figure 3B. One dimensional carbon NMR spectra of DDP in deuterated methanol. A total of 6 carbon environments were noted. The carbon peaks at 139, 156, 158, and 161 ppm correspond to the aromatic carbons of DDP molecule. Carbon peaks at 14.4 and 100.5 ppm correspond to methyl and olefinic carbon, respectively.

Single crystal x-ray diffraction (crystallography). Pale yellow crystals of DDP formed within 10 days and reached their final size after several additional days at –20°C. The supernatant was removed to analyze DDP crystal structure by single crystal x-ray diffraction. As expected, no crystals were observed from the control sample kept at –9°C for a month.

The DDP crystals diffracted x-rays isotropically to at least 0.8 Å in resolution. The lattice parameters of the crystals belong to the orthorhombic system with space group $Cmc2_1$ and cell dimensions a = 12.87 (1) Å, b = 10.81 (1) Å, c = 13 (1) Å, alpha = 90 Å, beta = 90 Å, and gamma = 90 Å. The most striking structural features of DDP were the symmetrical, rigid nature of the DDP, and the stacking of the DDP molecules. DDP is a very stable aromatic molecule consisting of 11 carbon atoms, 8 hydrogen atoms, and 4 oxygen atoms (Figure 4A and B). Each molecule has an axis of symmetry (mirror plane) and consists of a methylated furan molecule. The 2-methylated furan molecules are arranged symmetrically on the opposite ends of the central pyrone ring.

DISCUSSION

An articular cartilage-specific aromatic compound with IUPAC nomenclature of 2,6-dimethyldifuro-8-pyrone (DDP) was isolated from calf metacarpophalangeal joint. The molecular structure of DDP was determined and confirmed from its liquid phase by mass spectrometry and NMR spectroscopy and its solid phase by single crystal x-ray diffraction. Both low resolution and high resolution mass spectrometry, as well as crystallography, showed that the parent peak of purified DDP molecule has a molecular weight of 204 and a molecular formula of $C_{11}H_8O_4$. NMR spectroscopy experiments, in conjunction with the mass spectrometry data, clearly showed that the molecule in question has a very high degree of symmetry with 6 unique carbon environments and 2 unique proton environments. Since each half of the DDP molecule is a mirror image of the other, only 6 carbon atoms and 4 hydrogen atoms were assigned from the ^1H and ^{13}C NMR spectra. The single crystal x-ray diffraction of DDP conclusively provided the structure of DDP in solid phase. Therefore, the results from various analytical techniques used to identify DDP are well corroborated, confirming that the molecular structure and atomic connectivity of DDP were similar in both liquid and solid phases, indicating that there was no structural change upon crystallization of DDP.

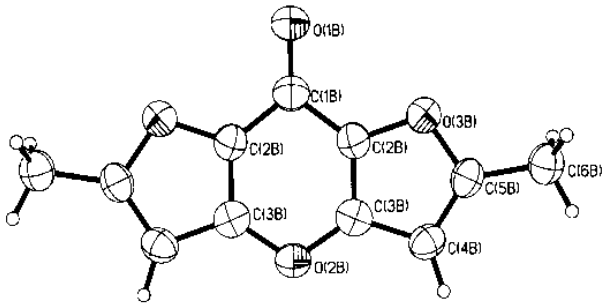

Figure 4A. Schematic of molecular structure of DDP (204 Dalton) determined from single crystal x-ray diffraction. A DDP molecule has a total of 11 carbons, 8 hydrogens, and 4 oxygens.

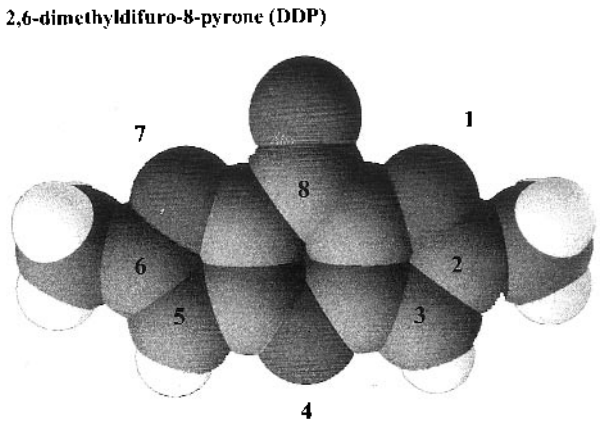

Figure 4B. Molecular structure of DDP (204 Dalton) with IUPAC nomenclature of 2,6-dimethyldifuro-8-pyrone. There are 11 carbon atoms, 8 hydrogen atoms, 4 oxygen atoms.

The control sample, consisting of 6 M HCl without AC and prepared similarly to the purification method for DDP, did not show crystal formation when seeded for crystallization for up to a period of one month. The absence of DDP in the control sample showed that DDP is not merely an artifact from 6 M HCl itself.

Based on its chromatographic elution times, DDP is different from pyridinoline (optimal absorption and emission wavelengths at 295 and 395 nm) and certainly different from glycated products that exhibit similar fluorescent emission spectra such as pyridoxamine (a vitamin B6 metabolite). DDP has optimal absorption and emission wavelengths at 306 and 395 nm. For pentosidine, absorption and emission wavelengths are optimal at 335 and 395 nm, respectively[15-17]. Further, DDP occurs principally in AC, making it unlikely to be pentosidine or conventional amino acids because these compounds also exist in noncartilagenous tissues. DDP is different from the fluorophores and chromophores reported by Hormel, *et al*[18]. They reported collagen CNBr-peptide, which was measured at fluorescent profiles of 370 nm excitation and 440 nm emission. Uchiyama, *et al*[16] also studied fluorescent materials in AC and concluded that 2 types of molecules, pyridinoline and pentosidine, contributed to the fluorescence. Hence, our research concludes that DDP is yet another AC fluorescent compound that is different from other known AC fluorescent compounds. Further, DDP (50% methanol solution) at maximal UV absorption (λ = 306 nm) has a molar extinction coefficient of 4700 $Au.M^{-1}.cm^{-1}$, whereas the values for Pyd and Dpyd are 5700 $Au.M^{-1}.cm^{-1}$ and 5000 $Au.M^{-1}.cm^{-1}$, respectively.

DDP is an aromatic molecule consisting of 2 methylated furan rings and a central pyrone ring. Because of the extraction method, HCL hydrolysis, it is likely that DDP is a derivative of a natural cartilage molecule. As the molecule does not

contain nitrogen, the precursor molecule is unlikely to be a peptide or protein. DDP may represent a hydrolysis product of a larger molecule or a condensation product of a small molecule or a small moeity of a larger molecule. The peculiar and hitherto unknown structure of this compound and its small concentration in cartilage makes it unlikely that DDP is derived from a known cartilage matrix substance that can be readily assayed by other methods. Based upon the structure of DDP, initially we had 2 theories regarding the natural precursor molecule. First, based on Fennema's integrative flow chart[19] of the major reactions that carbohydrates can undergo during the processing and handling of food, we considered that DDP could be a carbohydrate derived from molecules such as the sucrose/starch component of AC. Since DDP was detected from the carbohydrate fraction of AC[20], it is very likely that a component of one or more glycan fractions could be the precursor molecule of DDP. Second, conceptually, DDP could be derived from the lipid component of AC[21-23], such as from the diketide/polyketide molecules. Diketides/polyketides are naturally occurring and structurally diverse compounds, most often produced by microorganisms such as fungi and bacteria. The route by which these compounds are formed is one of the most common in nature. Polyketides are precursor molecules for a vast array of natural products with structures varying from simple aromatic compounds to large polycyclic compounds[24]. They are derived from highly functionalized carbon chains and are known to give modified or totally novel compounds mainly through condensation reactions[25].

It is not known whether DDP or its precursor is necessary to maintain cartilage integrity. DDP represents a small, chemically stable molecule, present consistently in very small concentrations in AC. As such, it has potential application as a unique biochemical marker for evaluation of cartilage injury and repair in arthritic diseases.

ACKNOWLEDGMENT

We sincerely thank Dr. Timothy Gray and Dr. Patricia Aroca for conducting the NMR experiments and providing us with the insights to the NMR spectrocopic results, as well as Dr. Alex Young for the mass spectrometry experiments and interpretation. We thank Maria Mendes and Jindra Tupy for technical assistance and Theresa Pirogowicz for assistance with the manuscript.

REFERENCES

1. Lohmander LS, Lark MW, Dahlberg L, Walakovits LA, Roos H. Cartilage matrix metabolism in osteoarthritis: Markers in synovial fluid, serum, and urine. Clin Biochem 1992;25:167-74.
2. Hormel SE, Eyre DR. Collagen in the ageing human intervertebral disc: an increase in covalently bound fluorophores and chromophores. Biochim Biophys Acta 1991;1078:243-50.
3. Grandhee SK, Monnier VM. Mechanism of formation of the Maillard protein cross-link pentosidine. J Biol Chem 1991;266:11649-653.
4. Fujimoto D, Moriguchi T, Ishida T, Hayashi H. The structure of pyridinoline, a collagen crosslink. Biochem Biophys Res Commun 1978;84:52-7.
5. Fujimoto D. Isolation and characterization of a fluorescent material in bovine Achilles tendon collagen. Biochem Biophys Res Commun 1977;76:1124-9.
6. Eyre DR, Dickson IR, Van Ness K. Collagen cross-linking in human bone and articular cartilage. Biochem J 1988;252:495-500.
7. Sell DR, Monnier VM. Structure elucidation of a senescence cross link from human extracellular matrix. J Biol Chem 1989;264:21597-602.
8. Sell DR, Monnier VM. End stage renal disease and diabetes catalyse the formation of a pentose-derived crosslink from ageing human collagen. J Clin Invest 1990;85:380-4.
9. Chen JR, Takahashi M, Suzuki M, Kushida K, Miyamoto S, Inoue T. Pentosidine in synovial fluid in osteoarthritis and rheumatoid arthritis: relationship with disease activity in rheumatoid arthritis. J Rheumatol 1998;25:2440-4.
10. Takahashi M, Suzuki M, Kushida K, Miyamoto S, Inoue T. Relationship between pentosidine levels in serum and urine and activity in rheumatoid arthritis. Br J Rheumatol 1997;36:637-42.
11. Takahashi M, Kushida K, Hoshino H, et al. Concentrations of pyridinoline and deoxypyridinoline in joint tissues from patients with osteoarthritis or rheumatoid arthritis. Ann Rheum Dis 1996;55:324-7.
12. Takahashi M, Kushida K, Ohishi T, et al. Quantitative analysis of crosslinks pyridinoline and pentosidine in articular cartilage of patients with bone and joint disorders. Arthritis Rheum 1994;37:724-8.
13. Sheldrick GM. SHELXTL\PC. Madison, WI: Siemens Analytical X-ray Instruments Inc; 1994.
14. Wehrli FW, Merchand AP, Wehrli S. In: Wehrli FW, editor. Interpretation of carbon-13 NMR spectra. 2nd ed. Chichester, New York: Wiley; 1988.
15. Bellmunt MJ, Portero M, Pamplona R, Muntaner M, Prat J. Age-related fluorescence in rat lung collagen. Lung 1995;173:177-85.
16. Uchiyama A, Ohishi T, Takahashi M, et al. Fluorophores from ageing human articular cartilage. J Biochem 1991;110:714-8.
17. Odetti P, Pronzato A, Noberasco G, et al. Relationships between glycation and oxidation related fluorescence in rat collagen during ageing. An in vivo and in vitro study. Lab Invest 1994;70:61-7.
18. Hormel SE, Eyre DR. Collagen in the ageing human intervertebral disc: an increase in covalently bound fluorophores and chromophores. Biochim Biophys Acta 1991;1078:243-50.
19. Fennema O. Chemical changes in food during processing — An overview. In: Richardson T, Finley JW, editors. Chemical changes in food during processing. Westport, CT: AVI; 1995:1-16.
20. Gahunia HK, Veith R, Pritzker KP. A novel fluorescent compound (DDP) in calf, rabbit and human articular cartilage and synovial fluid. J Rheumatol 2002:29:154-60.
21. Kirkpatrick CJ, Mohr W, Haferkamp O. Lipid storage in cultured articular chondrocytes due to prostanoid precursors and a prostanoid synthesis inhibitor. Cell Tissue Res 1982;224:441-8.
22. Le Lous M, Corvol MT, Maroteaux P. Lipid composition of two types of chondrocytes in primary culture. Calcif Tissue Int 1981;33:403-7.
23. Rabinowitz JL, Gregg JR, Nixon JE, Schumacher HR. Lipid composition of the tissues of human knee joints. I. Observations in normal joints (articular cartilage, meniscus, ligaments, synovial fluid, synovium, intra-articular fat pad and bone marrow). Clin Orthop 1979;143:260-5.
24. Simpson TJ. The biosynthesis of polyketides [review]. Nat Prod Rep 1987;4:339-76.
25. Staunton J, Wilkinson B. The biosynthesis of aliphatic polyketides. In: Leeper FJ, Vederas JC, editors. Biosynthesis — polyketides and vitamins. Berlin, Heidelberg: Springer-Verlag; 1998:49-92.