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Cholestanol Esters of Amino Acids*1

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The cholestanol (5α-cholestan-3β-ol) esters of amino acids were synthesized with an interest for their possible biochemical activities. The cholestanol esters of glycine, L-alanine, β-alanine, L-leucine and L-aspartic acid were synthesized by a direct fusion of the two components under the passing of hydrogen chloride through the reactants. That of glutamic acid was prepared by using quinoline hydrochloride as a solvent. From the condensation product of aspartic acid and cholestanol, mono- and di-ester were isolated. The monocholestanol ester of aspartic acid was also synthesized by the Gabriel process.

The preparation of the esters of cholestanol (5α-cholestan-3β-ol) or other aliphatic higher alcohols with amino acids was carried out with the interest that the esters with both hydrophilic and lipophilic moieties in a molecule may be expected to possess certain biochemical activities because the both components are structural materials of or closely connected with natural products.

Thus the knowledge about such esters would contribute to the field of biochemistry of complicated lipids. As to cholestanol, however, only several esters such as acetate, benzoate and the like have been reported.

Preparations of cholestanol esters of monobasic amino acids such as glycine, L-alanine, β-alanine and L-leucine, and those of dibasic amino acids such as L-aspartic acid and L-glutamic acid are described in this paper.

On heating a mixture of hydrochloride of amino acid and fused cholestanol for a few hours at 160—180°C, the hydrochloride of cholestanol ester of the corresponding amino acid was obtained. It is favorable to pass dry hydrogen chloride through the reaction system during the course of the reaction.

Racemization of amino acid or epimerization of cholestanol was not observed under the conditions employed.

Fusion of hydrochloride of L-aspartic acid or L-glutamic acid with cholestanol either in an equimolecular proportion or in 1:2 mole ratio always led to the formation of mixture of mono- and dicholestanol esters. The separation of the resulted esters from the unreacted amino acid was effected by the extraction of the reaction mixture with chloroform, in which the hydrochloride of the unreacted amino acid remained undissolved. On adding an excess of acetone to the filtrate, the hydrochlorides of the esters precipitated out, and the unreacted cholestanol remains in the mother liquor.

The last trace of cholestanol can be eliminated from the product on subjecting the chloroform solution of the crude product to silicic acid column chromatography. Cholestanol was eluted with chloroform and the ester hydrochloride was thereafter eluted with the chloroform-methanol mixture (3 : 1 by volume).

The purified esters of the monobasic amino acids were characterized by thin layer chromatography. The spot gave a positive ninhydrin test and got charred in a tan color with spraying sulfuric acid. With the esters of dibasic amino acids, a thin layer chromatography showed two (sometimes three) spots which indicated the presence of at least two kinds of esters (mono- and di-cholestanol esters) in the reaction products. The chloroform solution of the mixture of the hydrochlorides of mono- and di-esters on passing through alumina column and eluting with chloroform-methanol mixture gave the free base of the di-esters, entirely free from the mono-esters, the latter being recovered in low yield by treating the alumina with chloroform-glacial acetic acid mixture afterwards. However, purification of the mono-esters was tedious because of the contaminating inorganic matter.

Since the preparation of the dicholestanol ester of glutamic acid by the fusion method was much more difficult than that of aspartic acid, and was not successful except one trial out of several experiments, the glutamic acid ester was prepared mainly by fusion with a little modification, that is, by fusing the two components in quinoline hydrochloride.

The monoester of aspartic acid with cholestanol was successfully prepared by the modified Gabriel process.[1,2] The aspartic acid was condensed with phthalic anhydride to form phthalyl aspartic acid which was then transformed to phthalimide of

*1 Parts of this work were orally disclosed on 16th (in Tokyo, 1963), 17th (in Tokyo, 1964) and 18th (in Osaka, 1965) Annual Meeting of the Chemical Society of Japan.

1) H. R. Ing and R. H. F. Manske, *J. Chem. Soc.*, **1926**, 2348.

2) J. C. Sheehan and K. R. Henry-Logan, *J. Am. Chem. Soc.*, **81**, 3089 (1959).

aspartic acid anhydride by heating with acetic anhydride. The product was fused with cholestanol to give mono-cholestanol ester of phthalyl aspartic acid, which was treated with hydrazine hydrate in dioxane, followed by treatment with hydrochloric acid in glacial acetic acid to give hydrochloride of monocholestanol ester of aspartic acid.

One of the two spots appeared in a thin layer chromatogram of the product obtained by fusion of aspartic acid with cholestanol coincided both in R_f value and ninhydrin color test with those of the product obtained by Gabriel method, showing that one of the reaction products obtained by fusion method would be the monocholestanol ester hydrochloride.

This monocholestanolester is now assumed to be of a sort in which β-carboxy to the amino group was esterified. In a few lots of fusion experiments the crude reaction mixture showed another spot which might be thought to be that of another isomeric monoester on thin layer chromatogram, but it failed to be isolated.

Therefore, as to the determination of the definite constitutions of monoesters not only of aspartic but also of glutamic acid, further study is now in progress.

Experimental

Cholestanol Ester of Monobasic Amino Acid

A mixture of monobasic amino acid hydrochloride (0.11 mol) and cholestanol (0.11 mol) was heated for different periods of reaction time and at different temperatures with gentle stirring under a current of dry hydrogen chloride until the melt becomes a thick mass. After cooling, the reaction mixture was dissolved in a chloroform-methanol (10: 1 by volume) mixture, filtered by gravity from a white solid and washed with chloroform. From the combined filtrate and washings, on concentration if necessary *in vacuo* and on addition of acetone, the hydrochloride of the ester separated as a white precipitate which was recrystallized from an appropriate solvent.

The chloroform insoluble solid, after purification, is identified as the unreacted amino acid hydrochloride, and from the chloroform-acetone mixture (filtrate of the ester hydrochloride) on further working unreacted cholestanol, mp 141—142°C, was recovered.

To the solution of the ester hydrochloride in a chloroform-methanol (10: 1) mixture, an excess of finely powdered anhydrous sodium carbonate was added, the mixture warmed with gentle shaking for several minutes, filtered, the filtrate evaporated in vacuum and the residue was recrystallized to give the free base of the ester. (Table 1)

Cholestanol Ester of Dibasic Amino Acid

Hydrochloride of Cholestanol Ester of L-Aspartic Acid. A mixture of L-aspartic acid hydrochloride (0.1 mol) and cholestanol (0.22 mol) was heated at about 170°C with gentle stirring under a current of dry hydrogen chloride for about 2 hr. After cooling, the reaction mixture was dissloved in chloroform-methanol (10: 1 by volume) mixture, and filtered from unreacted aspartic acid hydrochloride. On addition of acetone to the filtrate, the mixture of di- and mono-cholestanol ester hydrochlorides separated as a white precipitate.

The amounts of di- and mono-ester hydrochlorides produced are roughly estimated to be in a proportion of about 3: 1 in weight taking account of the amount of diester recovered in alumina column chromatography. On subjecting to silicic acid thin layer chromatography developed with a mixture of chloroform-methanol-toluene (3: 1: 1 by volume), the ester mixture gave two spots showing the presence of di- and mono-ester hydrochlorides.

The Separation of the Each Component from the Mixture of Di- and Mono-Esters of Aspartic Acid. *By Alumina Column.* The diester free base was obtained by subjecting the crude mixture of ester hydrochlorides to an alumina column chromatography from which the contaminating cholestanol was first eluted with chloroform and then the free base of the diester with chloroform-methanol (3: 1 by volume) mixture, and it was recrystallized from benzene. The diester hydrochloride was prepared by treating the chloroform solution of the diester free base with dilute hydrochloric acid, drying the chloroform layer with anhydrous sodium sulfate, evaporating the solvent and the residue crystallizing from benzene.

By Silicic Acid Column. The monoester hydrochloride was separated by subjecting a small amount of the ester mixture to silicic acid column chromatography from which the diester hydrochloride, and the monoester hydrochloride were eluted successively with chloroform-methanol-toluene (3: 1: 1) mixture.

By Solvent Method. A mixture (2.6 g) of the di- and mono-ester hydrochlorides was dissolved in chloroform (20 m*l*), finely powdered anhydrous sodium carbonate (1 g) added to it and the mixture well triturated with warming.

After standing overnight it was evaporated to dryness on a water bath, treated with benzene (10 m*l*), filtered with suction (difficult), washed with benzene and finally with ether. The solid (A) on the filter containing sodium salt of the monoester and inorganic salts was reserved for later separation.

From the benzene filtrate on addition of acetone, precipitated out the crude free base (1.7 g) of the diester. On repeated recrystallizations from benzene, the diester free base (0.4 g) formed colorless needles, mp 246—247°C, lacking the spot of monoester on the thin layer chromatography and agreeing in mp with that of the free base of the diester obtained by passing the mixture of di- and mono-ester hydrochlorides through alumina column.

The solid (A) was suspended in chloroform, the pH of the mixture lowered, with external cooling, to 3 by adding dropwise methanol solution of hydrogen chloride and the mixture was filtered with suction. The filtrate was evaporated to dryness at below 30°C and the residue purified by chloroform-acetone method; yeild 0.15 g, mp 221°C with foaming and with sintering at 217°C. Silicic acid thin layer chromatogram indicated the absence of diester and its identity with the monoester derived

TABLE 1. CHOLESTANOL ESTERS

No.	Amino acid	Ester	Reaction time (Temp.)	Yield %	Mp °C
I	Glycine	Free base		44	178—180
		Hydrochloride	1.5 hr (160°C)	45.9	245—246 (dec.)
II	L-Alanine	Free base		30	120—121
		Hydrochloride	0.25 hr (180°C)	30.8	258 (dec.)
III	β-Alanine	Free base		21	104—106 (dec.)
		Hydrochloride	0.5 hr (180°C)	21.7	266 (dec.)
IV	L-Leucine	Free base		33	117—120 (dec.)
		Hydrochloride	1 hr (170°C)	32.6	286—287 (dec.)

Abbreviations: bz, benzene; chl, chloroform; MeOH, methanol; EtOH, ethanol; s., soluble; i., insoluble.

TABLE 2. CHOLESTANOL ESTERS

No.	Amino acid	Ester		Reaction time (Temp.)	Yield %	Mp °C
V	L-Aspartic acid	Diester	Free base			246
			Hydrochloride	1.5 hr (150—170°C)	32	265
		Monoester Hydrochloride		Direct fusion	11	221
				Gabriel process		226
VI	L-Glutamic acid	diester	Free base	1.5 hr (150°C) (Quinoline)	33	165—166
			Hydrochloride	1.5 hr (150°C) (Quinoline)		226—227

Abbreviations: bz, benzene; chl, chloroform; MeOH, methanol; EtOH, ethanol; s., soluble; i., insoluble.

by the Gabriel process. Analytical results are shown in Table 2.

Hydrochloride of Cholestanol Ester of L-Glutamic Acid. A mixture of glutamic acid hydrochloride (1.25 g) and quinoline hydrochloride (8 g) was heated at 150°C (bath temperature) for 15 min with passing of hydrogen chloride to remove moisture as far as possible, cholestanol (3 g) added to the resulted solution and the mixture stirred at the same temperature for further 1.5 hr with constant passing of hydrogen chloride until homogeneous viscous mass obtained. After cooling, the reaction mixture was treated with dilute hydrochloric acid, filtered with suction, washed with acetone, and then, treated successively with hot acetone and hot dioxane, each time being filtered with suction.

Yield of the crude mixture of di- and mono-ester hydrochlorides was 1.9 g, mp 225—226°C with preliminary sintering. The silicic acid thin layer chromatography of this product, when developed with chloroform-methanol-toluene (3: 1: 1 by volume), showed four spots corresponding to di- and mono-cholestanol esters of glutamic acid, unreacted cholestanol, and glutamic acid. For separating the diester of glutamic acid, the chloroform solution of the crude product (1.9 g) was subjected to alumina column and eluted with the chloroform-methanol (6: 1 by volume) mixture. On addition of acetone to the elute after concentrating, precipitated 1.5 g of a solid, mp 170—180°C, which proved on thin layer chromatography to be the diester contaminated with cholestanol but free from the monoester. It was dissolved in a small amount of chloroform, and on addition of acetone to the solution, the free base of dicholestanol ester of glutamic acid, mp 165—166°C, separated. It is easily soluble in chloroform, benzene, and slightly in acetone.

Hydrochloride of the dicholestanol ester of glutamic acid was precipitated from the chloroform solution of the above free base by adding a calculated amount of methanol-hydrogen chloride, and then acetone. It formed prisms, mp 226—227°C with foaming.

OF MONOBASIC AMINO ACIDS

Solubility	Formula (Formular weight)	Carbon % Found (Calcd.)	Hydrogen % Found (Calcd.)	Nitrogen % Found (Calcd.)
s. in chl, bz, hot acetone.	$C_{29}H_{51}O_2N$ 445.7	78.33 (78.16)	10.98 (10.66)	3.20 (3.15)
s. in chl-MeOH (6 : 1), hot MeOH, hot bz. i. in acetone, ether.	$C_{29}H_{51}O_2N \cdot HCl$ 482.2	71.75 (72.23)	10.70 (10.87)	2.93 (2.91)
s. in chl, acetone.	$C_{30}H_{53}O_2N$ 459.7			
s. in chl, bz, MeOH, hot EtOH, chl-MeOH. i. in acetone, ether.	$C_{30}H_{53}O_2N \cdot HCl$ 496.2	71.1 (72.6)	10.53 (10.76)	2.86 (2.83)
s. in acetone, *n*-hexane.	$C_{30}H_{53}O_2N$ 459.7	77.1 (78.3)	11.76 (11.52)	
s. in MeOH, hot EtOH, chl-MeOH (10 : 1), bz. i. in acetone, ether.	$C_{30}H_{53}O_2N \cdot HCl$ 496.2	71.46 (72.59)	10.82 (10.76)	2.80 (2.83)
s. in chl. acetone.	$C_{33}H_{59}O_2N$ 501.8	78.53 (78.9)	11.32 (11.65)	2.87 (2.79)
s. in chl, hot bz, MeOH. i. in acetone, ether.	$C_{33}H_{59}O_2N \cdot HCl$ 538.3	73.9 (73.7)	11.18 (11.25)	2.58 (2.60)

OF DIBASIC AMINO ACIDS

Solubility	Formula (Formular weight)	Carbon % Found (Calcd.)	Hydrogen % Found (Calcd.)	Nitrogen % Found (Calcd.)
s. in chl, chl-MeOH, bz. i. in MeOH, EtOH, ether, acetone.	$C_{58}H_{99}NO_4$ 874.3	78.4 (79.7)	11.2 (11.3)	1.63 (1.60)
s. in chl, chl-MeOH, bz. i. in acetone, ether, MeOH, EtOH.	$C_{58}H_{99}NO_4 \cdot HCl$ 910.8			1.55 (1.54)
s. in chl (not completely), chl-MeOH (10 : 1), hot bz. i. in acetone, MeOH, EtOH.	$C_{31}H_{53}O_4N \cdot HCl$ 540.2	69.2 (68.9)	10.0 (10.1)	2.50 (2.59)
s. in chl (not completely), chl-MeOH (10 : 1), hot bz. i. in acetone, MeOH, EtOH.	$C_{31}H_{58}O_4N \cdot HCl$ 540.2	69.6 (68.9)	10.2 (10.1)	2.50 (2.59)
s. in chl, bz. i. in acetone (slightly soluble).	$C_{59}H_{101}NO_4$ 888.4	79.1 (79.6)	11.2 (11.4)	1.59 (1.6)
s. in chl, chl-MeOH. i. in acetone, dioxane.	$C_{59}H_{101}NO_4 \cdot HCl$ 924.9	75.6 (76.5)	10.6 (11.0)	1.64 (1.5)

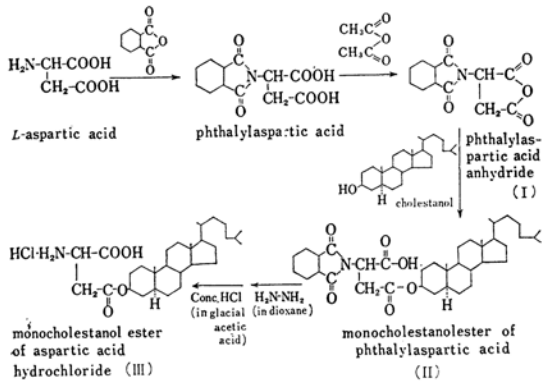

Fig. 1. Preparation of monocholestanol ester of aspartic acid hydrochloride through a modified Gabriel process.

Hydrochloride of Monocholestanolester of Aspartic Acid through a Modified Gabriel Process. *Pathalimide of Aspartic Acid Anhydride (I).* A solution of 13.7 g of phthalimide of the aspartic acid, mp 228°C, in 100 m*l* of acetic anhydride was gently refluxed for 1.5 hr. After standing o vernight, the separated crystal were filtered, washed first with a small amount of acetic anhydride and then with ether; yield 11.5 g. It forms colorless needles, mp 225°C with preliminary darkening; mixed mp with phthalimide of aspartic acid (mp 228°C) 210—218°C. It is soluble in acetone, difficultly in benzene, chloroform, ether and petroleum ether.

Found: N, 5.63%. Calcd for $C_{12}H_7O_5N$: N, 5.71%.

Cholestanol Ester of Phthalylaspartic Acid (II). A mixture of finely pulverized phthalylaspartic acid anhydride (4.9 g, 0.02 mol) and cholestanol (7.8 g, 0.02 mol) was heated at 145°C for 1.5 hr with stirring until the reaction mass become viscous. Longer period of heating produced a larger amount of diester. After cooling, the

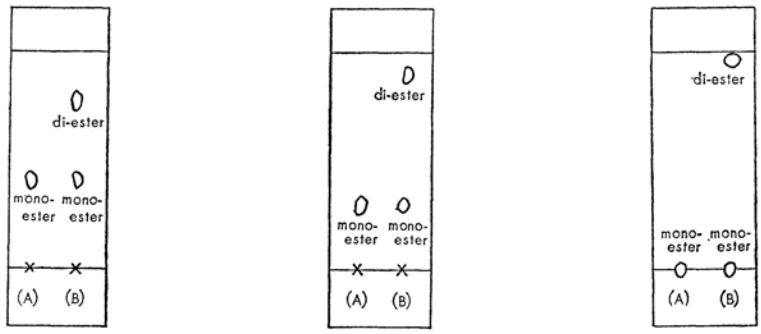

Plate:	silicic acid	silicic acid	activated alumina
Developer:	chloroform+methanol (6:1)	chloroform+methanol+toluene (3:1:1)	chloroform+methanol (6:1)

(A): Monocholestanolester of aspartic acid hydrochloride from the Gabriel process
(B): A mixture of di- and mono-cholestanolester hydrochlorides derived from the direct fusion method

Fig. 2. Thin layer chromatograms showing the correspondence between the both mono-esters, one from the Gabriel process and the other from the direct fusion method.

reaction mixture was dissolved in benzene (80 m*l*), filtered from the unreacted anhydride (1 g, mp 219—221°C with darkening and foaming) and the filtrate on long standing separated crude monoester. The filtrate of the crude monoester was evaporated to dryness and the residue was dissolved in hot acetone from which, on standing, separated crude diester. On further working, the average yields of products were obtained as follows:

Dicholestanol ester of phthalylaspartic acid crystallized from acetone as prisms, yield 1.5 g, mp 199°C. Found: C, 79.16; H, 10.16; N, 1.38%. Calcd for $C_{66}H_{101}O_6N$: C, 78.9; H, 10.2; N, 1.39%.

Infrared Absorption in KBr (wave number, cm^{-1}):
2950, 2870, 1780, 1740, 1727, 1620, 1470, 1455, 1390, 1340, 1270, 1255, 1240, 1180, 1155, 1135, 1110, 1090, 1015, 1005, 960, 930, 890, 880, 790, 720

Monocholestanolester of phthalylaspartic acid crystallized from benzene as prisms, yield 6.8 g, mp 169°C. Found: C, 74.72; H, 9.52; N, 2.05%. Calcd for $C_{39}H_{55}O_6N$: C, 73.9; H, 8.76; N, 2.21%.

Infrared Absorption in KBr (wave number, cm^{-1}):
2920, 2850, 2750—2450, 1775, 1725, 1620, 1470, 1440, 1390, 1340, 1280, 1260, 1240, 1205, 1172, 1155, 1135, 1110, 1090, 1022, 1005, 962, 930, 890, 875, 785, 720

Hydrochloride of Monocholestanolester of Aspartic Acid (III). Two grams (about 1.5 mmol) of the monocholestanolester of phthalylaspartic acid (mp 169°C) was dissolved in a mixture of dioxane (40 cc) and water (0.4 cc). To the cooled solution (13°C) was added dropwise 0.46 cc of 80% hydrazine hydrate during 1 min, the white precipitate separating immediately. The reaction mixture was allowed to stand at 13—15°C for 3 hr and at room temperature for 21 hr with frequent triturating. The precipitate was filtered, washed with dioxane and dried; yield, 1.6 g. It was finely powdered and suspended in glacial acetic acid (30 cc). To the cooled suspension (13°C) was added concentrated hydrochloric acid (0.8 cc) and the mixture stirred for 30 min at room temperature. It was centrifuged, the precipitate washed once with acetic acid containing conc. hydrochloric acid, three times with dilute hydrochloric acid and once with water, successively. It was dissolved in chloroform, and the solution after being dried with anhydrous sodium sulfate was evaporated to dryness, yield 0.5 g, mp 226°C. This compound was identified by analysis and the agreement of R_f value in silicic acid thin layer chromatography with one of the two kinds of ester products from the direct fusion method. (Fig. 2)

Infrared Absorptions of Cholestanol Esters in KBr (wave number, cm^{-1}):

Glycine Ester Hydrochloride,
3450, 2950, 2900, 2600, 1750, 1470, 1410, 1385, 1245, 1175, 1150, 1125, 1055, 1000, 950, 930

L-Alanine Ester Hydrochloride,
3400, 2910, 2850, 2700—2500, 1743, 1605, 1510, 1465, 1375, 1330, 1255, 1210, 1120, 1000, 955, 920

β-Alanine Ester Hydrochloride,
3450, 2950, 2870, 2750—2500, 1735, 1610, 1470, 1410, 1385, 1350, 1215, 1150, 1135, 1110, 1010, 960, 920, 790

L-Leucine Ester Hydrochloride,
3440, 2920, 2860, 2640—2360, 1740, 1585, 1510, 1470, 1380, 1275, 1220, 1170, 1130, 1000, 950, 920

L-Aspartic Acid Diester Hydrochloride,
3400, 2900, 2830, 2750—2350, 1740, 1725, 1575, 1500, 1465, 1410, 1385, 1330, 1260, 1230, 1205, 1150, 1100, 1070, 1000, 955, 930, 900, 790

L-Aspartic Acid Monoester Hydrochloride (Gabriel process)
3400, 2900, 2840, 2750—2350, 1735, 1615, 1510, 1463, 1445, 1380, 1330, 1300, 1200, 1170, 1150, 1130, 1075, 1000, 960, 930, 900

L-Glutamic Acid Diester
3400, 2920, 2860, 2750—2350, 1740, 1590, 1505, 1470, 1445, 1383, 1336, 1300, 1230, 1190, 1150, 1130, 1080, 1000, 960, 930

Test for the Ester Formation

The synthesized products were tested in the following four ways in proof of the identity to the intended esters.

Hydrolysis Test. Both of the esters of glycine and aspartic acid were subjected to either acid and alkaline

hydrolysis, and one of the hydrolysates from the alkaline hydrolysis after purification through silicic acid column proved to be identical with cholestanol by elementary analysis, melting point and mixed melting point method and infrared absorption spectrum; and one water soluble hydrolysate from the acid hydrolysis was identified on the two dimentional paper chromatography as the corresponding amino acid, glycine and aspartic acid respectively.

Infrared Spectra (in Potassium Bromide, cm^{-1}). In the infrared spectrum, all of the above synthesized esters have shown the absorption of carboxylic acid ester linkage at (1735—1750) and lacked that attributed to 3β-hydroxyl group in the cholestanol at (1040).

Hydroxamic Acid Test. Hydroxamic acid test (Hestelins test) was positive for all of the cholestanol esters of amino acids prepared.

Ninhydrin Color Test. All esters prepared were positive except those of phthalylamino acids.

Reservation of the Optical Activity

With regard to the cholestanol ester of L-aspartic acid prepared by the fusion method, reservation of the optical activity of amino acid was confirmed. The $[\alpha]_D$ values measured were as follows:

1. L-aspartic acid hydrochloride (1% in 5 N HCl)

$$[\alpha]_D^{20} = 19$$

2. aspartic acid hydrochloride from the hydrolysate of the cholestanolester of aspartic acid hydrolysed by 6 N HCl (1% as aspartic acid hydrochloride in 5 N HCl)

$$[\alpha]_D^{20} = 18$$

3. The recovered aspartic acid hydrochloride which remained unreacted on 3 hr fusing of the components at 150°C (1% in 5 N HCl)

$$[\alpha]_D^{20} = 17-19$$

The value 19 for the sample of 1% aspartic acid hydrochloride solution deserves to 24 for the sample of 1% in concentration of aspartic acid itself.

As to the monochlolestanol ester of aspartic acid from the Gabriel process as well as the starting phthalylaspartic acid which had been derived from L-aspartic acid according to the drastic conditions of L. Reese,[3] the optical activity will be examined in detail in succeeding work.

The authors are much grateful to Dr. Konomu Matsumura for help and advice throughout this work.

3) L. Reese, *Ann.*, **242**, 1 (1887).