

Chemical Structure and Some Properties of Roseoflavin

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Roseoflavin (I) is identified as 7-methyl-8-dimethylamino-10-(1'-D-ribityl)-isoalloxazine (IX) by comparing I with four diastereoisomers of 7-methyl-8-dimethylamino-10-D-n-pentitylisoalloxazine, which were synthesized from 2-dimethylamino-4-amino-toluene, D-pentoses, and violuric acid. The coincidence of chemical and physical properties—elemental analysis, UV-visible absorption spectrum, melting point, optical rotation, R_f value in thin layer chromatography, and NMR spectrum—of free and acetylated I with those of the free and acetylated IX, respectively, verified the identity. Some of the properties of I and acetylated I (pK_a , oxidation-reduction, absorption spectra, and extinction coefficients of various ionic species, and photosensitivity) are also described.

Recently many derivatives of riboflavin have been found to be constituents of coenzymes. In most cases, 8-CH₃ of riboflavin is modified, for example, to $\text{>N-CH}_2\text{-}^1$ $\text{-S-CH-}^2,3$ or 8-CH₃ is substituted with an HO-group.⁴ Otani *et al.*⁵ isolated an antibacterial red pigment, roseoflavin (I), from a culture medium of a *Streptomyces* strain No. 768, and it was revealed that in I, 8-CH₃ of riboflavin is substituted with a (CH₃)₂N-group. In this report we describe the details of the determination of the chemical structure and some properties of I.

Results and Discussion

Properties of I. I forms dark reddish-brown fine needles, which are slightly soluble in water producing a grenadine red solution. The UV-visible absorption spectrum in water, shown in Fig. 1 (curve 1), is analogous to that of 8-hydroxyflavin⁴ and dissimilar to that of riboflavin in water. I is not fluorescent in water in contrast with riboflavin and 8-hydroxyflavin. The results of mp, $[\alpha]_D$, and elemental analyses are shown in Table 1. The molecular weight is estimated to be 405 from the molecular ion peak in the mass spectrum. This value coincides with the molecular weight of C₁₈H₂₃N₅O₆.

As described in a previous communication,⁶ I was oxidized with periodate forming 7-methyl-8-dimethylamino-10-formylmethylisoalloxazine C₁₅H₁₅N₅O₃. From this fact it is deduced that the 10-side chain should be tetrahydic pentityl chain. I was acetylated

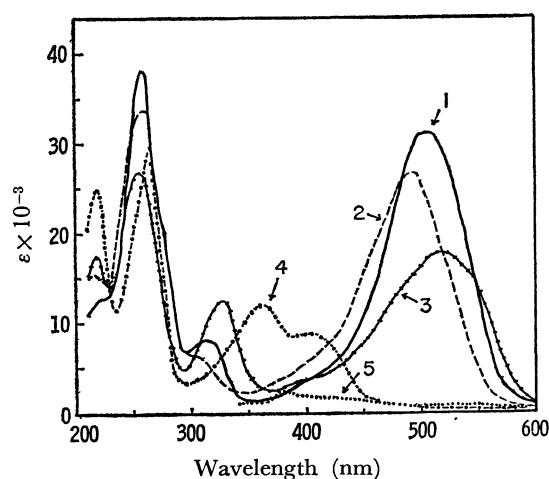


Fig. 1. UV-Visible absorption spectra of I and IX. Curve 1, I in water and IX at pH 6.99; curve 2, IX at pH 11.99; curve 3, IX at pH 0.60; curve 4, IX in 11 M HCl; curve 5, reduced IX in Na₂S₂O₄ solution (2.5 g/100 ml).

by the usual method with acetic anhydride producing tetraacetate (Ia).

Properties of Ia. Ia was recrystallized from ethanol (dark reddish-brown fine needles). The results of mp, $[\alpha]_D$, and elemental analyses are shown in Table 1. The observed values coincide with those calculated for C₂₆H₃₁N₅O₁₀ which corresponds to a tetraacetyl compound. The molecular weight ob-

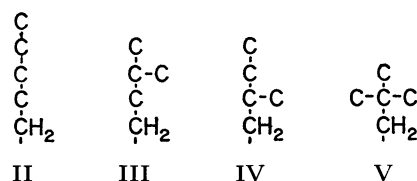
TABLE 1. MP, $[\alpha]_D$, AND ELEMENTAL ANALYSIS

Substance	mp °C	$[\alpha]_D$ in 0.1 M NaOH	Molecular formula	Elemental analysis %					
				Found			Calculated		
				C	H	N	C	H	N
I	274—276	−315	C ₁₈ H ₂₃ N ₅ O ₆ ·H ₂ O	51.17	5.84	16.62	51.06	5.95	16.54
IX	274—276	−320	C ₁₈ H ₂₃ N ₅ O ₆ ·H ₂ O	51.83	5.88	16.44			
X	282—284	+640	C ₁₈ H ₂₃ N ₅ O ₆ ·H ₂ O	51.84	5.83	17.00			
XI	280—282	−470	C ₁₈ H ₂₃ N ₅ O ₆ ·2H ₂ O	48.66	6.15	15.77	48.97	6.17	15.87
XII	273—275	+295	C ₁₈ H ₂₃ N ₅ O ₆ ·1.5H ₂ O	50.08	6.08	16.15	49.99	6.07	16.20
		in CHCl ₃							
Ia	279—280	+264	C ₂₆ H ₃₁ N ₅ O ₁₀	54.12	5.51	12.09	54.44	5.45	12.21
IXa	279—280	+260	C ₂₆ H ₃₁ N ₅ O ₁₀	54.21	5.42	12.42			
Xa	258—259	−24	C ₂₆ H ₃₁ N ₅ O ₁₀	53.77	5.36	12.75			
XIa	250—253	+44	C ₂₆ H ₃₁ N ₅ O ₁₀	54.12	5.40	12.75			
XIIa	255—256	−124	C ₂₆ H ₃₁ N ₅ O ₁₀	53.68	5.68	12.33			

TABLE 2. THE CHEMICAL SHIFTS OF ASSIGNABLE PROTONS OF Ia AND SYNTHESIZED ISOALLOXAZINE TETRAACETATES

Substance	Chemical shift of protons of							
	CH ₃				Aryl			
	Acetyl		7-	8 α N-				
Ia	1.80	2.07	2.13	2.27	2.47	3.13	6.80	7.87
IXa	1.80	2.07	2.13	2.27	2.47	3.13	6.80	7.87
Xa	1.83	1.98	2.03	2.28	2.47	3.12	6.88	7.85
XIa	1.92	1.95	2.07	2.25	2.47	3.13	6.97	7.83
XIIa	1.77	2.05	2.05	2.23	2.48	3.12	6.80	7.85

Solvent, CDCl₃. The chemical shifts are expressed in ppm with tetramethylsilane as an internal standard.



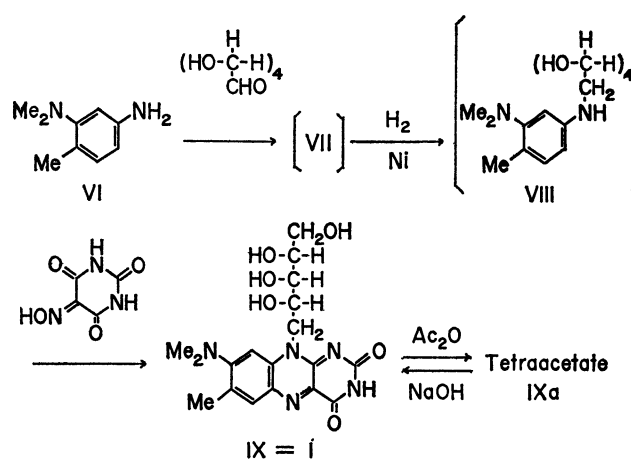
Scheme 1.

tained from mass spectrometry was 574, which coincide with the value calculated for the above compound. UV(CHCl₃), λ_{max} (ϵ_{mM}): 260 (40.7), 500 nm (40.9).

The chemical shifts of the protons in Ia are shown in Table 2. Twelve protons were assigned to (CO-CH₃)₄, 3 to C-CH₃, 6 to N(CH₃)₂, 2 to the H linked to the aromatic nucleus, and one to acidic H. Seven unassignable protons were found in the range from 4.23 to 5.70 ppm. These results agree with the structure of 7-methyl-8-dimethylamino-10-*n*-pentityl-isoalloxazine tetraacetate. The NMR spectrum shows 4 different shifts for the protons of CO-CH₃ (the ratio of the intensity of the signals being 1 : 1 : 1 : 1). This suggests that the pentityl side chain is unbranched, because among the possible structures of the pentityl side chain (scheme 1), II satisfies three requirements: (1) optical activity, (2) the formation of a formylmethyl compound by oxidation with periodate and (3) the formation of tetraacetate having the characteristic NMR spectrum described above.

The *n*-pentityl structure is consistent with the peaks of the fragment ions in the mass spectrum of I. In addition to the peak of the molecular ion (*m/e* 405), small peaks for *m/e* of 388, 374, 344 and 314, and large peaks at 284, 271 and 270 were observed. They are assumed to be residual fragments deprived of -OH, -CH₂OH, -(CHOH)₂-H, -(CHOH)₃-H, -(CHOH)₄-H, -CH₂-(CHOH)₄-H(+H) and -CH₂-(CHOH)₄-H, respectively.

Synthesis of 7-methyl-8-dimethylamino-10-n-pentitylisoalloxazine and Its Tetraacetates. *n*-Pentitylisoalloxazine has eight diastereoisomers. We synthesized four *D*-isomers and their tetraacetates for comparison with I and Ia. The process is shown in Scheme 2. VI⁷⁾ was condensed with *D*-ribose to VII, for which the structure was not confirmed, but is assumed to be ribopyranoside.⁸⁾ VII was reduced with H₂ in the presence of Raney nickel to VIII. As VII and VIII were too labile to be isolated in pure states, the reaction mixtures were subjected to succeeding steps as



Scheme 2.

soon as possible without purification. VIII was condensed with violuric acid to isoalloxazine IX (Piloty's alloxazine synthesis⁹⁾), which was purified through acetate, *i.e.*, IX was acetylated with acetic anhydride and pyridine producing tetraacetate (IXa), which was recrystallized from ethanol and hydrolyzed with 1 M NaOH forming IX.

Three other *D*-diastereoisomers and their tetraacetates, *D*-arabinityl- (X, Xa), *D*-xylyl- (XI, XIa), and *D*-lyxityl- (XII, XIIa), were synthesized in the same way as IX.

Comparison of I and Ia with Synthesized Isoalloxazines and Their Acetates.

Of the four synthetic isoalloxazines (IX—XII) only IX coincides with I for all three properties (mp, $[\alpha]_D$, and elemental analyses) and of the four tetraacetates (IXa—XIIa) only IXa coincides with Ia (Table 1). Mixed examinations of the mp of I-IX and Ia-IXa also demonstrated the identities of I-IX and Ia-IXa.

For NMR (Table 2), the chemical shifts of protons of Ia coincide with those of IXa. For thin-layer chromatography, the *R_f* value of I (0.45) coincides with that of IX (0.45) and not with those of X, XI and XII (0.37, 0.24 and 0.29, respectively) (the solvent being acetone-water 9 : 1 by volume; Merck's Precoated TLC Plate Silica Gel 60F-245). UV-visible absorption spectrum of I in water was coincident with that of IX at a pH of 6.99 (neutral species, Fig. 1) and those in 6 M HCl and in 0.1 M NaOH (not shown in Fig. 1) also coincide with those of IX under the

same conditions. However, the differences in absorption spectra of the diastereoisomers of IX are so small that this does not provide definite proof of the identification of diastereoisomer.

Therefore, I was identified to be 7-methyl-8-dimethylamino-10-(1'-D-ribityl)-isoalloxazine (Scheme 2).

The identification of I as 7-methyl-8-dimethylamino-10-(1'-D-ribityl)-isoalloxazine using physicochemical methods was consistent with bacteriological data, *i.e.*, IX was antibacterially as active as I and more active than the other diastereoisomers.¹⁰⁾

The mp of I recrystallized from water was 274—276 °C as described above. However, in a previous paper,⁵⁾ the authors reported that the mp of I was 276—278 °C. It was ascertained by repeated experimentation that this discrepancy was caused by the presence of water of crystallization, that is, the previous sample was dried over P₂O₅ *in vacuo* at 110 °C and the sample used in this paper was dried over CaCl₂ *in vacuo* at room temperature and contained one mole of water of crystallization.

Other Properties of I and IX. Some properties of I are analogous to those of riboflavin and 8-hydroxyflavin, *e.g.*, pK_a, reducibility-oxidizability, and photosensitivity. pK_a was measured spectrophotometrically using IX. The observed values, 10.8 and 1.6, correspond to those of riboflavin, 10.2 and 1.7,¹¹⁾ and of 8-hydroxyflavin, 11.5 and 0.7.⁴⁾ The absorption spectra of IX at pH 11.99, 6.99, and 0.60 (Fig. 1) show these for the anion, the neutral species, and the cation. When the solution was acidified to less than pH 0.1, the absorption spectrum changed even more (see, for example, absorption curve 4 (11 M HCl) shown in Fig. 1). Therefore, IX should have another pK_a < 0.1, and in this range IX should be protonated doubly. The absorption properties are as follows: λ_{max} (ε_{mM}): (pH 6.99, neutral species) 225 sh (12.7), 259 (38.2), 275 sh (20.3), 314 (8.0), 505 nm (31.1); (pH 0.60, cation) 219 (17.3), 255 (26.6), 328 (12.5), 519 nm (17.8): (11 M HCl, divalent cation) 219 (24.8), 265 (29.4), 364 (12.1), 402 nm (8.8), (pH 11.99, anion) 215 (15.2), 259 (33.7), 308 (6.0), 492 nm (26.6).

I was reducible with sodium dithionite to a yellow substance, which was autoxidizable to the original form. During the reduction with dithionite, no color of the intermediate (semiquinone) was recognized in contrast with riboflavin. The visible absorption spectrum of the reduced form is shown in Fig. 1. The oxidation-reduction potential of I, E_{m7}, was estimated to be -0.466 V, while that of riboflavin to be -0.428 V (*vs.* SCE, 20 °C, by the polarographic method). These values indicate that I is more difficult to reduce than riboflavin.

I was photosensitive as is riboflavin. When an alkaline solution of I was illuminated, the solution turned yellow and fluoresced. Two of the photolytic products of I were identified as 7-methyl-8-methylamino-10-(1'-D-ribityl)-isoalloxazine (a demethylated compound) and 7-methyl-8-dimethylaminoalloxazine. The details have been published elsewhere.¹²⁾

Some properties of I are qualitatively different from those of riboflavin, for example, the absorption

spectra. The neutral species of I has only one absorption peak (505 nm) in the range from 350 to 600 nm, while riboflavin has two (375 and 445 nm). The cation of I has one peak (509 nm) similar to that of riboflavin (396 nm). However, the divalent cation of I (in 11 M HCl) has two peaks (364 and 402 nm). In fluorescence, I was different from riboflavin. The neutral species of I does not fluoresce in water and ethanol, while that of riboflavin is fluorescent. However, Ia is apparently green fluorescent in chloroform, at a λ_{max} (emission) of 534 nm (uncorrected), as is riboflavin tetraacetate. However, it is curious that I was not fluorescent while Ia was fluorescent, and moreover, that other 8-dialkylamino-isoalloxazine tetraacetates were not fluorescent in chloroform (from unpublished data). The λ_{max} for excitation of Ia was 494 nm, which does not coincide with the λ_{max} for absorption of Ia (500 nm). Therefore, it would be reasonable to assume that the Ia preparation was fluorescent because of some impurity (or impurities).

Otani¹³⁾ reported that I inhibited the growth of *Lactobacillus casei* by competing with riboflavin. I has similar and dissimilar properties to riboflavin—this could be the cause of the antibacterial activity of I.

Experimental

Materials and Methods. I from *Streptomyces* strain No. 768 was provided by Mr. Takatsu of Nissin Food Products Co., Ltd.⁵⁾ As necessary, it was recrystallized from water. 2-Dimethylamino-4-aminotoluene⁷⁾ (VI) was prepared from *N*-dimethyl-*o*-toluidine by nitration and reduction with SnCl₂.

Absorption spectra, extinction coefficients, and fluorescence emission spectra were measured with a Hitachi recording spectrophotometer EPS-2U, fluorescence excitation spectra with a Jasco spectrofluorometer FP-4, specific rotation with a Jasco recording spectropolarimeter J-20, proton NMR spectra with a JEOL NMR spectrometer C-60 HL (60 MHz) (by Dr. Kinoshita), mass spectra with a Hitachi mass spectrometer RM-50 (direct injection method), oxidation reduction potential with a Yanaco Polarograph P-8 (by Mr. Ichimura), and pH with a Corning digital pH meter model 109.

Roseoflavin Tetraacetate (Ia). I (85 mg) was suspended in pyridine (10 ml) and dissolved by adding acetic anhydride (2 ml). The mixture was left standing overnight at room temperature. After the addition of cold water (about 30 ml) with ice cooling, the reaction mixture was evaporated in a rotary evaporator. The product was recrystallized from ethanol five times. Yield, 60.7 mg, reddish dark brown fine needles with metallic luster. Mp, elemental analysis, [α]_D, see Table 1; NMR, see Table 2.

Synthesis of Flavins. 7-Methyl-8-dimethylamino-10-(1'-D-ribityl)isoalloxazine (IX): D-Ribose (3.0 g) and 3.3 g of freshly distilled VI were suspended in 6 ml of absolute methanol. The suspension was refluxed to dissolve the materials, and after complete dissolution the reflux was further continued for 2 hr. Immediately after cooling, the reaction mixture was transferred into an autoclave and mixed with 8 g of freshly prepared Raney nickel. After hydrogenation at 100 °C at a pressure of 120 atm for 1 hr, the catalyst was removed by filtration. The solvent was removed in a rotary evaporator, and the residual syrup was again dissolved in

10 ml of methanol. The solution was poured into a hot solution of violuric acid (3.2 g in 50 ml of methanol). After reflux for 2 hr, the reaction mixture was chilled in a refrigerator overnight. The deposited crystals were filtered and washed with cold methanol. The filtrate, combined with washings, was evaporated and the residue was extracted with hot water. The second crop from the concentrated extract was recrystallized from water-methanol. The combined crystals (first and second crops) were further recrystallized from water. Yield, 4.4 g, 50%.

The purest material was obtained by hydrolysis of the recrystallized IXa (see following). IXa (3.0 g) was dissolved in 30 ml of 1 M NaOH, and after 10 min, the solution was neutralized with acetic acid (about 3 ml). The precipitates were filtered and washed with water. If necessary, it was recrystallized from water. Yield, 2.0 g, 90% of dark reddish-brown fine needles with metallic luster. Mp, elemental analysis, $[\alpha]_D$, see Table 1.

IX Tetraacetate (IXa): IX (2.0 g) dissolved in 20 ml of pyridine was mixed with 8 ml of acetic anhydride. After stirring at room temperature for several hours, the reaction mixture was evaporated to dryness in a rotary evaporator. The residue was dissolved in chloroform, and washed with water. The chloroform layer was dried over anhydrous magnesium sulfate, and was then evaporated. The product was recrystallized from ethanol. Yield, 2.0 g, 74% of dark reddish-brown fine needles with metallic luster. Mp, elemental analysis, $[\alpha]_D$, see Table 1; NMR, see Table 2.

7-Methyl-8-dimethylamino-10-(1'-D-arabinityl)-isoalloxazine (X): This compound was prepared from 3.0 g of D-arabinose, 3.3 g of VI, and 3.2 g of violuric acid (yield of crude preparation, 4.4 g, 50%), and purified through tetraacetate in the same way as IX. Yield, 2.0 g from 3.0 g of Xa, 90% of dark reddish-brown fine needles with metallic luster. Mp, elemental analysis, $[\alpha]_D$, see Table 1.

X Tetraacetate (Xa): This substance was prepared by acetylation of X in the same way as IXa. Yield, 2.0 g from 2.0 g of X, 74% of dark reddish-brown fine needles with metallic luster. Mp, elemental analysis, $[\alpha]_D$, see Table 1; NMR, see Table 2.

7-Methyl-8-dimethylamino-10-(1'-D-xylityl)-isoalloxazine (XI): This compound was prepared from 3.0 g of D-xylose, 3.3 g of VI, and 3.2 g of violuric acid (yield of crude preparation, 4.3 g, 48%), and purified through tetraacetate in the same way as IX. Yield, 2.0 g from 3.0 g of XIa, 87% of dark reddish-brown fine needles with metallic luster. Mp, elemental analysis, $[\alpha]_D$, see Table 1.

XI Tetraacetate (XIa): This compound was prepared by acetylation of XI in the same way as IXa. Yield, 1.9 g from 2.0 g of XI, 75% of dark reddish-brown fine needles with metallic luster. Mp, elemental analysis, $[\alpha]_D$, see

Table 1; NMR, see Table 2.

7-Methyl-8-dimethylamino-10-(1'-D-lyxityl)-isoalloxazine (XII): This compound was prepared from 3.0 g of D-lyxose, 3.3 g of VI, and 3.2 g of violuric acid (yield of crude preparation, 4.6 g, 53%), and purified through tetraacetate in the same way as IX. Yield, 2.0 g from 3.0 g of XIIa, 88% of dark reddish-brown fine needles with metallic luster. Mp, elemental analysis, $[\alpha]_D$, see Table 1.

XII Tetraacetate (XIIa): This compound was prepared by acetylation of XII in the same way as IXa. Yield, 1.9 g from 2.0 g of XII, 75% of dark reddish-brown fine needles with metallic luster. Mp, elemental analysis, $[\alpha]_D$, see Table 1; NMR, see Table 2.

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