an acid hydrolysate: Arg 0.97, Pro 2.06, Lys 0.96, Asp 1.08, Glu 1.16, Phe 2.21, Gly 1.02, Leu 1.00, Met 0.95 (average recovery 80%). *Anal.* Calcd. for $C_{62}H_{96}N_{18}O_{13}S \cdot 3CH_3COOH \cdot 9H_2O$: C, 48.73; H, 7.58; N, 15.05. Found: C, 48.62; H, 7.05; N, 15.09.

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Preparation of Specific Antibodies to Catecholamines and L-3,4-Dihydroxyphenylalanine. II.[1] The Site of Attachment on Catechol Moiety in the Conjugates

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In order to estimate the site of attachment on hapten in the conjugates of catecholamines to protein prepared by the Mannich reaction, a model compound was prepared from 4-methylcatechol and ethylamine. The structure of the compound was confirmed as 5-(ethylamino)methyl-4-methylcatechol from the infrared, ^{1}H- and ^{13}C-nuclear magnetic resonance spectra.

Keywords——catechol; Mannich reaction; catecholamine; formaldehyde; C-13-NMR;

In the previous papers[1,3] we reported a method for preparation of the antigens of catecholamines and L-3,4-dihydroxyphenylalanine, in which the haptens were conjugated to bovine serum albumin by the Mannich reaction with formaldehyde perhaps through ε-amino groups of lysine. The site of attachment on the hapten molecule in a conjugate is closely related to the specificity of the antibody to the hapten. Burckhalter and Leib suggested that *ortho*-substitution was predominant in aminomethylation by the Mannich reaction on phenols.[4] On catechols, *ortho*-substitution was also proposed, but no strict demonstration on the structures of the products was performed.[5] In order to estimate the site of attachment on catecholamines in the conjugates, a model compound (HC–EA) was prepared from 4-methylcatechol and ethylamine, and investigated for its structure.

Experimental

Materials——4-Methylcatechol, ethylamine hydrochloride, ethylamine hydrobromide, N-methylbenzylamine and Silica gel plates (2.5 × 10 cm) were obtained from Tokyo Chemical Industries Co., Ltd. N-Methylbenzylamine was converted to its hydrochloride crystal. Formaldehyde was obtained from Wako Pure

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Chemical Industries Ltd., Tokyo. Other general reagents were guaranteed of Kanto Kagaku Co., Ltd., Tokyo.

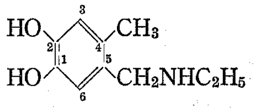
^{13}C-Nuclear Magnetic Resonance (^{13}C-NMR) Spectrometry——A sample was dissolved in D_2O (200 mg/ml). A spectrum was measured with a pulsed Fourier transform spectrometer JNM-FX 60. The condition of measurements are following: temperature, 45°; pulse width, 4 μsec; external standard, tetramethylsilane (TMS).

Synthesis of Model Compound——In a test tube, 2.48 g (0.02 mol) of 4-methylcatechol and 1.63 g (0.02 mol) of ethylamine hydrochloride were dissolved in 5 ml of water. The solution was added with 3.3 ml of 37% formaldehyde (0.04 mol) and added with water to make 10 ml. After substitution of air with nitrogen the tube was closed with a ground stopper. The reaction mixture was allowed to stand overnight at room temperature. The completion of the reaction was confirmed by thin-layer chromatography described in the previous paper.[1] In the present case the *Rf* values on the silica gel plate developed by *n*-butanol/acetic acid/water (3: 1: 1) were 0.8 and 0.45 for 4-methylcatechol and the single reaction product respectively.

After the reaction, the solution was condensed under reduced pressure, the residue was dissolved in a small volume of methanol and precipitated by adding a large volume of ethyl ether. The precipitate was collected by centrifugation and the precipitation was repeated in the same manner. A white crystalline powder of the hydrochloride of the model compound (HC-EA·HCl) was obtained. The yield was 3.18 g (73%). The hydrobromide of the model compound (HC-EA·HBr) was prepared by the same procedure using 2.52 g (0.02 mol) of ethylamine hydrobromide. The recrystallization of HC-EA·HBr was performed by adding ethylether to the methanol solution and standing at 4° overnight, and a slightly yellowish prism was obtained. HC-EA·HCl: mp 204—205° (uncorr.) *Anal.* Calcd. for $C_{10}H_{16}ClNO_2$: C, 55.17; H, 7.41; N, 6.43. Found: C, 54.95; H, 7.43; N, 6.48. ^1H-NMR (10% solution in D_2O) δ: 1.22 (3H, triplet) 2.10 (3H, singlet) 3.02 (2H, quartet) 3.93 (2H, singlet) 6.62 (1H, singlet) 6.72 (1H, singlet). HC-EA·HBr: mp 197—199° (uncorr.) *Anal.* Calcd. for $C_{10}H_{16}BrNO_2$: C, 45.82; H, 6.15; N, 5.34. Found: C, 45.72; H, 6.31; N, 5.28. ^1H-NMR spectrum was the same as that of HC-EA·HCl.

Results and Discussion

The IR spectra (Fig. 1) of 4-methylcatechol and HC–EA·HBr showed 1,2,4 and 1,2,4,5 substitution respectively.[6] The result suggested that the site of attachment of ethylamine to the 4-methylcatechol was the 5 position of the catechol as in the proposed structure [5-(ethylamino)methyl-4-methylcatechol]:



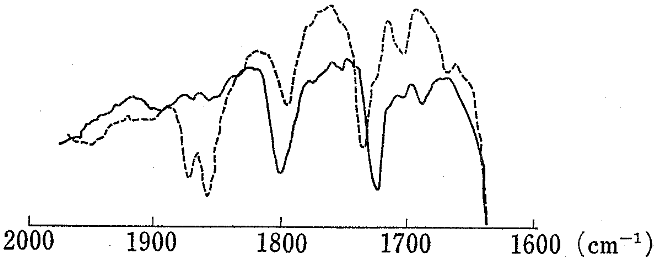


Fig. 1. IR Spectra of the Model Compound and 4-Methylcatechol

A KBr tablet containing 2—3 fold amount of the test compound usually used was prepared.
——, model compound; ------, 4-methylcatechol.

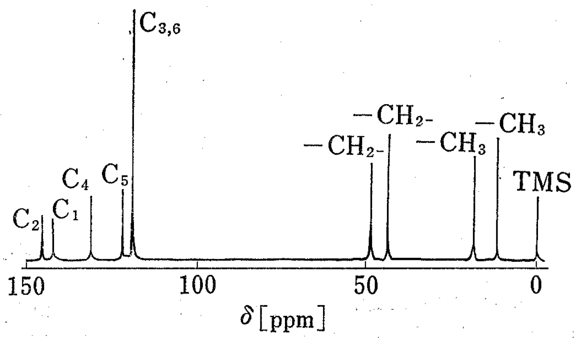


Fig. 2. ^{13}C–NMR Spectrum of the Model Compound

The structure was further confirmed by the ^{13}C–NMR spectrum (Fig. 2), which was assigned on the basis of the law of additivity[7] and the signal intensity as follows: C_2, found, 145.9 (calculated, 145.2); C_1, 143.0 (142.3); C_4, 131.6 (133.5); C_5, 122.3 (126.6); $C_{3,6}$, 119.2 (120.4, 119.2). In the calculation the effect of ethylaminomethyl group on the chemical

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shift was estimated by ^{13}C–NMR spectrum of N-methylbenzylamine hydrochloride as follows: C_{1}' +3.3; C_{o}' +2.0; C_{m}' +2.5; C_{p}' +2.4. The signals of C_o and C_m were not certainly assigned, but the small difference between the two shifts did not give a large influence on the calculated values. The ^{1}H–NMR spectrum of the model compound showed the singlets at 6.62 and 6.72 ppm, supporting the proposed structure. Thus the site of attachment on catechol will be different from that suggested on phenols in their conjugates.

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Synthesis of Xanthines by Dehydrogenative Cyclization of 6-Amino-5-benzylideneaminouracils with Diethyl Azodicarboxylate

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The treatment of 6-amino-5-benzylideneaminouracil derivatives with diethyl azodicarboxylate gave the corresponding xanthines in high yields.

Keywords——xanthine derivatives; 6-amino-5-benzylideneaminouracils; diethyl azodicarboxylate; dehydrogenative cyclization; oxidation

It is well known that diethyl azodicarboxylate (DAD) is a strong hydrogen acceptor.[2] For instance, DAD is capable of dehydrogenating alcohols,[2] mercaptans,[2,3] hydrazobenzene,[2] hydroxylamines,[4,5] phenols,[6] secondary formamides,[7] and tertiary amines[8,9] and converting them into carbonyl compounds, disulfides, azobenzenes, nitroso compounds, biphenyls, isocyanates, and enamines. In this process DAD undergoes hydrogenation to diethyl hydrazodicarboxylate. DAD is also an effective reagent for the oxidation of several sulfur-containing amino acids[10] to sulfoxides, and for the dehydrogenation of imines of amino acid esters[11] to imines of the corresponding dehydroamino acid esters.

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