Chem. Pharm. Bull.
30(4)1352—1357(1982)

Unexpected Conversion of Epinephrine into Tetrahydroisoquinolines in a Solution containing Ascorbic Acid

AKIRA SHIRAHATA,*[1] MASANORI YOSHIOKA, and ZENZO TAMURA

Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, 7-3-1, Bunkyo-ku, Tokyo

(Received August 14, 1981)

Degradation of ^{3}H-epinephrine during incubation for 14 h at 37°C in a phosphate buffer, pH 7.0, containing ascorbic acid was observed in a study on the radioimmunoassay of epinephrine. Chromatographic separations of the products were carried out on a large scale. They were identified as 1,2,3,4-tetrahydro-4,6,7-trihydroxy-2-methylisoquinoline (**1**) and 1,2,3,4-tetrahydro-4,7,8-trihydroxy-2-methylisoquinoline (**2**). These products were synthesized from epinephrine and formaldehyde for the first time. Our results suggested that ^{3}H-epinephrine reacted with formaldehyde generated through the oxidation of ascorbic acid by oxygen from air under the incubation conditions used above to yield **1** or **2**. Care is clearly required in using ascorbic acid as an antioxidant in catecholamine studies.

Keywords——catecholamine; epinephrine; ascorbic acid; formaldehyde; tetrahydroisoquinoline; antioxidant

Catecholamines contain an oxidizable catechol and a basic amine, and a rapid oxidative degradation which proceeds *via* the amino-chrome in neutral and alkaline solutions is well known. To protect catecholamines from such degradation, ascorbic acid is often used as an antioxidant and is added to analytical systems.[2] In the previous study on radioimmunoassay of epinephrine,[3] we used ascorbic acid for this purpose, and found that an unusually long period was required for completion of the reaction of ^{3}H-epinephrine with the antiserum. We suspected a degradation of ^{3}H-epinephrine under the conditions of the radioimmunoassay, and also the presence of an antibody against the degradation product(s) in the antiserum. The products were expected to be different from adrenochrome, the amino-chrome of epinephrine, and the degradation seemed to depend on the presence of ascorbic acid. To determined the characteristics of the products, chromatographic separations were carried out on a large scale in the present work. The products were identified as tetrahydroisoquinolines (TIQ), condensation products of epinephrine with formaldehyde.

Experimental

Reagents and Materials——L-Epinephrine bitartrate (Nakarai Chemicals Co., Ltd. (Tokyo)), L-ascorbic acid, sodium L-ascorbate (Kanto Chemicals Co., Ltd. (Tokyo)) and DL-epinephrine (Sigma Inc.) were used. DL-Epinephrine-[7-^{3}H]bitartrate, 11.0 Ci/mmol, was purchased from New England Nuclear Inc. Other reagents were of guaranteed grade. Boric acid gel (Aldrich Chemicals), Amberlite CG-50, type II, Amberlite XAD-4 (Rohm and Haas) and Dowex 1X8 (Dow Chemicals) were used. Hitachi gel 3011-C was kindly given by Mr. A. Narita, Nissei Sangyo Co., Ltd.

Amberlite XAD-4 was ground and grains of 150—300 mesh were obtained by sieving. Samples for binding assay were dissolved in modified Bray's solution[3] and the radioactivity was measured in an on-line scintillation spectrometer, Packard Model 3255 Tricarb System.

^{1}H- and ^{13}C-nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were obtained on a JEOL FMN-FX 100 Fourier-transform NMR spectrometer. Ultraviolet spectra were taken on a JASCO UVIDEC 505 spectrophotometer. Melting points were determined with a Yanagimoto melting point apparatus and are uncorrected.

Binding Assay of ^{3}H-Epinephrine——The assay was carried out according to the radioimmunoassay procedure for epinephrine.[3] The antiserum[3] and ^{3}H-epinephrine (6000 dpm) diluted with 0.1 M phosphate buffer, pH 7.0, containing 10 mM ascorbic acid were taken in a siliconized test tube. After incubation at

NII-Electronic Library Service

37°C, the solution was filtered through a Millipore filter. The antibody-bound radioactivity on the filter was measured.

Fractionation on a Boric Acid Gel Column——A catecholamine fraction was obtained according to Higa's method[4] with some modifications. One-tenth sample volume of 1.0 M phosphate buffer, pH 7.0, containing 0.1 M ascorbic acid was added to a sample and the pH of the solution was adjusted to 7.0 with 1 N NaOH. The solution was applied to a column of boric acid gel. The column was washed with 20 gel volumes of water, then the catecholamine fraction was eluted with 0.025 N HCl. Each fraction was monitored by observing its OD_{280} or its color reaction with Doty's reagent.[5]

Chromatography on an Amberlite CG-50 Column——The catecholamine fraction eluted from the boric acid gel was evaporated to dryness under reduced pressure at 40°C, and 1.0 ml of buffer (0.2 M ammonium acetate, pH 6.1) containing 10 mM ascorbic acid was added. The solution was applied to a column of Amberlite CG-50 equilibrated with the buffer. Chromatography was carried out according to Kirshner's method.[6]

High Performance Liquid Chromatography (HPLC) on a Column of Hitachi Gel 3011-C——The chromatograph employed consisted of a mini micro pump, Kyowa Seimitsu type KSU-16H, a UV monitor, Mitsumi Science Industry LDC type ST-1205, and a column (2×500 mm) in a glass tube packed with Hitachi gel 3011-C, a porous polymer cation exchanger. The temperature of the column was maintained at 45°C. The eluent was 0.4 M ammonium acetate buffer, pH 5.0. The flow rate was 0.50 ml/min. A sample was injected with a 50-μl microsyringe.

Chromatography on an Amberlite XAD-4 Column——A sample was mixed with the same volume of 0.01 N HCl saturated with NaCl, applied to a column of Amberlite XAD-4 (1.0×20 cm) equilibrated with 20% NaCl in 0.01 N HCl, and eluted with an NaCl gradient (20—0% NaCl in 0.01 N HCl) as shown in Fig. 4. The effluent was fractionated and monitored by HPLC.

Determination of Formaldehyde——Formaldehyde was determined as its 2,4-dinitrophenylhydrazone (HCHO-DNP) by HPLC. The reaction with 2,4-dinitrophenylhydrazine (DNP) and the extraction of the product, HCHO-DNP, were carried out according to Hino's method[7] with some modifications. One hundred ml of a sample solution or standard solution containing formaldehyde (0—5.0 μM) was mixed with 5 ml of conc. HCl and 10 ml of 0.01% DNP solution in 2 N HCl. After the addition of 0.05 mg of triphenylene as an internal standard in carbon tetrachloride, HCHO-DNP in the solution was extracted twice with carbon tetrachloride (20 ml and 10 ml). The organic phases were combined, washed with 20 ml of 1 N HCl, and dried over sodium sulfate (anhydrous). The organic solution was filtered and concentrated to dryness under reduced pressure at 45°C. The dried residue was dissolved in 2.0 ml of dichloromethane. Ten μl of this solution was then analyzed by HPLC. The chromatographic conditions were chosen according to Mansfield's method.[8] The column of Licrosorb SI-100, 4×250 mm, was operated at 25°C.

The minimum concentration of formaldehyde detectable by the present method was 0.1 μM.

Synthesis of 1,2,3,4-Tetrahydro-4,6,7-trihydroxy-2-methylisoquinoline Hydrochloride (1) and 1,2,3,4-Tetrahydro-4,7,8-trihydroxy-2-methylisoquinoline Hydrochloride (2)——1: DL-Epinephrine (1.0 g) was suspended in a mixture of 5 ml of methanol and 0.5 ml of 35% formaldehyde. The suspension was stirred for 1 h at room temperature, then the precipitate was filtered off, washed with chilled methanol, and dissolved in 1 ml of 6 N HCl to obtain the chloride form. The solution was added to methanol to precipitate **1**, which was recrystallized from methanol as needle crystals (300 mg, 23.7%). mp 200—201°C. *Anal.* Calcd for $C_{10}H_{13}NO_3$, C, 51.84; H, 6.09; N, 6.05. Found: C, 51.77; H, 6.08; N, 5.92. ^{1}H-NMR (2% solution in D_2O) δ:[9] 3.09 (3H, s, N–CH_3), 3.40—3.80 (2H, m, –CH_2–), 4.06—4.50 (2H, m, –CH_2–), 4.95 (1H, m, –C$\underline{H}$OH–), 6.69 (1H, s, Ar–H), 6.91 (1H, s, Ar–H). ^{13}C-NMR (2% solution in D_2O) δ:[9] 46.1 (C-9); 57.1, 61.0 (C-1, C-3); 65.8 (C-4); 115.6, 118.9 (C-4a, C-8a); 122.6, 126.6 (C-5, C-8); 146.9, 147.8 (C-6, C-7). UV $\lambda_{max}^{0.1N HCl}$ nm (ε): 282.5 (2650).

2: DL-Epinephrine (0.5 g) dissolved in 2 ml of 6 N HCl was added to 100 ml of Tris-HCl buffer, pH 7.0, containing 10 mM ascorbic acid with 1.0 ml of 35% formaldehyde. The solution was adjusted to pH 7.0, and stirred at room temperature for 1 h, then 25 g of NaCl was added and the whole was applied to an Amberlite XAD-4 column as described above to separate **2** from **1**. The fractions containing only **2** were desalted through a boric acid gel column and evaporated to dryness under reduced pressure at 40°C. The residue was crystallized from 1 N HCl and acetone as needle crystals (51 mg, 8.1%). mp 200—201°C (darkening from 180°C). *Anal.* Calcd for $C_{10}H_{13}NO_3$: C, 51.84; H, 6.09; N, 6.05. Found: C, 51.74; H, 5.98; N, 5.95. ^{1}H-NMR (2% solution in D_2O) δ: 3.15 (3H, s, N-CH_3), 3.40—3.75 (2H, m, –CH_2–), 3.97—4.75 (2H, m, –CH_2–), 5.02 (1H, m, –C$\underline{H}$OH–), 6.96 (2H, s, Ar–H). ^{13}C-NMR (2% solution in D_2O) δ: 46.3 (C-9); 53.6, 60.5 (C-1, C-3); 65.9 (C-4); 118.7 (C-4a, C-8a); 124.4, 126.9 (C-5, C-6); 142.9, 147.0 (C-7, C-8). UV $\lambda_{max}^{0.1N HCl}$ nm (ε): 279 (2460).

Results

The binding equilibrium of ^{3}H-epinephrine with the antiserum[5] took more than 10 h, but when ^{3}H-epinephrine was preincubated for 14 h at 37°C in the assay buffer without the antiserum, equilibrium was attained rapidly, as shown in Fig. 1. Like ^{3}H-epinephrine, the binding-

NII-Electronic Library Service

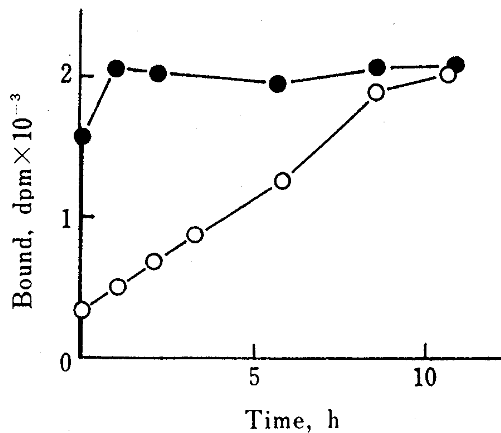

Fig. 1. Time Course of the Reaction of ^{3}H-Epinephrine with the Antiserum

—○—: ^{3}H-epinephrine was used.
—●—: ^{3}H-epinephrine (1×10^{-8} M) preincubated in the assay buffer at 37°C for 14 h was used.
300-fold diluted antiserum was used.

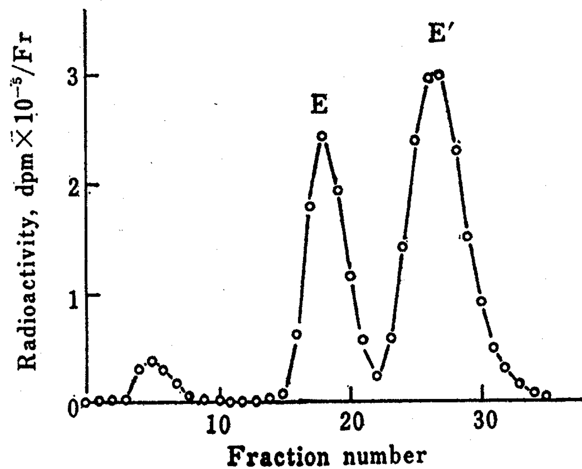

Fig. 2. Chromatogram of the Degradation product of ^{3}H-Epinephrine on Amberlite CG-50

The catecholamine fraction obtained from 25 ml of the preincubation solution in Fig. 1 was used as a sample.

active compound was adsorbed on the boric acid gel column. When ^{3}H-epinephrine thus incubated was chromatographed on a cation exchange column, three radioactive peaks were found, as shown in Fig. 2. The first peak seemed to consist of oxidation products of ^{3}H-epinephrine, adrenochrome and so on. The second peak was ^{3}H-epinephrine (E) itself. The third peak had binding activity and was named E′. The ^{3}H-E′ did not appear when 10 mM cysteine or 10 mM metabisulfite was used as the antioxidant instead of 10 mM ascorbic acid, or when the assay buffer was first bubbled through with nitrogen gas and incubated in an enclosed flask with a ground glass stopper. On the other hand, bubbling of oxygen gas through the buffer enhanced the formation of ^{3}H-E′. These results suggested that the degradation required ascorbic acid and oxygen. When the peak of ^{3}H-E′ collected was separated by HPLC, two peaks were obtained as shown in Fig. 3. These peaks were named E-1 and E-2. E-1 and E-2 were also separated on an Amberlite XAD-4 column with a salt concentration gradient as shown in Fig. 4.

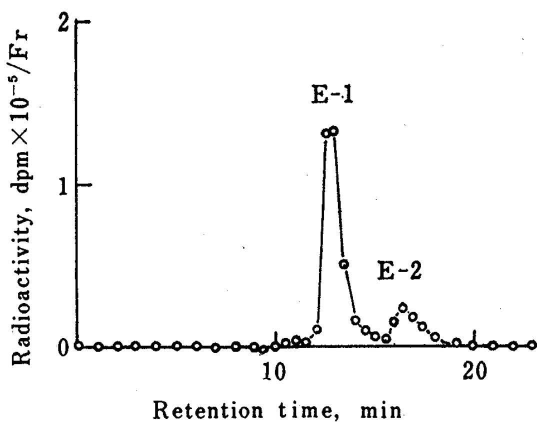

Fig. 3. Chromatogram of ^{3}H-E′ on Hitachi gel 3011-C(HPLC)

The fractions (No. 23—30) in Fig. 2 were collected and used as a sample after being desalted on boric acid gel.

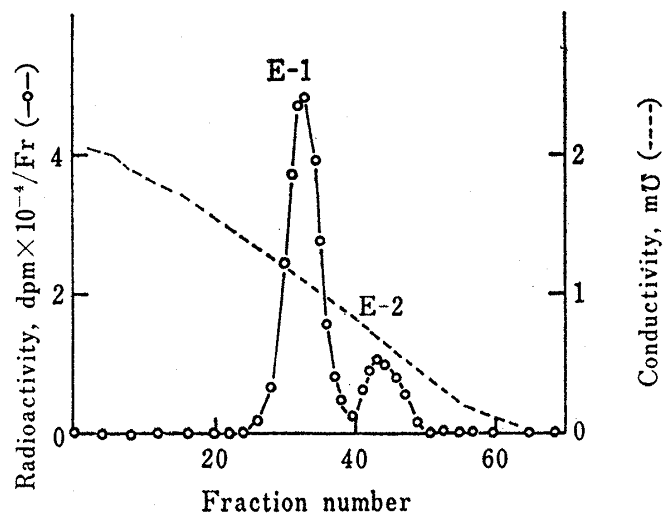

Fig. 4. Chromatogram of ^{3}H-E′ on Amberlite XAD-4

The same sample as in Fig. 3 was used.

NII-Electronic Library Service

For the preparation of E′ on a large scale, the reaction was carried out as follows: a solution of L-epinephrine bitartrate (2×10^{-4} M) and L-ascorbic acid (1×10^{-2} M) in 1 liter of 0.1 M phosphate buffer, pH 7.0, was incubated at 60°C for 3 h under 95% O_2–5% CO_2 gas bubbling.The higher the concentration of L-epinephrine, the lower the yield of E′. A concentration of 2×10^{-4} M was chosen, where the yield was several percent. A high concentration of ascorbic acid slightly increased the yield, but inhibited the adsorption of E′ on the boric acid gel used, for desalting. Thus, 1×10^{-2} M ascorbic acid was used. Higher temperature increased not only the production but also the other degradation. Thus, 60°C for 3 h was adopted.

The incubated mixture was cooled to room temperature and passed through a Dowex 1×8 column to remove polymerized material derived from ascorbic acid. After being desalted through a boric acid gel column, the effluent was separated on Amberlite CG-50 (1.6×30 cm). E′ fractions were pooled and separated on the Amberlite XAD-4 column (1.6×25 cm) into E-1 and E-2. Each fraction was pooled and desalted. White crystals were obtained from both pools. From 12 liters starting volume, 3 mg of crude E-1 and 1 mg of crude E-2 were obtained.

E-1 and E-2 both gave a positive color reaction with Doty's reagent and were adsorbed on a boric acid gel column, demonstrating that their catechol moieties were intact. From the ^{1}H-NMR data in D_2O, the presence of two pairs of geminal protons, protons of the N-methyl group and two protons in the benzene ring was suggested. As possible structures, we considered **1** and **2** in Chart 1. Therefore, **1** and **2** were synthesized from epinephrine and formaldehyde for the first time and analyzed as described in "Experimental." A comparison of the characteristics of E-1, E-2 and **1**, **2** is shown in Table I. ^{1}H-NMR spectra of **1** and **2** were in good accord with those of E-1 and E-2, respectively. E-1 was identical with **1**, and E-2 was identical with 2.

We next examined which component of the epinephrine solution was involved in the reaction. Since the production of E-1 and E-2 required the presence of ascorbic acid and air,

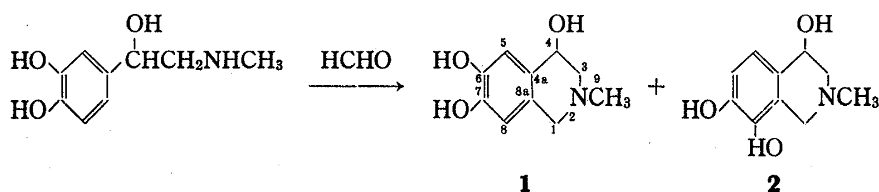

Chart 1. Reaction of Epinephrine with Formaldehyde

TABLE I. Identification of Degradation Products of Epinephrine with Tetrahydroisoquinolines

Product	^{3}H-E-1[a]	E-1	**1**	^{3}H-E-2[a]	E-2	**2**
Chromatography						
Hitachi gel 3011-C(HPLC) t_R,[b] min	12.8	12.8	12.8	16.5	16.5	16.5
Silica gel (TLC), *Rf*						
BuOH: AcOH: H_2O=3: 1: 1	0.38	0.37	0.37	0.44	0.44	0.44
Electrophoresis[c]						
Cellulose acetate, Rm[d]						
pH 8.6	0.2	0.2	0.2	0.2	0.2	0.2
pH 5.0		1.0	1.0		1.0	1.0
UV spectrum λ_{max} nm		282.5	282.5		279.0	279.0
Color with						
Doty's reagent		Purplish red			Deep purple	
Gibbs reagent		Blue			Blue	

a) ^{3}H-E-1 and ^{3}H-E-2 in Fig. 3 were collected, concentrated and used.
b) Retention time.
c) Electrophoresis was carried out for 10 min at 1 mA/cm. The electrolytes were 0.07 M veronal-sodium veronal buffer, pH 8.6, and 0.1 M acetate-sodium acetate buffer, pH 5.0.
d) Relative mobility with respect to epinephrine.

NII-Electronic Library Service

TABLE II. Formation of Formaldehyde in Ascorbic Acid Solution incubated at 37°C for 14 Hours

Sample	Air[a]	HCHO Found, μM
Water	+	ND[d]
	−	ND
Buffer I[b]	+	ND
	−	ND
Buffer II[c]	+	0.5
	−	ND
+10 mM sodium metabisulfite	+	ND
+0.1 mM $FeCl_3$	+	1.4
	−	0.3
+0.1 mM $CuCl_2$	+	1.5
	−	0.2

a) − : The sample was enclosed in a 200 ml flask with a ground glass stopper during the incubation.
+ : The sample was not enclosed.
b) 10 mM phosphate buffer, pH 7.0.
c) 10 mM phosphate buffer containing 10 mM ascorbic acid, pH 7.0.
d) ND: not detected, <0.1 μM.

we checked the formation of formaldehyde in ascorbic acid solution in contact with air.

As colorimetric or fluorometric determination of formaldehyde was interfered with by excessive ascorbic acid, form aldehyde was determined as 2,4-dinitrophenylhydrazone by HPLC. The method was not affected by 10 mM ascorbic acid even after incubation for 14 h at 37°C. As shown in Table II, formaldehyde was found in the incubated solution. Its formation depended on air, and was enhanced by cupric and ferric ions. The control experiment ruled out the possibility of contamination by formaldehyde before the incubation. These results suggested that this formaldehyde formation might be involved in metal-catalyzed oxidation of ascorbic acid. As the amount of formaldehyde detected was not affected by change of the lot or form (free acid or sodium salt) of ascorbic acid, possible impurities in the reagent can be neglected.

Discussion

Our results indicate that TIQs are formed from epinephrine in the presence of ascorbic acid in neutral solution (Table I). The formation of TIQs requires ascorbic acid and oxygen (air), and under the same conditions, formaldehyde is detected in ascorbic acid solution (Table II). Furthermore, formaldehyde readily reacts with epinephrine to give TIQs (Chart 1). Therefore, the formations of TIQs can be explained in terms of the reaction of epinephrine with formaldehyde which is formed through the oxidation of ascorbic acid by oxygen from air to yield TIQs.

Though the present study is concerned with epinephrine, the conversion of other catecholamines, norepinephrine and dopamine, would probably occur under conditions where formaldehyde is formed. Actually, when ^{3}H-norepinephrine was incubated under the same conditions Fig. 2, the degradation product was detected by Amberlite CG-50 column chromatography.

In a further study of the binding assay, ^{3}H-E-1 had much higher binding activity to the antiserum in Fig. 1 than ^{3}H-epinephrine or ^{3}H-E-2, and **1** showed 10-fold higher activity than epinephrine in inhibition of the reaction of ^{3}H-E-1 with the antiserum. These observations show that the antiserum used in this study contained antibodies against TIQ, which may have been formed from epinephrine in the antigen by which the antiserum was raised during the preparation of the antigen or in the circulation in the immunized rabbit body.

NII-Electronic Library Service

Ascorbic acid has been used in many catecholamine studuies, such as enzyme study,[10] binding assay,[11] determination,[12] and so on. If conversion under mild conditions occurred in such cases, it would have influenced the experimental data, besides the direct effect of formaldehyde formed. Even when ascorbic acid is not used, the possibility remains that endogeneous ascorbic acid would influence *in vitro* experiments with serum or organs through these reactions. Furthermore, the conversion might occur in the body, because the reaction proceeds at physiological pH and temperature.

Though the reaction of epinephrine with formaldehyde is known, the products have not previously been identified definitly[13] and the characteristics of the reaction are not well known. Thus, in a preliminary experiment, the pH dependency of the ratio of the amounts of produced TIQ isomers, **1** and **2**, was examined. The ratio (**2**/**1**) in the reaction using ^{3}H-epinephrine was obtained by measuring the radioactivities after separation by HPLC. When the reaction was carried out in acetate buffer (pH 3.5—5.5) or phosphate buffer (pH 5.5—8.0) below pH 6.0, the ratio was constant and about 1/7, while above pH 6.0, the ratio increased with increase of pH. At pH 7.0, the ratio was 1/6, comparable to that obtained from Fig. 3. On the other hand, when the reaction was carried out in Tris buffer (pH 7.0—9.0) or glycine buffer (pH 8.5—10.5), the ratio increased with decrease of pH, and at pH 7.0, it was 3. The reason for the change of the ratio in different buffer solutions is not clear. In alcoholic solution, the ratio was almost the same as in the acetate buffer.

Our preparation method for **2** described in "Experimental" should be applicable to other TIQ isomers, such as the condensation product of epinephrine or norepinephrine with acetaldehyde, whose formation *in vivo* has been reported, though its structure has not been confirmed by synthesis.[14]

Acknowledgement This work was supported by Grants-in-Aid for Scientific Research (Nos. 487145 and 557505) from the Ministry of Education, Science and Culture, Japan. We would like to thank Mr. Y. Arakawa of this faculty for the gift of fround and sieved Amberlite XAD-4. A fellowship from Iatrochemical Foundation to A. Shirahata during his doctoral course is gratefully acknowledged.

References and Notes

1) Present address: *Tokyo Biochemical Research Institute, 41-8, Takada 3, Toshima-ku, Tokyo.*
2) T. Nagatsu, "Biochemistry of Catecholamines," Univ. of Tokyo Press, Tokyo, 1973, Chapter IV.
3) A. Miwa, M. Yoshioka, and Z. Tamura, *Chem. Pharm. Bull.*, **26**, 3347 (1978).
4) S. Higa, T. Suzuki, A. Hayashi, I. Tsuge, and Y. Yamamura, *Anal. Biochem.*, **77**, 18 (1977).
5) A. Miwa, A. Shirahata, M. Yoshioka, and Z. Tamura, *Chem. Pharm. Bull.*, **25**, 1904 (1977).
6) N. Kirshner and McC. Goodall, *J. Biol. Chem.*, **226**, 207 (1957).
7) T. Hino and S. Kaito, *Bunseki Kagaku* (*Tokyo*), **26**, 451 (1977).
8) C.T. Mansfield, B.T. Hodge, R.B. Hege, Jr., and W.C. Hamlin, *J. Chromatogr. Sci.*, **15**, 301 (1977).
9) Chemical shifts are represented in ppm from methyl protons (^{1}H NMR) or methyl carbons (^{13}C-NMR) of trimethylsilylpropanesulfonate sodium salt as the internal standard ($\delta=0$). The assignments of the signals of the carbons were achieved by the off-resonance technique. The carbons of the structure of **1** were numbered as in Chart 1.
10) C.R. Creveling, J.W. Daly, B. Witkop, and S. Udenfriend, *Biochem. Biophys. Acta*, **64**, 125 (1962).
11) R.J. Lefkowitz and L.T. Williams, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 515 (1977).
12) K. Engelman and B. Portony, *Circ. Res.*, **26**, 53 (1970).
13) G. Cohen and M. Collins, *Science*, **167**, 1749 (1970).
14) G. Cohen, *Biochem. Pharmacol.*, **25**, 1123 (1976).

NII-Electronic Library Service