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Isolation and Structural Investigation of the Chromophore in the Fujiwara Reaction as Applied to Chloramphenicol

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The structure of a red chromogen which is produced by applying the Fujiwara reaction to chloramphenicol was determined by means of NMR, mass spectroscopy and reasonance Raman spectroscopy. The red product was identified as 5-hydroxymethyl-4-(4-nitrobenzeno)-2,3-pyrrolidindione. This compound is different from the nitrogenated polyene which is produced in the Fujiwara reaction applied to such gem-trichloro compounds as chloroform and benzotrichloride.

Keywords——Fujiwara reaction; 5-hydroxymethyl-4-(4-nitrobenzeno)-2,3-pyrrolidindione; chloramphenicol; ^{1}H NMR spectrum; ^{13}C NMR spectrum

An intense red color develops when a mixture of a gem-polyhalogen compound and pyridine is heated for a few minutes in a strongly alkaline medium. This reaction is called the Fujiwara reaction.[1] The structures of the red chromogens produced in the Fujiwara reaction for such trichloroalkyl compounds ($RCCl_3$) as benzotrichloride[2] and chloroform[3] have been determined. The red chromogens decomposed to glutaconaldehyde, which shows an absorption maximum at around 360 nm.[4] Kakemi *et al.*[5] used this reaction for colorimetric determination of chloramphenicol, since this compound has a $-CHCl_2$ group. The reaction solution, however, did not give an absorption band at around 360 nm, indicating that no glutaconaldehyde is formed. Therefore, it is suggested that the color-producing mechanism in the case of chloramphenicol is completely different from that in the case of gem-trichloro compounds, although both substances show a similar red color in the Fujiwara reaction.

The purpose of the present study was to isolate the red chromogen produced from chloramphenicol and to clarify the structure and the color-producing mechanism by means of nuclear magnetic resonance (NMR), mass, and resonance Raman spectroscopies.

Experimental

Reagents and Chemicals——Pyridine of analytical reagent grade was further purified by distillation, and protected against light and moisture. Chloramphenicol (Nakarai Chemicals Ltd.), isoquinoline (Nakarai Chemicals Ltd.), *N*-dichloroacetyl-D-serine (Tokyo Kasei Kogyo Co., Ltd.), and *N*-dichloroacetyl-L-serine (Tokyo Kasei Kogyo Co., Ltd.) of analytical reagent grade were used as supplied. *N*-Methyl-dichloroacetyl amide was synthesized by aminolysis of the corresponding ethyl ester using 40% aqueous methyl amine solution, and recrystallized. The purity was checked by means of NMR.

Apparatus——The ^{1}H chemical shifts, which were measured on Varian HA-100D and Nicolet NT-360 NMR spectrometers, are given in parts per million (ppm) from internal tetramethylsilane or from 3-(trimethylsilyl)-propionic acid sodium salt, and are reported in δ values. The ^{13}C chemical shifts, which were measured on JEOL FX-100 and FX-200 NMR spectrometers, are given in ppm from internal tetramethylsilane. Mass spectra (MS) were taken with a JEOL JMS-01SG mass spectrometer. Field desorption (FD) MS were measured with an emitter current of 12 mA. High-resolution MS were recorded with a JEOL JMS-2000 mass data analysis system. The resonance Raman spectra between 1650 and 1000 cm^{-1} were recorded on a JEOL S-1 laser Raman spectrophotometer by using

the 488 nm excitation line of a Coherent Radiation 52G argon laser with an output power of 50 mW at the sample position. Column chromatography was carried out over silica gel with the solvent system of methanol–benzene (7 : 3, v/v). Lobar column size B LiChroprep RP-8 (Merck) was used for further purification, with methanol–water (1 : 2, v/v).

Isolation of the Color Product I——Chloramphenicol was added to a mixture of pyridine and 20% sodium hydroxide. The whole was heated for 5 min and then cooled at 0—5 °C. The pyridine layer was collected and the solvent was removed by evaporation under reduced pressure. The residue was dissolved in a small amount of metahnol–benzene (7 : 3, v/v) and subjected to silica gel column chromatography. The fraction corresponding to the colored zone was collected, and the solvent was removed by evaporation. The residue was dissolved in a small amount of methanol, and water was added until the colored material precipitated. The precipitate was collected on a microfilter and washed with water. Reversed phase column chromatography was used for further purification and an orange powder I was obtained. The FD-MS of I showed the molecular ion peak at *m/z* 250, and the high-resolution MS showed a parent ion peak at *m/z* 250.0588 which is consistent with the formula $C_{11}H_{10}N_2O_5$.

Reduction Product II from I——I was reduced with sodium borohydride on palladium carbon (Pd 5%) in methanol solution.[6] The solvent was evaporated off under reduced pressure, and the residue was dissolved in methanol–benzene (1 : 2, v/v) then subjected to silica gel column chromatography with the same solvent system. Thin-layer chromatography (TLC) was carried out over silica gel with methanol–benzene (7 : 3, v/v) for further purification (*Rf* 0.62). The FD-MS of II showed the molecular ion peak at *m/z* 222, and the high-resolution mass spectrum showed a parent ion peak at *m/z* 222.1002 which is consistent with the formula $C_{11}H_{14}N_2O_3$.

Results and Discussion

Structure of the Isolated Compound I

Observed chemical shifts and spin-spin coupling constants of ^1H and ^{13}C NMR spectra are listed in Table I. The ^1H NMR spectrum shows the presence of aromatic protons and three CH protons (H_a, H_b, and H_c), whereas the ^{13}C NMR spectrum shows the presence of a –CH carbon (55.4 ppm), a $-CH_2$ carbon (65.6 ppm), a quaternary carbon (103.5 ppm), benzene ring carbons (120.3—144.9 ppm), and two carbonyl carbons (166.1 and 172.2 ppm). Thus, H_a and H_b can be ascribed to the methylene group. Since the ^1H and ^{13}C chemical shifts for the benzene ring are almost the same as those of chloramphenicol (—7.9 ppm in ^1H NMR, and 122.7—150.4 ppm in ^{13}C NMR in dimethyl-d_6 sulfoxide), the aromatic nitro group of chloramphenicol may be retained in I. The infrared (IR) spectrum also supports the presence of the nitro group *i.e.*, $\nu_{as\,N-O}$ at 1560 cm^{-1} and $\nu_{s\,N-O}$ at 1332 cm^{-1}. The molecular formula

Table I. Observed Chemical Shifts (in ppm) and *J* values (in Hz) of I and Their Assignments

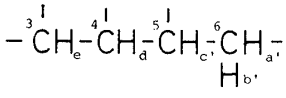
^1H NMR of I[a]		^{13}C NMR of I[a]	
Chemical shift and *J* value	Assignment	Chemical shift and multiplicity[c]	Assignment
3.08	H_a	55.4 (d)	5C
3.92	H_b	65.6 (t)	6C
4.25	H_c	103.5 (s)	4C
4.98	OH	120.3 (d)	2′C, 2″C
—7.90 (—8.10)[b]	Phenyl	123.6 (d)	3′C, 3″C
8.58	NH	138.3 (s)	4′C
		144.9 (s)	1′C
11.5[b]	J_{HaHb}	166.1 (s)	3C
8.5[b]	J_{HaHc}	172.2 (s)	2C
2.9[b]	J_{HbHc}		

a) In dimethyl-d_6 sulfoxide.
b) In methanol-d_4.
c) Multiplicities determined by off-resonance decoupling (s = singlet, d = doublet, t = triplet).

TABLE II. Observed Chemical Shifts (in ppm) and J Values (in Hz) of II and Their Assignments

^{1}H NMR of II[a]		^{13}C NMR of II[b]	
Chemical shift and J value	Assignment	Chemical shift and multiplicity[c]	Assignment
3.50	$H_{a'}$	50.6 (d)	4C
3.73	$H_{b'}$	58.4 (d)	5C
3.75	$H_{c'}$	62.8 (t)	6C
3.90	H_d	75.8 (d)	3C
4.59	H_e	116.6 (d)	2′C, 2″C
—7.09 (—6.90)[b]	Phenyl	128.9 (d)	3′C, 3″C
		141.6 (s)	4′C
12.5	$J_{Ha'Hb'}$	145.5 (s)	1′C
4.3	$J_{Ha'Hc'}$	177.4 (s)	2C
2.9	$J_{Hb'Hc'}$		
9.9	$J_{Hc'Hd}$		
10.7	J_{HdHe}		

a) In deuterium oxide.
b) In dimethyl-d_6 sulfoxide.
c) Multiplicities determined by off-resonance decoupling (s=singlet, d=doublet, t=triplet).



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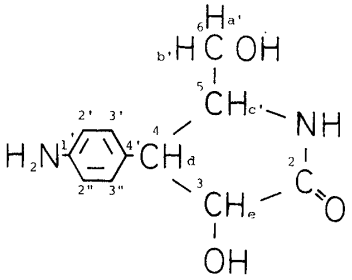

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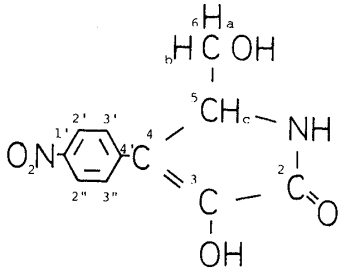

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suggested by high-resolution mass spectrometry, $C_{11}H_{10}N_2O_5$, involves eight sites of unsaturation. Accordingly, another ring structure can be expected, since seven sites of unsaturation can be counted from the already deduced partial structures (*i.e.*, two from the two carbonyl groups and five from the aromatic nitro group).

When I was reduced with sodium borohydride, a colorless product II, which has a molecular formula of $C_{11}H_{14}N_2O_3$, was obtained. This formula involves six sites of unsaturation. The ^{1}H and ^{13}C NMR spectra (Table II) suggest a partial structure such as **1** and the fragment peak at m/z 93 in the mass spectrum suggests the presence of an aromatic amine, *i.e.*, the nitro group of I seems to have been reduced to an amino group (see Figure 1). The fragment peaks shown in Figure 1 suggest a structure such as **2**. The ^{13}C NMR spectrum of II (Table II) shows the presence of three –CH carbons, a $-CH_2$ carbon, benzene ring carbons, and a carbonyl carbon. Their chemical shifts are also consistent with the above structure. Consequently, the structural formula of I can be determined to be **3** by analogy to that of II. The molecular formula and molecular weight obtained from the mass spectrum exactly coincide with those of **3**.

In the Fujiwara reaction involving gem-trichloro compounds, pyridine acts not only as a solvent but also as a starting material.[2] The pyridine ring is cleaved and incorporated in the structure of the red product.[2,3] On the other hand, the pyridine moiety is not incorporated in **3**. Indeed, **3** was also obtained when isoquinoline was used instead of pyridine.

The quaternary carbon observed at 103.5 ppm in the ^{13}C NMR spectrum of I can be assigned to 4C, and the ^{1}H signal at 8.58 ppm which disappeared on addition of deuterium oxide can be assigned to the NH proton adjacent to the carbonyl group (2C). The ^{13}C signal at 172.2 ppm may be assigned to 2C, and the remaining signal at 166.1 ppm may be assigned to 3C, since carbonyl carbons of lactams are usually observable in the range of 170—180 ppm.[7]

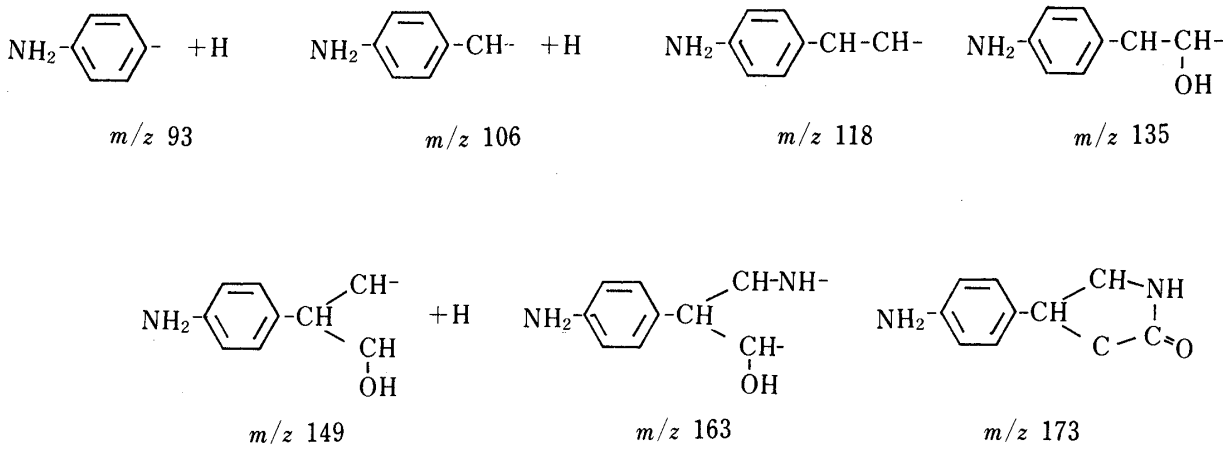


Fig. 1. Assignments of the Fragment Peaks

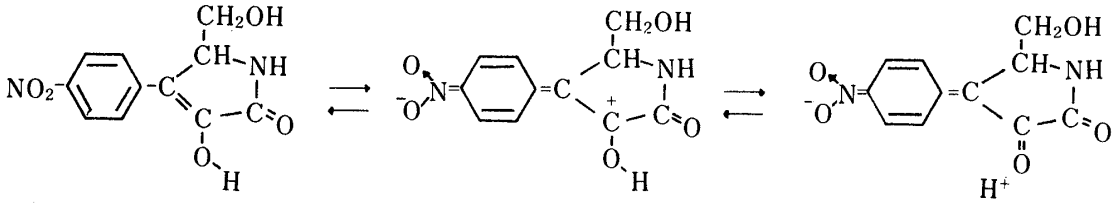
Molecular Structure Responsible for the Development of Red Color

Methanol solution of I shows a yellow color and has an absorption maximum at 433 nm. However, in alkaline pyridine, I shows a red color and has an absorption maximum at 505 nm. In alkaline pyridine, I may take a quinoidal structure **4** (see Figure 2), thus showing a red color[8] in the solution. The presence of this quinoidal structure can be confirmed by resonance Raman spectroscopy. Resonance Raman spectroscopy is a powerful technique for structural investigation of chromophores[9] and if a quinoidal structure such as **4** is present, a strong resonance Raman band due to the quinoide ring C–C stretching vibration is expected to appear at around 1600 cm^{-1}.[10]

In fact, the resonance Raman spectrum of I in alkaline pyridine, showed a strong band at 1611 cm^{-1}. Accordingly, the structure of I in alkaline pyridine can be considered to be **4**. However, the resonance Raman spectrum of I in methanol showed no band at around 1600 cm^{-1}. Thus, it can be considered that I in methanol does not take the quinoidal structure, and therefore shows a yellow color. The structure of I in dimethyl sulfoxide seems to be almost identical to **4**, because the band due to the quinoidal structure was observed at 1615 cm^{-1}. The ^{13}C signal at 166.1 ppm (3C) measured in dimethyl-d_6 sulfoxide shifted markedly to 155.9 ppm on addition of methanol (28%). The signals due to the benzene ring carbons and 4C also shifted in the range of 5.5—7.5 ppm. Accordingly, on addition of methanol to dimethyl-d_6 sulfoxide, the structure of I might be change from **4** to **3**, that is, from the quinoidal form to the normal benzene ring structure.

A Plausible Mechanism for the Formation of I

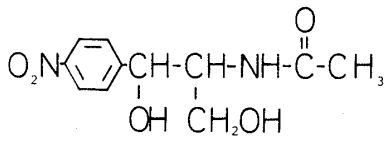
In the case of the Fujiwara reaction for gem-trichloro compounds, the color-producing mechanism is the replacement of two chlorine ions by two pyridines followed by cleavage of the pyridine rings.[2] However, in the present case, the color development seems to be due to



(yellow) (red)

3 4

Fig. 2. Molecular Structure Responsible for the Development of Color



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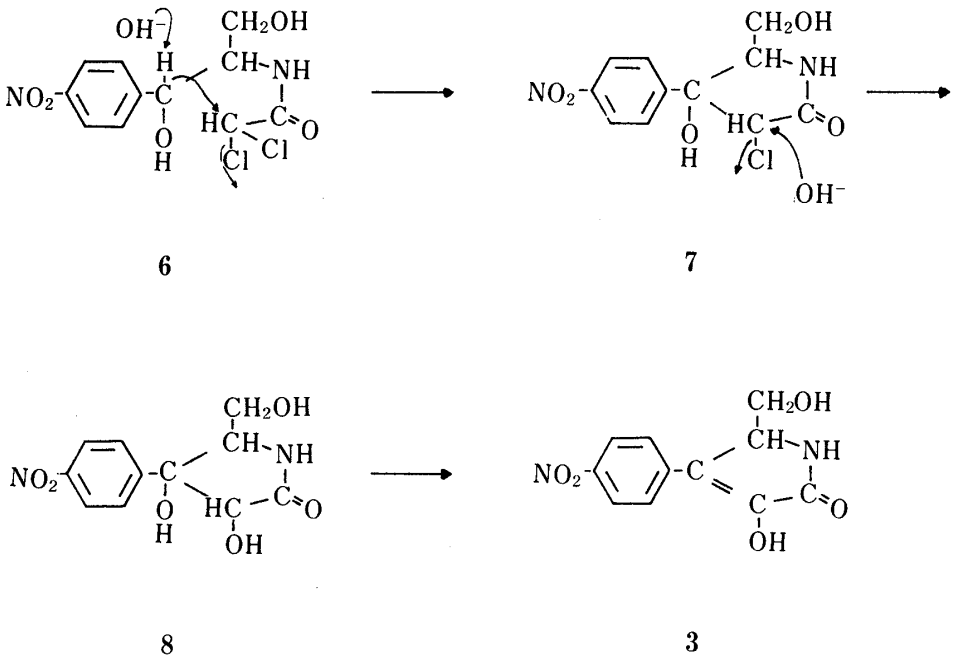
the presence of the aromatic nitro moiety. In fact, when *N*-dichloroacetyl serine and *N*-methyl-dichloroacetyl amide, whose partial structures are similar to that of chloramphenicol as gem-polyhalogen compounds, were used in the Fujiwara reaction, they did not develop any color.

l-Threo-*p*-nitrophenyl-2-acetamide-1,3-propanediol (**5**) also did not develop a red color in the Fujiwara reaction.[5] This result means that the $-CHCl_2$ group in chloramphenicol plays an important role in initiation of this reaction.

A plausible mechanism for the formation of **3** in the reaction of chloramphenicol with alkaline pyridine is outlined in Chart 1.



6 7

8 3

Chart 1

Conclusion

The use of chloramphenicol as a chromogenic reagent in the Fujiwara reaction leads to formation of **3**. In the reaction solution, this compound takes the anionic form **4**, which is responsible for the development of the red color.

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