Structural Determination of Unknown Subsidiary Colors in Commercial Food Red Nos. 2 and 102

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Major subsidiary colors A (Sub A) and B (Sub B) in commercial Food Red No.102, and colors C (Sub C) and D (Sub D) in commercial Food Red No.2 were isolated. These structures were characterized by physicochemical evidence. The structures of Sub A, B, and C are trisodium salt of 7-hydroxy-8-(6-sulfonaphthyl-2-azo)-1,3-naphthalenedisulfonic acid, disodium salt of 4-amino-3-(4-sulfonaphthyl-1-azo)-1-naphthalenesulfonic acid and trisodium salt of 3-hydroxy-4-(6-sulfonaphthyl-2-azo)-2,7-naphthalenedisulfonic acid, respectively. Sub D was identified as Sub B.

Key words Food Red No.102; New Coccine; Food Red No.2; Amaranth; subsidiary color; coal-tar dye

There has been growing demand for international harmonization of regulations as food and drug distribution becomes global. Food Red No.40 was approved for use in Japan on January 17, 1991 as part of this coordination effort. There are specifications for raw materials and subsidiary dyes for this food coloring as analyzed by HPLC[1] which have not been imposed for other coal-tar dyes approved in Japan as food colors and coloring agents for pharmaceuticals. Recently, however, such specifications for the other coal-tar dyes have been demanded in this country with levels similar to or stricter than those set by FAO/WHO.[2]

We have reported studies on the identification and quantification of impurities such as raw materials, intermediates, and subsidiary colors in these dyes.[3–6] Food Red No.102 (R102; C.I.16255, New Coccine) is a mono-azo dye obtained by diazotizing naphthionic acid (NA, sodium salt of 4-amino-1-naphthalenesulfonic acid) and coupling it with G salt (GS, disodium salt of 7-hydroxy-1,3-naphthalenedisulfonic acid). Food Red No. 2 (R2; C.I.16185, Amaranth) is a mono-azo dye obtained by diazotizing NA and coupling it with R salt (RS, disodium salt of 3-hydroxy-2,7-naphthalenedisulfonic acid). Several impurities and subsidiary colors in R102 and R2 have been reported (Fig. 1).[7–11] From the results of HPLC analyses of these two colors, we recognized the

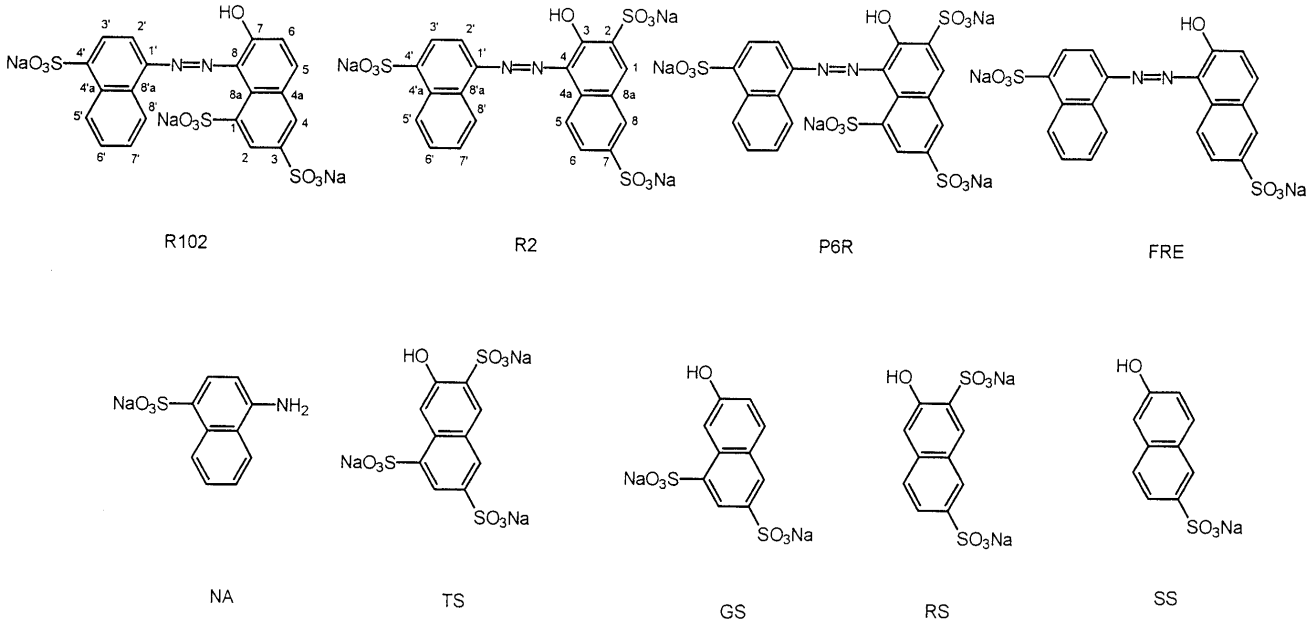

Fig. 1. Structures of Raw Materials and Known Subsidiary Colors in Commercial R2 and R102

R102: New Coccine, trisodium salt of 7-hydroxy-8-(4-sulfonaphthylazo)-1,3-naphthalenedisulfonic acid
R2: Amaranth, trisodium salt of 3-hydroxy-4-(4-sulfonaphthylazo)-2,7-naphthalenedisulfonic acid
P6R: Ponceau 6R, tetrasodium salt of 7-hydroxy-8-(4-sulfonaphthylazo)-1,3,6-naphthalenetrisulfonic acid
FRE: Fast Red E, disodium salt of 6-hydroxy-5-(4-sulfonaphthylazo)-2-naphthalenesulfonic acid
NA: naphthionic acid, sodium salt of 4-amino-1-naphthalenesulfonic acid
TS: trisodium salt of 7-hydroxy-1,3,6-naphthalenetrisulfonic acid
GS: G salt, disodium salt of 7-hydroxy-1,3-naphthalenedisulfonic acid
RS: R salt, disodium salt of 3-hydroxy-2,7-naphthalenedisulfonic acid
SS: Schaeffer's salt, sodium salt of 6-hydroxy-2-naphthalenesulfonic acid

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existence of other previously unknown subsidiary colors. In this paper, we present the structure of the major unknown subsidiary colors in R2 and R102.

Experimental

Chemicals Authentic R2 and R102 were standard products distributed by NIHS. Ponceau 6R (P6R), Fast Red E (FRE), trisodium salt of 7-hydroxy-1,3,6-naphthalenetrisulfonic acid (TS), GS, RS, Schaeffer's salt (SS, sodium salt of 6-hydroxy-2-naphthalenesulfonic acid), and NA were used in previous studies.[3,5] Commercially available R2 (12 samples, 2 manufacturers) and R102 (10 samples, 5 manufacturers) products were analyzed by HPLC. Sephadex LH-20 (Pharmacia Co. Ltd.) was used to remove ammonium acetate. Other reagents used were of analytical grade.

Instruments HPLC was carried out with a JASCO 900 system equipped with a Sic system instrument chromatocoder 21. Electrospray ionization (ESI)-MS was obtained using a VG Biotech Platform spectrometer. NMR spectra were measured with a JEOL A600 or a JEOL A500 spectrometer. UV-Visible (UV-Vis) spectrum was determined with a Shimadzu UV-260 spectrometer. IR spectra were measured by a JASCO FT/IR-350 spectrometer.

Analytical HPLC Analytical HPLC was performed under the same conditions as earlier reported.[5]

Isolation of Subsidiary Colors A, B, C, and D (Sub A, B, C, and D) from Commercial Food Color Products Preparative HPLC to separate Sub A was performed on a Develosil ODS-5 (20 mm i.d. × 250 mm; Nomura Chemical Co.) at 30 °C with detection at 510 nm. The mobile phase of 0.01 M ammonium acetate–acetonitrile (90 : 10) was used at a flow rate of 6.0 ml/min. Commercial R102 was dissolved in water at a concentration of 50 mg/ml and a 50 μl aliquot of the solution was injected for each HPLC run. A small peak eluted between 14.0 and 16.0 min, just before the main peak, was collected. After 250 runs the collected solutions were combined, evaporated to dryness, redissolved in water, and rechromatographed using the same conditions to give Sub A fraction. After evaporation to dryness, the Sub A fraction was dissolved in a small amount of water and charged on a Sephadex LH-20 column (10 mm i.d. × 350 mm) to remove ammonium acetate. After the column purification, the eluate was evaporated to dryness and dried in a vacuum oven at 40 °C for 48 h to give Sub A (3.5 mg).

Preparative HPLC to separate Sub B was performed on an L-column ODS (10 mm i.d. × 250 mm; Chemicals Inspection and Testing Institute) at 30 °C with detection at 510 nm. The mobile phase of 0.02 M ammonium acetate–acetonitrile (84 : 16) was used at a flow rate of 4.0 ml/min. Commercial R102 was dissolved in water at a concentration of 50 mg/ml and a 50 μl aliquot of the solution was injected for each HPLC run. A small peak eluted between 16.0 and 18.5 min, after the main peak, was collected. After 700 runs the collected solutions were combined. After evaporation to dryness, the Sub B fraction was treated as described for Sub A, to give Sub B (5.5 mg).

Preparative HPLC to separate Sub C was performed under the same conditions as those for Sub B except for the mobile phase condition as follows. A 20 min linear gradient progressed from 4% to 12% acetonitrile in 0.02 M ammonium acetate. Commercial R2 was dissolved in water at a concentration of 50 mg/ml and a 20 μl aliquot of the solution was injected for each HPLC run. A small peak eluted between 16.0 and 17.5 min, just after the main peak, was collected. After 400 runs the collected solutions were combined, evaporated to dryness, redissolved in water, and rechromatographed using the same conditions to give Sub C fraction. After evaporation to dryness, the Sub C fraction was treated as described for Sub A, to give Sub C (2.5 mg).

Preparative HPLC to separate Sub D was performed under the same conditions as those for Sub B except for the mobile phase. Twenty mM ammonium acetate–acetonitrile (82 : 18) was used as the mobile phase. Commercial R2 was dissolved in water at a concentration of 50 mg/ml and a 50 μl aliquot of the solution was injected for each HPLC run. A small peak eluted between 18.0 and 19.0 min, after the main peak, was collected. After 300 runs the collected solutions were combined. After evaporation to dryness, the Sub D fraction was treated as described for Sub A, to give Sub D (3.2 mg).

Spectroscopic Analyses ESI-MS analyses were performed under the same conditions as described in the preceding paper.[6]

^{1}H- and ^{13}C-NMR spectra were measured in CD_3OD with tetramethylsilane as the internal standard. The signals of the ^{1}H- and ^{13}C-NMR spectra of R102, Sub A, and Sub B were assigned on the basis of their coupling pattern and chemical shifts, and the correlation spectroscopy (COSY), differential nuclear Overhauser effect (dif-NOE), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) spectra. The signals of the ^{1}H- and ^{13}C-NMR of R2 and Sub C were assigned on the basis of their coupling pattern and chemical shifts, and the COSY and phase sensitive nuclear Overhauser effect spectroscopy (NOESY), HMQC and HMBC spectra.

R102; ESI-MS m/z: 580.8 $[M-Na]^-$, 279.0 $[M-2Na]^{2-}/2$, 178.4 $[M-3Na]^{3-}/3$.

R2; ESI-MS m/z: 580.8 $[M-Na]^-$, 279.0 $[M-2Na]^{2-}/2$, 268.0 $[M-3Na+H]^{2-}/2$, 178.4 $[M-3Na]^{3-}/3$.

Sub A; UV-Vis λ_{max} (H_2O) nm (ε): 492 (29300). IR (KBr) cm^{-1}: 3447, 3136, 1628, 1489, 1400, 1218, 1036.

Sub B and Sub D; UV-ViS λ_{max} (H_2O) nm (ε): 325 (16500), 482 (19100). IR (KBr) cm^{-1}: 3448, 3169, 1612, 1400, 1182, 1047.

Sub C; UV-Vis λ_{max} (H_2O) nm (ε): 508(29900). IR (KBr) cm^{-1}: 3446, 1616, 1495, 1341, 1194, 1036.

Results and Discussion

Analytical HPLC Chromatograms of commercial R102 and R2 products are shown in Fig. 2 and Fig. 3, respectively. Those of R102 showed the existence of TS (retention time (t_R): 2.3 min), GS (t_R: 6.4 min) and NA (t_R: 13.8 min) as non-coloring impurities, P6R (t_R: 4.3 min), R2 (t_R: 23.2 min) and FRE (t_R: 45.1 min) as subsidiary colors and two major unidentified subsidiary colors (t_R: 27.3 min, 39.2 min). Therefore, chromatographic purification was performed to isolate Sub A (t_R: 27.3 min) and Sub B (t_R: 39.2 min).

The chromatograms of R2 in Fig. 3 showed the existence of TS, RS (t_R: 7.1 min), and NA as non-coloring impurities, R102 (t_R: 31.6 min) and FRE as subsidiary colors, and two major unidentified subsidiary colors (t_R: 24.0 min, 39.2 min). Therefore, chromatographic purification was also performed to isolate Sub C (t_R: 24.0 min) and Sub D (t_R: 39.2 min).

Structure of Sub A In the visible absorption spectrum of Sub A, the absorption maximum was observed at 494 nm which was shorter than that for R102, and the other absorption band was similar to that of R102 as shown in Fig. 4. The similarity of the spectrum of Sub A to that of R102 suggests that both compounds have the same resonance system. The IR spectrum exhibited an absorption band due to hydroxy (3447 cm^{-1}) and sulfonic groups (1218, 1036 cm^{-1}). In the ESI-MS of Sub A , three quasi-molecular ion peaks at m/z 536.7 $[M-3Na+2H]^-$, 268.0 $[M-3Na+H]^{2-}/2$ and 178.3 $[M-3Na]^{3-}/3$ were observed. The ^{1}H- and ^{13}C-NMR spectra exhibited 10 aromatic proton signals and 20 aromatic carbon signals as shown in Tables 1 and 2. These data indicate that Sub A is a structural isomer of R102 with the formula $C_{20}H_{11}N_2Na_3O_{10}S_3$.

Comparing the ^{1}H-NMR spectrum of Sub A with that of R102, two pairs of aromatic proton signals [δ 9.06 (d, J=2.4 Hz) and δ 8.21 (d, J=2.4 Hz), and δ 7.91 (d, J=9.0 Hz) and δ 6.92 (d, J=9.0 Hz)] due to the hydroxynaphthalene moiety were very similar to those of R102 (Table 1). In the ^{13}C-NMR spectrum, 10 carbon signals of Sub A were also very similar to those due to the hydroxynaphthalene moiety of R102 (Table 2). These observations confirmed that Sub A possesses 8-azo-7-hydroxy-1,3-naphthalenedisulfonic acid moiety. The remaining ^{1}H- and ^{13}C-NMR data of Sub A indicated the

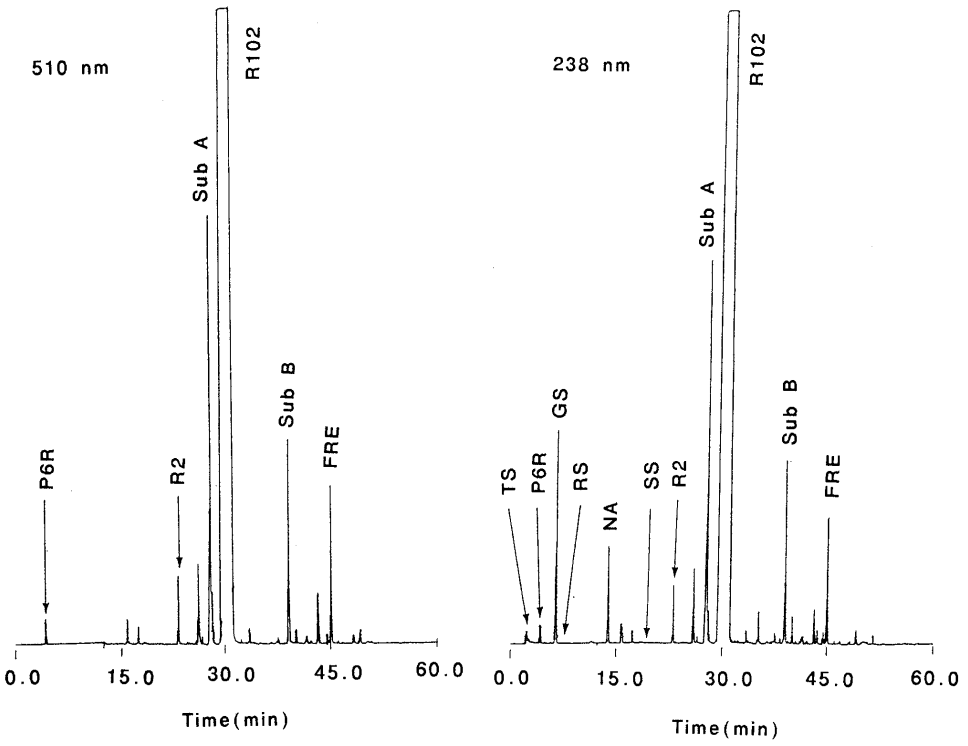

Fig. 2. Analytical HPLC of Commercial R102 at 510 nm (Left) and 238 nm (Right)

HPLC conditions : column, L-column ODS (4.6 mm i.d. × 250 mm); flow rate, 1.0 ml/min; temperature, 30 °C; detection, 510 nm and 238 nm; mobile phase, 0—5 min, 0.02 M ammonium acetate, 5—55 min, a linear gradient from 0% to 30 % acetonitrile in 0.02 M ammonium acetate.

See legend of Fig.1 for abbreviations.

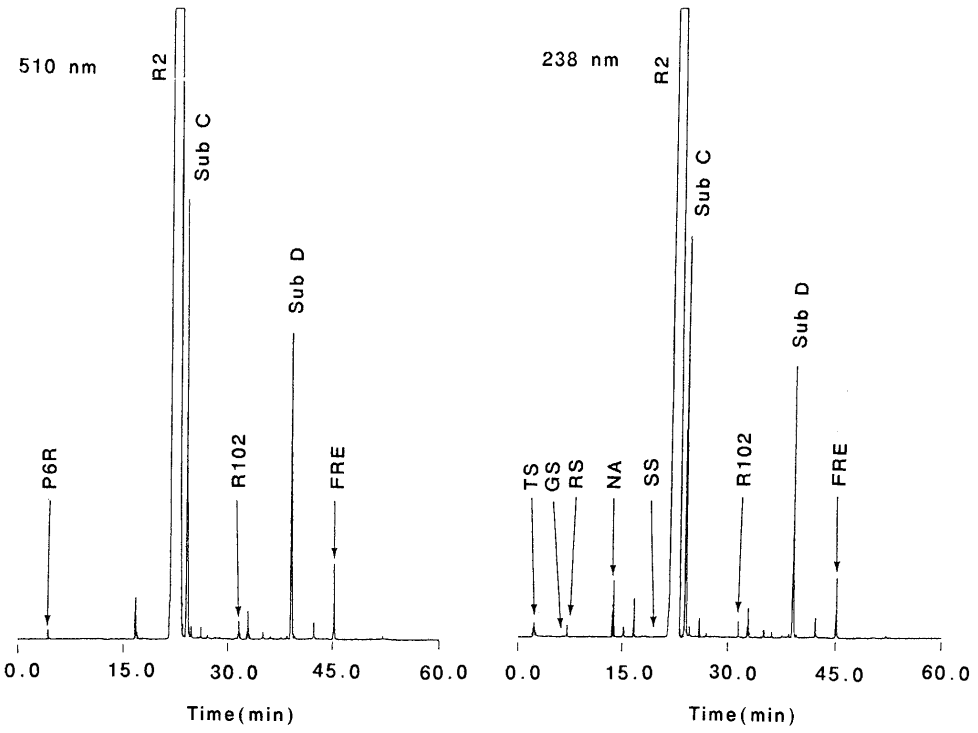

Fig. 3. Analytical HPLC of Commercial R2 at 510 nm (Left) and 238 nm (Right)

See legend of Fig. 2 for HPLC conditions.

presence of additional 2,6- or 2,7-substituted naphthalene. The defined formula showed that the substitutes in the naphthalene moiety were azo and sulfonato groups. The substituted positions of these groups were determined by dif-NOE experiment. Namely, when the *meta*-coupling signal at δ 8.34 (assigned to the 1′-position) was irradiated, NOE was observed at the vicinal- coupling signal of δ 8.03 (assigned to the 8′-position). This revealed that the two substituents were located at 2- and 6-positions. On the basis of the above evidence, the structure of Sub A

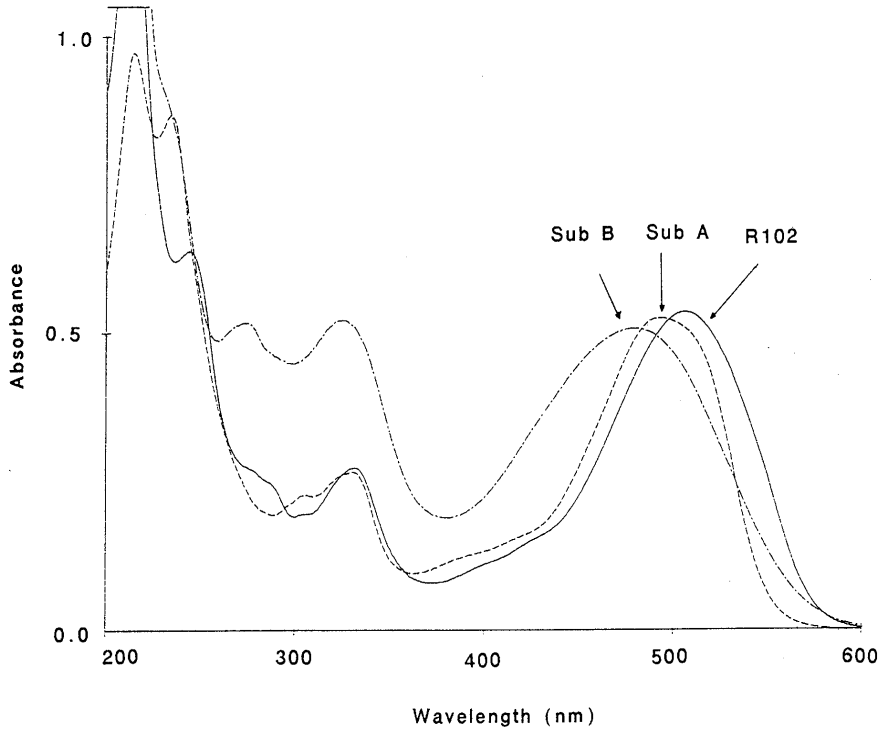

Fig. 4. UV-VIS Spectra of R102, Sub A, and Sub B

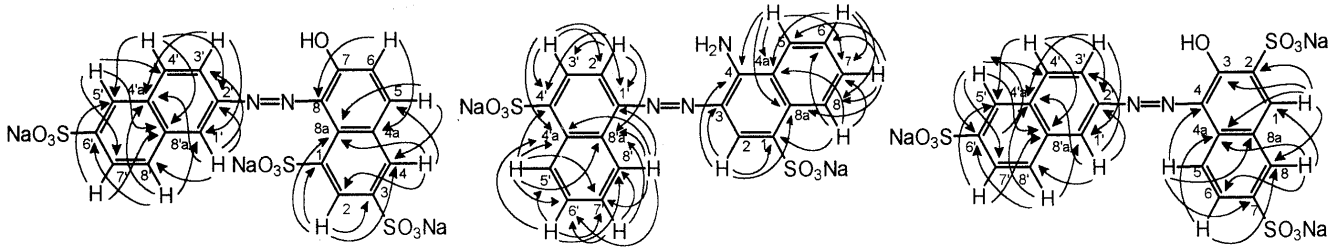

Fig. 5. Long-Range Correlations in HMBC Spectra of Sub A, Sub B, and Sub C

was elucidated as trisodium salt of 7-hydroxy-8-(6-sulfonaphthyl-2-azo)-1,3-naphthalenedisulfonic acid. The long-range correlations in the HMBC spectrum also support the structure (Fig. 5).

Structure and Origin of Sub B In the visible spectrum for Sub B, two strong absorption maxima at 485 nm and 325 nm were observed and the spectrum was appreciably different from that of R102. The IR spectrum of Sub B indicated the absorption due to NH_2 group (3448, 3169 cm^{-1}) and sulfonic group (1182, 1047 cm^{-1}). In the ESI-MS of Sub B, two quasi-molecular ion peaks at *m/z* 455.9 $[M-2Na+H]^-$ and 227.5 $[M-2Na]^{2-}/2$ were observed. The ^1H- and ^{13}C-NMR spectra exhibited 11 aromatic proton signals and 20 aromatic carbon signals (Tables 1 and 2). These facts suggested that the molecular formula of Sub B was $C_{20}H_{13}N_3Na_2O_6S_2$, and that Sub B possesses two sulfonic, one amino, and one azo group.

The ^1H-NMR spectrum of Sub B showed one singlet signal (δ 8.72), one *ortho*-located AB type coupling signal [δ 8.28 (d, $J=8.2$ Hz) and δ 7.87 (d, $J=8.2$ Hz)], and two sets of AA′BB′ coupling signals [δ 8.35 (br d, $J=8.4$ Hz), δ 7.57 (ddd, $J=7.2, 8.4, 1.2$ Hz), δ 7.68 (m) and δ 8.80 (br d, $J=8.4$ Hz); and δ 8.95 (m), δ 7.68 (m), δ 7.68 (m) and δ 8.95 (m)]. The presence of 1,4-substituted and 1,2,4-substituted naphthalene moieties was confirmed by HMBC data as shown in Fig. 5. On the basis of the combined data described above, Sub B was elucidated as a disulfonato-monoamino-azonaphthalene (disulfonato-monoamino-dinaphthyldiazene).

Considering the synthetic origin, both naphthalene moieties of Sub B, which is the main subsidiary color of R102, would be derived from NA and azo-coupling between the diazotized NA, and the non-diazotized one would produce Sub B. Steric hindrance due to the sulfonato group inhibits the attack of the diazo compound at the vicinal-position. Consequently, the locations of sulfonato and azo groups in one naphthalene moiety were estimated to be at the 4- and 1-positions, respectively, and the locations of sulfonato, azo, and amino groups of the other one were at 1-, 2- and 4-positions, respectively. Therefore, the structure of Sub B was thought to be disodium salt of 4-amino-3-(4-sulfonaphtyl-1-azo)-1-naphthalenesulfonic acid. The chemical shift data of ^1H- and ^{13}C-NMR spectra (Tables 1 and 2) also support the structure. Uematsu *et al.*[11] earlier reported the structure of Sub B as a contaminant of R102 and R2,

though they did not describe how they ascertained the structure.

Structures of Sub C and Sub D The absorption maximum for Sub C in the visible spectrum was observed at 507 nm, which was shorter than that for R2 (521 nm) as shown in Fig. 6. The similarity of the UV-Vis absorption of Sub C to that of R2 suggests that both compounds have the same conjugate system. The IR spectrum of Sub C indicated that the absorption was due to hydroxy group (3447 cm^{-1}) and sulfonic group (1194, 1036 cm^{-1}). In the ESI-MS of Sub C, the quasi-molecular ion peaks were observed at m/z 536.9 $[M-3Na+2H]^-$, 279.0 $[M-2Na]^{2-}/2$ and 178.3 $[M-3Na]^{3-}/3$, which were similar to those of R2. ^{1}H- and ^{13}C-NMR exhibited 10 aromatic proton signals and 20 aromatic carbon signals as shown in Tables 3 and 4. These data suggest that Sub C is a structural isomer of R102 with the formula $C_{20}H_{11}N_2Na_3O_{10}S_3$.

In the ^{1}H-NMR spectrum of Sub C, four aromatic protons were observed, which have a similar chemical shift and coupling pattern to those of the hydroxynaphthalene moiety of R2 (Table 3). Also, in the ^{13}C-NMR spectrum of Sub C, corresponding signals to the hydroxynaphthalene were recognized (Table 4). These data indicated Sub C possess 4-azo-3-hydroxy-2,7-naphthalenedisulfonic acid moiety. The presence of an additional 2,6-substituted or 2,7-substituted naphthalene was suggested by the remaining ^{1}H- and ^{13}C-NMR data. The defined formula indicated the substitutes in the naphthalene moiety were azo and sulfonato groups. The locations of the substituted groups were determined by phase-sensitive NOESY spectrum, which exhibited NOE correlation between the vicinal coupling signal at δ 8.14

Table 1. ^{1}H-NMR Data (600 MHz/TMS) of R102, Sub A, and Sub B in CD_3OD

Position[a]	R102	Sub A	Sub B
2	9.08 (d, J=2.4 Hz)	9.06 (d, J=2.4 Hz)	8.72 (s)
4	8.21 (d, J=2.4 Hz)	8.21 (d, J=2.4 Hz)	—
5	7.95 (d, J=9.6 Hz)	7.91 (d, J=9.0 Hz)	8.35 (br d, J=8.4 Hz)
6	6.95 (d, J=9.6 Hz)	6.92 (d, J=9.0 Hz)	7.57 (ddd, J=7.2, 8.4, 1.2 Hz)
7	—	—	7.68 (m)
8	—	—	8.80 (brd, J=8.4 Hz)
1′	—	8.34 (d, J=1.8 Hz)	—
2′	9.23 (d, J=8.4 Hz)	—	8.28 (d, J=8.2 Hz)
3′	8.33 (d, J=8.4 Hz)	7.90 (dd, J=1.8, 8.4 Hz)	7.87 (d, J=8.2 Hz)
4′	—	8.02 (d, J=8.4 Hz)	—
5′	8.99 (br d, J=7.8 Hz)	8.58 (d, J=2.4 Hz)	8.95 (m)
6′	7.68 (td, J=7.8, 1.2 Hz)	—	7.68 (m)
7′	7.74 (td, J=7.8, 1.2 Hz)	8.59 (dd, J=2.4, 8.4 Hz)	7.68 (m)
8′	8.30 (br d, J=7.8 Hz)	8.03 (d, J=8.4 Hz)	8.95 (m)

a) See numbering of the structures in Fig. 1 and Fig. 5.

Table 2. ^{13}C-NMR Chemical Shifts (δ Value, 150 MHz/TMS) of R102, Sub A, and Sub B in CD_3OD

Position[a]	R102	Sub A	Sub B	Position[a]	R102	Sub A	Sub B
1	143.2 (Ss)[b]	142.8[c]	131.2	1′	140.9 (Sm)	118.6	151.2
2	131.0 (Dd)	130.7	122.1	2′	115.8 (Ds)	143.8	111.2
3	142.5 (Ss)	142.3[c]	131.8	3′	128.0 (Ds)	120.7	127.1
4	130.5 (Dt)	130.5	148.1	4′	140.4 (Sm)	131.5	142.7
4a	131.2 (Sd)	130.9[d]	126.1	4′a	131.1 (Sq)	132.9	132.5
5	143.2 (Dd)	142.4	124.7	5′	129.0 (Dd)	126.5	128.0
6	128.2 (Ds)	127.9	126.9	6′	127.8 (Dd)	143.7	127.7[e]
7	179.4 (Sd)	177.6	130.4	7′	128.2 (Dd)	125.1	127.9[e]
8	133.1 (Sd)	131.8[d]	128.7	8′	121.1 (Dd)	130.0	124.7
8a	133.1 (Sq)	133.2	133.0	8′a	126.0 (Sq)	135.9	131.5

a) See numbering of the structures in Fig. 1 and Fig. 5. *b*) The multiplicities of carbon signals in one bone and two or more bone couplings are indicated by capital letters (S and D) and small letters (s, d, t, q and m), respectively. *c*)—*e*) Signal may be interchanged within each column.

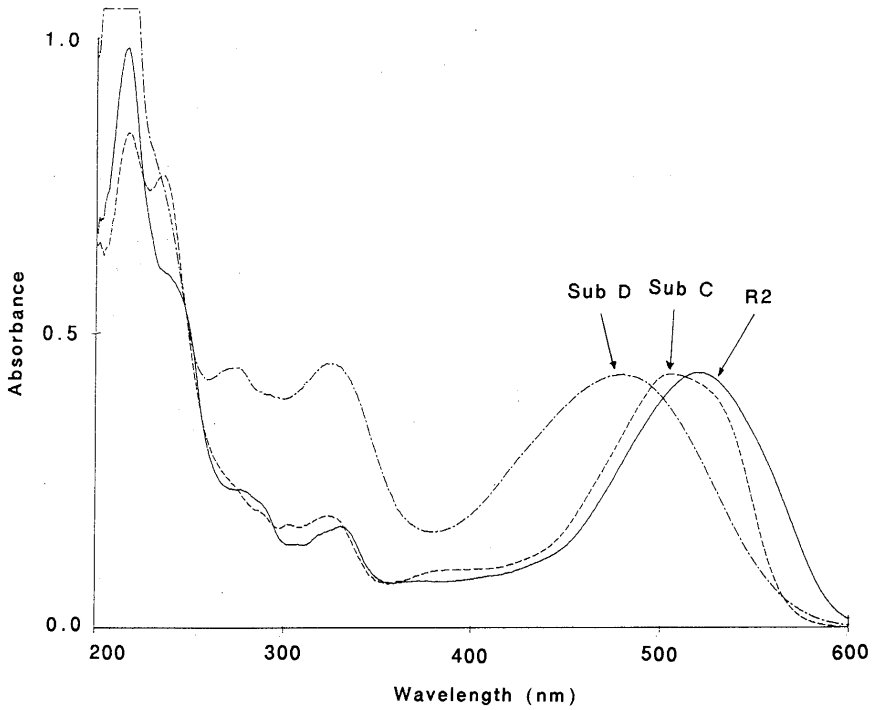

Fig. 6. UV-VIS Spectra of R2, Sub C, and Sub D

Table 3. ^{1}H-NMR Data (600 MHz/TMS) of R2 and Sub C in CD_3OD

Position[a)]	R2	Sub C
1	8.55(s)	8.51(s)
2	—	—
5	8.68 (d, J=8.5 Hz)	8.67 (d, J=8.4 Hz)
6	8.10 (dd, J=8.5, 1.9 Hz)	8.09 (dd, J=8.4, 2.0 Hz)
7	—	—
8	8.22 (d, J=1.9 Hz)	8.21 (d, J=1.8 Hz)
1′	—	8.39 (br d, J=1.8 Hz)
2′	8.29 (d, J=8.1 Hz)	—
3′	8.36 (d, J=8.1 Hz)	7.97 (dd, J=8.5, 1.8 Hz)
4′	—	8.06 (d, J=8.5 Hz)
5′	9.01 (br d, J=8.7 Hz)	8.28 (d, J=2.4 Hz)
6′	7.72 (td, J=8.7, 1.2 Hz)	—
7′	7.77 (td, J=8.7 Hz)	8.17 (dd, J=9.0, 2.4 Hz)
8′	8.42 (br d, J=8.7 Hz)	8.14 (d, J=9.0 Hz)

a) See numbering of the structures in Fig. 1 and Fig. 5.

Table 4. ^{13}C-NMR Chemical Shifts (δ Value, 150 MHz/TMS) of R2 and Sub C in CD_3OD

Position[a)]	R2	Sub C	Position[a)]	R2	Sub C
1	142.1	141.1	1′	141.2	126.7
2	140.6	140.4	2′	112.5	144.2
3	172.4	173.2	3′	127.6	125.7
4	131.4	131.5	4′	141.1	129.7
4a	136.8	137.0	4′a	132.0	135.9
5	123.4	123.1	5′	128.9	118.1
6	129.2	129.0	6′	128.4	143.8
7	145.2	144.8	7′	128.6	118.3
8	128.9	128.7	8′	121.5	132.2
8a	127.2	127.0	8′a	126.1	133.2

a) See numbering of the structures in Fig. 1 and Fig. 5.

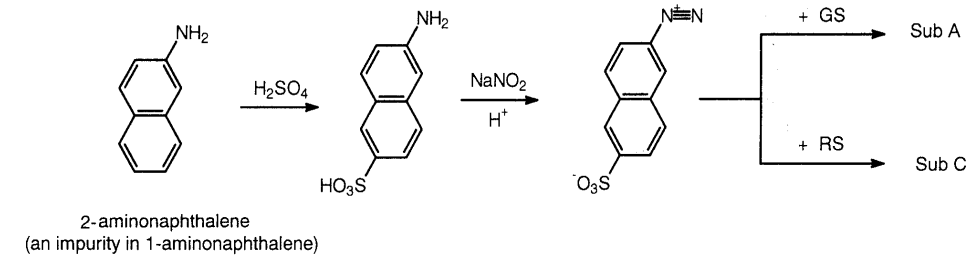

Fig. 7. Proposed Pathways of Formation of Sub A and Sub C

See legend of Fig. 1 for abbreviations.

and the *meta*-coupling signal at δ 8.39, and between the other vicinal coupling signal at δ 8.29 and the other *meta*-coupling signal at δ 8.06. On the basis of this combined data, the structure of Sub C was elucidated as trisodium salt of 3-hydroxy-4-(6-sulfonaphthyl-2-azo)-2,7-naphthalenedisulfonic acid. The long-range correlations in HMBC spectrum also support this structure (Fig. 5).

All the spectral data of Sub D were identical with those of Sub B. Therefore, the structure of Sub D was confirmed as disodium salt of 4-amino-3-(4-sulfonaphthyl-1-azo)-1-naphthalenesulfonic acid.

Origin of Sub A and Sub C NA is the coupling source common to R102 and R2, and is manufactured by sulfonation of 1-aminonaphthalene. When 2-aminonaphthalene exists as an impurity in 1-aminonaphthalene, 6-amino-2-naphthalenesulfonic acid should be produced as a side product under the sulfonation. Chromatographic purification is not carried out during the manufacturing process. Consequently, the fate of the side product does not differ from NA. Therefore, Sub A seemed to be produced as an impurity of R102, and Sub C as an impurity of R2. Proposed pathways of formation of Sub A and Sub C are shown in Fig. 7.

Quantitative Analysis We isolated major subsidiary colors from commercial R102 and R2. The quantitative HPLC analyses of Sub A, Sub B, other known subsidiary colors, and impurities were performed on commercial R102 (10 samples, 5 manufacturers). The average contents of Sub A and Sub B were 0.256% and 0.187% calculated as R102. Also, the quantitative HPLC analyses of Sub C, Sub D (Sub B), and others were performed on commercial R2 (12 samples, 2 manufacturers). The average contents of Sub C and Sub D (Sub B) were 0.803% and 0.349% calculated as R2. Detailed results of the analyses will be reported elsewhere.

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