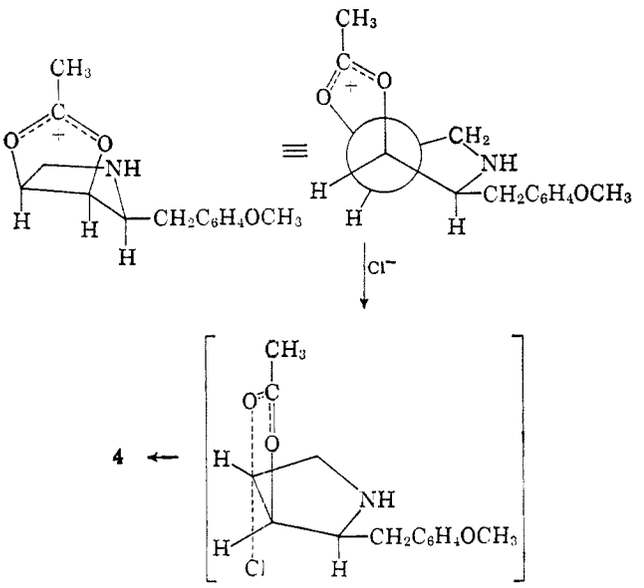
SCHEME II



Reaction of **1** with thionyl chloride gave a crystalline chlorosulfite, **6**, which decomposed on heating to a mixture of two chloroacetates, **4** and **7**; the composition of this mixture was dependent upon the reaction conditions. Reaction of **7** with alcoholic potassium hydroxide gave a diol, **8**, which was identical with the product obtained on saponification of **1**. Since the conversion of **7** to **8** involves a Walden inversion, **7** must have the all-*cis* configuration.

The remarkable stereoselectivity of the ring-opening reaction described above is also manifested in the reaction of the epoxide with a variety of nucleophiles. When **5** was treated with acetic acid and hydrolyzed, only diol **8** was obtained. Reaction of **5** with seventeen other nucleophiles (alkoxides, amines, and mercaptans) proceeded smoothly. And, in every case, only a single product was isolated in high yield. From this series of experiments, it appears that reactions of epoxides of cyclopentane derivatives are subject to the same stereoelectronic factors which are operative in the cyclohexane series and that these are highly selective.

Registry No.—**1**, 2322-08-9; **2**, 14669-64-8.

Acknowledgment.—We wish to thank Dr. Kenneth Butler for bringing this problem to our attention and for supplying us with crystals of compound **2**.

Peptide Synthesis *via* Oxidation of N-Acyl-α-amino Acid Phenylhydrazides. II. Benzyloxycarbonyl Peptide Phenylhydrazides[1]

H. BAYARD MILNE AND CLARK F. MOST, JR.

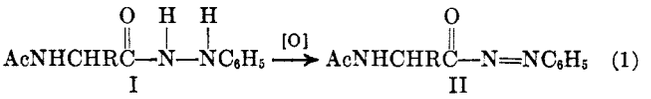
Department of Chemistry, Washington State University, Pullman, Washington

Received July 11, 1967

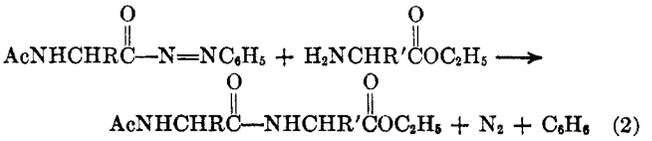
Several benzyloxycarbonyl-α-amino acid phenylhydrazides were oxidized to give crystalline benzyloxycarbonyl-α-amino acid phenyldiimides. These compounds have an n → π* transition at 450 mμ which causes a positive Cotton effect in their optical rotatory dispersion curves and a positive ellipticity in their circular dichroism curves. The benzyloxycarbonylamino acid phenyldiimides reacted with amino acid phenylhydrazides to give benzyloxycarbonyl dipeptide phenylhydrazides in good yield with little or no racemization. The physical properties (infrared, ORD, melting point, and tlc R_f values) of these compounds were the same as those of benzyloxycarbonyl dipeptide phenylhydrazides prepared by a papain-catalyzed reaction between benzyloxycarbonylamino acids and -amino acid phenylhydrazides. The application of this method to higher peptides was shown by the reaction between benzyloxycarbonylglycyl-L-phenylalanine phenyldiimide with glycyl-L-phenylalanine phenylhydrazide to give benzyloxycarbonylglycyl-L-phenylalanylglycyl-L-phenylalanine phenylhydrazide.

It is becoming apparent that the phenylhydrazide group is an effective carboxyl blocking group for amino acids. N-acyl-α-amino acid phenylhydrazides (I) are easily prepared from the acid chloride, the azide or by papain-catalyzed reactions. This group is stable toward mild acid or base. There have been several procedures reported for the removal of the phenylhydrazide group from N-acyl-α-amino acid phenylhydrazides. These all consist of oxidizing the phenylhydrazides in aqueous solutions to give essentially quantitative yields of the acylated amino acids or peptides.[3] We have recently shown that N-acyl-α-amino acid phenylhydrazides may be oxidized in nonaqueous solvents to give N-acyl-α-amino acid phenyldiimides (II).[2]

The usefulness of these N-acyl-α-amino acid phenyldiimides (II) in peptide synthesis was demonstrated by coupling them with amino acid ethyl esters giving N-acyl dipeptide ethyl esters. Several dipeptides were prepared by the method with little racemization. The reactions are formulated in eq 1 and 2.



(1)



(2)

We now wish to report the extension of this method of peptide synthesis to include the reaction of N-acyl-α-amino acid phenyldiimides with α-amino acid phenylhydrazides to give N-acyl dipeptide phenylhydrazides,

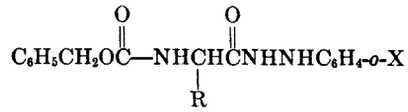
(1) Supported in part by U. S. Public Health Service Research Grant G. M. 11835 from the National Institutes of Health. For part I of this series, see ref 2.

(2) H. B. Milne and W. D. Kilday, *J. Org. Chem.*, **30**, 64 (1965).

(3) (a) E. Waldschmidt-Leitz and K. Kühn, *Ber.*, **84**, 381 (1951); (b) H. B. Milne, J. E. Halver, D. S. Ho, and M. S. Mason, *J. Am. Chem. Soc.*, **79**, 637 (1957); (c) R. B. Kelley, *J. Org. Chem.*, **28**, 453 (1963).

TABLE I

BENZYLOXYCARBONYL-L-AMINO ACID PHENYLHYDRAZIDES AND *o*-CHLOROPHENYLHYDRAZIDES

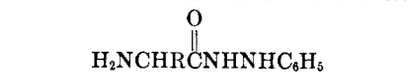


Amino acid	X	Recrystn solvent	Yield,[a] %	Mp, °C	$[\alpha]_D$, deg	Formula	Calcd, % C	H	N	Cl	Found, % C	H	N	Cl
L-Proline[b]	H	Ethyl acetate–ligroin	50	127–128	−79.0[c]	$C_{19}H_{21}N_3O_4$[i]	67.24	6.24	12.38		67.36	6.16	12.44	
L-Tyrosine	H	Ethanol–water	37	209–209.5	−37.6[d]	$C_{23}H_{23}N_3O_4$[j]	68.13	5.72	10.36		68.30	5.58	10.33	
L-Tryptophan	H	Dioxane–water	82	213.5–214	−31.6[e]	$C_{25}H_{24}N_4O_4$[k]	70.08	5.65	13.07		69.86	5.72	12.91	
L-Alanine	Cl	Ethanol–water	59	176–177	−49.0[f]	$C_{17}H_{18}ClN_3O_3$[l]	58.71	5.22	12.08	10.20	58.55	5.25	11.91	10.25
Glycine	Cl	Methanol–water	68	141.5–142.5		$C_{16}H_{16}ClN_3O_3$[m]	57.57	4.83	12.59	10.62	57.68	4.89	12.71	10.76
L-Leucine	Cl	Ethyl acetate–legroin	72	108–109	−48.0[g]	$C_{20}H_{24}ClN_3O_3$[n]	61.61	6.20	10.78	9.09	61.80	6.35	10.79	9.21
L-Phenylalanine	Cl	Methanol–water	82	178.5–179.5	−45.0[h]	$C_{23}H_{22}ClN_3O_3$[o]	65.17	5.23	9.91	8.36	65.27	5.31	9.72	8.44

[a] After one crystallization. [b] Prepared by azide method. [c] *c* 0.228, dioxane. [d] *c* 0.359, dioxane. [e] *c* 0.114, dioxane. [f] *c* 0.334, dioxane. [g] *c* 0.519, dioxane. [h] *c* 0.324, dioxane. [i] Registry no.: 14723-78-5. [j] 14723-79-6. [k] 14723-80-9. [l] 14723-81-0. [m] 14746-07-7. [n] 14746-08-8. [o] 14723-82-1.

TABLE II

AMINO ACID PHENYLHYDRAZIDE HYDROIODIDES AND AMINO ACID PHENYLHYDRAZIDES



Amino acid phenylhydrazide	Yield,[a] %	Mp, °C	Formula	Calcd, % C	H	N	I	Found, % C	H	N	I
Glycine, HI	96	216–227[b]	$C_8H_{12}IN_3O$[e]	32.78	4.13	14.34	43.29	32.97	4.25	14.51	43.41
L-Leucine, HI	79	221–222	$C_{12}H_{20}IN_3O$[f]	41.27	5.77	12.03	36.34	41.44	5.90	11.93	36.12
L-Phenylalanine, HI	95	198–202	$C_{15}H_{18}IN_3O$[g]	47.00	4.74	10.97	33.11	46.87	4.89	10.79	32.91
Glycine	82[c]	138.2–139.5	$C_8H_{11}N_3O$[h]	58.17	6.71	25.44		57.88	6.74	25.30	
L-Leucine	93[d]	122–123	$C_{12}H_{19}N_3O$[i]	65.13	8.65	18.99		65.31	8.76	18.82	
L-Phenylalanine	94[c]	134.5–135	$C_{15}H_{17}N_3O$[j]	70.56	6.71	16.46		70.22	6.83	16.19	

[a] After three recrystallizations from ethanol–ether. [b] Lit.[6] mp 215–220. [c] Recrystallized from ethanol. [d] Recrystallized from chloroform–petroleum ether (bp 30–60°). [e] Registry no.: 14723-83-2. [f] 14723-84-3. [g] 14723-85-4. [h] 14723-86-5. [i] 14723-87-6. [j] 14723-88-7.

and the reaction of N-acyl dipeptide phenyldiimides with dipeptide phenylhydrazides to give N-acyl tetrapeptide phenylhydrazides.

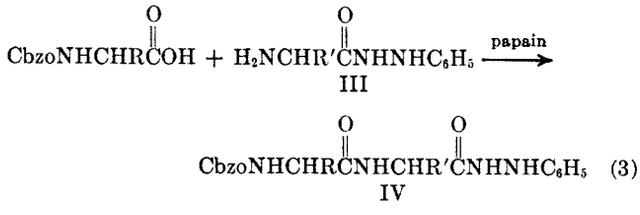
Discussion

In order to have pure benzyloxycarbonyl-L-amino acid phenylhydrazides (I) as starting materials benzyloxycarbonyl-L-amino acids were used in the papain-catalyzed reaction with phenyl hydrazine.[4] Also because the *o*-chlorophenyl anion has been reported[5] to be more stable than other phenyl anions it was felt that the *o*-chlorophenylazo anion might be a better leaving group with less tendency to decompose by free-radical reactions than the phenylazo anion.

For this reason a series of benzyloxycarbonyl-α-amino acid *o*-chlorophenylhydrazides were prepared by the papain-catalyzed reaction between benzyloxycarbonyl-L-amino acids and *o*-chlorophenylhydrazine. As shown in Table I, the yields of benzyloxycarbonyl-α-amino acid *o*-chlorophenylhydrazides were as high as or higher than those reported for the corresponding phenylhydrazides.

α-Amino acid phenylhydrazides (III) were prepared from the benzyloxycarbonyl-α-amino acid phenylhydrazides in a two-step synthesis, using a modification of the method of Waldschmidt-Leitz and Kühn.[3a] The benzyloxycarbonyl group was cleaved with hydrogen iodide in anhydrous acetic acid to give amino acid phenylhydrazide hydroiodides. The hydroiodides were converted to the free bases by the action of dry ammonia gas. This method gave high yields of the α-amino acid phenylhydrazides which were stable crystalline solids (see Table II).

In order to prepare optically pure benxyloxycarbonyl dipeptide phenylhydrazides (IV) to compare with the products from the reaction of benzyloxycarbonylamino acid phenyldiimides with amino acid phenylhydrazides, the enzymatic method used by Waldschmidt-Leitz and Kühn[3a] to prepare benzyloxycarbonyl glycylglycine phenylhydrazide was modified (eq 3). They reported



a 53% yield of benzyloxycarbonylglycylglycine phenylhydrazide after 40 days in a citrate buffer at pH 5. We obtained an 82% yield after 30 hr at pH 6.4 in a solution of high ionic strength (2.7 *M* potassium chloride). The preparation and properties of the benzyloxycarbonyl dipeptide phenylhydrazides (IV) are shown in Table III.

In the enzymatic synthesis of benzyloxycarbonyl-L-phenylalanyl-L-phenylalanine phenylhydrazide transamidation resulted in 39 mole % of benzyloxycarbonyl-L-phenylalanine phenylhydrazides and only 9 mole % of the desired benzyloxycarbonyl-L-phenylalanyl-L-phenylanine phenylhydrazide. This observation is similar to that of Jannsen, Winitz, and Fox,[6] who reported that

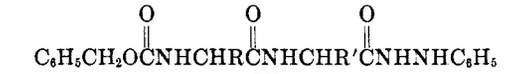
(4) M. Bergmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **119**, 707 (1937); H. B. Milne and C. M. Stevens, *J. Am. Chem. Soc.*, **72**, 1742 (1950).

(5) J. A. Zoltewicz and J. F. Bunnett, *ibid.*, **87**, 2640 (1965).

(6) F. Jannsen, W. Winitz, and S. W. Fox, *ibid.*, **75**, 704 (1953).

TABLE III

ENZYMATIC PREPARATION AND PROPERTIES OF BENZYLOXYCARBONYL DIPEPTIDE PHENYLHYDRAZIDES



IV

Benzyloxycarbonyl dipeptide phenylhydrazide	pH of reacn mixture	Recrystn solvent	Yield,[a] %	Mp, °C	$[\alpha]$D, deg
Glycylglycine[e]	6.0	Ethanol	82	164–165	
Glycyl-L-phenylalanine[f]	4.8	Ethanol	82	168.5–169.5	−45.0[b]
L-Leucyl-L-phenylalanine[g]	5.2	Ethanol	52	205.5–206.5	−50.1[c]
L-Phenylalanyl-L-phenylalanine[h]	6.2	Acetone–water	14	207–209	−23.4[d]

[a] After one recrystallization. [b] *c* 0.49, dioxane. [c] *c* 0.42, dioxane. [d] *c* 0.5, dioxane. [e] Registry no.: 14723-89-8. [f] 14723-90-1. [g] 14723-91-2. [h] 14746-09-9.

TABLE IV

SOME SPECTRAL PROPERTIES OF BENZYLOXYCARBONYLAMINO ACID PHENYLDIIMIDES AND *o*-CHLOROPHENYLDIIMIDES

Benzyloxycarbonyl-amino acid phenyldiimides	Extrema of ORD Cotton effects							Circular dichroism		ORD and CD	Electronic spectra			
	λ_1, mμ	$[\alpha]$	$[\phi]^a$	μ_2, mμ	$[\alpha]$	$[\phi]^a$	A[b]	λ, mμ	$[\theta]^c$	*c*, dioxane	λ, mμ[d]	ε	λ, mμ[d]	ε
L-Alanine[e]	518	255°	793°	415	−219°	−683°	14.8	455	1110°	0.3016	445	106	290	11,500
Glycine[f]											450	90	293	10,400
L-Leucine[g]	518	261°	918°	415	−232°	−820°	17.4	455	1375°	0.4532	450	101	291	11,350
L-Phenylalanine[h]	510	221°	855°	413	−157°	−608°	14.6	455	1140°	0.3887	452	106	292	11,500
L-Proline[i]	500	249°	839°	410	−274°	−922°	17.6	450	1195°	0.1925	445	114	287	10,070
o-Chlorophenyldiimides														
L-Alanine[j]	486	457°	1585°	400	−457°	−1585°	31.7	445	2145°	0.3084	458	106	292	10,720
Glycine[k]											460	86	292	9,350
L-Leucine[l]	490	322°	1250°	390	−438°	−1420°	26.7	448	1900°	0.3964	460	90	292	9,050
L-Phenylalanine[m]	490	296°	1250°	400	−316°	−1335°	25.8	450	1808°	0.2932	460	107	293	9,540

[a] Molecular rotation. [b] Molecular amplitude: $[\phi]\lambda^{t_1} - [\phi]\lambda^{t_2}/100$. [c] Molecular ellipticity, $[\theta]\lambda^t = 3298\ \Delta OD/lC$ where OD is optical density, *l* is path length in dm, and *C* is molar concentration. [d] λ_{max} in dioxane. [e] Registry no.: 14723-92-3. [f] 3005-90-1. [g] 14723-94-5. [h] 14721-30-3. [i] 14746-10-2. [j] 14721-31-4. [k] 14721-32-5. [l] 14721-33-6. [m] 14721-34-7.

the papain-catalyzed reaction of benzoyl-L-phenylalanine with glycine anilide gave a mixture of benzoyl-L-phenylalanine anilide and benzoyl-L-phenylanylglycylglycine anilide. Because the enzymatic method gave a low yield of product of doubtful purity, the desired benzyloxycarbonyl-L-phenylalanyl-L-phenylalanine phenylhydrazide was also synthesized by the azide method. The infrared spectra, the elemental analysis, the optical rotatory dispersion (ORD) spectra, and the thin layer chromatography (tlc) R_f values of the benzyloxycarbonyl-L-phenylalanyl-L-phenylalanine phenylhydrazide prepared by the two methods were the same.

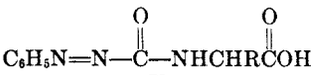
In the previous publication of this series[3] we reported the preparation of crystalline benzyloxycarbonylglycine phenyldiimide. We have now prepared crystalline benzyloxycarbonylamino acid phenyldiimides of L-alanine and L-phenylalanine, and crystalline benzyloxycarbonylamino acid *o*-chlorophenyldiimides of glycine, L-alanine, L-leucine, and L-phenylalanine. These were prepared from the corresponding phenylhydrazide by oxidation with N-bromosuccinimide or lead tetraacetate. The lead tetraacetate oxidation was simple and it was easier to purify the diimide. However, the difficulty of preparation and the tendency of lead tetracetate to decompose in the presence of atmospheric moisture made the use of N-bromosuccinimide more convenient.

The benzyloxycarbonyl-α-amino acid phenyldiimides were unstable at room temperature, but kept satisfactorily for several days at Dry Ice temperature. Satisfactory elemental analyses were obtained by shipping the samples in evacuated sealed ampoules.

The visible and ultraviolet spectra of the benzyloxycarbonyl-α-amino acid phenyldiimides showed maxima at 290 ± 3 mμ (ε 10,500 ± 10%) and 448 ± 3 (90–114). See Table IV.

The benzyloxycarbonyl-L-amino acid phenyldiimides all gave optical rotatory dispersion curves which exhibited single positive Cotton effects at 448 mμ with amplitudes of 14.8 to 17.6. Their circular dichroism (CD) curves had molecular ellipticities from 1195 to 1375°.

The presence of the *o*-chloro substituent in the benzyloxycarbonyl-L-amino acid *o*-chlorophenyldiimides lead to a bathochromic shift in the n → π * transition from 448 to 460 mμ. There was little change in the extinction coefficient. However, the amplitudes of the Cotton effect and the molecular ellipticities were from 1.5 to 2 times the value for the nonchlorinated phenyldiimides. On the other hand the optical rotatory dispersion curve of phenylazocarbonyl-L-leucine[7] (V)



V

showed a negative Cotton effect at *ca.* 450 mμ. These results suggest that the "optical activity" of the phenylazocarbonyl group around 450 mμ is strong enough to overcome the "optical activity" of other structured factors in the α-amino acids. When it is on the carbonyl end of an amino acid, the L series give strong positive Cotton effects. When it is on the α-amino group of an L-amino acid, it gives a negative Cotton effect.

Because of their ease of preparation these phenyldiimides should serve as useful "chromophoric" deriva-

(7) H. B. Milne and W. Kilday, *J. Org. Chem.*, **30**, 67 (1965).

tives in the investigation of the stereochemistry of other optically active acids by means of optical rotatory measurements of the Cotton effect associated with the phenyldiimide group.

The large difference in optical rotation at 520 mμ between an acyl-α-amino acid phenyldiimide and the corresponding acyl-α-amino acid phenylhydrazide or other amino acid derivatives makes it possible to use changes in optical rotation to study the kinetics of oxidation of the phenylhydrazide or the reaction of an optically active N-acyl-α-amino acid phenyliimide with a nucleophile.

The reactions between benzyloxycarbonylamino acid phenyldiimides and -amino acid phenylhydrazides (eq 4) were followed by observing the changes in ab-

$$\underset{\text{II}}{\text{Cbzo-NHCHRC(=O)N=NC}_6\text{H}_5} + \underset{\text{III}}{\text{H}_2\text{NCHR'C(=O)NHNHC}_6\text{H}_5} \longrightarrow \underset{\text{IV}}{\text{Cbzo-NHCHRC(=O)NHCHR'C(=O)NHNHC}_6\text{H}_5} + \text{N}_2 + \text{C}_6\text{H}_5 \quad (4)$$

sorbance at 450 mμ. After the reactions were complete the yield of benzene in the reaction mixture was determined by gas chromatography and the benzyloxycarbonyl dipeptide phenylhydrazide was determined by thin layer chromatography.

The highest yields of benzyloxycarbonyl dipeptide phenylhydrazides (IV) were obtained when a pure benzyloxycarbonylamino acid phenyldiimide (II) was added to 2 equiv of the amino acid phenylhydrazide (III) in freshly purified dry dioxane. The use of triethylamine with amino acid phenylhydrazide hydrogen iodide resulted in a lower yield and increased racemization.

The conditions that give the highest yields of benzyloxycarbonyl dipeptide phenylhydrazides (IV) resulted in a 46–60% yield of benzene. Along with benzene the gas chromatography of reaction mixtures showed the presence of biphenyl and phenol. Kelly[2c] also observed phenol along with biphenyl when phenylhydrazides were oxidized with manganese dioxide in aqueous acetic acid. He suggested that the phenyldiimide formed in the reaction was oxidized to the benzenediazonium ion which decomposed to give the phenol.

The use of benzyloxycarbonylamino acid *o*-chlorophenyldiimides in the reaction with amino acid phenylhydrazides did not increase the yield of benzyloxycarbonyl dipeptide phenylhydrazides or reduce the by-products of the reaction. Thus there is no apparent advantage in using the *o*-chlorophenylhydrazides as a blocking group.

Anderson's method[8] was used to determine the extent of racemization in the reaction between benzyloxycarbonylglycyl-L-phenylalanine phenyldiimide and glycine ethyl ester. The extent of racemization depends upon the reaction conditions. When the reaction was in dichloromethane using glycine ethyl ester hydrochloride and triethylamine, 4.50% of the racemate was isolated from benzyloxycarbonylglycylphenylalanylglycine ethyl ester. When glycine ethyl ester in dichloromethane was used, 2.4% of the racemate was isolated. On the other hand, when glycine ethyl ester in dioxane was used, only the L isomer was isolated.[9,10]

The benzyloxycarbonylglycyl-L-phenylalanine phenylhydrazide and the benzyloxycarbonyl-L-leucyl-L-phenylalanine phenylhydrazide prepared by the phenyldiimide method had the same melting point and optical rotation as when prepared by the enzymatic method, indicating little if any racemization. However, the benzyloxycarbonyl-L-phenylalanyl-L-phenylalanine phenylhydrazide had a melting point of 212–214° and a specific rotation of −34° when it was prepared by the phenyldiimide method, compared to a melting point of 207–209° and specific rotation of −24° when it was prepared by the enzymatic method. The melting point of benzyloxycarbonyl-D-phenylalanyl-L-phenylalanine phenylhydrazide prepared by the azide method was 231–233° and the specific rotation −49°. As all three compounds gave the same infrared spectra and the same elemental analysis the discrepancy may be an indication of racemization in this phenyldiimide reaction.

Benzyloxycarbonylglycyl-L-phenylalanine phenylhydrazide was used to prepare the tetrapeptide benzyloxycarbonylglycyl-L-phenylalanylglycyl-L-phenylalanine phenylhydrazide (VIII). The benzyloxycarbonylglycyl-L-phenylalanine phenylhydrazide was treated with hydrogen bromide in anhydrous dioxane. The product was then treated with dry ammonia to give glycyl-L-phenylalanine phenylhydrazide (VI) (see eq 5).

$$\text{Cbzo-Gly-L-Phe-NHNHC}_6\text{H}_5 \xrightarrow[\text{2. NH}_3]{\text{1. HBr–dioxane}} \underset{\text{VI}}{\text{Gly-L-Phe-NHNHC}_6\text{H}_5} \quad (5)$$

The benzyloxylcarbonylglycyl-L-phenylalanine phenylhydrazide was converted to the diimide (VII) with N-bromosuccinimide (eq 6). The benzyloxycarbonyl-

$$\text{Cbzo-Gly-L-Phe-N(H)—N(H)—C}_6\text{H}_5 \xrightarrow{\text{NBS}} \underset{\text{VII}}{\text{Cbzo-Gly-L-Phe—N=N—C}_6\text{H}_5} \quad (6)$$

glycyl-L-phenylalanine phenyldiimide was then treated with glycyl-L-phenylalanine phenylhydrazide in dioxane to give the benzyloxycarbonylglycyl-L-phenylalanylglycyl-L-phenylalanine phenylhydrazide (VIII) (see eq 7).

$$\underset{\text{VII}}{\text{Cbzo-Gly-L-Phe—N=N—C}_6\text{H}_5} + \underset{\text{VI}}{\text{Gly-L-Phe—NHNHC}_6\text{H}_5} \xrightarrow{\text{dioxane}} \underset{\text{VIII}}{\text{Cbzo-Gly-L-Phe-Gly-L-Phe-NHNHC}_6\text{H}_5} \quad (7)$$

(8) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **80**, 2902 (1958).

(9) That the phenyldiimide method does not lead to extensive racemization was confirmed using Halpern's gas chromatography method.[10] *t*-Butyloxycarbonyl-L-alanine phenyldiimide and *t*-butyloxycarbonyl-L-leucine phenyldiimide were treated with (−)-2-amino-4-methylpentane in dioxane and the reaction mixtures were analyzed by gas chromatography. The results indicated that there was no racemization.

(10) B. Halpern, L. F. Chew, and J. W. Westley, *Anal. Chem.*, **39**, 399 (1967).

Experimental Section[11,12]

Enzymatic Synthesis of Benzyloxycarbonyl-α-amino Acid Phenylhydrazides.—The benzyloxycarbonyl-α-amino acid phenylhydrazides were prepared by the enzymatic method reported previously.[4] The properties and analysis of the previously unreported phenylhydrazides are listed in Table I.

α-Amino Acid Phenylhydrazide Hydroiodides.—The α-amino acid phenylhydrazide hydroiodides were prepared by a modification of the method used by Waldschmidt-Leitz and Kühn.[3a] The yields, melting points and analyses are shown in Table II.

α-Amino Acid Phenylhydrazides.—The α-amino acid phenylhydrazide hydroiodides (2.0 mmoles) were suspended in 150 ml of methylene chloride and treated with dry ammonia gas until all solids dissolved. The resulting solution was evaporated *in vacuo* at 30° to one-half its original volume and the precipitated ammonium iodide removed by filtration. The filtrate was evaporated to dryness and the residue was recrystallized from the solvents indicated in Table II. The elemental analyses are given in Table II.

Enzymatic Synthesis of Benzyloxycarbonyl Dipeptide Phenylhydrazides.—The benzyloxycarbonyl dipeptide phenylhydrazides were prepared by the enzymatic method previously reported.[2b] The properties and elemental analyses are listed in Table III and VI. The infrared spectra of these compounds showed N–H stretch, 3.03; C=O stretch, 5.95 and 6.07; anilino NH, 6.25; amide II, 6.51; and phenyl C—H bend, 13.38 and 14.40 μ. The ultraviolet spectra were $\lambda_{max}^{dioxane}$ 234 mμ (ε 10,750) and 281 mμ (ε 1550).

Benzyloxycarbonyl-α-amino Acid Phenyldiimides and *o*-Chlorophenyldiimides. Method A.—A benzyloxycarbonyl-α-amino acid phenylhydrazide or *o*-chlorophenylhydrazide (1.00 mmole) and pyridine (1.03 mmoles) were dissolved in 75 ml of methylene chloride at room temperature. The reaction flask was wrapped so as to exclude light, and N-bromosuccinimide[13] (1.03 mmoles) was added. The solution was stirred rapidly for about 20 min and was then washed successively with 75 ml each of distilled water, 1 *N* hydrochloric acid, distilled water, 1 *M* sodium bicarbonate, and distilled water. It was dried thoroughly over anhydrous magnesium sulfate and filtered. The filtrate was evaporated at about 15 mm almost to dryness over a water bath at room temperature, then at about 1 mm over a water bath at 0° for 1–2 hr. The product was further treated as described below. Spectral properties are indicated in Table IV.

Method B.—A benzyloxycarbonyl-α-amino acid phenylhydrazide (1.00 mmole) was dissolved in 80 ml of methylene chloride at room temperature. The reaction flask was wrapped so as to exclude light, and lead tetraacetate (0.456 g, 1.03 mmoles) was added. The solution was stirred for 10 min, and the precipitated lead diacetate was removed by filtration. After washing successively with 20 ml each of distilled water, 1 *M* sodium bicarbonate, and distilled water, it was dried with anhydrous magnesium sulfate and evaporated as described in method A.

Benzyloxycarbonyl-L-alanine Phenyldiimide.—Evaporation of the reaction mixture from method A yielded 0.28 g of bright orange cake with some needle clusters. It was dissolved in 3.5 ml of recently distilled *p*-xylene at room temperature, and 3.5 ml of petroleum ether (30–60°) was added. The solution was placed in an ice–salt bath for about 1.5 hr. A 71% yield of bright orange, blunt needles was obtained, mp 66–67°.

Anal. Calcd for $C_{17}H_{17}N_3O_3$: C, 65.58; H, 5.50; N, 13.31. Found: C, 65.35; H, 5.68; N, 13.26.

Benzyloxycarbonylglycine Phenyldiimide.—The dark red cake obtained from evaporation of the reaction mixture from method A or method B was dissolved in 9.0 ml of recently distilled *p*-xylene at room temperature. To the solution was added 15.0 ml of petroleum ether (30–60°) and the solution was placed in an ice bath for about 2 hr. A 72% yield of purple needles was obtained, mp 74.5–75°.

Anal. Calcd for $C_{16}H_{15}N_3O_3$: C, 64.64; H, 5.08; N, 14.13. Found: C, 64.74; H, 5.20; N, 13.95.

Benzyloxycarbonylglycyl-L-phenylalanine Phenyldiimide.—An orange glassy solid (0.34 g, 77%) was obtained from evaporation of the solution pepared by method A and was not recrystallized, mp 20.5–32°.

Benzyloxycarbonyl-L-leucine Phenyldiimide.—A red oil (0.346 g, 98%) resulted from evaporation of the reaction solution prepared by method A or method B and was not further purified.

Benzyloxycarbonyl-L-phenylalanine Phenyldiimide.—A dark orange-red cake containing some needle clusters was obtained from method A. It was dissolved in 20 ml of recently distilled *p*-xylene at room temperature. The solution was filtered through Whatman No. 1 paper, and 80 ml of petroleum ether (30–60°) was added. The solution was placed in an ice–salt bath for about 2 hr. A 79% yield of pale orange needles was obtained, mp 101–103°.

Anal. Calcd for $C_{23}H_{21}N_3O_3$: C, 71.30; H, 5.46; N, 10.85. Found: C, 71.10; H, 5.58; N, 10.75.

Benzyloxycarbonyl-L-proline Phenyldiimide.—The product (0.32 g) from method A was a red-orange oil. It was dissolved in 1.5 ml of cold methylene chloride, and 60 ml of cold petroleum ether (30–60°) was added. The solution was placed in an ice–salt bath until a thin film of oil had begun to deposit on the flask wall. The solution was decanted and evaporated under reduced pressure at 5° until only 5 ml of liquid remained. The liquid was poured off and the oil was washed with 20 ml of cold petroleum ether (30–60°), yielding 0.21 g (62%) of red-orange oil.

Benzyloxycarbonyl-L-alanine *o*-Chlorophenyldiimide.—A 0.32-g purple cake with needle clusters was obtained from method A and was dissolved in 6.0 ml of recently distilled *p*-xylene at room temperature. To this solution was added 8.5 ml of petroleum ether (30–60°). After sitting in an ice–salt bath for 1.5 hr, 0.21 g (61%) of purple needles was obtained, mp 76–77°.

Anal. Calcd for $C_{17}H_{16}ClN_3O_3$: C, 58.71; H, 5.22; Cl, 10.20; N, 12.08. Found: C, 58.83; H, 4.85; Cl, 9.97; N, 11.90.

Benzyloxycarbonylglycine *o*-Chlorophenyldiimide.—The pale orange cake (0.31 g) from method A was dissolved in 15.0 ml of recently distilled *p*-xylene at room temperature, and 9.7 ml of petroleum ether (30–60°) was added. After 2 hr in an ice–salt bath, 0.20 g (59%) of bright orange needle clumps was obtained, mp 92–94°.

Anal. Calcd for $C_{16}H_{14}ClN_3O_3$: C, 57.93; H, 4.25; Cl, 10.69; N, 12.67. Found: C, 57.66; H, 4.20; Cl, 10.43; N, 12.47.

Benzyloxycarbonyl-L-leucine *o*-Chlorophenyldiimide.—A purple cake or film containing needles in a radial pattern was obtained from method A, 0.362 g (93%). To obtain this as a solid rather than an oil, the evaporative process was often extended to periods up to 8 hr. Physical constants were obtained for these radially patterned crystals, mp 59–66°.

Benzyloxycarbonyl-L-phenylalanine *o*-Chlorophenyldiimide.—The 0.38-g purple cake from method A was dissolved in 10.0 ml of recently distilled *p*-xylene and filtered. Addition of 40.0 ml of petroleum ether (30–60°) caused an almost immediate precipitate of fine purple needles in 63.5% yield. Cooling the solution in an ice–salt bath increased the yield to 83%, mp 84–86°.

Anal. Calcd for $C_{23}H_{20}ClN_3O_3$: C, 65.48; H, 4.78; Cl, 8.40; N, 9.96. Found: C, 65.51; H, 4.90; Cl, 8.47; N, 9.99.

Thin Layer Chromatograpic Analysis of Benzyloxycarbonyl Dipeptide Phenylhydrazides.—The analyses of benzyloxycarbonyl dipeptide phenylhydrazides were carried out on 16 × 20 cm chromatoplates covered with a 0.25-mm layer of Silic AR tlc-7 GF. The plates were spotted with known amounts of benzyloxycarbonyl dipeptide phenylhydrazides prepared from the enzymatic synthesis and aliquots from the reaction mixtures of benzyloxycarbonyl amino acid phenyldiimides and amino acid phenylhydrazides. The chromatograms were developed using a chloroform–methyl alcohol solution. The plates were sprayed with an aqueous solution of 70% sulfuric acid saturated with potassium dichromate, and then baked at 200° for 20 min. The density of the carbonized spots was determined with a densitometer (Photovolt Corp., Model 52C and Gilford Model 220 absorbance indicator and optical density converter). The concentrations of benzyloxycarbonyl dipeptide phenylhydrazides in the reaction mixture was determined by comparing the area under the optical density curves with the optical density curves from known amounts of benzyloxycarbonyl dipeptide phenyl-

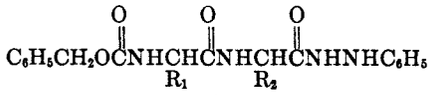
(11) The melting points are corrected. The microanalytical work was performed by the Galbraith Laboratories, Knoxville, Tenn. The infrared spectra were determined with a Beckman IR-5 or IR-8 spectrophotometer, and assignments were based on values cited by Bellamy.[12] Visible and ultraviolet spectra were determined with a Cary Model 14 recording spectrophotometer. Optical rotatory dispersion and circular dichroism curves were determined with a Jasco Model O.R.D./U.V.-5 optical rotatory dispersion recorder with a Durrum-Jasco circular dichroism attachment.

(12) L. J. Bellamy, "The Infrared Spectra of Complex Molecules," 2nd ed, John Wiley and Sons, Inc., New York, N. Y., 1958.

(13) Purified by the method of Fieser: L. F. Fieser and S. Rajagopalan, *J. Am. Chem. Soc.*, **71**, 3935 (1949).

TABLE V

PREPARATION AND PROPERTIES OF BENZYLOXYCARBONYL DIPEPTIDE PHENYLHYDRAZIDES *via* THE PHENYLDIIMIDE METHOD



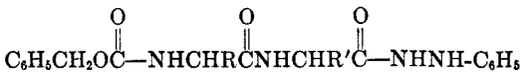
A

R_1	R_2	Vol. of reacn mixture, ml	Tlc, R_f[a]	Yield, % Tlc	Yield, % Isolated	Mp, °C	Time for 90% reacn, min	Benzene, %	$[\alpha]^{25}D$, deg
H	H	30	0.41	93					
H	H	30	0.41	97				46	
H	H	249			66	164–165	9	53	
H	$-CH_2C_6H_5$	288	0.58	64	40	168–169	34	55	−46.0
H	$-CH_2C_6H_5$	350	0.58	66	49	168–169		60	
$-CH_2CH(CH_3)_2$	$-CH_2C_6H_5$	225	0.69	78	54	204–206	123	51	−50.0
$-CH_2CH(CH_3)_2$	$-CH_2C_6H_5$	350			66	204–206			
$-CH_2C_6H_5$	$-CH_2C_6H_5$	225	0.67	73	60	212–214	56	46	−34.0
$-CH_2C_6H_5$	H	50			56	179–180			

[a] Developing solution, $CHCl_3$–MeOH (v/v, 95:5).

TABLE VI

ANALYSES OF BENZYLOXYCARBONYL DIPEPTIDE PHENYLHYDRAZIDES



Benzyloxycarbonyl dipeptide phenylhydrazide	Formula	Calcd, % C	Calcd, % H	Calcd, % N	Found,[a] % C	Found,[a] % H	Found,[a] % N	Found,[b] % C	Found,[b] % H	Found,[b] % N
Glycylglycine	$C_{18}H_{20}N_4O_4$	60.67	5.66	15.72	60.79	5.66	15.59	60.69	5.73	15.64
Glycyl-L-phenylalanine	$C_{25}H_{26}N_4O_4$	67.24	5.87	12.55	67.46	5.75	12.67	67.06	5.98	12.68
L-Leucyl-L-phenylalanine	$C_{29}H_{34}N_4O_4$	69.30	6.82	11.15	69.06	6.68	11.17	69.48	6.89	11.09
L-Phenylalanyl glycine	$C_{25}H_{26}N_4O_4$	67.24	5.87	12.55				66.98	5.72	12.72
L-Phenylalanyl-L-phenylalanine	$C_{32}H_{32}N_4O_4$	71.62	6.01	10.44	71.33	6.20	10.36	71.42	5.94	10.36

[a] Prepared by the enzymatic method. [b] Prepared by the phenyldiimide method.

hydrazides prepared by the enzymatic method. The chromatographic R_f values and the analysis of the reaction mixtures are given in Table V.

Glycyl-L-phenylalanine Phenylhydrazide.—Benzyloxycarbonylglycyl-L-phenylalanine phenylhydrazide (0.89 g, 2.0 mmoles) was dissolved in 125 ml of absolute methanol and subjected to catalytic hydrogenolysis over palladium on powdered chracoal (0.5 g, 10% Pd) for 6 hr at room temperature and atmospheric pressure. The catalyst was removed by filtration and the filtrate was concentrated to dryness *in vacuo* at 30°, yielding a glass, mp 41–51°. This product was recrystallized once from ethyl acetate–petroleum ether (30–60°), mp 69–73°.

Anal. Calcd for $C_{17}H_{20}N_4O_2$: C, 65.37; H, 6.45; N, 17.94. Found: C, 65.15; H, 6.61; N, 17.90.

The infrared spectra gave N—H stretch, 3.06; C=O stretch, 6.05; anilino N—H, 6.26; and phenyl C—H bend, 13.35 and 14.40 μ.

Benzyloxycarbonyl-Gly-L-Phe-Gly-L-Phe Phenylhydrazide.—A solution of benzyloxycarbonyl-gly-L-phe phenyldiimide (0.267 g, 0.600 mmole) in 30.0 ml of anhydrous dioxane was added to a solution of gly-L-phe phenylhydrazide (0.474 g, 1.20 mmoles) in 60.0 ml of anhydrous dioxane. The reaction was 90% complete in 8 min. The solution was stirred for 3 hr at room temperature; then was evaporated *in vacuo* to dryness at 50°. The solid obtained was dissolved in 50 ml of methylene chloride and washed successively with 25-ml portions of 1 *N* hydrochloric acid, distilled water, 1 *N* sodium hydroxide, and distilled water. The solution was dried with anhydrous magnesium sulfate and again evaporated to dryness. The product was recrystallized from ethanol, filtered, and washed with a small amount of cold ether, yielding 0.252 g (64.5%) with mp 225–226.5° dec. Principal peaks in the infrared were at λ_{max}^{KBr} 3.03 (s) (N—H stretch), 5.92 (s) (shoulder, urethan C=O), 6.08 (s) (amide and phenylhydrazide C=O), 6.25 (w) (anilino N—H), 6.51 (m) (amide II), 13.35 (w), and 14.35 μ (m) (phenyl C—H bend). Ultraviolet spectrum was $\lambda_{max}^{dioxane}$ 236 mμ (ϵ 11,920) and 283 mμ (ϵ 1685). The optical rotation was −7.8° (*c* 0.0640). Yields of benzene and nitrogen were 64 and 33%, respectively.

Anal. Calcd for $C_{36}H_{38}N_6O_6$: C, 66.45; H, 5.89; N, 12.92. Found: C, 66.21; H, 5.89; N, 12.69.

The phenylhydrazide was oxidized with N-bromosuccinimide to benzyloxycarbonyl-gly-L-phe-gly-L-phe phenyldiimide: $\lambda_{max}^{dioxane}$ 293 mμ (ϵ 11,720) and 448 mμ (ϵ 113); $[\alpha]_{515}$ (max) +71.8°, $[\alpha]_{416}$ (min) −67.2°; $[\theta]_{455}$ 637° (*c* 0.638, dioxane).

Benzyloxycarbonyl-Gly-L-Phe-Gly Ethyl Ester.—All experiments were conducted in semidarkness and were worked up in a manner analogous to those for preparation of the benzyloxycarbonyl dipeptide phenylhydrazides prepared by the phenyldiimide method. The crude product was recrystallized several times by dissolution in hot ethanol followed by addition of water to incipient precipitation. Fractional crystallization by the method of Anderson and Callahan[9] was then used to determine the extent of racemization. Three preparations were made.

Preparation 1.—Benzyloxycarbonylglycyl-L-phenylalanine phenyldiimide (0.98 g, 2.24 mmoles) was dissolved in 100 ml of methylene chloride and added dropwise to a solution of glycine ethyl ester hydrochloride (0.62 g, 4.47 mmoles) and triethyl amine (0.45 g, 4.47 mmoles) in 100 ml of methylene chloride at 40°. The reaction was allowed to proceed at 40° for 1 hr before work-up. The crude yield was 0.45 g (46%) which yielded 4.5% of the racemic compound, mp 132–133.5°, lit.[9] mp 132–134°. Infrared spectrum was λ_{max}^{KB} 3.04 (s) (N—H stretch), 5.75–6.10 (7 peaks, with that at 6.10 being the strongest), and 13.50 (w) and 14.40 μ (w) (phenyl C—H bend).

Anal. Calcd for $C_{28}H_{27}N_3O_6$: C, 62.57; H, 6.16; N, 9.52. Found: C, 62.45; H, 6.12; N, 9.51.

Preparation 2.—Glycine ethyl ester (0.24 g, 1.73 mmoles) in 50 ml of methylene chloride was added dropwise to a stirring solution of benzyloxycarbonylglycyl-L-phenylalanine phenyldiimide (0.38 g, 0.85 mmole) in 50 ml of methylene chloride at room temperature. The reaction was allowed to proceed 15 min before work-up. The crude yield was 0.13 g (35%), which gave 2.4% of racemic compound, mp 131–133°.

Preparation 3.—Glycine ethyl ester (0.21 g, 2.0 mmoles) in 100 ml of dry dioxane was added to a stirred solution of benzyloxycarbonylglycyl-L-phenylalanine phenyldiimide (0.44 g, 1.0 mmole) in 50 ml of dry dioxane. The reaction was continued for

3 hr before work-up. Crude yield was 0.34 g (77%) from which only the L isomer was recovered, mp 118–119°, lit.[9] mp 118–119°. The infrared spectrum showed absorption at λ_{max}^{KBr} 3.04 (m) (N–H stretch), 5.74 (s) (ester C=O stretch) 5.87 (s) (C=O stretch), 6.10 (s) (amide C=O stretch), 8.18 (s) and 8.34 (s) (C–O stretch), and 13.60 (w), 14.30 μ (m) (phenyl C—H) bend).

Registry No.—A (Table V; $R_1 = CH_2C_6H_5$; $R_2 = H$), 14746-00-0; glycyl-L-phenylalanine phenylhydrazide, 14721-35-8; benzyloxycarbonyl-Gly-L-Phe-Gly-L-Phe phenylhydrazide, 14721-36-9; benzyloxycarbonyl-Gly-L-Phe-Gly ethyl ester, 2073-59-8.

Bile Acids. XXIII. A New Direct Synthesis of Allocholic Acid and Its 3β Isomer[1]

M. N. MITRA AND WILLIAM H. ELLIOTT

Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

Received August 22, 1967

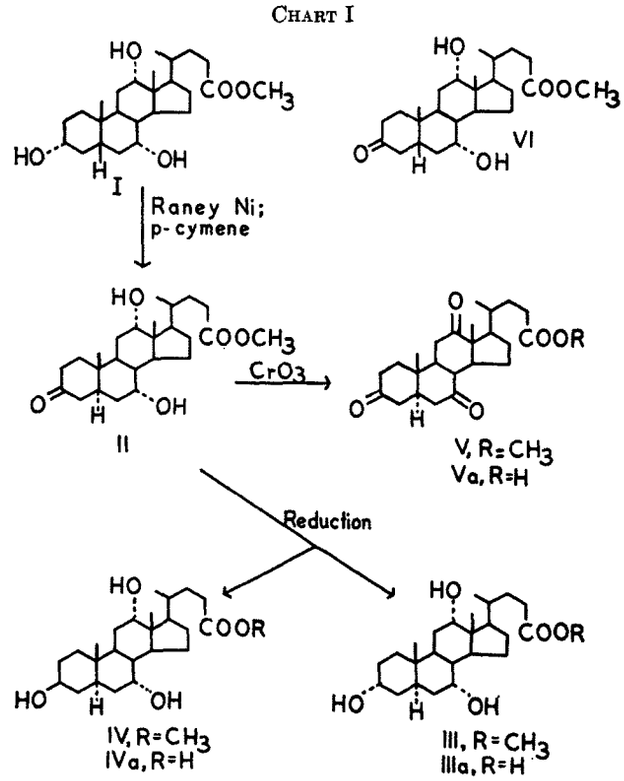
Treatment of methyl cholate with Raney nickel in boiling *p*-cymene afforded a mixture from which methyl 3-keto-7α,12α-dihydroxy-5α-cholanoate could be separated. Catalytic reduction of the latter substance provided allocholic acid as the major product; reduction with sodium borohydride afforded a better yield of the 3β epimer. Supporting evidence for the structures of these substances is provided by mass spectrometry and other physical properties and by chemical degradation. Correlation of the structures of the products of degradation with the parent substances is discussed.

Allocholic acid (3α,7α,12α-trihydroxy-5α-cholanoic acid) has recently been of great interest because of its wide-spread occurrence in a number of sources, *e.g.*, several species of fish,[2,3] snakes,[4] the salamander,[5] penguin,[6] leopard seal,[4] chicken,[7] and several mammals including man.[8,9] We have demonstrated that allocholic acid is a major biliary metabolite[10,11] in the rat after administration of cholestan-3β-ol-4-^{14}C. Continuing studies in this laboratory have shown the need for larger quantities of this material than are normally obtained from natural sources.

Synthetic allocholic acid was first reported by Anderson and Haslewood[12a] as a mixture with cholic acid from catalytic reduction of methyl 3α,12α-diacetoxy-7-keto-Δ^5-cholenoate. Subsequently[12b] they prepared allocholic acid from 3α,7β,12α-trihydroxy-6-keto-5α-cholanoic acid, a substance derived from methyl cholate. However, the latter method involves a number of steps and in our hands[10] provided a low yield of final product. The method described here consists essentially of two steps: (i) the conversion of methyl cholate (I) to methyl 3-keto-7α,12α-dihydroxy-5α-cholanoate (II) and (ii) reduction of II to methyl 3α,7α,12α-trihydroxy-5α-cholanoate (methyl allocholate) (III) followed by alkaline hydrolysis to the free acid. The melting points of the methyl and ethyl esters of allocholic acid prepared by this method agree with those of Haslewood,[4,12b] although the free acid melts at a higher temperature. In view of this difference additional studies are reported which support the assignment of structure of the intermediates and their derivatives.

The first step in this synthesis (see Chart I) was conveniently carried out utilizing the method of Chakravarti, Chakravarti, and Mitra[13] who reported that Raney nickel isomerizes *cis*-A/B to *trans*-A/B steroids

CHART I



(1) (a) This investigation was supported in part by the National Institutes of Health (Grant No. HE-07878 and AM-09992) and by an American Cancer Society Institutional Grant. (b) Presented in part at the 152nd Meeting of the American Chemical Society, New York, N. Y., Sept 1966. (c) For Paper XXII in this series, see H. J. Karavolas, W. H. Elliott, S. L. Hsia, E. A. Doisy, Jr., J. T. Matschiner, S. A. Thayer, and E. A. Doisy, *J. Biol. Chem.*, **240**, 1568 (1965). (d) The following abbreviations have been used: tlc, thin layer chromatography; plc, preparative layer chromatography; glpc, gas–liquid partition chromatography; TMSi, trimethylsilyl derivatives; R_t, retention time relative to methyl deoxycholate (methyl 3α,12α-dihydroxy-5β-cholanoate; absolute time = 29 min); R_t (TMSi), retention time relative to trimethylsilyl derivative of methyl deoxycholate (absolute time = 16.8 min).

(2) G. A. D. Haslewood, *Ann. N. Y. Acad. Sci.*, **90**, 877 (1960).

(3) T. Sasaki, *J. Biochem.* (Tokyo), **60**, 56 (1966).

(4) G. A. D. Haslewood, *Biochem. J.*, **78**, 352 (1961).

(5) K. Amimoto, *J. Biochem.* (Tokyo), **59**, 340 (1966).

(6) I. G. Anderson, G. A. D. Haslewood, and I. D. P. Wootton, *Biochem. J.*, **67**, 323 (1957).

(7) G. A. D. Haslewood in "The Biliary System," W. Taylor, Ed., F. A. Davis Co., Philadelphia, Pa., 1965, pp 106–116.

(8) A. R. Tammer, *Biochem. J.*, **98**, 25p (1966).

(9) P. Eneroth, B. Gordon, and J. Sjövall, *J. Lipid Res.*, **7**, 524 (1966).

(10) See ref given in 1c.

(11) H. J. Karavolas and W. H. Elliott in "The Biliary System," W. Taylor, Ed., F. A. Davis Co., 1965, pp 175–181.

(12) (a) I. G. Anderson and G. A. D. Haslewood, *Biochem. J.*, **74**, 37 (1960). (b) I. G. Anderson and G. A. D. Haslewood, *ibid.*, **85**, 236 (1962), reported mp 229–232°, $[\alpha]^{22}D$ +28 ± 1°, for Va; mp about 225° for III; and mp 239–41°, $[\alpha]^{22}D$ +23 ± 1°, for IIIa.

(13) D. Chakravarti, R. N. Chakravarti, and M. N. Mitra, *Nature*, **193**, 1071 (1962).