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Reaction Products of 2-Oxoglutaric Acid with Diazotized Sulfanilamide[1]

Yosuke Ohkura,[2a] Masatoshi Yamaguchi,[2] and Tsutomu Momose[2b]

Faculty of Pharmaceutical Sciences, Kyushu University[2]

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In the previous paper,[3] a microcolorimetric method for the determination of 2-oxoglutaric acid (2-OG) was presented on the basis of a selective and sensitive color reaction of the acid with diazotized sulfanilic acid in sodium hydroxide solution in the presence of sodium sulfite and sodium hypophosphite. Then, the method was applied to the determination of serum transaminase activities[4] and isocitrate dehydrogenase activity.[5] Subsequently, diazotized sulfamethizole was shown to be also used as a reagent instead of diazotized sulfanilic acid.[6]

In order to elucidate the structures of products formed in the color reactions of 2-OG with diazotized sulfanilic acid and with diazotized sulfamethizole, an attempt was first made to isolate the products in crystalline forms, but this could not be successfully carried out because of difficulties in treatment of the reaction mixtures. Thus, diazotized sulfanilamide was selected for simple treatment of the reaction mixture for the present purpose, which gave quite similar coloration to that of diazotized sulfanilic acid.

Isolation of Reaction Products

A diazotized sulfanilamide solution was mixed with 2-OG solution and an alkaline solution[7] and warmed at 37° for appropriate time, as described in Experimental. The molar ratio of 2-OG to the diazotized amine, 1:2, was employed in the reaction. When a more excess of the diazotized amine was used, a large amount of it remained unreacted, which might interfere the separation of reaction products.

The reaction mixture was neutralized with hydrochloric acid, at which time carbon dioxide gas was observed to evolve, and then extracted with ethyl acetate. The dried extract on evaporating the solvent was dissolved in a small amount of methanol and chromatographed on an acidic alumina[8] column to give two major fractions which were eluted with methanol.[9] The resulting first and second eluates left orange prisms of mp 266° (I) and colorless needles of mp 116° (II), respectively. Several other fractions were observed in the chromatogram, but they were so small and could not be treated successfully. From the mother liquor which was left after the ethyl acetate extraction, on the other hand, oxalic acid was obtained.

The I showed an absorption band with the maximum at 525 nm when dissolved in an alkaline solution,[10] which was almost identical in shape and maximum with the band of the

1) This forms "Organic Analysis XCI." Part XC: Y. Ohkura and K. Zaitsu, *Talanta*, "in press."
2) Location: *Katakasu, Higashi-ku, Fukuoka*; *a*) *To whom all inquiries should be addressed*; *b*) Present address: *Higashimitsugi 102-7, Sayama, Saitama, Emeritus Professor of Kyushu University*.
3) Ts. Momose, Y. Ohkura, and Ta. Momose, *Talanta*, **16**, 1451 (1969).
4) Y. Ohkura, Ta. Momose, and Ts. Momose, *Enzymol. Biol. Clin.*, **10**, 343 (1969).
5) Y. Ohkura, H. Hamada, and T. Momose, *Chem. Pharm. Bull.* (Tokyo), **20**, 56 (1972).
6) Y. Ohkura, K. Matsumura, H. Hamada, and T. Momose, *Bunseki Kagaku*, **20**, 480 (1971).
7) 8% sodium hydroxide solution containing 1% each of sodium sulfite and sodium hypophosphite monohydrate, whose concentration and composition were identical with those of the alkaline solution used in the practical method of 2-OG determination.[3] Sodium sulfite and sodium hypophosphite served to stabilize the developed color.
8) The reaction products were strongly adsorbed on neutral or basic alumina.
9) These fractions could not be eluted with non-polar or weakly polar solvents such as benzene, chloroform, ethyl acetate and ethanol.
10) A mixture of 1 part of water and 4 parts of the alkaline solution,[7] whose composition and concentration were the same as those in the reaction mixture of the practical method of 2-OG determination.

reaction mixture in a longer wave length region as shown in Fig. 1, a and b, indicating that I contributed mainly to the coloration. The reaction mixture had another absorption band with the maximum at 413 nm, but compound responsible for the band was not separated from the reaction mixture. On the other hand, II dissolved in the alkaline solution[10] had no absorption band in the visible region (Fig. 1, d), showing II was not directly concerned with the coloration.

Structures of the Reaction Products

The data of elemental analysis and molecular weight determination of I were consistent with the formula of $C_{14}H_{16}O_4N_6S_2$, suggesting that the compound might be resulted from the reaction of two moles of the diazotized amine with one mole of 2-OG. The infrared (IR) spectrum of I indicated the presence of *p*-sulfamoylphenyl group in the molecule by the characteristic bands due to the vibrations of SO_2, NH_2, aromatic C=C and CH of 1,4-disubstituted benzene ring, which was confirmed by the signals caused by the aromatic and amide protons in the nuclear magnetic resonance (NMR) spectrum (Table I). The NMR spectrum also indicated the existence of methyl group though it could not be distinctly clarified from the IR spectrum.

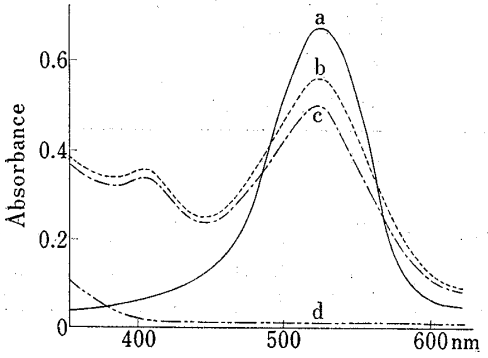

Fig. 1. Visible Absorption Spectra of I, II, and the Color Reaction Mixtures of 2-OG with Diazotized Sulfanilamide and with Diazotized Sulfanilic Acid

a: 5.3 μg/ml solution of I in the alkaline solution.[10] Measured against the alkaline solution.
b: 1.0 ml of 29.2 μg/ml solution of 2-OG was treated with 1.0 ml of diazotized sulfanilamide solution (prepared by diazotizing 0.7 g of sulfanilamide dissolved in 100 ml of 1.5% HCl with 10 ml of 3% $NaNO_2$ at 5°) and 8 ml of the alkaline solution[7] at 37° for 45 min. Measured against the reagent blank.
c: 1.0 ml of 29.2 μg/ml solution of 2-OG was treated as in b using diazotized sulfanilic acid solution (prepared by diazotizing 0.7 g of sulfanilic acid dissolved in 100 ml of 1.5% HCl with 10 ml of 3% $NaNO_2$ at 5°) instead of diazotized sulfanilamide solution. Measured against the reagent blank.
d: 20.0 μg/ml solution of II in the alkaline solution.[10] Measured against the alkaline solution.

The bands ascribable to NH, C=N, N=N and C–N groups in the IR spectrum and the signal assigned to NH proton in the NMR spectrum (Table I) suggested the presence of a yellow form of formazan moiety.[11]

From the evidences described above, I was characterized as the yellow form of 3-methyl-1,5-di(*p*-sulfamoylphenyl)formazan (Chart 1).

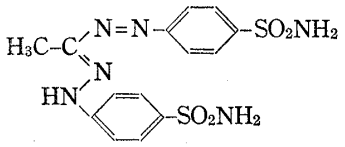

Chart 1. Structure of I

The molecular formula of II was determined by the elemental analysis and the molecular ion (M^+) in the mass spectrum (MS) as $C_6H_6O_2N_4S$. The IR and NMR spectra of II showed that *p*-sulfamoylphenyl group was involved in the molecule (Table I). The IR spectrum also indicated the existence of azide group, which was supported by the intense fragment ions of M^+-N_2, M^+-N_3+H and M^+-N_3-NH in the MS. Therefore, II should be *p*-sulfamoylphenylazide.

11) The IR spectrum of the yellow form of 3-methyl-1,5-diphenylformazan had the characteristic bands due to NH, C=N, N=N and C-N groups at 3220, 1575, 1410 and 1235 cm^{-1}, respectively, in the solid state, and generally the band of NH group in the yellow form of formazan was located at 3200—3500 cm^{-1} though the corresponding band in the red form was not observed in this region; The NMR spectrum of the yellow form of 3-methyl-1,5-diphenylformazan dissolved in dimethylsulfoxide-d_6 showed the signal of NH proton at δ (ppm) 10.22, but that of the red form at about δ 14: W. Otting and F.A. Neugebauer, *Z. Naturforsch.*, 23B, 1064 (1968).

Table I. IR and NMR Spectral Data of I and II

	I	II
IR ν_{max}^{KBr} cm^{-1}	3360, 3260 (NH_2) 1335, 1160 (SO_2) 1600 (arom. C=C) 829 (arom. CH) 3330 (NH) 1510 (C=N) 1412 (N=N) 1245 (C–N)	3340, 3260 (NH_2) 1335, 1160 (SO_2) 1590 (arom. C=C) 842 (arom. CH) 2120, 2150 ($N=N^+=N^-$)
NMR (δ, ppm)[a] in $(CD_3)_2SO$	10.70 s (1H)[b] (NH) 7.75 m (8H) (arom. H) 7.20 b (4H)[b] (SO_2NH_2) 2.34 s (3H) (CH_3)	7.85 d (2H), J = 9.0 7.25 d (2H), J = 9.0 (arom. H, adjacent two protons) 6.60 b (2H)[b] (SO_2NH_2)

a) J was expressed in Hz. Abbreviations: s, singlet; d, doublet; m, multiplet; b, broad
b) The signal disappeared on adding D_2O.

Now, the reaction to form I may be considered as follows: Two moles of diazotized sulfanilamide combine successively with the active methylene group of 2-OG to form an azo compound (III) under the alkaline conditions of the reaction, which is hydrolyzed to afford I and oxalic acid with eliminating carbon dioxide[12] (Chart 2).

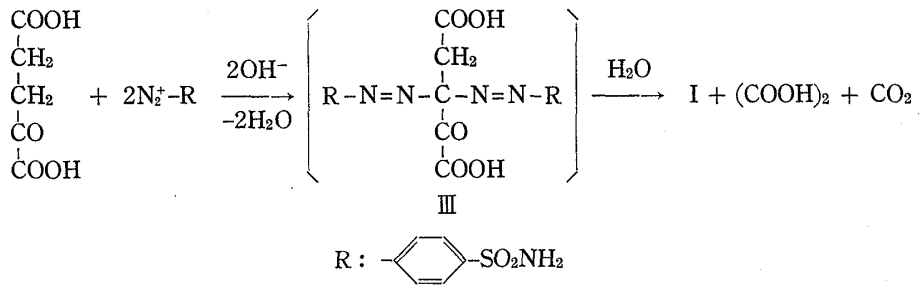

Chart 2. Reaction Schema of Formation of I

The route to form II could not be elucidated successfully from the experimental evidence, but II might be postulated to form by dehydration of *p*-sulfamoylphenyl-α-nitrosohydrazine which was resulted from diazotized sulfanilamide by reduction and nitrosation with excessive nitrite remained unreacted in the preparation of the diazotized amine.[13]

Coloring Matter in the Practical Method of 2-OG Determination

The absorption spectrum of the reaction mixture with diazotized sulfanilic acid in the practical method had the maximum at 525 nm and was quite similar to that of the reaction mixture with diazotized sulfanilamide as shown in Fig. 1. Furthermore, carbon dioxide gas was observed to evolve on neutralizing the reaction mixture, from which oxalic acid was also obtained by treating the mixture in the same way as described in Isolation of Oxalic Acid in Experimental. These facts explained that the coloring matter in the practical method should be a formazan derivative corresponding to I.

12) Similar reactions of active methylene compound with diazotized aromatic amine in alkaline media were already reported: A.W. Nineham, *Chem. Review*, **55**, 355 (1955).
13) An analogy was described in the formation of phenylazide from diazotized aniline: S.E.C. Biffin, J. Miller, and D.B. Paul, "The Chemistry of the Azide Group," ed. by S. Patai, Interscience, John Wiley & Sons, New York, 1971, Chapter II.

Experimental[14]

Isolation of I and II——To a solution of 2.4 g of sulfanilamide in 10 ml of 10% HCl, 0.95 g of $NaNO_2$ freshly dissolved in 5 ml of H_2O was added with stirring under ice-water cooling at 5°. To the resulting diazotized sulfanilamide solution, 1 g of 2-OG dissolved in 15 ml of H_2O and 120 ml of the alkaline solution[7] were successively added. The mixture was warmed at 37° for 2 hr, neutralized with 10% HCl with cooling in ice-water and then extracted with 300 ml portions of AcOEt three times. The combined extract was dried over Na_2SO_4 and concentrated below 50° *in vacuo* to almost dryness. The residue was dissolved in 10 ml of MeOH with warming, poured onto a column packed with about 100 g of HCl-treated Al_2O_3[15] and eluted with MeOH to afford two main fractions, from which I and II were separated when the each eluate was concentrated.

I——Orange prisms, mp 266° (from MeOH), yield 50 mg. *Anal.* Calcd. for $C_{14}H_{16}O_4N_6S_2$: C, 42.43; H, 4.07; N, 21.21; MW, 396. Found: C, 42.64; H, 4.17; N, 21.01; MW, 400. UV λ_{max}^{MeOH} nm (ε): 270 (2.1×10^4), 420 (3.2×10^4); λ_{max} (in the alkaline solution[10]) nm (ε): 280 (2.0×10^4), 525 (6.6×10^4).

II——Colorless needles, mp 116° (from benzene), yield 20 mg. *Anal.* Calcd. for $C_6H_6O_2N_4S$: C, 36.37; H, 3.05; N, 28.28. Found: C, 36.43; H, 3.07; N, 27.95. Mass Spectrum *m/e*: 198 (M^+), 170 (M^+-N_2), 157 (M^+-N_3+H, $(C_6H_5SO_2NH_2)^+$), 141 (M^+-N_3-NH, $(C_6H_5SO_2)^+$). UV λ_{max}^{EtOH} nm (ε): 210 (1.6×10^4), 260 (1.7×10^4); λ_{max} (in the alkaline solution[10]) nm: 265, 325.[16]

Isolation of Oxalic Acid——The neutralized reaction mixture remained after the AcOEt extraction of I and II was made slightly alkaline with 10% NH_4OH, to which 10 ml of 10% $CaCl_2$ was added. The precipitate thus separated was filtered, washed with H_2O and dissolved in 30 ml of 10% HCl. The resulting solution was continuously extracted with ether for 5 hr. The extract was dried over Na_2SO_4, concentrated to dryness, and the residue was sublimed above 159° to colorless fine prisms of mp 187° (measured in a sealed capillary tube), whose IR spectrum was entirely identical with that of anhydrous oxalic acid. Yield 30 mg.

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14) UV spectra were measured by a Shimadzu SV-50A Spectrophotometer in a cell of 10 mm optical length, IR spectra by a Nihonbunko 701G IR Spectrometer in KBr pellets, NMR spectra by a JEOL-C60H NMR Spectrometer at 60 MHz with tetramethylsilane as an internal standard and MS by a JEOL 01SG Mass Spectrometer. Molecular weight (MW) was determined by the Rast method using camphorquinone as a solvent. All melting points were uncorrected.

15) Commercial activated Al_2O_3 (Merck, 100 meshes) was dispersed in 20% HCl, allowed to stand for about 50 hr and filtered. After thorough washing with H_2O, it was air-dried and used without activation.

16) II was unstable in the alkaline solution and so the molar absorption coefficient could not be determined precisely.

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Free Radical formed during the Reaction of L-Ascorbic Acid with Hydrazine

Hideo Utsumi, Yutaka Kirino, and Takao Kwan

Faculty of Pharmaceutical Sciences, The University of Tokyo[1]

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It has been reported by Burlamacchi, *et al.*[2] that an electron spin resonance (ESR) spectrum is observed during the autooxidation of the alkaline aqueous solution of L-ascorbic acid with hydrazine. The spectrum consisted of nine resonance lines probably due to hyperfine

1) Location: *Hongo, Bunkyo-ku, Tokyo.*

2) L. Burlamacchi, P. Sarti-Fantoni, and E. Tiezzi, *Tetrahedron Letters*, **1969**, 5005.