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Structural Determination of an FD & C Red No. 3 Contaminant

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ABSTRACT

A manufacturing side-reaction product that is present in most certified lots of FD & C Red No. 3 at levels of 1–5% was isolated by semipreparative HPLC. Structural identification, as determined by proton and ^{13}C NMR and mass spectrometry, showed the contaminant to be 4′-chloro-2′,5′,7′-triiodofluorescein.

1 INTRODUCTION

FD & C Red No. 3 (R3), a xanthene color additive that is currently listed in the U.S. Code of Federal Regulations (CFR) for use in foods and ingested drugs, is subject to batch certification by the Food and Drug Administration (FDA).[1] The dye is synthesized by the condensation of phthalic anhydride with resorcinol to yield fluorescein, which is then iodinated to tetraiodofluorescein (**1**), also known as erythrosine (Scheme 1). Regulations specify that certified lots of erythrosine, which are designated as 'FD & C Red No. 3', may contain no more than 10% of lower-iodinated subsidiary colors. A previously unidentified manufacturing impurity has been shown to be

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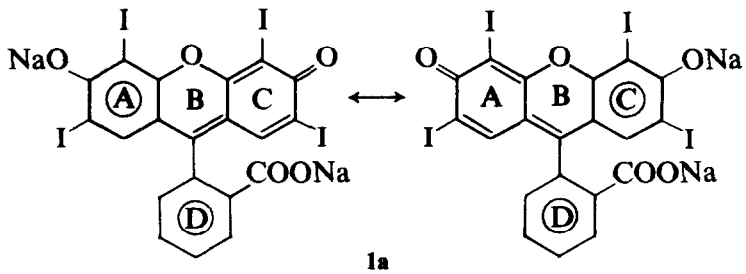
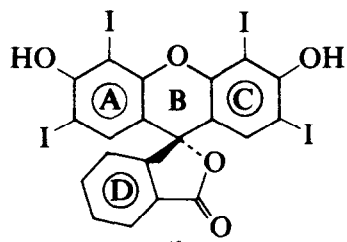


1a

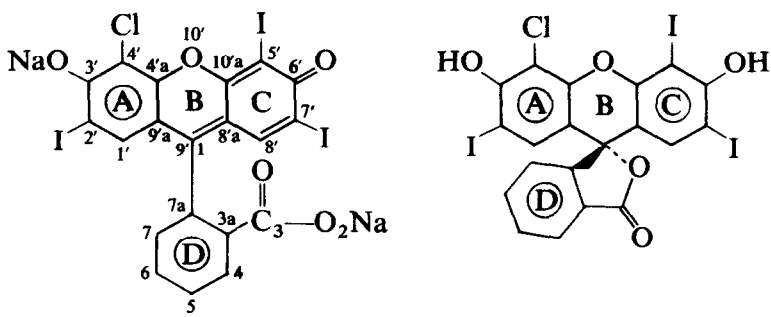


1b

Scheme 1

present at the 1–5% level in most certified lots of the color additive. The contaminant (R3X, **2**) (Scheme 2) was designated by FDA (and referred to by the color industry) as the 'X-fraction' (see chromatogram, Fig. 1, and Experimental Section) because its structural identification was not known. That designation is used in the present study.

R3X was isolated from a commercial, uncertified lot of erythrosine by using semipreparative reversed-phase HPLC. On the basis of mass spectrometric analysis, the isolated material was believed to be a monochlorotriiodofluorescein. ^{13}C NMR was then employed to test this hypothesis and, once confirmed, to determine the exact location of the chlorine atom.



2a **2b**

R3X

Scheme 2

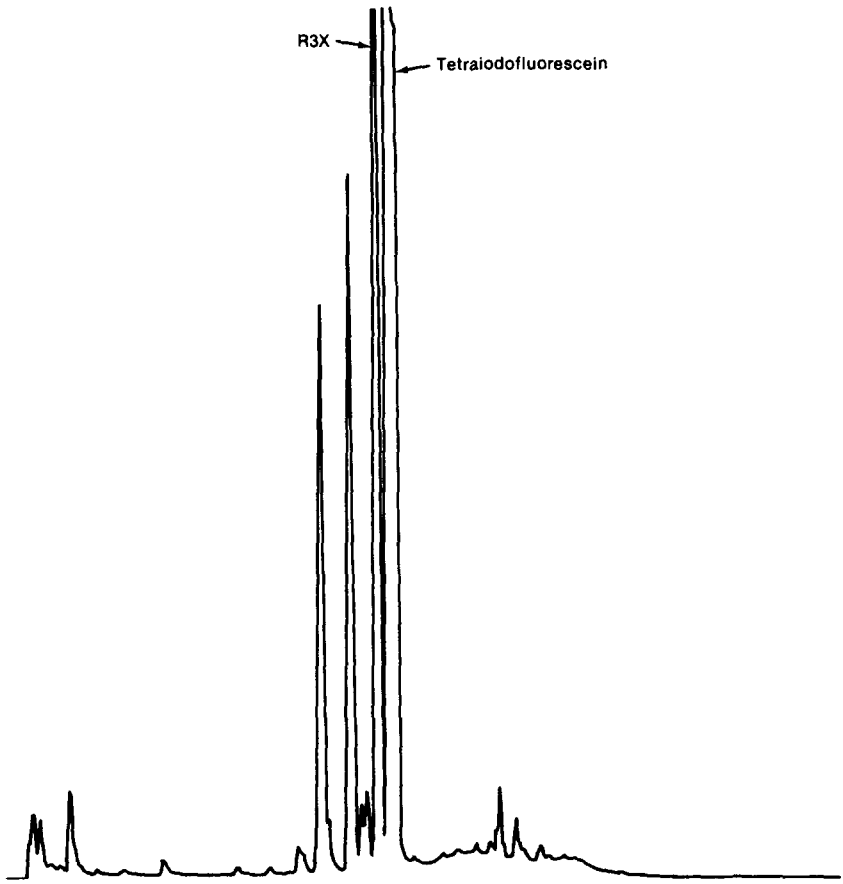


Fig. 1. High-performance liquid chromatogram of a commercial lot of FD & C Red No. 3.

2 EXPERIMENTAL

2.1 Materials

The starting material used for the isolation of R3X was a commercial lot of erythrosine, provided by Warner Jenkinson Co. (St. Louis, MO 63106, USA). Commercial HPLC-grade methanol and water were used for both the semipreparative and analytical HPLC separations. The ammonium acetate buffer was also HPLC grade (Fisher Scientific, Fair Lawn, NJ 07410, USA). Hydrochloric acid was ACS reagent grade.

2.2 Semipreparative isolation and purification of R3X

Semipreparative HPLC was performed by using a Beckman/Altex (Berkeley, CA, USA) Model 420 gradient liquid chromatograph with two

Model 110A pumps fitted with preparative pump heads and operated at 3·64 ml/min. A Microsorb C-18 column (5 μm, 10 mm ID × 25 cm) (Rainin Instrument Co., Woburn, MA, USA) was used for chromatographic separations. A Beckman/Altex Model 153 UV detector and a 405-nm filter that was chosen to give decreased sensitivity for semipreparative R3X concentrations were used. The detector was fitted with a special preparative flow cell of 0·5-mm pathlength (Beckman/Altex). A Rheodyne Model 7125 injector (Rheodyne Inc., Cotati, CA 94928, USA) fitted with a 5-ml loop was used. Ammonium acetate was dissolved in HPLC-grade water (15·42 g/2 litre, 0·1M), filtered (0·2 μm, nylon 66), and used as solvent A for gradient chromatography. HPLC-grade methanol was solvent B.

The column was equilibrated for 15 min with 55% solvent B (45% solvent A). Multiple injections (3 ml each) were performed by using an aqueous solution of erythrosine containing the R3X contaminant at a level of several percent. A linear gradient, from 55 to 100% solvent B over 25 min, was used to achieve separations. Fractions were collected according to the detector response. At the end of each run, a 10–20-min wash with 100% methanol eluted remaining components from the column.

It was determined that, for the initial injections, in which the compound of interest was a minor component, R3X eluted in the range of 15·7–17·3 min, immediately before the primary dye (**1**). Corresponding fractions for multiple semipreparative runs were combined, rotary-evaporated, and rechromatographed under the same conditions. In these subsequent runs, R3X was the major component, and the large quantity of **1** was no longer present. This change in composition extended the retention time for the elution of R3X to approximately 20 min. Fractions were collected and analyzed by analytical HPLC, and those containing pure R3X were combined.

2.3 Analytical HPLC analysis of semipreparative fractions

Selected fractions collected during the semipreparative HPLC runs were further analyzed by analytical HPLC to determine the relative quantities of the component of interest (R3X). On the basis of these results, fractions were saved and combined, or discarded. The chromatography was performed by standard methodology used by FDA for the determination of subsidiary colors in R3, namely, the reversed-phase HPLC procedure for batch certification.[2]

2.4 Conversion of isolated R3X sodium salt to the corresponding lactone

Under neutral conditions, tetraiodofluorescein and its related R3X impurity are water-soluble disodium salts (**1a** and **2a**, respectively), which, by virtue of

their polarity, are poorly volatilized under typical conditions employed for mass spectrometry. Fractions isolated by semipreparative HPLC that contained the purified R3X contaminant and the primary dye component, tetraiodofluorescein, were therefore converted to their corresponding organic-soluble lactones (**1b** and **2b**, respectively),[3] to facilitate mass spectral examinations. Each of the combined HPLC fractions containing these components was rotary-evaporated to approximately 25 ml and then transferred to a 250-ml separatory funnel. Chloroform (100 ml) and 10% (v/v) hydrochloric acid (3 ml) were added to the funnel. The mixture was shaken, and then the chloroform layer was removed and saved. The aqueous layer was re-extracted three times with chloroform (25-ml portions), and the extracts were combined. A small portion of each combined extract was subjected to mass spectral analysis. The remaining solutions were rotary-evaporated to 20 ml and then brought to dryness with a stream of nitrogen. Portions of each residue were neutralized with deuterated sodium hydroxide solution to form the disodium salt and dissolved in deuterium oxide for analyses by proton and ^{13}C NMR.

2.5 Mass spectral analysis of isolated R3X

Mass spectra were obtained by using a Finnigan MAT TSQ-46 quadrupole mass spectrometer interfaced to an INCOS 2300 data system with TSQ software (revision C). Electron-ionization data were obtained under the following instrumental conditions: 70-eV electron energy; 0·35-mA emission current; 140°C source temperature; pre-amp, multiplier, and conversion dynode at 10^{-8} A/V, −900 V, and −5 kV, respectively. Full-scan data were acquired by scanning Q_1 from $m/z = 50$ to 1250 in 3·5 s. Tris(perfluorononyl)-5-triazine was used as the calibration standard. Test samples were deposited on a direct-exposure probe wire and volatilized in the instrument at a heating rate of 20 mA/s.

2.6 Proton and ^{13}C NMR analyses of isolated R3X

Proton and ^{13}C-NMR spectra were recorded at 400 and 100 MHz, respectively, in D_2O at pH 7, on a Varian Associates VXR-400 spectrometer. The proton NMR spectra were described by 32 768 data points (real part). Pulse widths of 31 μs were employed, which correspond to tip angles of 90° with 5-mm sample tubes. Spectral widths of 4000 Hz were used, corresponding to acquisition times of 4·1 s.

Proton-decoupled ^{13}C-NMR spectra, described by 32 768 data points (real part), were obtained with broadband irradiation at 400 MHz. Pulse widths of 5 μs were employed, which correspond to tip angles of 35° with 5-mm sample tubes. Spectral widths of 20 kHz were used, corresponding to

acquisition times of *c.* 0·8 s. Dioxane was employed as an internal standard, and chemical shifts are reported relative to TMS. A fully coupled ^{13}C-NMR spectrum was determined under conditions identical to those described above but without broadband irradiation.

Spin-lattice relaxation times, T_1, were determined for two particular, equivalent pairs of ^{13}C-nuclei (2′/7′ and 4′/5′) of **1a** by using the inversion-recovery method.[4] The delay time, T, between sequences was 20 s; the intervals between 180° and 90° pulses, τ, were 0·01, 0·02, 0·04, 0·08, 0·16, 0·30, 0·625, 1·25, 2·5, 5·0, 10·0, and 20·0 s.

Selective heteronuclear NMR-decoupling experiments were conducted for protons A (7·56 ppm) and D (6·65 ppm) of **1a** by using minimum-decoupling power levels. Partial or complete (depending on the spin system) collapse of appropriate ^{13}C signals could be clearly observed at 3000 transients.

A COSY NMR spectrum was recorded with a spectral width of 400 Hz in each domain and with 256 data points in the F2 dimension. A ^{1}H pulse width of 30 μs (90°) and a 1-s repetition rate were used to acquire 128 incremented proton NMR spectra of 16 scans each. Free induction decays were processed as a 512×512 matrix with appropriate zero-filling and pseudo-echo weighting.

A directly bonded, heteronuclear chemical-shift-correlation (HETCOR) NMR spectrum was obtained with spectral widths of 1383 and 549 Hz in the carbon and proton dimensions, respectively, and with 2048 data points in the ^{13}C dimension. Pulse widths of 13 μs (^{13}C, 90°) and 21·3 μs (^{1}H decoupler, 90°) and a 1-s repetition rate were employed to acquire 128 incremented ^{13}C-NMR spectra of 64 scans each. Free induction decays in both dimensions were processed as a 256×2048 matrix with appropriate zero-filling and modified pseudo-echo weighting. A value of $^1J(CH) = 165$ Hz was employed for calculating the delays Δ_1 and Δ_2.

An indirectly bonded, heteronuclear chemical-shift-correlation (FLOCK)[5] NMR spectrum was obtained with spectral widths of 10278 and 549 Hz in the carbon and proton dimensions, respectively, and with 1024 data points in the ^{13}C dimension. Pulse widths of 13 μs (^{13}C, 90°) and 21·3 μs (^{1}H decoupler, 90°) and a 1-s repetition rate were employed to acquire 128 incremented ^{13}C-NMR spectra of 416 scans each. Free-induction decays in both dimensions were processed as a 512×2048 matrix with appropriate zero-filling and modified pseudo-echo weighting. The delays Δ_1 and Δ_2 were set to 100 ms, which corresponds to a value of $^nJ(CH) = 5$ Hz. The 3-bond C–H couplings in question are 3–4 Hz, but it was felt that delays greater than 100 ms would risk unacceptable loss of phase coherence prior to magnetization transfer. A value of $^1J(CH) = 165$ Hz was used for τ in the BIRD pulses.[6]

3 RESULTS AND DISCUSSION

Electron-impact mass spectra indicated a molecular ion of $m/z = 836$ for purified **1b**, the major component of R3. Under similar conditions, a molecular ion of $m/z = 744$ was obtained for the isolated **2b**; with this compound, however, a large $m + 2$ peak was present, at a relative abundance of *c.* 30%. This value, which is characteristic of a chlorinated compound, is due to the presence of a ^{37}Cl isotope, with a normalized natural abundance of 32·5%. A difference of 92 mass units between the molecular weights of **1b** ($m/z = 836$) and **2b** is equal to that between the atomic weights of iodine and chlorine, suggesting that one of the four iodine substituents in **1** had been effectively replaced by chlorine in **2**.

This conclusion was supported by proton NMR, which revealed the presence of four contiguous and two isolated protons in **2a**. Because of the mirror plane of symmetry that exists in **1a** (perpendicular to the B-ring and passing through C–9′ and O–10′ of this ring and C–5 and C–7a of the D-ring), two isomeric structures are then possible for **2**, i.e. the chlorine atom could be situated at either the 2′- or 4′-position of the substituted fluorescein molecule.

These isomeric possibilities can be distinguished by ^{13}C NMR spectroscopy. The ^{13}C-NMR spectrum of **1a** exhibits double-intensity signals at 77·9 and 97·4 ppm, which can be ascribed to the four iodine-substituted carbons on the basis of the heavy-atom effect of iodine.[7] Moreover, two lines of reasoning led to assignment of the low-field resonance (97·4 ppm) to C–2′/7′ and the high-field signal to C–4′/5′. First, in the fully coupled ^{13}C-NMR spectrum, the resonance at 97·4 ppm was observed as a 2-Hz doublet, while that at 77·9 ppm appeared as a slightly broadened singlet. Since aromatic, 2-bond C–H coupling constants are larger than the corresponding 4-bond C–H couplings when a halogen is substituted on the coupling carbon,[8] the low-field resonance line was tentatively assigned to C–2′/7′. Second, spin-lattice relaxation times, T_1, were determined for the two signals. The resonance at 97·4 ppm has a T_1 value of 1·4 s, while that of the 77·9 ppm signal is 2·6 s. Examination of a Dreiding model of **1a** showed that C–2′ is situated *c.* 2 Å from H–1′ (and C–7′ similarly from H–8′) while C–4′ is located *c.* 4 Å from H–1′ (and C–5′ likewise from H–8′). Because of substantial decrease in the effectiveness of the dipole–dipole relaxation mechanism with increasing inter-nuclear distance,[9] the T_1 values of carbons 2′ and 7′ are expected to be shorter than those of carbons 4′ and 5′. The low-field resonance was therefore assigned to C–2′/7′, consistent with the initial designation.

Compounds **1** and **2** were inferred to be structurally similar from their mass and proton NMR spectra as well as from their electronic spectra in the

ultra-violet and visible ranges (R. J. Calvey, unpublished results). Furthermore, *effective* substitution of a chlorine for an iodine atom in **1** should not greatly change the over-all electronic environment of the aromatic ring in which this replacement occurs.[10] Excluding the site of substitution (2′ or 4′), the five remaining carbons of the chlorine-substituted ring are each expected to have chemical shifts that are similar to those of the corresponding carbons of the diiodoaryl ring in which no chlorine replacement has occurred. Closely situated pairs of signals should then be observed for carbons 1′ and 8′, 3′ and 6′, 4′*a* and 10′*a*, and 8′*a* and 9′*a*. An additional pair should be seen for either carbons 2′ and 7′ (close to 97 ppm) or carbons 4′ and 5′ (near 78 ppm).

The ^{13}C-NMR spectrum of **2a** exhibits 20 resonances (Table 1), confirming the structural similarity of **1** and **2**. Moreover, signals that were assigned to C–1′/8′, C–3′/6′, C–4′*a*/10′*a*, and C–8′*a*/9′*a* in the ^{13}C-NMR spectrum of **1a** do, in fact, appear as close-lying pairs of resonance lines in

TABLE 1
^{13}C and ^{1}H NMR Data at pH 7 (ppm)[a]

	1a			*2a*		
Position	^{13}C	^{n}H	^{1}H (*J*)	^{13}C	^{n}H	^{1}H (*J*)
1(9′)[b]	157·2	0		158·5	0	
3	177·4	0		177·5	0	
3*a*	142·5	0		142·4	0	
4	131·1	1	7·56 dd (7·6, 1·2)	131·1	1	7·51 d (8)
5	132·5	1	7·36 dt (7·6, 1·2)	132·6	1	7·38 t (8)
6	132·3	1	7·25 dt (7·6, 1·2)	132·3	1	7·31 t (8)
7	132·9	1	6·65 dd (7·6, 1·2)	133·0	1	6·80 d (8)
7*a*	132·8	0		132·8	0	
1′	141·6	1	7·48	140·0	1	7·40
2′	97·4	0		97·1	0	
3′	176·5	0		174·0	0	
4′	77·9	0		109·3	0	
4′*a*	160·4	0		155·9	0	
5′	77·9	0		77·6	0	
6′	176·5	0		176·8	0	
7′	97·4	0		99·0	0	
8′	141·6	1	7·48	141·9	1	7·40
8′*a*	116·2	0		116·3	0	
9′*a*	116·2	0		115·7	0	
10′*a*	160·4	0		159·9	0	

[a] Referenced to dioxane at 67·4 ppm.
[b] This carbon is numbered twice: 9′ with respect to the xanthene-ring system and 1 with respect to the D-ring.

the spectrum of **2a**, as predicted. More importantly, two signals are observed at 97 and 99 ppm, whereas only one is found at 77·6 ppm. This spectroscopic evidence clearly demonstrates that the chlorine atom is located at the 4′-position and not the 2′-position of **2**.

Chemical-shift assignments for the remaining carbon atoms of the xanthene-ring system of **1a** were made on the basis of (i) their multiplicity in the fully coupled ^{13}C-NMR spectrum, (ii) chemical-shift relationships, and (iii) substituent-effect considerations. Assignments for the protons and carbons of the D-ring of **1a** were made in the following manner. A COSY NMR spectrum established that H_A is *ortho*-coupled to H_B and that H_D is similarly coupled to H_C. A HETCOR NMR spectrum permitted assignment of the four carbons that are directly attached to these protons (A–D). Finally, selective heteronuclear decoupling experiments and a FLOCK NMR spectrum demonstrated that H_A is vicinally coupled to C–3 and that H_D is similarly coupled to C–1(9′). Proton and ^{13}C chemical shifts of **1a** are virtually identical to those of **2a** and were assigned by analogy.

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