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Studies on Drug Metabolism by Use of Isotopes. XXI.[1] Synthesis of 3-Phenylpropyl Carbamate labeled with Deuterium and Carbon-13, and Physicochemical Properties of These Labeled Drugs[2]

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For the investigation of metabolism of 3-phenylpropyl carbamate in man, drugs labeled with deuterium and carbon-13 were synthesized. Physicochemical properties of labeled compounds, especially mass spectrometric behavior, were examined. The deuterium-hydrogen exchange reaction was observed in 3-phenylpropyl[arom.-d_5] carbamate but in the case of carbon-13 labeled compounds, there was no isotope effect occurring in mass chamber. Calibration curve for dilution analysis was examined by use of methyl benzoate single-labeled with carbon-13 and linear correlation was obtained between 1 and 300 fold dilutions.

Keywords——stable isotope; deuterium; carbon-13; ion cluster; isotope effect; 3-phenylpropyl carbamate

Gas chromatography–mass spectrometry (GC–MS) has been widely used in the study on drug metabolism due to its inherent high specificity and sensitivity. The stable isotope has also been extensively used in recent years because it is not necessary to consider the radiation hazard and it is possible to distinguish an exogenous compound from an endogenous one from mass difference and quantify the amount of metabolite by application of reverse dilution analysis. Deuterated compounds are generally used but there are several problems in using deuterated compounds as a tracer, such as an isotope effect in metabolic reaction[4] and gas chromatographic retention time,[5] and deuterium-hydrogen exchange reaction in fragmentation process in the ionization chamber.[6] On the other hand, in the case of carbon-13 label, there may not be a fear of isotope effect as observed in deuterium label. For this reason, carbon-13 label will be more useful than deuterium label when a tracer technique is applied to drug metabolic studies, in spite of difficulty of multi-labeling. The present paper deals with the synthesis of 3-phenylpropyl carbamate (I) labeled with deuterium and carbon-13 at first, and some of the physicochemical characteristics of these drugs which are important in

1) Part XX: S. Baba and S. Morishita, *J. Pharm. Sci.*, in press.
2) A part of this work was presented at the 94th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, April, 1974.
3) Location: *1432-1, Horinouchi, Hachioji, Tokyo, 192-03, Japan.*
4) M. Tanabe, F. Yasuda, S. Levalley, and C. Mitoma, *Life Sci.*, **8**, 1123 (1969).
5) C.C. Sweeley, W.H. Elliot, I. Fries, and R. Ryhage, *Anal. Chem.*, **38**, 1549 (1969).
6) S. Baba, K. Kawai, and Y. Shida, *Yakugaku Zasshi*, **94**, 826 (1974).

mass spectrometric measurements, and some problems which should be taken into consideration in qualitation and quantitation of metabolites of drugs labeled with stable isotope are discussed.

In preparation of drugs labeled with stable isotope which is usable for tracer experiment, it is necessary to be careful about a biological isotope effect, elimination of the label, and so on. Our previous study[7] in deuterated ephedrine metabolism indicated that a drug labeled with deuterium in the benzene ring can be used as a tracer for metabolic studies. Therefore, benzene ring was chosen as labeled position in deuterated I (I-d_5). 3-Phenylpropyl carbamate-[3-^{13}C] (I-^{13}C) was labeled in specific position of benzylic carbon which would not eliminate during metabolic reaction.

By introducing stable isotopes in the molecule, several characteristic changes appeared in the infrared (IR) spectrum. The band ($\nu C_{arom.}$-H) at 3030—3090 cm^{-1} observed in protio species disappeared completely and shifted to 2270 cm^{-1} region in benzyl[arom.-d_5] alcohol (III-d_5), benzyl[arom.-d_5] chloride (IV-d_5), and I-d_5. The bands (δ $C_{arom.}$-H) at 770—730 and 710—690 cm^{-1} also disappeared completely in deuterated species. The band at 1600 cm^{-1}, which was assigned to –C=C– streching vibration of benzene ring in benzoic acid (II), shifted to 1550 cm^{-1} region in II-d_5. The absorption at 1690 cm^{-1} in II and 1710 cm^{-1} in phenylacetic acid (V) due to –C=O streching vibration shifted to a lower region by about 40 cm^{-1} in both II[α-^{13}C] (II-^{13}C) and V[1,2-$^{13}C_2$] (V-$^{13}C_2$), but no shift was observed in V[1-$^{13}C_1$] (V-$^{13}C_1$).

As the signals due to protons on benzene ring disappeared completely in nuclear magnetic resonance (NMR) spectra of deuterated species, it was clarified that deuterium was not eliminated during synthetic steps. Owing to ^{13}C–H coupling, satellite peaks having a coupling constant of about 144 Hz and 153 Hz, respectively, were observed in NMR spectra of III-^{13}C and IV-^{13}C. In I-^{13}C, triplet signal due to protons on the C-3 carbon was observed as a satellite peak having a coupling constant of about 132 Hz (Fig. 1).

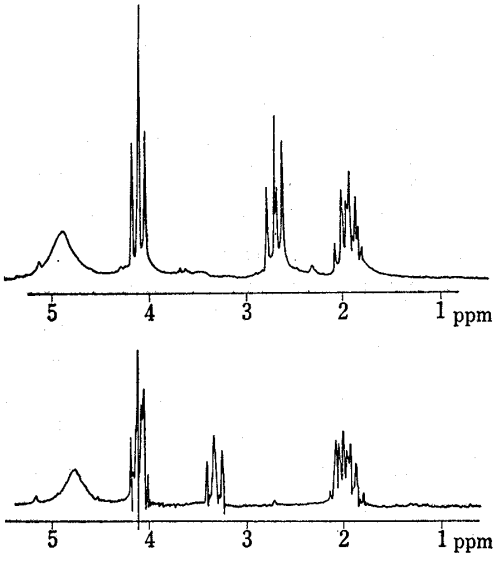

Fig. 1. Partial NMR Spectra of 3-Phenylpropyl Carbamate (Upper) and 3-Phenylpropyl[3-^{13}C] Carbamate (Lower)

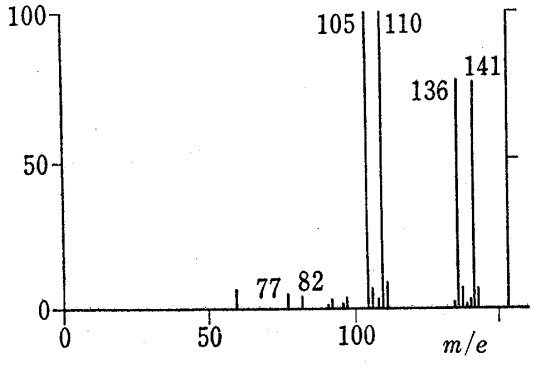

Fig. 2. Mass Spectrum of an Equimolar Mixture of Methyl Benzoate and Methyl-Benzoate[arom.-d_5]

Coupled with stable isotope tracer technique, mass spectrometric analysis is used almost without exception in drug metabolic studies. For this reason, it is necessary to examine the mass spectrometric behavior of labeled compounds before metabolic studies. After methylation of II and V to methyl benzoate (VIII) and methyl phenylacetate with diazomethane,

7) K. Kawai and S. Baba, *Chem. Pharm. Bull.* (Tokyo), **24**, 2728 (1976).

the mass spectra of these esters were measured. Mass spectrum of an equimolar mixture of VIII and VIII-d_5 is shown in Fig. 2. The ion cluster peaks corresponding to the molecular ion (*m/e* 136 and 141) and benzoyl ion (*m/e* 105 and 110) appeared typical ion clusters having an equal peak intensity. We reported previously[6] that an intramolecular exchange reaction between deuterium on the benzene ring and hydrogen on the side chain was recognized slightly in the phenyl ion and remarkably in the benzoyl ion from I-d_5. However, no exchange reaction between deuterium and hydrogen was observed in VIII-d_5. This means that fragmentation pathway that produces a benzoyl ion from I is not the same as that from VIII. It may be concluded that the carboxyl hydrogen was equilibrating with the two *ortho* deuterium atoms and then fragmentation to benzoyl ion was accompanied with exchange reaction in I-d_5,[8] but benzoyl ion was produced by α- fission which released an methoxyl group ($-OCH_3$) in VIII-d_5. The mass spectrum of an equimolar mixture of III and III-d_5 is shown in Fig. 3. The ion cluster corresponding to the molecular ion (*m/e* 108 and 113) appeared as a typical ion cluster having an equal intensity, but the peak intensity of each corresponding M-1 ion differed markedly between III and III-d_5. The ratios of *m/e* 107 to 108 and *m/e* 106 to 108 in the mass spectrum of III were 0.35 and 0.03, respectively. In contrast, the ratios of *m/e* 112 to 113 and *m/e* 111 to 113 in III-d_5 were 0.20 and 0.20, respectively. These phenomena agreed with the observation of Fisher[9] that hydrogens on all carbon atoms are subtracted without distinction when hydrogen is eliminated from the molecular ion. In benzenium ion (*m/e* 79

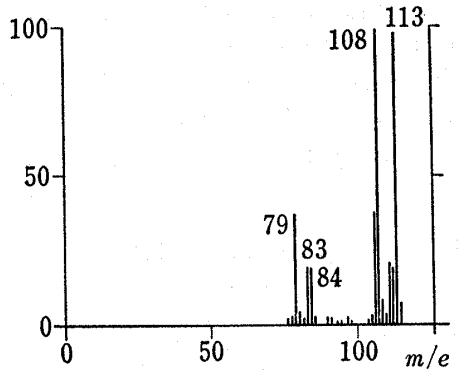

Fig. 3. Mass Spectrum of an Equimolar Mixture of Benzyl Alcohol and Benzyl[arom.-d_5] Alcohol

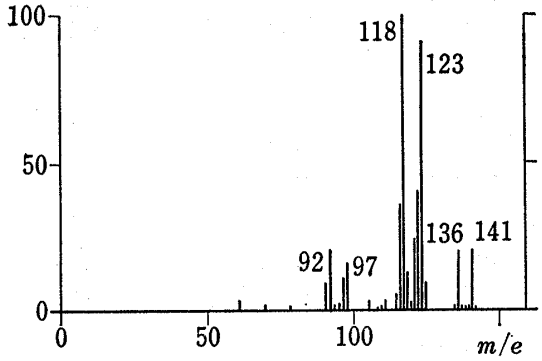

Fig. 4. Mass Spectrum of an Equimolar Mixture of 3-Phenylpropyl Carbamate and 3-Phenylpropyl [arom-d_5] Carbamate

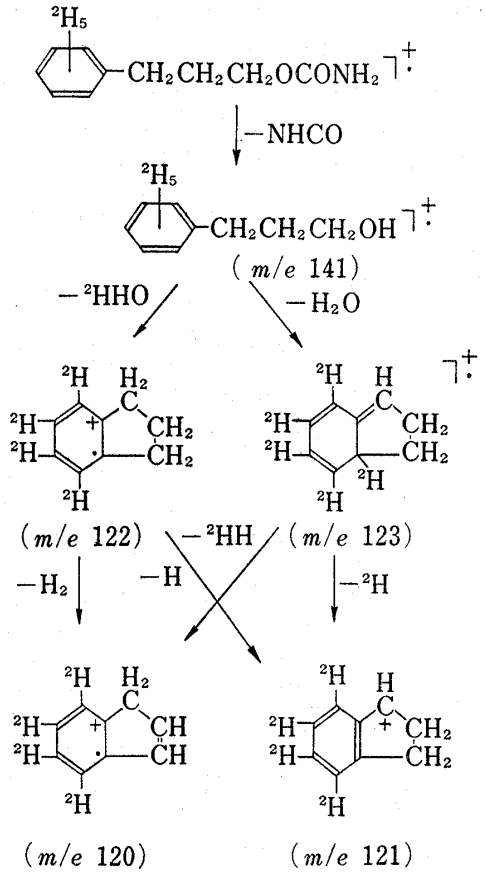

Chart 1. Partial Fragmentation Pathway of 3-Phenylpropyl [arom.-d_5] Carbamate

8) J.H. Beynon, B.E. Job, and A.E. Williams, *Z. Naturforsh.*, **20**, 883 (1965).
9) M. Fischer and C. Djerassi, *Chem. Ber.*, **99**, 750 (1966).

and 84) which resulted from loss of a neutral CO molecule from M–H or M–^{2}H, the ratio of each ion intensity in the cluster (*m/e* 79 and 84) changed drastically by about 0.5, but the sum of ion intensity at *m/e* 83 and *m/e* 84 was nearly equal to the intensity of ion at *m/e* 79. Such ion clusters not keeping the original mixture ratio is defined as an "abnormal ion cluster".

The mass spectrum of an equimolar mixture of I and I-d_5 and partial fragmentation pathway of I-d_5 are shown in Fig. 4 and Chart 1, respectively. As shown in spectrum, I did not give rise to the detectable molecular ion (*m/e* 179) and the highest mass ion appeared at *m/e* 136, which may be due to the elimination of NHCO group with the rearrangement of proton of the amide group from the molecular ion. The ion cluster (*m/e* 136 and *m/e* 141) corresponding to this ion appeared as a typical ion cluster of 1 : 1 intensity, but the other ion peaks at *m/e* 118 and 123 and *m/e* 92 and 97 were determined as abnormal ion clusters. Then, each peak intensity was compared briefly. The ratios of *m/e* 123 to 118 was lower than that of *m/e* 141 to 136, while the ratios of *m/e* 122 to 117 and *m/e* 121 to 116 were higher than that of *m/e* 141 to 136. This fact indicated that the elimination of water from the ion at *m/e* 136 proceeded through two different pathways. In one, the hydroxyl group substracts hydrogen from 3-position and in the other, from the *ortho* position of benzene ring. The same phenomenon was observed in the case of 3-phenylpropanol fragmentation.[10] These studies on the use of multiple-deuterium labeled drugs mentioned above led to an important conclusions as follows. When the ion cluster technique is applied to identify the metabolites by using 1 : 1 mixture of deuterated and non-labeled drug, care must be taken for the ion clusters appearing in the mass spectrum. Sometimes they appear not as typical 1 : 1 clusters but as abnormal clusters, when the elimination of deuterium and/or deuterium-hydrogen exchange reaction occurs in mass spectrometer. It is necessary to give careful attention to the peaks which appear in minus one and two of each fragment ion, and careful investigation of the mass spectrum to determine the ion usable for quantitative analysis is needed before quantitative analysis by the dilution technique.

Table I. Comparison of Ion Intensities of Non-labeled Compound with Labeled Compound

Methyl benzoate					
	$(M-COOCH_3)$	$(M-CO_2)$	$(M-OCH_3)$	(M)	
^{12}C	8.1±1.1[a]	1.9±0.0	53.3±0.9	35.8±1.2	
^{13}C	8.2±0.3	1.9±0.1	53.5±1.1	36.4±0.9	
3-Phenylpropyl carbamate					
	$(M-CH_2CH_2-OCONH_2-CH_2CH_2-H)$	$(M-CH_2CH_2-OCONH_2)$	$(M-CH_2-OCONH_2)$	$(M-NHCO-H_2O)$	(M–NHCO)
^{12}C	1.4±0.1	15.8±0.4	2.9±0.1	72.4±0.6	7.5±0.2
^{13}C	1.6±0.1	15.8±0.7	3.0±0.2	71.5±1.5	8.1±0.7
Methyl phenylacetate					
	$(M-COOCH_3-C_2H_2)$	$(M-COOCH_3)$	$(M-CO_2)$	$(M-OCH_3)$	(M)
^{12}C	3.0±0.2	49.2±1.1	1.1±0.0	2.5±0.2	44.2±1.2
$^{13}C_1$	3.2±0.3	49.2±0.3	1.4±0.2	2.5±0.2	43.7±0.8
$^{13}C_2$	3.0±0.2	50.8±1.4	1.4±0.1	2.5±0.1	42.1±1.4
1-Hydroxyethylbenzene					
	$(M-CH_2OH-C_2H_2)$	$(M-CHCH_2OH)$	$(M-CH_2OH)$	(M–OH)	(M)
^{12}C	0.6±0.2	1.5±0.1	67.6±0.5	1.8±0.1	28.6±0.8
$^{13}C_2$	0.6±0.1	1.5±0.1	67.5±1.0	1.6±0.1	28.9±0.7
1-Chloroethylbenzene					
	$(M-CH_2Cl-C_2H_2)$	$(M-CHCH_2Cl)$	$(M-CH_2Cl)$	(M–Cl)	(M)
^{12}C	0.3±0.1	1.2±0.1	63.1±1.1	3.5±0.2	31.8±0.9
$^{13}C_2$	0.2±0.0	1.0±0.1	63.1±0.5	3.7±0.2	32.0±0.9

a) Percentage of each ion group per total ions.
Three times measurement (mean ±S.D.) (20 eV).

10) N.M.M. Nibbering and Th. J. de Boer, *Tetrahedron*, **24**, 1415 (1968).

Then, the mass spectrometric behavior of compounds labeled with carbon-13 were examined. A mass spectrum of an equimolar mixture of non-labeled and single-labeled compound will give difficulty in analysis because mass peaks corresponding to the labeled compounds will overlap those of non-labeled compounds. In order to exclude this complexity, mass spectra of labeled and non-labeled compounds were measured individually, and the ratios of peak intensities of each ion group against total ion intensities were calculated to examine whether carbon-13 label induced any isotope effect on the process of mass fragmentation or not. If the isotope effect occurs, which is due to the different nature of bonds, such as ^{12}C–^{12}C, ^{12}C–^{13}C, and ^{13}C–^{13}C, these ratios of labeled compounds must be different from those of non-labeled compounds. As shown in Table I, the peak intensity ratios observed in each compound showed no significant difference. This fact indicated that there was no isotope effect occurring in the ionization chamber in the case of carbon-13 labeled compounds.

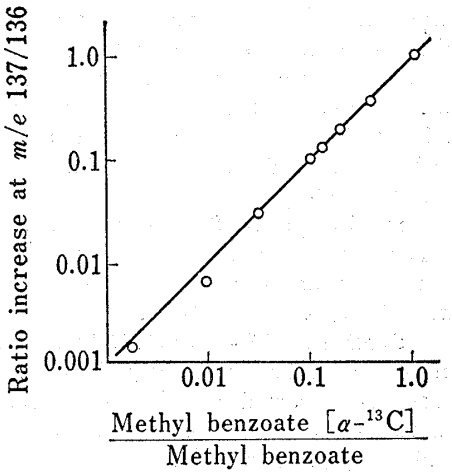

Fig. 5. Calibration Curve of Methyl Benzoate [α-^{13}C] and Methyl Benzoate

Then, how accurately the dilution analysis can be done was examined by using I-^{13}C. Calibration curve for dilution analysis is shown in Fig. 5. Linear correlation was obtained between 1 and 300 fold dilutions and the quantitative accuracy was over 97% in this range. This indicates that drugs single-labeled with carbon-13 can be used for quantitative analysis of drug metabolites.

Experimental

IR spectra were obtained in $CHCl_3$ or CCl_4 solution with a Nihon Bunko Model IR-1 spectrophotometer. NMR spectra were measured in $CDCl_3$ solution, with tetramethylsilane as an internal standard, with JNM-PS-100 spectrometer. Mass spectra were obtained with Shimadzu LKB-9000 GC–MS interfaced multiple-ion-detector (MID) and OKITAC 4300 computer at 20 eV. GC was carried out with 1.5% OV-1 column (glass, 2 m × 3 mm) and He was used as a carrier gas (20 ml/min).

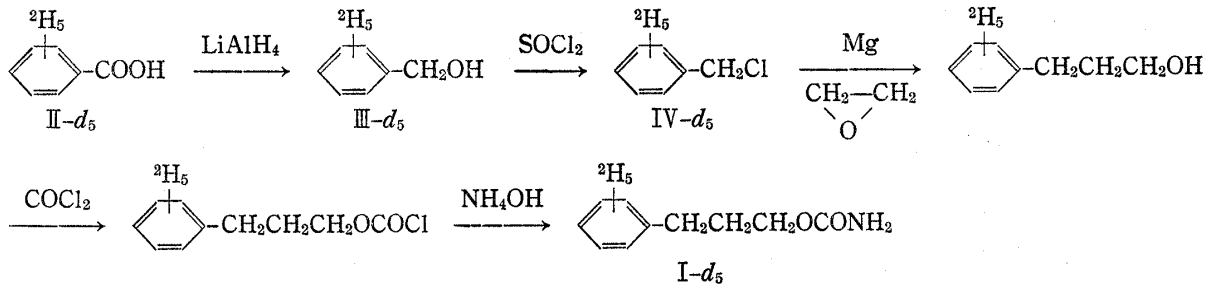

Chart 2. Synthetic Route of 3-Phenylpropyl[arom.-d_5] Carbamate

Synthesis of I-d_5——The synthetic route for the preparation of I-d_5 is shown in Chart 2. An ether solution of 9.5 g of II-d_5 (99.0 atom %, E. Merck, Germany) was added dropwise to an ether solution of $LiAlH_4$ under an anhydrous condition. The mixture was refluxed for 2 hr, cooled in an ice-bath, and acidified with dil. HCl. The aqueous layer was extracted with ether. The combined ether extract was washed with 5% Na_2CO_3 and dried over $MgSO_4$. After evaporation of ether, the reactant was purified by distillation under a reduced pressure (83°/7 mmHg), giving a III-d_5 in 90.0% yield based on II-d_5.

An ether solution of III-d_5 obtained as above was added to a mixture of 6 ml of $SOCl_2$, 0.15 ml of dry pyridine, and 20 ml of ether. After refluxing for 2 hr, the mixture was diluted with 20 ml of ether and filtered off. The filtrate was concentrated and distilled under a reduced pressure (75°/8 mmHg), giving IV-d_5 in 78.7% based on II-d_5.

$C_6D_5CH_2MgCl$ was prepared by adding 7.8 g of IV-d_5 to 1.8 g of Mg in ether. A solution of 5.6 g of $(CH_2)_2O$ in ether was added to this reaction mixture, keeping below 12° in an ice-bath with stirring. After standing overnight at a low temperature, the reactant was decomposed with 70 ml of 35% H_2SO_4. The aqueous layer was extracted with ether. The combined extract was washed with 5% $NaHCO_3$, dried over $MgSO_4$, concentrated to a small volume, and then dissolved in 10.1 g of dimethylaniline. This solution was added dropwise to 40 ml of 20% phosgene-toluene cooled in an ice-bath during 2 hr and the reaction mixture was stirred further for 20 hr at room temperature. After dimethylaniline-HCl formed was filtered off, the filtrate was added dropwise to 100 ml of 28% NH_4OH during 30 min and stirred for 7 hr at room temperature. The aqueous layer was extracted with toluene, the reactant was dried over $MgSO_4$, and the solvent was distilled off. The concentrated extract was subjected to column chromatography over a Wakogel C-100 and the column eluted first with benzene and then with ether. After concentration of the fraction corresponding to I-d_5, the product was recrystallized from a mixture of ether and petr. ether, giving I-d_5 in 18.2% yield based on II-d_5.

Synthesis of I-^{13}C——II-^{13}C was prepared by carbonation of C_6H_5MgBr with $^{13}CO_2$ obtained by addition of 20 ml of conc. H_2SO_4 to 5.9 g of $Ba^{13}CO_3$ (97.5 atom %, Los Alamos Scientific Laboratory, U.S.A.) according to the method of Dauben,[11] in 90—95% yield. I-^{13}C was synthesized by the same method as above. Yield was 16.4% based on II-^{13}C.

Synthesis of V-$^{13}C_1$ and V-$^{13}C_2$——$C_6H_5{}^{13}CH_2MgCl$ was prepared from 2.0 g of IV-^{13}C and 0.46 g of Mg in absolute ether under an anhydrous condition. The reaction mixture was divided into two parts. One part was converted to V-$^{13}C_1$ by carbonation with CO_2 generated from Dry ice, and the other part was converted to V-$^{13}C_2$ by carbonation with $^{13}CO_2$ generated from $Ba^{13}CO_3$ and conc. H_2SO_4. Yields were 53.1% and 65.6%, respectively.

Synthesis of 1-Hydroxyethylbenzene[1,2-$^{13}C_2$] (VI-$^{13}C_2$) and 1-Chloroethylbenzene[1,2-$^{13}C_2$] (VII-$^{13}C_2$)—— VI-$^{13}C_2$ was obtained by reduction of V-$^{13}C_2$ with $LiAlH_4$ and VII-$^{13}C_2$ was prepared by chlorination of VI-$^{13}C_2$ with $SOCl_2$.

Calibration Curve——Fixed amount of II from 1 to 300 mg was mixed with 1 mg of II-^{13}C and each mixture was dissolved in ether. After methylation with CH_2N_2, the mixture was applied to GC-MS. MID was focused to *m/e* 136 and 137, and peak height ratio was measured manually.

11) W. Dauben, J.C. Reid, and P.E. Yankwich, *Anal. Chem.*, **19**, 828 (1974).