normal urine, consisting of glycine, alanine and glutamic acid (2:1:1). The remaining peptides were all dipeptides. It is interesting to observe that all peptides identified contained one of the dicarboxylic amino acids. This may be one of the sources of the considerable increase in the amounts of dicarboxylic amino acids after hydrolysis of urine, found by Dunn and co-workers.[18]

Calculations have been made of the theoretical retardation volumes of several of those amino acids which emerged with the 2 *M* HCl eluate. The equation used for these calculations was given.[10] The theoretical retardation volumes for the amino acids behaving as single solutes are compared with the observed retardation volumes on column III in Table V.

In view of the complexity of the ampholyte fraction, agreement is surprisingly good.

TABLE V

RETARDATION VOLUMES OF AMINO ACIDS ELUTED WITH 2 *M* HCl FROM 25 G. COLUMN OF DOWEX 50

Run	II, 0.285 mmole of ampholyte fraction of normal urine		IV, 0.379 mmole of ampholyte fraction of pathological urine	
Amino acid	Retardation volume Obsd.	Calcd.	Retardation volume Obsd.	Calcd.
Threonine	46	46	44	47
Hydroxy-proline	64	54	62	53
Glycine	74	73	72	73
Alanine	82	83	84	73
Proline	140	118	142	114

Acknowledgment.—The author is indebted to Dr. R. Keith Cannan for his advice and criticism during the course of this work.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, AND THE CLAYTON FOUNDATION FOR RESEARCH]

Synthesis of N-Phosphoryl Amino Acid Esters

BY SI-OH LI[1]

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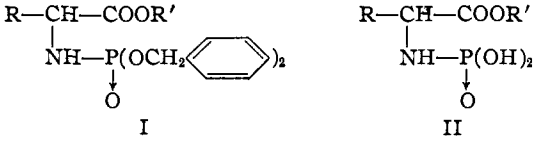
The synthesis of N-phosphoryl amino acid methyl esters of DL-phenylalanine and DL-tryptophan has been carried out by hydrogenolysis of the corresponding dibenzylphosphoryl compounds. The isolation of four dibenzylphosphoryl amino acid esters is reported.

Because of the increasing realization of the importance of phosphate compounds in biological systems, we have undertaken the preparation of some N-phosphoryl amino acid derivatives to be used as substrates for the study of the properties and physiological functions of phosphoamidase, an enzyme which specifically splits the P–N bond of the phosphoamide.[2] The direct N-phosphorylation of amino acids has been done by Neuberg and Oertel,[3] and later by Winnick and Scott,[4] using phosphorus oxychloride as the phosphorylating agent; however, the yields were low and the compounds thus isolated were readily hydrolyzed in acid solution.[4] Amino acid esters have been phosphorylated with diphenylphosphoryl chloride by Sciarini and Fruton[5] and with diisopropylphosphoryl chloride by Wagner-Jauregg, *et al.*[6] In both methods, the reaction took place without difficulty; however, the preparation of pure N-phosphoryl amino acid esters through hydrogenolysis of diphenylphosphoryl amino acid esters was not entirely successful.[5]

The method described in the present investigation is based on the reaction of dibenzylphosphoryl chloride (DBPCl) with an amine in organic solvents and the subsequent removal of the benzyl groups by hydrogenolysis with palladium oxide[7,8] to give compounds with structures (I) and (II), respectively.



I II

Thus an amino acid ester was first liberated from its hydrochloride salt according to the procedure of Hilmann[9] with slight modification. The free ester was then condensed with DBPCl in the presence of triethylamine. By this method the phosphorylation of about ten amino acid esters have so far been tried, but only four dibenzylphosphoryl amino acid esters have been isolated in good yield (Table I). These compounds are soluble in organic solvents but insoluble in water. Evidence for the attachment of the phosphoryl group onto the nitrogen atom in the phosphorylated compounds is given by the fact that these substances do not show on paper chromatograms treated with ninhydrin, whereas the unphosphorylated amino acid esters give characteristic spots.[10]

(1) Rosalie B. Hite Predoctorate Fellow, The University of Texas.

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(10) Contrary to general belief and to statements appearing in several standard reference books, many amino acid derivatives and other amines that do not possess a free α-carboxyl group will react with ninhydrin to give a distinct color reaction, *cf.* A. H. Cook and A. L. Levy, *J. Chem. Soc.*, 646 (1950). It is only when evolution of CO_2 is measured that the ninyhdrin reaction is specific for free α-amino acids.

TABLE I

DIBENZYLPHOSPHORYL AND N-PHOSPHORYL AMINO ACID ESTERS

Products	R and R′ in I R	R′	Yield, %	M.p.,[a] °C.	Formula	Carbon,[b] % Calcd.	Found	Hydrogen,[b] % Calcd.	Found	Nitrogen,[c] % Calcd.	Found	Phosphorus,[d] % Calcd.	Found
Dibenzylphosphoryl glycine ethyl ester[e]	-H	$-C_2H_5$	85	43–45	$C_{18}H_{22}O_5NP$	59.4	59.3	6.10	6.11	3.86	3.78	8.54	8.55
Dibenzylphosphoryl DL-alanine methyl ester[e]	$-CH_3$	$-CH_3$	88	40–41	$C_{18}H_{22}O_5NP$	59.4	59.92	6.10	6.37	3.86	3.91		
Dibenzylphosphoryl DL-phenylalanine methyl ester	$-C_7H_7$	$-CH_3$	93[a] 84[b]	82–83	$C_{24}H_{26}O_5NP$	65.7	65.54	5.97	6.05	3.18	3.04	7.06	6.88
Dibenzylphosphoryl DL-tryptophan methyl ester	$-C_9H_8N$	$-CH_3$	83	104.5–105	$C_{26}H_{27}O_5N_2P$	65.3	65.51	5.68	5.69	5.85	5.73	6.58	6.44
	R and R′ in II R	R′											
N-Phosphoryl DL-phenylalanine methyl ester	$-C_7H_7$	$-CH_3$	78	143–145	$C_{10}H_{14}O_5NP$	43.3	43.69	5.81	5.73	5.05	5.03	11.2	11.6
N-Phosphoryl DL-tryptophan methyl ester	$-C_9H_8N$	$-CH_3$	72.2	130–132	$C_{12}H_{17}O_5N_2P$	45.5	46.36	5.42	6.02	8.85	8.30	9.81	9.83

[a] Corrected. [b] Analyzed by the Clark Microanalytical Laboratory, Urbana, Illinois. [c] Determined by micro-Kjeldahl method.[11] [d] Determined by the method of Lowry and Lopez[12] after preliminary digestion with sulfuric acid and nitric acid.[13] [e] Prepared in the research Laboratory of Sadolin and Holmblad A/S, Copenhagen, Denmark, and analyzed by Mr. Conali. The author is indebted to Dr. N. Clauson-Kaas for his kind interest in this work.

The phosphorylated compounds of the methyl esters of DL-valine and DL-leucine crystallized out in needles at −18° but melted again during the filtration of crystals. DL-Histidine methyl ester gave only a viscous liquid with DBPCl, which could be neither crystallized nor distilled in vacuum without decomposition.

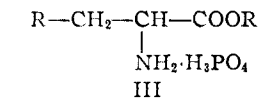
The reaction with serine methyl ester produced an oily substance which has not been isolated in crystalline form but gives the elementary analytical data corresponding to the dibenzylphosphoryl compound. We are not sure whether the dibenzylphosphoryl group is bound to the oxygen atom of the hydroxyl group or to the nitrogen atom of the amino group, since the compound does show a weak spot of ninhydrin reaction on the paper chromatogram. Further work is being done along this line.

With the product obtained from tyrosine methyl ester we have found on the basis of phosphorus content that both the α-amino group and the phenolic group can react with DBPCl and the ninhydrin reaction of this product was negative.

The phosphorylation with DBPCl in the presence of two moles of triethylamine was found to proceed equally well if the hydrochloride salt of the amino acid ester was used directly.

The hydrogenolysis of the dibenzylphosphoryl compounds was carried out in the usual manner.[7,8] However, only two of the hydrogenolysis products have been isolated in crystalline form (Table I). These compounds are hygroscopic, soluble in methanol and water but insoluble in organic solvents. In case of glycine ethyl ester and DL-alanine methyl ester, all attempts to isolate the product from hydrogenolysis either as the free phosphoamide or as pure sodium or barium phosphates have been unsuccessful. This may be due to the instability of the compound, thus rendering the isolation difficult.

On the basis of elementary analysis, the structure of N-phosphoryl amino acid methyl ester would be either II, a phosphoamide with one mole of water of crystallization or III, a phosphate salt of the amino acid ester; both have the same elementary composition. In order to distinguish between II



III

and III a known phosphate salt of DL-phenylalanine methyl ester was prepared. The properties of the two compounds are compared in Table II.

TABLE II

PROPERTIES OF PHOSPHOAMIDE AND PHOSPHATE SALT OF DL-PHENYLALANINE METHYL ESTER

Properties	Phosphoamide (presumed)	Phosphate salt
Melting point, °C.	143–145	184–185 (dec.)
Content of free phosphate,[14] %	0.15	11.3 (calcd. 11.2)
Solubility in MeOH	Freely sol.	Sol. in hot MeOH
Subject to enzyme hydrolysis[15]	+	.....

It is evident from Table II that the salt structure is excluded. Similarly, the hydrogenolysis product of dibenzylphosphoryl DL-tryptophan methyl ester should be the corresponding phosphoamide compound.

Preliminary attempts to saponify the carboxyl ester groups of the dibenzylphosphoryl amino acid esters were unsuccessful and since the saponification of the corresponding diphenylphosphoryl amino acid esters have been reported to be difficult,[5] we are now in the process of preparing the benzyl esters in the hope that the cleavage of these esters may be more successfully carried out by hydrogenolysis.

It is interesting to note that the N-phosphoryl-amino acid esters have been found quite stable in acid solution and have since been shown to be suitable substrates for phosphoamidase. The studies dealing with the enzymatic hydrolysis of such compounds will be reported later.

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(15) Unpublished data.

Experimental

Reaction of Dibenzylphosphoryl Chloride with Amino Acid Esters. (a).—Amino acid ester hydrochloride (1 mole) was suspended in dry chloroform and dry ammonia was then passed into the solution under cooling (ice–salt-bath at 0°) till saturation (about 20 minutes). Ammonium chloride was filtered off and washed with fresh chloroform. The excess ammonia was removed by passing dry nitrogen into the cold solution. To the ammonia-free chloroform solution at 0° was added triethylamine (1 mole) followed by slow addition, with stirring, of DBPCl (1 mole) (freshly prepared from dibenzyl hydrogen phosphite and sulfuryl chloride according to Atherton, Howard and Todd.[16] All the apparatus and reagents were protected from moisture. After the addition had been completed (about 20 minutes), the reaction mixture was taken out of the cooling bath and stirring continued for 30 minutes, at which time the white precipitate separated out. Next day the precipitate was filtered off and the filtrate was washed successively with water, N-hydrochloric acid, 10% sodium hydrogen carbonate and water, then dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure, and the residue was dissolved in benzene and the benzene evaporated. After cooling in the ice-box at −18° the residue crystallized in needles and was recrystallized from benzene and petroleum ether. The results are presented in Table I.

(b).—To a suspension of 1 mole of amino acid ester hydrochloride in chloroform was added at 0°, slowly with stirring, DBPCl (1 mole) in the presence of 2 moles of triethylamine. The compound was isolated as in (a).

Preparation of N-Phosphoryl Amino Acid Esters.—One gram of dibenzylphosphoryl amino acid ester was subjected to hydrogenolysis in dry methanol in the presence of about 0.04 g. of palladium oxide. After the reaction had been completed (in 1 hour), the catalyst was filtered off and the solvent removed under reduced pressure in a water-bath of about 50°. The residue crystallized on cooling and was recrystallized from methanol and ether. The results are presented in Table I.

Preparation of DL-Phenylalanine Methyl Ester Phosphate.—One gram of DL-phenylalanine methyl ester hydrochloride dissolved in dry methanol was neutralized under cooling with freshly prepared sodium methoxide using phenolphthalein as indicator. The precipitate of sodium chloride was filtered off. To the filtrate was added about 0.4 ml. (in excess) of phosphoric acid drop by drop, whereby a white precipitate separated out. Ether was added to obtain more crystals. The crude product was recrystallized from methanol and ether in needles; m.p. 184–185° (decomposed with gas evolution).

Anal. Calcd. for $C_{10}H_{16}O_6NP$ (277.2): N, 5.05; P, 11.2. Found: N, 4.91; P, 11.3.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTH TEXAS STATE COLLEGE]

Antitubercular Studies. V. 4-Aminobenzamides and 4-Aminobenzenesulfonamides

BY PRICE TRUITT, GEORGE SAMMONS[1] AND DAVID ZACHRY[1]

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Series of 1-(4-nitrobenzoyl)- and 1-(4-aminobenzoyl)-4-alkylpiperidines, 1-(4-nitrobenzenesulfonyl)-1-(4-acetylaminobenzenesulfonyl-1-(4-aminobenzenesulfonyl)-4-alkylpiperidines and 1,2,3,4-tetrahydroquinolines are described. A partial evaluation of the antituberculous activity of these amides is given. One derivative, 1-(4-aminobenzoyl)-4-(1-octyl)-piperidine, is rather active against the tubercle bacilli.

Recent reports from this Laboratory[2,3] have indicated that certain substances which contain the piperidine or 4-alkylpiperidine moiety may possess limited antitubercular activity. These compounds include 1-phenacylpiperidines and 1-diphenylmethylpiperidines.

1-(4-Aminophenyl)-piperidine[4] is reported to be active against the tubercle bacilli. Also, 4-nitrobenzoylpiperidine[5] has been found to possess a slight antistreptococcal and antipneumococcal activity. 1-(4-Aminobenzoyl)-piperidine[6] has been prepared and tested for local anesthetic activity. The 4-nitro- and 4-aminobenzoyl-4-alkylpiperidines are not described.

Thus, it seemed desirable to prepare a number of the 1-(4-nitrobenzoyl)-4-alkylpiperidines and 1-(4-aminobenzoyl)-4-alkylpiperidines in which there was some range in size of the alkyl group. Of particular interest were the preparations where this alkyl group contained from five to nine carbons.

To further examine the influence of the 1-(4-alkylpiperidyl) radical on antituberculous activity, a number of 1-(4-aminobenzenesulfonyl)-4-alkylpiperidines were prepared to test for antitubercular activity. Sargent and Small[7] synthesized 1-(4-acetylaminobenzenesulfonyl) - 1,2,3,4 - tetrahydroquinoline and certain methoxy substituted tetrahydroquinolines. These compounds were not tested for antitubercular activity. For this reason several methyl substituted tetrahydroquinoline derivatives were included in this study.

The condensation of 4-nitrobenzoyl chloride with the various 4-alkylpiperidines proceeded with ease and the subsequent reduction with iron gave good yields of the expected amines.

The preparation of 4-aminobenzenesulfonyl derivatives was achieved by two routes. 4-Nitrobenzenesulfonyl chloride was treated with the secondary amines and the resultant amide reduced with iron and acetic acid. The yields with catalytic reduction were not as satisfactory as with iron reduction. The second procedure involved the hydrolysis of the 1-(4-acetylaminobenzenesulfonyl) derivative of the 4-alkylpiperidines and tetrahydroquinolines. The latter route gave much better yields but the purifications were somewhat more tedious.

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