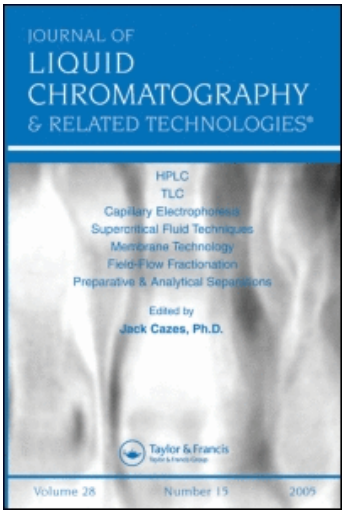

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Isolation and Characterization of Fluoro-Jade B, a Selective Histochemical Stain for Neuronal Degeneration

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ABSTRACT

Fluoro-Jade B is a novel fluorescent dye, and since its introduction in 1999 it has been widely used in neuroscience research for selectively staining degenerating neurons in brain tissue sections. However, the chemical composition of Fluoro-Jade B has not been previously resolved. We here report successful separation and identification of eight isomers and structural analogues of Fluoro-Jade B. Two analytical HPLC methods, consisting of a reversed-phase C18 column and a mobile phase with either a pH gradient or an acetonitrile gradient, were

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developed. A quantitative separation was performed by a semi-preparative reversed phase C_{18} HPLC column. Each individual component was characterized by LC/ESI mass spectrometry and NMR spectroscopy. The compounds 5-(6′-hydroxy-3′-oxo-3H-xanthen-9′-yl)benzene-1,2,4-tricarboxylic acid, 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-(2,4-dihydroxybenzoyl)terephthalic acid, and 4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-6-(2,4-dihydroxybenzoyl)isophthalic acid represent three new fluorescent compounds discovered in Fluoro-Jade B. They are, presumably, responsible for the dye's ability to detect degenerating neurons.

Key Words: Fluorescent dye; Fluoro-Jade B components; Neuronal degeneration; Derivatives of carboxyfluorescein.

INTRODUCTION

The development of a histochemical stain for the detection of neuronal degeneration is of interest to neuroscientists studying brain cell degeneration resulting from a variety of insults, including neurotoxicant exposure, trauma, ischemia, developmental apoptosis, and neurodegenerative diseases, such as Parkinson's and Alzheimer's disease. Traditional histological staining methods, such as hematoxylin and eosin or Nissl type stains,[1–5] are not selective for the staining of degenerating neurons. About 50 years ago, Nauta and associates pioneered the development of suppressed silver stains that exhibit the ability to label only degenerating neurons.[6–9] However, the labor intensive and capricious nature of the method precluded a more widespread acceptance of the technique. The use of fluorochromes, such as Fluoro-Jade and Fluoro-Jade B, represents a new milestone in the development of histochemical stains, which are capable of the direct and selective labeling of degenerating neurons.[10] Fluoro-Jade has been fully characterized as a mixture of 5-carboxyfluorescein di-sodium salts and 6-carboxyfluorescein di-sodium salts. In addition to staining degenerating neurons, numerous other important biological applications of these compounds and its derivatives have been reported.[11–16] Fluoro-Jade B was developed as the structural analogue of Fluoro-Jade and found to produce an even greater staining contrast and resolution than Fluoro-Jade on degenerating neurons.[17–19] However, the composition and the molecular structure of Fluoro-Jade B had not been fully explored before this study. This is most likely due to the challenging task of separating so many isomers and structural analogues (see Fig. 1).

Scientifically, it is important to identify each major component of Fluoro-Jade B. This will lead to discovering new fluorescent compounds that are the efficacious fluorescent staining component(s) of Fluoro-Jade B. This will also provide a scientific basis for understanding the selective staining mechanism

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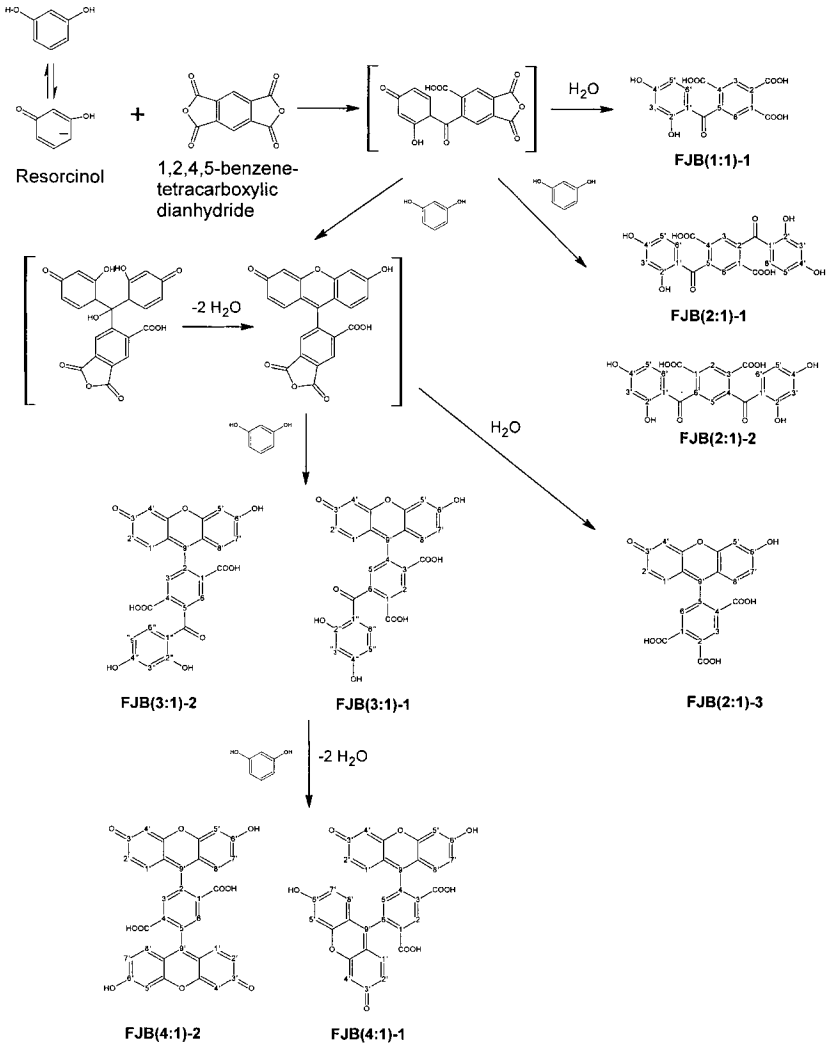

Figure 1. Reaction mechanism and products of Flow-Jade B. *Note:* The numbering schemes shown in each structure of the products are for the purpose of NMR chemical shift assignments.

of Fluoro-Jade B, as well as the relationship between the chemical structure and fluorescence properties of this type of molecule. Furthermore, this could potentially lead to expend the applications of the identified new fluorescent compounds of Fluoro-Jade B in more biological targets. The present research

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focused on isolation, identification, and characterization of the major components of Fluoro-Jade B and reports the discovery of three new fluorescent compounds.

EXPERIMENTAL

Chemicals

Fluoro-Jade B was obtained from Histo-Chem Inc. (Jefferson, AR). The synthesis of Fluoro-Jade B has been described in a US Patent.[20] The predicted possible components of Fluoro-Jade B are shown in Fig. 1. Acetonitrile (ACN) of HPLC quality, acetic acid, glacial (HOAc) were obtained from J.T. Baker (Phillipsburg, PA). Formic acid (HF), 99%, was obtained from Sigma Chemicals Co. (St. Louis, MO, USA). High purity water (18 M*) was used in all HPLC experiments and was prepared in-house by a Millipore Milli-Q water system (Millipore Corp., Bedford, MA). The deuterated NMR solvents methanol-d_4 (D 99.8%), deuterated oxide (D 99.96%), and sodium deuteroxide (D 99.5%, 30% w/w in D_2O) were all from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Nitrogen gas used in the LC/ESI MS experiment was produced in-house using a Nitrox™ UHPLCMS25 nitrogen generator (Dominick Hunter Inc., Charlotte, NC).

Preparation of HPLC Mobile Phase Solutions

Mobile phase solution A: 5% ACN aqueous solution with 0.1% HF; mobile phase solution B: 50% ACN aqueous solution with 0.1% HF; mobile phase solution C: 30% ACN aqueous solution; mobile phase solution D: 30% ACN aqueous solution with 0.1% HF; mobile phase solution E: 5% ACN aqueous solution with 0.1% HOAc; mobile phase solution F: 50% ACN aqueous solution with 0.1% HOAc; mobile phase solution G: 33% ACN aqueous solution with 0.03% HOAc; were prepared by the following method: ACN and water were measured separately with graduated cylinders and mixed together. The mixed solution was then filtered using 0.45 μm Nylon membrane filters and degassed under vacuum. A concentration of formic or acetic acid (0.1%) was made by adding 1 mL of formic or acetic acid into 1000 mL of ACN aqueous solution without further pH adjustment.

HPLC Analysis

The HPLC apparatus used for analytical separation was equipped with two Waters 510 HPLC Pumps and a Waters 486 UV Detector. A Discovery® C18 25 cm × 10 mm column (Supelco, Bellefonte, PA) was employed.

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Two HPLC methods were established. In method 1, the mobile phase solutions A and B were employed. The elution mobile phase was a 0.1% formic acid aqueous solution with a linear gradient of ACN concentrations ranging from 18.5% (A : B = 70 : 30) to 45.5% (A : B = 10 : 90) over 40 min. In the HPLC method 2, the mobile phase solutions C and D were employed. The elution mobile phase was a 30% ACN aqueous solution with a linear gradient of HF concentrations ranging from 0.005% (C : D = 95 : 5) to 0.07% (C : D = 30 : 70) over 45 min. The flow rate was 4 mL/min for both methods. The wave length of the UV detector was set at 260 nm. The injection sample was at the concentration of 0.1 mg/mL and was dissolved in ACN aqueous solution in accordance with the HPLC initial condition, except that no acid was added. The injection volume was 80 μL using a 100 μL injection loop. Each fraction from HPLC methods 1 and 2 was collected and then was detected using a LC/ESI/MS spectrometer.

Preparative HPLC Fractionation

A semi-preparative scale HPLC method was developed using the mobile phase solution E and F. The elution mobile phase was a 0.1% acidic acid aqueous solution with a linear gradient of ACN concentrations ranging from 14% (E : F = 80 : 20) to 41% (E : F = 20 : 80) over 10 min, and then holding for 12 min at the constant ratio of 20 : 80 (E : F). The experiments were carried out on the same instrument with the same HPLC column. A maximum injection volume of 100 μL with a 100 μL injection loop was employed. The injection sample concentration was 5 mg/mL. The flow rate was 4 mL/min. The two compounds, FJB (2 : 1)-2 and FJB (2 : 1)-3 (Fig. 1), could not be fully separated by this preparative scale. They were collected as one fraction and further separated by an isocratic mobile phase solution G. The two compounds eluted at a retention time of approximately 20 min. To save time, serial injections were performed every 7 min. Each fraction collected from the preparative HPLC was subjected for MS and NMR spectrometry studies.

LC/ESI Mass Spectrometry

Liquid chromatography consisted of a HP 1100 HPLC (Agilent, Palo Alto, CA) with a Prodigy ODS (3) 2 × 30 mm^2, 5 μm guard column (Phenomenex, Torrance, CA). The mobile phase, delivered at a rate of 0.2 mL/min, was a 10 min linear gradient from 5% to 95% of ACN with constant 0.1% formic acid and holding for 5 min at the top of the gradient.

LC/ESI/MS analyses were performed on a TSQ 7000 triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA) with an electrospray

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ion source operated in negative-ion mode. The in-source CID offset (SID) was set at 35 V to induce neutral losses of 44 Da (CO_2) and 110 Da (resorcinol) from the $[M - H]^-$ ions. Full scans were acquired with Q1 from m/z 155 to 750 sec^{-1}. Other mass spectrometer conditions: capillary temperature, 275°C; sheath gas, 70 psi; auxillary gas flow, 5 units; electrospray voltage, 4.5 kV.

NMR Instrumentation

A Gemini 300 mHz NMR apparatus (Varian, Palo-Alto, CA) equipped with a Gemini-300 computer was employed for all NMR experiments. Each fraction collected from the preparative HPLC was concentrated and dried under vacuum for at least 24 hr before NMR analysis. The deuterated methanol-d_4 was used as a solvent for all compounds except for the compounds FJB (4 : 1)-1, and -2, in which a mixture of deuterated oxide with a small amount of sodium deuteroxide was used as the solvent. Tetramethylsilane (TMS) and ACN were used as a reference for the ^{1}H NMR spectrum.

RESULTS AND DISCUSSION

Theoretical Prediction of Possible Components of Fluoro-Jade B

In order to successfully develop the HPLC separation method for Fluoro-Jade B, it is better to have some understanding of how many components are likely to be contained in the material, and what kind of chemical structures and physical properties these compounds might possess. To answer these questions, we proposed a reaction mechanism of Fluoro-Jade B synthesis based on a known similar reaction, that has been previously reported,[21,22] in which fluorescein was formed by fusing resorcinol and phthalic anhydride together. This mechanism was demonstrated by the product analysis presented later in this paper.

Figure 1 shows the proposed reaction mechanism and products of Fluoro-Jade B synthesis. Fluoro-Jade B was synthesized by mixing resorcinol with 1,2,4,5-benzenetetracarboxylic dianhydride and heating together. The reaction mechanism involves enol–keto tautomerization of the resorcinol molecule and its nucleophilic attack on the carbonyl group(s) of 1,2,4,5-benzenetetracarboxylic dianhydride. The hydroxyketoxanthone group, which is a known fluorophore and present in several predicted Fluoro-Jade B components, is formed by condensation and dehydration with the loss of two

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water molecules. The hydrolysis of the anhydride group is involved in the formation of FJB-(1 : 1)-1 and FJB-(2 : 1)-3 molecules. Depending on the number and positions of the resorcinol molecule(s) that are added to one 1,2,4,5-benzenetetracarboxylic dianhydride molecule, the formation of eight different reaction products are predicted. The 1 : 1 ratio addition gives one product, 5-(2,4-dihydroxybenzoyl)benzene-1,2,4-tricarboxylic acid (FJB-(1 : 1)-1). The 2 : 1 ratio addition gives three isomers, 2,5-*bis*(2,4-dihydroxybenzoyl)terephthalic acid (FJB-(2 : 1)-1), 4,6-*bis*(2,4-dihydroxybenzoyl)isophthalic acid (FJB-(2 : 1)-2), and 5-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzene-1,2,4-tricarboxylic acid (FJB-(2 : 1)-3). The 3 : 1 ratio addition gives two isomers, 2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-5-(2,4-dihydroxybenzoyl)terephthalic acid (FJB-(3 : 1)-1) and 4-(6-hydroxy-3-oxo-3H-xanthen-9-yl)-6-(2,4-dihydroxybenzoyl)isophthalic acid (FJB-(3 : 1)-2). The 4 : 1 ratio addition gives two isomers, 2,5-*bis*(6-hydroxy-3-oxo-3H-xanthen-9-yl)terephthalic acid (FJB-(4 : 1)-1) and 4,6-*bis*(6-hydroxy-3-oxo-3H-xanthen-9-yl)isophthalic acid (FJB-(4 : 1)-2).

HPLC Results

Figure 2 is a representative HPLC chromatogram of the Fluoro-Jade B components using HPLC method 1 (an ACN gradient described in Experimental Section). A total of 10 peaks were detected. The peak at the retention time 3.94 was identified as hydrolyzed starting compound 1,2,4,5-benzenetetracarboxylic dianhydride. The peak at the retention time 4.46 min represents starting compound resorcinol. The peak at 7.78 min is FJB-(1 : 1)-1. The peaks at the retention times of 15.22, 17.84, and 21.44 min represent FJB-(2 : 1)-3, -2, and −1, respectively. The peaks at 25.47 and 28.35 min represent FJB (3 : 1)-2 and -1, respectively. The peaks at 32.22 and 33.60 min represent FJB (4 : 1)-2 and FJB (4 : 1)-1, respectively. The assignment of each peak was originally made by mass spectral analysis. The resorcinol peak was assigned by co-injection with a commercially available product. The 1,2,4,5,-benzenetetracarboxylic acid peak was identified by co-injection with hydrolyzed 1,2,4,5,-benzenetetracarboxylic dianhydride.

Figure 3 is a representative HPLC chromatogram of the Fluoro-Jade B components using HPLC method 2 (an acid gradient described in Experimental Section). A total of 9 peaks were detected. The peak with the retention time of 4.68 min represents unreacted resorcinol. The peaks at 8.27 and 9.16 min represent two isomers of FJB-(4 : 1)-1 and -2, respectively. The peak at the retention times 9.90 and 11.02 min represent FJB-(3 : 1)-1 and -2, respectively. The peak at 15.55 min contains two isomers of the 2 : 1 addition products, FJB-(2 : 1)-1, and -2. The peaks at the retention time 20.70 min represent the

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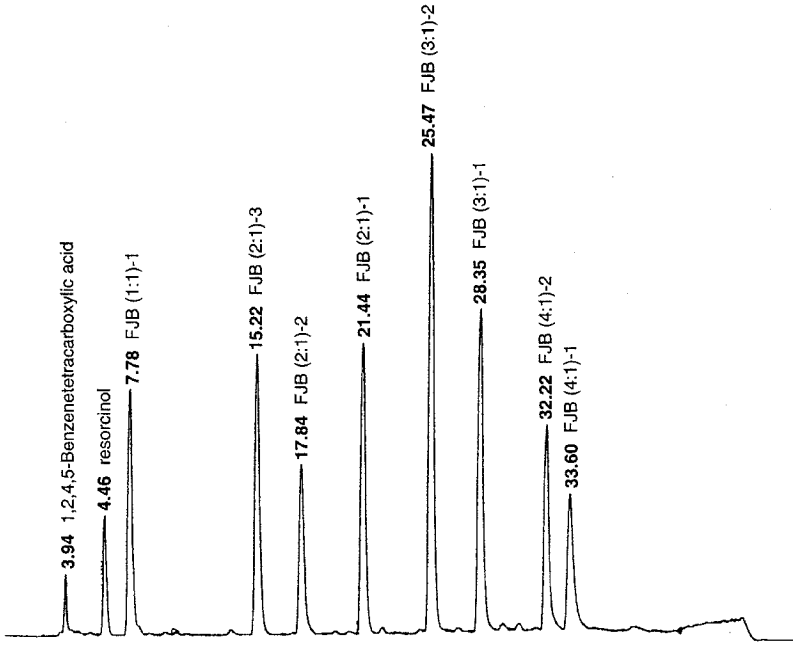

Figure 2. Separation of Fluoro-Jade B components using HPLC method 1 on a Discovery® reversed-phase C18 column. The elution mobile phase was a 0.1% formic acid aqueous solution with a linear gradient of ACN.

FJB-(2 : 1)-3. The peak at the retention time of 34.02 min represents FJB-(1 : 1)-1. The last peak at 41.21 min is benzenetetracarboxylic acid, which is formed from hydrolysis of unreacted benzenetetracarboxylic dianhydride. Each peak was assigned by mass spectral analysis and by co-injection.

It is interesting to note that the elution order of the components exhibited in the chromatography of HPLC method 2 is the reverse of that exhibited in the chromatography of HPLC method 1. In HPLC method 1, the elution order of the compounds is similar to that typically observed in reversed-phase chromatography. The compounds containing three carboxylic acid groups, such as FJB (1 : 1)-1 and FJB (2 : 1)-3, are relatively more hydrophilic and elute earlier with a more polar mobile phase solution, while the compounds containing two hydroxyketoxanthone groups, such as FJB (4 : 1)-1 and FJB (4 : 1)-2, are relatively more hydrophobic and elute later with a relatively less polar mobile phase. In contrast, in method 2, by increasing the gradient of formic acid in a constant 30% ACN aqueous

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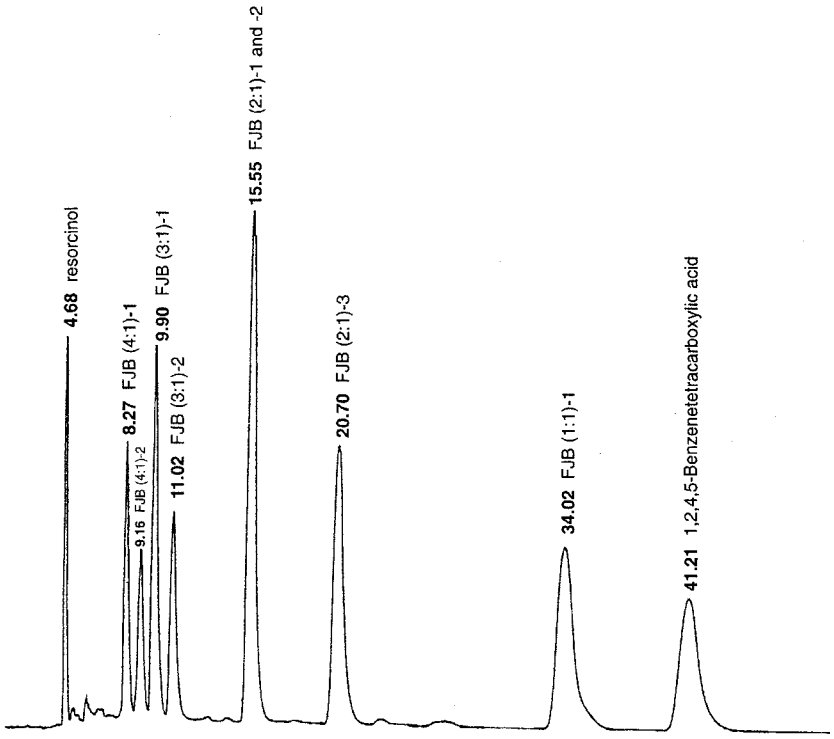

Figure 3. Separation of Fluoro-Jade B components using HPLC method 2 on a Discovery® reversed-phase C18 column. The elution mobile phase was a 30% acetonitrile aqueous solution with a linear gradient of formic acid.

solution, the mobile phase polarity was increased, which is opposite to method 1. In method 1, the polarity of the mobile phase was decreased by increasing ACN gradient in a constant formic acid aqueous solution. Therefore, the elution order of the compounds (except the resorcinol) was reversed compared to method 1. Method 2 produced a "double" reversed-phase chromatography.

The HPLC results of the detection of eight components of Fluoro-Jade B from HPLC method 1 were in agreement with the results of HPLC method 2 and are consistent with the theoretical prediction. The weights of each isolated compound obtained from preparative HPLC fractionation, indicate that the 2 : 1 addition and 3 : 1 addition products are the major components of Fluoro-Jade B sample.

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LC/ESI MS Spectral Analysis

Table 1 lists significant ions seen in the mass spectra of each compound separated using HPLC methods 1 and 2, respectively. The combined results are discussed.

For the compound FJB-(1 : 1)-1, the mass spectrum gives the expected $[M - H]^-$ at m/z 345. The three fragments corresponding to the loss of one, two, or three CO_2 groups ($CO_2 = 44$) indicate the existence of three carboxyl groups on the molecule. For the compound FJB-(2 : 1)-1 and -2, the mass spectra give the expected $[M - H]^-$ ion at m/z 437. The fragmentation indicates the presence of at least one resorcinol group (resorcinol = 110) and two carboxylic acid groups. For the compound FJB-(2 : 1)-3, the $[M - H]^-$ ion at m/z 419, and the three ions corresponding to the loss of one, two, or three CO_2 groups, provide strong evidence for the suggested molecular structure possessing three acid groups. For compounds FJB-(3 : 1)-1 and -2, the $[M - H]^-$ ions at m/z 511, and the fragments corresponding to the loss of resorcinol and two CO_2 groups support the suggested molecular structure. For the compound FJB-(4 : 1)-1 and -2, the $[M - H]^-$ ions at m/z 585 and the fragmentation indicating the presence of two carboxyl groups, support the suggested molecular structure.

Characterization of ^{1}H NMR Spectra

^{1}H NMR spectral data and assignments of each compound are listed below:

FJB-(1 : 1)-1 (CD_3OD): δ 6.21 (dd, 1H, $J_{5',6'} = 8.7$ Hz, $J_{3',5'} = 2.25$ Hz, $H_{5'}$), δ 6.30 (d, 1H, $J_{3',5'} = 2.25$ Hz, $H_{3'}$), δ 7.04 (d, 1H, $J_{5',6'} = 8.7$ Hz, $H_{6'}$), δ 8.00 (s, 1H, H_6), δ 8.70 (s, 1H, H_3).

FJB-(2 : 1)-1 (CD_3OD): δ 6.30 (dd, 2H, $J_{5',6'} = 8.7$ Hz, $J_{3',5'} = 2.25$ Hz, $H_{5'}$, $H_{5'}$), δ 6.35 (d, 2H, $J_{3',5'} = 2.25$ Hz, $H_{3'}$, $H_{3'}$), δ 7.05 (d, 2H, $J_{5',6'} = 8.7$ Hz, $H_{6'}$, $H_{6'}$), δ 7.99 (s, 2H, H_3, H_6).

FJB-(2 : 1)-2 (CD_3OD): δ 6.26 (dd, 2H, $J_{5',6'} = 8.57$ Hz, $J_{3',5'} = 2.13$ Hz, $H_{5'}$, $H_{5'}$), δ 6.31 (d, 2H, $J_{3',5'} = 2.13$ Hz, $H_{3'}$, $H_{3'}$), δ 7.02 (d, 2H, $J_{5',6'} = 8.57$ Hz, $H_{6'}$, $H_{6'}$), δ 7.29 (s, 1H, H_2), δ 8.67 (s, 1H, H_5).

FJB-(2 : 1)-3 (CD_3OD): δ 6.56 (dd, 2H, $J_{1',2'}$, $J_{7',8'} = 8.7$ Hz, $J_{2',4'}$, $J_{5',7'} = 2.25$ Hz, $H_{2'}$, $H_{7'}$), δ 6.64 (d, 2H, $J_{1',2'}$, $J_{7',8'} = 8.7$ Hz, $H_{1'}$, $H_{8'}$), δ 6.70 (d, 2H, $J_{2',4'}$, $J_{5',7'} = 2.25$ Hz, $H_{4'}$, $H_{5'}$), δ 7.53 (s, 1H, H_3), δ 8.47 (s, 1H, H_6).

FJB-(3 : 1)-1 (CD_3OD): δ 6.19 (dd, 1H, $J_{5'',6''} = 8.7$ Hz, $J_{3'',5''} = 2.04$ Hz, $H_{5''}$), δ 6.26 (d, 1H, $J_{3'',5''} = 2.04$ Hz, $H_{3''}$), δ 6.57 (dd, 2H, $J_{1',2'}$, $J_{7',8'} = 8.67$ Hz, $J_{2',4'}$, $J_{5',7'} = 2.25$ Hz, $H_{2'}$, $H_{7'}$), δ 6.67 (d, 2H, $J_{2',4'}$, $J_{5',7'} = 2.25$ Hz, $H_{4'}$, $H_{5'}$), δ 6.70 (d, 2H, $J_{1',2'}$, $J_{7',8'} = 8.67$ Hz, $H_{1'}$, $H_{8'}$), δ 6.89 (d, 1H, $J_{5'',6''} = 8.7$ Hz, $H_{6''}$), δ 7.15 (s, 1H, H_3), δ 8.60 (s, 1H, H_6).

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Table 1. LC/ESI MS analysis of all fractions separated by HPLC methods 1 and 2.

HPLC RT (min)				
Method 1	Method 2	Ions (m/z) and their significance	MW	ID
3.94	41.21	$275[M-H+1H/Na]^-$ $253[M-H]^-$ $209[M-H\text{-}44]^-$ $165[M-H\text{-}2X44]^-$	254	Benzenetetra-carboxylic acid
7.78	34.02	$367[M-H+1H/Na]^-$ $345[M-H]^-$ $301[M-H\text{-}44]^-$ $257[M-H\text{-}2X44]^-$ $213[M-H\text{-}3X44]^-$ $191\ [M-H\text{-}44\text{–}110]^-$ 165	346	FJB-(1 : 1)-1
15.22	20.70	$441[M-H+1H/Na]^-$ $419[M-H]^-$ $375[M-H\text{-}44]^-$ $331[M-H\text{-}2X44]^-$ $287[M-H\text{-}3X44]^-$	420	FJB-(2 : 1)-3
17.84	15.55	$459[M-H+1H/Na]^-$ $437[M-H]^-$ $393[M-H\text{-}44]^-$ $349[M-H\text{-}2X44]^-$ $327[M-H\text{-}110]^-$ $283[M-H\text{-}44\text{–}110]^-$	438	FJB-(2 : 1)-2
21.44	15.55	$459[M-H+1H/Na]^-$ $437[M-H]^-$ $393[M-H\text{-}44]^-$ $349[M-H\text{-}2X44]^-$ $327[M-H\text{-}110]^-$ $283[M-H\text{-}44\text{–}110]^-$	438	FJB-(2 : 1)-1
25.47	11.02	$533[M-H+1H/Na]^-$ $511[M-H]^-$ $467[M-H\text{-}44]^-$ $423[M-H\text{-}2X44]^-$ $401[M-H\text{-}110]^-$ $357[M-H\text{-}44\text{–}110]^-$	512	FJB-(3 : 1)-2
28.35	9.90	$533[M-H+1H/Na]^-$ $511[M-H]^-$ $467[M-H\text{-}44]^-$ $423[M-H\text{-}2X44]^-$ $401[M-H\text{-}110]^-$ $357[M-H\text{-}44\text{–}110]^-$	512	FJB-(3 : 1)-1
32.22	9.16	$631[M+HCOO]^-$ $585[M-H]^-$ $541[M-H\text{-}44]^-$ $497[M-H\text{-}2X44]^-$	586	FJB-(4 : 1)-2
33.60	8.27	$631[M+HCOO]^-$ $585[M-H]^-$ $541[M-H\text{-}44]^-$ $497[M-H\text{-}2X44]^-$	586	FJB-(4 : 1)-1

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FJB-(3 : 1)-2 (CD_3OD): δ 6.30 (dd, 1H, $J_{5'',6''} = 8.7$ Hz, $J_{3'',5''} = 2.04$ Hz, $H_{5''}$), δ 6.34 (d, 1H, $J_{3'',5''} = 2.04$ Hz, $H_{3''}$), δ 6.59 (dd, 2H, $J_{1',2'}$, $J_{7',8'} = 8.67$ Hz, $J_{2',4'}$, $J_{5',7'} = 2.25$ Hz, $H_{2'}$, $H_{7'}$), δ 6.71 (d, 2H, $J_{2',4'}$, $J_{5',7'} = 2.25$ Hz, $H_{4'}$, $H_{5'}$), δ 6.73 (d, 2H, $J_{1',2'}$, $J_{7',8'} = 8.67$ Hz, $H_{1'}$, $H_{8'}$), δ 7.14 (d, 1H, $J_{5'',6''} = 8.7$ Hz, $H_{6''}$), δ 7.70 (s, 1H, H_5), δ 7.92 (s, 1H, H_2).

FJB-(4 : 1)-1 (D_2O + NaOD): δ 6.53 (dd, 4H, $J_{1',2'}$, $J_{7',8'} = 8.52$ Hz, $J_{2',4'}$, $J_{5',7'} = 2.06$ Hz, $H_{2'}$, $H_{2'}$, $H_{7'}$, $H_{7'}$), δ 6.61 (d, 4H, $J_{2',4'}$, $J_{5',7'} = 2.06$ Hz, $H_{4'}$, $H_{4'}$, $H_{5'}$, $H_{5'}$), δ 6.71 (d, 4H, $J_{1',2'}$, $J_{7',8'} = 8.52$ Hz, $H_{1'}$, $H_{1'}$, $H_{8'}$, $H_{8'}$), δ 7.02 (s, 1H, H_5), δ 8.57 (s, 1H, H_2).

FJB-(4 : 1)-2 (D_2O + NaOD): δ 6.55 (s, 4H, $H_{4'}$, $H_{4'}$, $H_{5'}$, $H_{5'}$), δ 6.63 (d, 4H, $J_{1',2'}$, $J_{7',8'} = 8.78$ Hz, $H_{2'}$, $H_{2'}$, $H_{7'}$, $H_{7'}$), δ 7.08 (s, 2H, H_3, H_6), δ 7.23 (d, 4H, $J_{1',2'}$, $J_{7',8'} = 8.78$ Hz, $H_{1'}$, $H_{1'}$, $H_{8'}$, $H_{8'}$), δ 8.57 and δ 8.75 (br, COOH and OH).

The NMR spectra of the hydroxyketoxanthone motif in each compound show their symmetrical character. This may be explained by the resonance of the conjugated aromatic electrons. The long-range coupling distinguishes between the two adjacent protons on 6-hydroxy-3H-xanthen-3-one motif. The structural information provided by the ^{1}H NMR spectra support each predicted structure.

CONCLUSION

The components of Fluoro-Jade B have been identified in this study. The results of HPLC separation using two separate methods are consistent and indicate that Fluoro-Jade B consists of eight reaction products, in addition to traces of the two starting materials. The preparative scale HPLC provided purified fractions of each individual compound. The MS and NMR spectrometry support the theoretically predicted chemical structure of each compound. Among those components, FJB-(3 : 1)-1, FJB-(3 : 1)-2, and FJB-(2 : 1)-3 all exhibit strong fluorescence and are found in relatively large quantities, suggesting that they are the efficacious components of Fluoro-Jade B regarding its selective staining of degenerating neurons in brain tissue. They also represent discovery of novel fluorescent compounds, and their fluorescent and staining properties are currently being studied. The successful identification of the components of Fluoro-Jade B provides the scientific basis for further studying this unique histochemical stain, as well as for expanding the use of Fluoro-Jade B in biological and neuroscientific research.

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