mic acid. One hundred and fifty mg. of oxidized insulin was hydrolyzed with 3 g. of Dowex 50 and 15 ml. of 0.05 N HCl for 24 hours at 105°. Estimation of free amino acids indicated that, among the neutral and acidic amino acids, only aspartic acid and glycine were completely liberated. Insulin, even in its oxidized form, represents a protein which is difficult to hydrolyze and would require temperatures of the order of 110° under the conditions employed.

Casein and Tryptophan.—An attempt was made to hydrolyze casein and at the same time to protect tryptophan which is destroyed under ordinary conditions of resin hydrolysis. Fifteen ml. of 0.296 N H_2SO_3 was substituted for 0.05 N HCl to provide reducing conditions according to Pederson and Baker.[12] To the H_2SO_3 were added 150 mg. of casein and 3 g. of Dowex 50; the mixture was sealed in a Pyrex tube and heated at 105° with rotation for 20 hours. The products of hydrolysis were separated on a 15-cm. column of Dowex 50 and the 1-ml. eluate fractions were examined for tryptophan by the method of Graham, *et al.*[13] No tryptophan is found under these conditions.

In order to obtain essentially complete hydrolysis of edestin, oxidized insulin and bovine serum albumin in a 24-hour period by ion-exchange resin catalysis, it is necessary to use a large excess of Dowex 50 and temperatures of the order of 110°. The peptides of glutamic acid, leucine, isoleucine and valine have relatively high stabilities and, as a result, they persist through the final stages of hydrolysis. The use of H_2SO_3 in conjunction with Dowex 50 does not protect tryptophan from destruction during the hydrolysis of casein.

Acknowledgment.—The authors gratefully acknowledge the assistance of Mr. J. R. Whitaker who prepared and furnished the nitrated derivative of Dowex 50.

(12) J. W. Pederson and B. E. Baker, Abstracts, American Chemical Society, 123rd meeting, Los Angeles, April (1953).

(13) C. E. Graham, E. P. Smith, S. W. Hier and D. Klein, *J. Biol. Chem.*, **168**, 711 (1947).

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Acetylation of Some Aminopyrimidines

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In the synthesis of various purines it is usual to combine 4,5-diaminopyrimidines, which may also have other substituents such as amino, hydroxyl, mercapto, etc., in the 2- and/or 6-position, with suitable acid derivatives. Invariably it is assumed that the acid derivative acylates the 5-amino position preferentially if not exclusively, and this 5-acylamino compound is subsequently cyclized to the purine. No report has been found describing simultaneous acylation of amino groups in the 2-, 4- or 6-positions of the pyrimidine ring. Since no systematic work has been reported on the acylation of amino groups in positions other than the 5-position, it seemed to be of interest to study the acetylation of several simple pyrimidines bearing one or more amino groups along with certain other substituents in the 2-, 4- and 6-positions of the pyrimidine ring.

Although no systematic work has been reported on the acylation of assorted aminopyrimidines occasional isolated instances have been found in which, incidental to other work, the preparation of some monoacetylaminopyrimidines has been described. Thus, for example, Wheeler[1] reported 4-acetylaminopyrimidine. Wheeler and Johnson[2] have made 2-acetylamino-4-hydroxypyrimidine which has also been made and included in this paper for comparison purposes. Although all the cases where an individual aminopyrimidine has been acetylated for protective or preparative purposes cannot be included here, one very recent example will be mentioned. Baker and co-workers[3] have acetylated 2-methylmercapto-4-amino-6-dimethylaminopyrimidine to give the 4-acetylamino product. This paper, important in adducing evidence for the structure of the purine antibiotic puromycin, is significant in the current work in that these authors[3] have seemingly established the position of acetylation as on the 4-amino group.

The substituted pyrimidines used in the acetylation experiments were 2-aminopyrimidine (I), 2-amino-4-hydroxypyrimidine (II), 2-amino-4-piperidino-6-methylpyrimidine (III), 2-amino-4,6-dihydroxypyrimidine (IV), 4-amino-2,6-dihydroxypyrimidine (V), 2,4-diamino-6-hydroxypyrimidine (VI) and 2,4,6-triaminopyrimidine (VII). A standard acetylation procedure was used for all the compounds, and this is described in the Experimental part. The results and analytical data are summarized in Table I.

In general it can be said that where the number of amino groups present was equal to or greater than the number of hydroxy groups in a particular pyrimidine molecule acetylation of all the amino groups resulted under the conditions used, and the products formed were stable to various purification procedures. When more hydroxyl groups than amino were present on a molecule as in compounds IV and V, acetylation was not easy or complete.

Compound IV, malonylguanidine, gave less than 50% yield of an insoluble substance different in certain properties from IV. This product was extremely difficult to purify since it was nearly insoluble in the variety of solvents tried. It gave analyses which did not check with the theoretical for either the starting compound or a monoacetyl derivative, but which were much closer to the values for the former. Malonylguanidine can be purified either by solution in alkali and reprecipitation with acid or by the reverse process. The acetylation product dissolved less readily in either acid or alkali than the parent compound, and then could not be reprecipitated by neutralization.

Compound V gave no acetylation product that could be isolated, and after several reprecipitations from dilute alkali with dilute acid all of the V was recovered unchanged.

The simpler monoacetylaminopyrimidines, obtained from compounds I, II and III, could be purified easily by recrystallization from common organic solvents. The products from VI and VII on the other hand were much more insoluble and more difficult to purify. The acetyl derivative of VI was soluble in cold alkali and could be reprecipitated unchanged by addition of acid. It was best purified, however, by dissolving it in a little cold concentrated hydrochloric acid from which it separated unchanged on simple dilution with water. The product so obtained analyzed well for the diacetylamino

(1) H. L. Wheeler, *J. Biol. Chem.*, **3**, 291 (1907).

(2) H. L. Wheeler and T. B. Johnson, *Am. Chem. J.*, **29**, 492 (1903).

(3) B. R. Baker, R. E. Schaub and J. P. Joseph, *J. Org. Chem.*, **19**, 638 (1954).

TABLE I

ACETYLAMINOPYRIMIDINES

Cmpd. to be acetylated	Product[a] X	Product[a] Y	Product[a] Z	Crystn. solvent[b]	M.p., °C.	Formula	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
I	CH_3CONH	H	H	Ea	144–145	$C_6H_7N_3O$	52.6	52.8	5.1	5.0
II	CH_3CONH	OH	H	M	249–250	$C_6H_7N_3O_2$[c]	47.1	47.3	4.6	4.8
III	CH_3CONH	$N(CH_2)_5$	CH_3	B.H	131–132	$C_{12}H_{18}N_4O$	61.5	61.6	7.7	7.5
IV	NH_2	OH	OH	M	>325	$C_4H_5N_3O_2$	37.8		3.9	
	CH_3CONH	OH	OH			$C_6H_7N_3O_3$	42.6	39.0, 39.3	4.1	4.0, 4.0
V	OH	OH	NH_2	Aq. NaOH + HCl	>325	$C_4H_5N_3O_2$	37.8 N, 33.1	38.0 N, 32.9	3.9	4.0
VI	CH_3CONH	CH_3CONH	OH	Aq. HCl	>340	$C_8H_{10}N_4O_3$	45.7	45.7	4.8	4.8
VII	CH_3CONH	CH_3CONH	CH_3CONH	HOAc	304–305	$C_{10}H_{13}N_5O_3$	47.8	47.9	5.2	5.1

[a] Yields of acetylated products usually exceeded 90%. In the case of malonylguanidine (IV) the yield of crude product was about 50%. Compound V was recovered unchanged. [b] B = benzene; Ea = ethyl acetate; H = hexane; HOAc = glacial acetic acid; M = methanol; Aq. NaOH + HCl = dissolved in dilute NaOH, reprecipitated by dilute HCl; Aq. HCl = dissolved in cold concd. HCl, precipitated by dilution with H_2O. [c] Reported by H. L. Wheeler and T. B. Johnson, *Am. Chem. J.*, **29**, 492 (1903).

compound and gave a negative Beilstein test for halogen. The triacetylamino compound from VII was recrystallized from a large volume of boiling glacial acetic acid.

An attempt to acetylate VII in aqueous solution failed to give the triacetylaminopyrimidine. The product isolated was presumably the acetate of VII for it was soluble in warm water and when basified with alkali gave back VII at once.

It is well known that there is a variation in the lability toward hydrolysis of amino groups in different positions on a pyrimidine ring. Thus 4- or 6-amino(or imino)-barbituric acids containing either hydrogen or alkyl groups in the 5-position can be converted easily to the corresponding barbituric acid by acid hydrolysis.[4–7] Although both imino (or amino) groups of 2,4-di-iminobarbituric acids can be replaced by hydroxyl groups upon boiling with dilute acid, the 4-amino is considerably more easily hydrolyzed than the 2-amino group.[7] Attempts were made to hydrolyze the triacetylamino product obtained from VII. Considering the above described differential hydrolysis of the aminobarbituric acids, it was hoped that mild aqueous acid hydrolysis might yield the 2-acetylaminobarbituric acid, which had not been obtained pure by the direct acetylation of IV (malonylguanidine). Even if hydrolysis went further barbituric acid might be isolated. In either of these results it should not matter whether the acetyl groups were first cleaved, followed by hydrolysis of the amino groups, or whether, possibly, the acetylamino groups came off as such. The triacetylamino compound from VII dissolved readily in five parts of 6 *N* hydrochloric acid to give a clear solution, but after heating for ten minutes at 100°, addition of ammonium hydroxide to *p*H 9–10 gave no precipitate. Upon slow evaporation of this clear solution the first and only crystals isolated were identified as ammonium chloride, thus suggesting that the pyrimidine had undergone extensive breakdown.

(4) M. Conrad, *Ann.*, **340**, 310 (1905).

(5) D. L. Tabern and E. H. Volwiler, THIS JOURNAL, **56**, 1139 (1934).

(6) J. S. Chamberlain, *et al.*, *ibid.*, **57**, 352 (1935).

(7) A. C. Cope and E. M. Hancock, *ibid.*, **61**, 776 (1939).

Cleavage of 2-acetylaminopyrimidine was accomplished easily by refluxing with methanolic hydrogen chloride. The product isolated was 2-aminopyrimidine hydrochloride.

The structures assumed for the acetylation products of the various aminopyrimidines of the present work bear the acetyl groups on the side-chain amino groups as indicated in Table I, rather than on the ring nitrogens, or rather than on oxygen, in the cases where oxygen was available. These assumed structures have not been subjected to rigid and complete structure proof, which was felt to be implicit, beyond any serious question, from numerous pieces of evidence and statements made in this paper.

(1) Earlier workers[1–3] have assumed and written the acetylation products of a number of aminopyrimidines with the acetyl on the amino rather than on the ring nitrogen. This assumption seems reasonable and is supported by several pieces of evidence. Firstly, the work of Baker, *et al.*,[3] seems to prove the location of acetyl on the 4-amino group in one particular case. Secondly, in the present study, the parallel between the number of acetyl groups introduced into the aminopyrimidines and the number of potential amino groups available is taken as strong evidence for the attachment of the acetyls to these side-chain amino groups in the products. Thirdly, the sulfonyl group is known to be attached to the 2-amino in certain sulfanilamide-type drugs derived from 2-aminopyrimidines.

(2) The second point above argues strongly against the improbable formation of derivatives in which two acetyls are attached to a single amino group. Although such compounds are known among aniline derivatives, they are less common, more difficult to prepare, and less stable than the monoacetylamino products. Thus, according to Tassinari,[8] these diacetylamino compounds easily lose one acetyl group in the presence of ammonia, aniline, water, alcohol or acetic acid. Thus even when two acetyls can be forced onto a single nitrogen, one of them tends to be quite labile, and, under

(8) G. Tassinari, *Gazz. chim. ital.*, **24I**, 62, 445 (1894).

many of the usual recrystallization procedures, would be lost leaving the more stable monoacetylamino product.

(3) There was no evidence of acetylation of hydroxyl groups, when present, under the conditions of the current study. The hydroxyl-bearing pyrimidines were all alkali soluble after acetylation, and were precipitated without any apparent change upon the addition of acid.

Experimental

Standard Acetylation Conditions.—A mixture of 0.02 mole of the aminopyrimidine and 25 cc. of acetic anhydride was refluxed for two hours in a metal-bath at 160°. After cooling, the insoluble product was collected by filtration. This crude material was suspended in 50 cc. of water and the mixture was brought to *p*H 8–9 with dilute ammonium hydroxide solution. The insoluble product was again collected by filtration, was washed with much cold water and was purified by recrystallization as described in Table I for the individual compounds. Yields of acetylamino compounds were all close to 100%.

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Synthesis of L-Phenylalanyl-L-glutaminyl-L-asparagine[1]

BY EDWIN A. POPENOE AND VINCENT DU VIGNEAUD

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The structure CyS.Tyr.Phe.Glu(NH_2).Asp(NH_2).CyS.Pro.Lys.Gly(NH_2) has recently been proposed[2,3] for lysine–vasopressin, the pressor-antidiuretic hormone which has been found to occur in extracts of hog posterior pituitary glands.[4] As an intermediate in a projected synthesis of an octapeptide amide with this structure, carbobenzoxy-L-phenylalanyl-L-glutaminyl-L-asparagine was desired. The preparation of L-glutaminyl-L-asparagine[5] and tosyl-L-isoleucyl-L-glutaminyl-L-asparagine[6] used in the synthesis of oxytocin[7] have recently been reported. The tosyl tripeptide was prepared by treatment of the magnesium salt of L-glutaminyl-L-asparagine in aqueous solution with tosyl-L-isoleucyl chloride in the presence of excess magnesium oxide. The present paper reports an analogous preparation of tosyl-L-phenylalanyl-L-glutaminyl-L-asparagine and the conversion of this compound to the desired carbobenzoxy derivative. The preparation of the free tripeptide is also described.

Experimental[8]

Tosyl-L-phenylalanyl Chloride.—To 5.4 g. of tosyl-L-phenylalanine[9] suspended in 75 ml. of anhydrous ether at 0° there was added 3.90 g. of phosphorus pentachloride. The mixture was shaken for 10 minutes at 0°, then for 10 minutes at room temperature and finally stored at 0° for 1 hour. The crystalline product was filtered off, washed on the funnel quickly with a little ether and then with ice-water and dried for 2 hours in a vacuum desiccator under the vacuum provided by a good oil pump. The yield was 5.07 g. (88%) of a product which melted with decomposition at 128–129°.

Anal. Calcd. for $C_{16}H_{16}O_3NSCl$: Cl, 10.5. Found: Cl, 10.6.

Tosyl-L-phenylalanyl chloride is considerably more stable than carbobenzoxy-L-phenylalanyl chloride. A sample stored over phosphorus pentoxide at room temperature for 5 months showed no change in melting point. This stability is advantageous for reactions of the type used here.

Tosyl-L-phenylalanyl-L-glutaminyl-L-asparagine.—A mixture of 3.44 g. of L-glutaminyl-L-asparagine,[5] 0.82 g. of magnesium oxide and 6 ml. of water was shaken on a machine for 20 minutes. The mixture was then chilled in an ice-bath and 4.46 g. of tosyl-L-phenylalanyl chloride was added in small portions during 1 hour. Stirring was best accomplished by hand. As the reaction proceeded the mixture became so thick that it was necessary to add 5 ml. of water after about half of the acid chloride had been added. After all of the acid chloride had been added, 15 ml. of water was added and the mixture was allowed to come to room temperature during the next half-hour. The gelatinous product which was obtained by acidification of the mixture to congo red with concentrated HCl, was filtered off after 1 hour, washed thoroughly with water and dried. To remove any tosyl-L-phenylalanine the solid was ground in a mortar with 15 ml. of ethyl acetate, filtered and dried; wt. 5.78 g. (78%).

For purification the product was stirred with an excess of $NaHCO_3$ in 100 ml. of water and treated with Darco to remove some turbidity. Sufficient water was then added to make 200–300 ml. per g. of substance. The product which separated slowly after acidification was amorphous, but was usually crystalline after the second or third similar reprecipitation. The yield of purified product, m.p. 193–195°, was usually about 60%; $[\alpha]^{21}D$ −26.0° (*c* 1.95, 0.5 *N*, $KHCO_3$).

Anal. Calcd. for $C_{25}H_{31}O_8N_5S$: C, 53.5; H, 5.56; N, 12.5. Found: C, 53.3; H, 5.68; N, 12.4.

L-Phenylalanyl-L-glutaminyl-L-asparagine.—The tosyl group was removed from the tosyl tripeptide with sodium and liquid ammonia according to the method of du Vigneaud and Behrens.[10] Four grams of the tosyl tripeptide was dissolved in 300 ml. of liquid ammonia and reduced at the boiling point by the addition of metallic sodium until a persistent blue color was produced. Approximately 1.4 g. of sodium was required. Three grams of ammonium chloride was added and then the ammonia was allowed to evaporate. The last traces of ammonia were removed on the water pump. The solid residue was dissolved in 50 ml. of water at 0°, the solution treated quickly with Darco, filtered, washed with ether and the *p*H adjusted to 6 by the cautious addition of concentrated HCl. The crystalline product which separated was filtered off after a few hours at 0°, washed with cold water and dried; wt. 2.15 g. (75%). This product was pure enough for use in the next step. For purification it was dissolved in a slight excess of 0.15 *N* HCl, filtered if necessary and reprecipitated by the addition of dilute NH_4OH to *p*H 6–7. The compound melted with decomposition between 226 and 230° when inserted at 215° into a bath, the temperature of which was rising 2° per minute; $[\alpha]^{21}D$ 0.1° (*c* 4.9, 0.15 *N* HCl).

Anal. Calcd. for $C_{18}H_{25}O_6N_5$: C, 53.1; H, 6.19; N, 17.2. Found: C, 53.0; H, 6.35; N, 17.1.

Carbobenzoxy-L-phenylalanyl-L-glutaminyl-L-asparagine.—A mixture of 2.0 g. of L-phenylalanyl-L-glutaminyl-L-asparagine, 0.25 g. of magnesium oxide and 15 ml. of water was shaken for 10 minutes. The mixture was cooled in ice and 1.23 g. of carbobenzoxy chloride was added in 4 portions. The flask was kept in ice and shaken vigorously between additions. Water was added occasionally to keep the mix-

(1) Appreciation is expressed to the Lederle Laboratories Division, American Cyanamid Company, for a research grant which has aided greatly in this work.

(2) E. A. Popenoe and V. du Vigneaud, *J. Biol. Chem.*, **206**, 353 (1954).

(3) V. du Vigneaud, H. C. Lawler and E. A. Popenoe, THIS JOURNAL, **75**, 4880 (1953).

(4) E. A. Popenoe, H. C. Lawler and V. du Vigneaud, *ibid.*, **74**, 3713 (1952).

(5) J. M. Swan and V. du Vigneaud, *ibid.*, **76**, 3110 (1954).

(6) P. G. Katsoyannis and V. du Vigneaud, *ibid.*, **76**, 3113 (1954).

(7) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954).

(8) Capillary melting points were determined for all compounds and are corrected.

(9) M. p. = 163–165°. E. Fischer and W. Lipschitz [*Ber.*, **48**, 360 (1915)] found m.p. 164–165° (cor.).

(10) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).