Experimental Section[8]

3-Phthalimido-4-(1'-formyl-1'-methylethylthio)-2-azetidinone (**4**). To a stirred solution of 3.68 ml. of 1 *N* hydrochloric acid in 300 ml. of 50% aqueous tetrahydrofuran at room temperature was added dropwise during 3.25 hr. a solution of 1.26 g. (3.68 mmoles) of 2,2-dimethyl-6-phthalimido-3-penamyl isocyanate (**2**)[5] in 200 ml. of tetrahydrofuran. The reaction mixture was stirred for an additional 0.5 hr. after the addition of the isocyanate was complete. The reaction mixture was then extracted with three 200-ml. portions of methylene chloride and the combined methylene chloride extracts were washed with two 150-ml. portions of water, dried over anhydrous magnesium sulfate, filtered, and concentrated under reduced pressure to an oil. From this oil was obtained by fractional crystallization from an ethanol–petroleum ether (b.p. 30–60°) mixture 0.02 g. of *N,N'-bis(2,2-dimethyl-6-phthalimido-3-penamyl)urea* (**5**), which, after recrystallization from acetone–petroleum ether, was obtained as a hygroscopic crystalline solid, m.p. 184–185.5°.

Anal. Calcd. for $C_{31}H_{28}N_6O_7S_2 \cdot 3H_2O$: C, 52.08; H, 4.80; N, 11.74; S, 8.97. Found: C, 52.10; H, 4.60; N, 11.52; S, 8.66.

The infrared spectrum (CH_2Cl_2) showed 3320–3410 (urea N—H), 1790 (β-lactam C=O), 1775 and 1725 (phthalimido group), 1685 (urea C=O), and 1525 cm.$^{-1}$ (urea N—H).

The n.m.r. spectrum ($CDCl_3$) showed τ 2.25 (8 H, multiplet), 3.95 (2 H, doublet, J = 9 c.p.s.), 4.25 (2 H, doublet, J = 9 c.p.s.), 4.45 (2 H, doublet, J = 4 c.p.s.), 4.60 (2 H, doublet, J = 4 c.p.s.), 8.32 (6 H, singlet), and 8.46 (6 H, singlet).

The filtrate after removal of the urea was concentrated to a viscous oil which on crystallization from benzene–ligroin (b.p. 90–100°) yielded in four crops 1.0 g. (76%) of the aldehyde **4**, m.p. 101–105°. Two recrystallizations from benzene–ligroin gave an analytical sample, m.p. 110–119°.

(8) Melting points were determined on a Kofler hot-stage microscope and are uncorrected. The infrared spectra were measured on a Perkin-Elmer Model 237 recording spectrophotometer. A Varian Associates A-60 instrument was used for recording nuclear magnetic resonance spectra and peak positions are reported in τ units (TMS = τ 10).

Anal. Calcd. for $C_{15}H_{14}N_2O_4S \cdot 0.5C_6H_6$: C, 60.47; H, 4.80; N, 7.85. Found: C, 60.21; H, 4.99; N, 7.71.

The infrared spectrum (CH_2Cl_2) showed 3400 (β-lactam N—H), 2810 and 2700 (aldehyde C—H), 1798 and 1730 (phthalimido group), 1770 (β-lactam C=O), and 1720 cm.$^{-1}$ (aldehyde C=O).

The n.m.r. spectrum ($CDCl_3$) showed τ 0.7 (1 H, singlet), 2.15 (4 H, multiplet), 2.65 (3 H, singlet, 0.5 mole of benzene solvate), 2.92 (1 H, broad), 4.32 (1 H, multiplet), 5.08 (1 H, doublet), 8.6 (3 H, singlet), and 8.7 (3 H, singlet).

3-Phthalimido-4-(1'-carboxy-1'-methylethylthio)-2-acetidinone (**6**). To a stirred solution of 2.0 g. (5.6 mmoles) of the aldehyde **4** in 50 ml. of acetone (distilled from potassium permanganate) at room temperature was added dropwise the Jones reagent[7] until decolorization no longer occurred. Approximately 2.3 ml. of the reagent was required. The reaction mixture was then diluted with 50 ml. of methylene chloride and 50 ml. of water was added. The organic layer was separated and the aqueous layer was extracted further with 100 ml. of 1:1 methylene chloride–acetone. The organic extracts were combined and washed with 50 ml. and then 20 ml. of water. The solution was then dried over anhydrous magnesium sulfate, filtered, and concentrated. The oily residue crystallized from benzene–petroleum ether (b.p. 30–60°) to yield 1.56 g. (83%) of the crude acid **6**, m.p. 135–160°. Recrystallization from methylene chloride gave 0.95 g. (51%) of the acid **6**, m.p. 190–195°. Two further recrystallizations from methylene chloride gave an analytical sample, m.p. 193.5–195.5°.

Anal. Calcd. for $C_{15}H_{14}N_2O_6S$: C, 53.94; H, 4.32; N, 8.39; S, 9.60. Found: C, 54.09; H, 4.36; N, 8.43; S, 9.59.

The infrared spectrum (KBr) showed 3400 (β-lactam N—H), 1775 and 1725 (phthalimido and carboxyl C=O), 1755 (β-lactam C=O), and 1275 cm.$^{-1}$ (carboxyl C—O).

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Benzhydryl Esters of Amino Acids in Peptide Synthesis[1]

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Contribution from the Department of Biochemistry, Yale University, New Haven, Connecticut. *Received September 23, 1965*

Benzhydryl esters of amino acids and peptides have been prepared in good yield by reaction of their p-toluenesulfonate salts with diphenyldiazomethane. In some cases, the salts of other aromatic sulfonic acids (e.g., β-naphthalenesulfonic acid) may be preferable. Examples are given of the use of the benzhydryl ester salts in representative coupling reactions to yield protected peptide derivatives, and of the removal of the benzhydryl group by catalytic hydrogenolysis.

Although significant progress has been made in the development of methods for the protection of carboxyl

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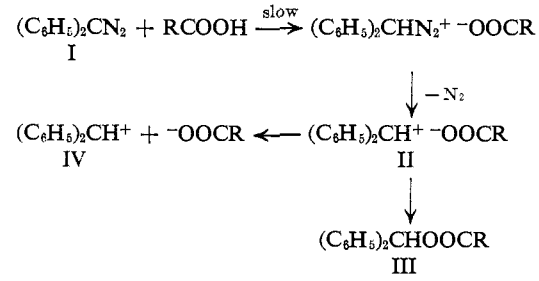
(2) Alexander Brown Coxe Fellow of the Yale School of Medicine, 1964–1965.

groups in the synthesis of peptides, the number of carboxyl-protecting groups is relatively small. In addition to the traditional methyl and ethyl esters, whose cleavage requires saponification, the benzyl (and *p*-nitrobenzyl) and *t*-butyl esters occupy the leading place because of their ready scission (by hydrogenolysis or acidolysis) under conditions that usually do not affect peptides adversely. Several newer methods are available for the preparation, in satisfactory yield, of benzyl esters,[3-5] of *p*-nitrobenzyl esters,[6,7] and of *t*-butyl esters[8,9] of free amino acids. The purpose of the present communication is to describe the facile preparation of diphenylmethyl (benzhydryl) esters of amino acids, and their use for the synthesis of peptides.[10]

Hardegger, *et al.*,[11] described the preparation of benzhydryl esters of various carboxylic acids by reaction with diphenyldiazomethane (DDM) and the ready removal of the blocking group by catalytic hydrogenolysis. This method does not appear to have been applied extensively in the amino acid field and has been limited thus far to the esterification of N-protected amino acids. Bethell, *et al.*,[12] prepared the benzhydryl ester of benzyloxycarbonyl-4-methylene-L-proline; after conversion of the product to the 4-hydroxymethyl-L-proline derivative, both blocking groups were removed by catalytic hydrogenolysis. Hiskey and Adams[13] prepared the benzhydryl esters of S,N-ditrityl-L-cysteine, benzyloxycarbonylglycine, phthaloylglycine, and hippuric acid; these investigators showed that the benzhydryl group of benzhydryl hippurate can be removed readily by acidolysis. For use in peptide synthesis, however, the benzhydryl esters of free amino acids are needed; previous attempts[13] to prepare such esters either by azeotropic distillation (as in the preparation of benzyl esters[3]) or by reaction with DDM appear to have been unsuccessful.

The mechanism of the reaction of DDM (I) with carboxylic acids has been investigated extensively.[14] The main features of the currently accepted mechanism may be represented as involving a rate-determining, proton-transfer reaction leading to the formation of an ion pair (II) which can either collapse to give the benzhydryl ester (III) or dissociate to form solvent-separated ions. The subsequent fate of the benzhydryl carbonium ion (IV) depends on the nucleophiles (including DDM) in the medium.

These features of the reaction indicate the conditions for the successful preparation of the amino acid benzhydryl esters. For optimal utilization of the carbonium ions for ester formation, it is necessary that the ion pair II be stabilized with respect to the solvent-separated components, and that no additional reactive nucleophiles (*e.g.*, chloride) be introduced into the



reaction medium. These two conditions are well met by the use of the poorly nucleophilic aryl sulfonate or perchlorate ion as the counterion to the NH_3^+ group of the amino acid, and of a dipolar aprotic solvent[15] such as dimethylformamide. The major impurities to be expected in these circumstances are benzophenone azine ($(C_6H_5)_2C{=}N{-}N{=}C(C_6H_5)_2$), formed by the reaction of the thermally generated carbene with DDM[16] or by the reaction of the benzhydryl cation with DDM, and tetraphenylethylene produced by acid-catalyzed decomposition of DDM. In the present work, the formation of the azine was noted, but no evidence was obtained for the formation of tetraphenylethylene.

In our synthesis of the benzhydryl esters of free amino acids, use was largely made of the *p*-toluenesulfonates of the amino acids to be esterified; the *p*-toluenesulfonate of glycylglycine also was prepared for this purpose. The studies of Bergmann, *et al.*,[17-19] on the salts of protein amino acids with a large variety of aromatic sulfonic acids have provided data that may be valuable for the modification of the general method described in this paper. Examples are given in the Experimental Section for the preparation of the β-naphthalenesulfonate salts of the benzhydryl esters of L-arginine and of L-histidine.

Preliminary studies on the rate of reaction of DDM with substituted acetic acids in dimethylformamide indicate a correlation between log *k* and the pK_a of the acids (in water). This leads to the expectation that the α-carboxyl group of aspartic acid or glutamic acid will be much more reactive than the β- (or γ-) carboxyl group. Experiments are in progress to determine the optimum conditions for the synthesis of the α-benzhydryl esters of L-aspartic and L-glutamic acid in pure form.

To test the utility of the amino acid benzhydryl esters for the peptide synthesis, two coupling reactions were examined: (1) benzyloxycarbonylglycine and L-phenylalanine benzhydryl ester by the mixed anhydride method (ethyl chloroformate); (2) benzyloxycarbonyl-L-phenylalanine and L-alanine benzhydryl ester by the dicyclohexylcarbodiimide method. The results (details in the Experimental Section) indicate that the bulky

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(11) E. Hardegger, Z. El. Heweihi, and F. G. Robinet, *Helv. Chim. Acta*, **31**, 439 (1948).
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diphenylmethyl group does not appear to prevent satisfactory coupling reactons, even with amino acid residues having large side chains. The hydrogenolysis of the benzyloxycarbonyl dipeptide benzhydryl esters was conducted in the usual manner with palladium black and proceeded smoothly; with the catalyst employed, the hydrogenolysis of both blocking groups usually was complete within 1 hr.

Experimental Section

Preparation of p-Toluenesulfonates. The amino acid or peptide (0.02–0.1 mole) was dissolved or suspended in water (10–100 ml.), and *p*-toluenesulfonic acid monohydrate (1.05 molar equiv.) was added. The mixture was stirred and, if necessary, heated to obtain a clear solution, which was then concentrated under reduced pressure. The resulting product was washed with cold acetone. The salts of glycine and L-alanine were recrystallized from water–acetone–ether; the other salts listed in Table I were recrystallized from hot acetone.

Table I. *p*-Toluenesulfonate Salts of Amino Acids and Peptides

p-Toluenesulfonate of	Yield, %	M.p., °C.	N Calcd.	N Found
Glycine	95	196–197	5.67	5.59
L-Alanine	95	210	5.36	5.31
L-Valine	86	145–146	4.84	4.76
L-Leucine	91	162–164	4.62	4.65
L-Methionine	98	159–161	4.36	4.37
L-Serine	86	173–175	5.06	4.98
L-Threonine	92	88–90	4.81	4.80
L-Glutamic acid	85	156–158	4.39	4.27
L-Phenylalanine	97	165–167	4.15	4.15
Glycylglycine	98	181–183	9.22	9.11

Preparation of Benzhydryl Ester p-Toluenesulfonates. DDM was prepared by the procedure of Smith and Howard.[20] The amino acid (or peptide) *p*-toluenesulfonate (0.01–0.02 mole) was dissolved in dimethylformamide (5 ml.) and warmed to 50°, and DDM (1.5 molar equiv. in *ca.* 10 ml. of dimethylformamide) was added with stirring. After 10 min., the reaction mixture was concentrated to dryness under reduced pressure, and the resulting product was recrystallized from acetonitrile (see Table II).

Table II. Benzhydryl Ester *p*-Toluenesulfonates

Ester salt of	Yield, %	M. p., °C.	N Calcd.	N Found
Glycine	90	140–142	3.47	3.50
L-Alanine	82	176–177	3.20	3.21
L-Valine	77	170–171	3.08	3.10
L-Leucine	88	197–198	2.98	3.13
L-Methionine	82	169–171	2.87	2.92
L-Serine	75	158–159	3.16	3.19
L-Threonine	82	153–154	3.06	3.19
L-Phenylalanine	86	195–197	2.78	2.85

(20) L. I. Smith and K. L. Howard, "Organic Syntheses," Coll. Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1955, p. 351.

L-Arginine Benzhydryl Ester Di-β-naphthalenesulfonate. L-Arginine di-β-naphthalenesulfonate[17] (5.9 g., 0.01 mole) was treated with DDM in the manner described above. The product was precipitated from acetonitrile by the addition of ether, and the resulting oil was crystallized from methanol, yield 6.0 g. (78%). *Anal.* Calcd. for $C_{39}H_{40}N_4O_8S_2$: N, 7.40. Found: N, 7.49.

L-Histidine Benzhydryl Ester Di-β-naphthalenesulfonate. L-Histidine di-β-naphthalenesulfonate[17] (4.3 g., 7.5 mmoles) was treated with DDM in the manner described above, yield, 2.9 g. (53%). *Anal.* Calcd. for $C_{39}H_{36}N_3O_8S_2$: N, 5.69. Found: N, 5.89.

Glycyl-L-phenylalanine. A mixed anhydride was prepared in the usual manner from benzyloxycarbonylglycine (1.05 g., 5 mmoles) in cold dimethylformamide (30 ml.) with triethylamine (0.51 g., 5 mmoles) and ethyl chloroformate (0.54 g., 5 mmoles). The reaction mixture was added to a solution of L-phenylalanine benzhydryl ester *p*-toluenesulfonate (2.5 g., 5 mmoles) and triethylamine (0.51 g., 5 mmoles) in dimethylformamide (30 ml.). After being stirred at room temperature overnight, the reaction mixture was concentrated to dryness, and the residue was dissolved in methylene chloride (70 ml.). The solution was washed successively with three 20-ml. portions each of dilute bicarbonate, dilute hydrochloric acid, and water, and then was dried over sodium sulfate. The residue obtained upon evaporation to dryness was recrystallized from ethyl acetate–ether to give 1.35 g. (52%) of product, which melted at 119–120°. *Anal.* Calcd. for $C_{32}H_{30}N_2O_5$: N, 5.36. Found: N, 5.31.

The above protected dipeptide derivative (0.41 g.) was hydrogenated in the usual manner over palladium in methanol (20 ml.). After 1 hr., the catalyst was removed by filtration, and the filtrate was concentrated to dryness to yield 0.16 g. (90%) of the dipeptide, $[\alpha]^{25}D$ 39.9° (*c* 1.6, water); lit.[21] $[\alpha]^{25}D$ +41.5° (*c* 2.0, water). *Anal.* Calcd. for $C_{11}H_{14}N_2O_3$: N, 12.6. Found: N, 12.8. Ascending paper chromatography (Whatman No. 1) showed one spot, R_f 0.60, with 1-butanol–acetic acid–water (4:1:5), and one spot, R_f 0.74, with phenol–water (4:1).

L-Phenylalanyl-L-alanine. Benzyloxycarbonyl-L-phenylalanine (1.49 g., 5 mmoles) was coupled in the usual manner with L-alanine benzhydryl ester *p*-toluenesulfonate (2.14 g., 5 mmoles) in the presence of dicyclohexylcarbodiimide (1.03 g., 5 mmoles) and triethylamine (0.5 g., 5 mmoles), with acetonitrile (120 ml.) as the solvent. The reaction mixture was allowed to stand overnight, filtered, and evaporated to dryness under reduced pressure. The residue was taken up in ethyl acetate, and the solution was washed successively with three 20-ml. portions of each of water, dilute hydrochloric acid, dilute bicarbonate, and water, and then dried over sodium sulfate. The solution was then concentrated to dryness, and the resulting product was crystallized from cyclohexane. Recrystallization from ethyl acetate gave 1.6 g. (61%) of the product, which melted at 120–121°. *Anal.* Calcd. for $C_{33}H_{32}N_2O_5$: N, 5.22. Found: N, 4.97.

The above protected dipeptide derivative (0.36 g.)

(21) J. P. Greenstein, M. Winitz, P. Gullino, S. M. Birnbaum, and M. C. Otey, *Arch. Biochem. Biophys.*, **64**, 342 (1956).

was hydrogenated in the usual manner over palladium in methanol (20 ml.) to yield 0.13 g. (86%) of the dipeptide: $[\alpha]^{25}D$ +13.2° (*c* 2.1, water); lit.[22] $[\alpha]^{20}D$ +12.8 (*c* 2.0, water). *Anal.* Calcd. for $C_{12}H_{16}N_2O_3$: N, 11.9. Found: N, 12.1. Paper chromatography showed one spot, R_f 0.62, with 1-butanol–acetic acid–water, and one spot, R_f 0.80, with phenol–water.

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A Nuclear Magnetic Resonance Study of the Structures of L- and *meso*-Cystine in Aqueous Solutions[1]

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The n.m.r. spectra of L-*cystine, meso-cystine, and their dimethyl esters in acidic and basic solutions are analyzed. The results indicate that the possible configurations for* L-*cystine are stablized by intramolecular interactions between the two moieties. This is in contrast to meso-cystine where no stabilization is observed.*

Introduction

There have been several analyses of the n.m.r. spectra of the L-amino acids, including L-cystine. Taddei and Pratt[2] have observed the chemical shift changes of the various protons as a function of pH. Pachler,[3,4] Fujiwara and Arata,[5,6] and Martin and Mathur[7] have analyzed the high-resolution spectra of these acids in a fashion which can be interpreted to give the relative populations of the three classical rotamers derived from considering the α-amino acids as derivatives of ethane.

The case of the cystines is, however, more complicated than these studies would indicate, and some of the peculiarities of their spectra may throw some light on the interpretation of the spectra of the other amino acids.

The structure of L-cystine in solution has been considered by others[8–10] theoretically, in connection with the very large optical activity exhibited by the molecule. This is a factor of 10 greater than any other amino acid. The disulfide ultraviolet absorption band is optically active,[10a] and studies of this phenomenon in solution indicate that there is only very highly hindered rotation about the disulfide bond. In fact it would appear that the disulfide portion of the structure of the cystines is represented by structures I and II, in which the dihedral angle is 90°.

I II

There are two cases which must be considered in a discussion of the cystines in solution. The first is when R = R′. This is either all L- or all D-cystine. The two structures I and II do not necessarily have the same energy. This is apparent from the use of space filling models and is, of course, theoretically valid. Thus, the possibility of one of the structures (I or II) being predominant in solution obtains.

The second cases arises in a cystine molecule where one moiety is of the L optical isomer, and the other is of the D variety. This is defined as *meso*, or internally compensated, cystine. For this case, by the same argument as above, both structures I and II have the same energy.

There have been speculations[11] concerning the possibility of an endocyclic configuration of L-cystine brought about by binding between the opposing amino and carboxyl groups of the two moieties. The presence of this structure would fit in with the observation that cystine has a strong temperature coefficient of molecular optical rotation, indicating an equilibrium between structural forms.

All previous analyses of the n.m.r. spectra of the simple amino acids in solution are based on the classical rotamer structures which neglect intramolecular interactions other than steric hindrance in the *trans* and *gauche* configurations. Absence of intramolecular interactions leads to the conclusion that both L- and *meso*-cystine should have the same n.m.r. spectra. It was of some interest to determine whether or not this is true.

Experimental Section

The n.m.r. spectra were taken with a DA-60 Varian n.m.r. spectrometer at an in-probe temperature of 26 ± 1° as measured by the ethylene glycol method. An internal standard of TSS (3-trimethylsilyl-1-propanesulfonic acid sodium salt) was used. pH measurements were made with a Beckman one-drop electrode useful in the range 0–11 pH units, and no attempt was

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