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Analogs of Tetrahydrofolic Acid. XIX. On the Mode of Binding of the Pyrimidyl Moiety of N-(2-Amino-4-hydroxy-6-methyl-5-pyrimidylpropionyl)-*p*-aminobenzoyl-L-glutamic Acid to 5,10-Methylenetetrahydrofolate Dehydrogenase (1,2)

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The synthesis of several analogs of the title compound (Ia) which had (a) replacement of the 4-hydroxy group by thiol, (XXVIIIa), (b) replacement of the 6-methyl group by 6-phenyl (Ib), (c) replacement of the 2-amino group by 2-thiol (XXVI), or (d) replacement of both the 4-hydroxyl and 6-methyl groups by 4-thiol and 6-phenyl, respectively, have been described. The key intermediate was the appropriate 2,4,6-trisubstituted 5-pyrimidylpropionic acids which were synthesized by suitable transformation of the 5-pyrimidylpropionitriles (VIII and XII). Replacement (a) or (b) gave an improvement in inhibition of 5, 10-methylenetetrahydrofolate dehydrogenase, but the two changes in the same molecule (d) did not further increase inhibition. The 2-amino group of the title compound (Ia) can be replaced by 2-thiol without decreasing the inhibitory properties of Ia.

In a previous study from this laboratory (3), it was shown that the tetrahydrofolate analog (Ia) was an inhibitor of 5,10-methylenetetrahydrofolate dehydrogenase (V $\longrightarrow$ VI) and that the intact carboxy-L-glutamate side-chain was needed for good binding to the enzyme; in fact, about 75% of the total free energy of binding of Ia - calculated from the enzyme-inhibitor constant (K_i) - could be accounted for by N-acetyl-*p*-aminobenzoyl-L-glutamate and about 25% by the pyrimidyl moiety. Since replacement of the 6-methyl group in the tetrahydrofolate analog (II) by 6-phenyl (III) (4) or the 4-hydroxyl group by 4-mercapto (IV) (5) led to an increase in binding to this enzyme, a study was initiated to determine if (a) the replacement of both the 4-hydroxy and 6-methyl groups of Ia by 4-mercapto and 6-phenyl (XXVIIIb), respectively, could give a further increment in binding to the enzyme. The synthesis of one other compound which could give information on the mode of binding of the pyrimidyl moiety was investigated, namely a 2-mercapto-4-pyrimidinol analog (XXVI). The synthesis and enzymic evaluation of the preceding compounds are the subjects of this paper.

Acetylation of the 2-amino-4-pyrimidinol (VIIIa) (3) at 90° with acetic anhydride in pyridine (6) gave XIIIa in 83% yield. Replacement of the 4-hydroxyl group of XIIIa by chloro with phosphorus oxychloride was quite sensitive to reaction conditions; the optimum conditions were ten minutes in boiling benzene followed by neutralization with cold aqueous sodium acetate (6), which afforded the crude, but crystalline chloropyrimidine (XVIIa) in a maximum of 77% yield.

The chloropyrimidine (XVIIa) reacted rapidly with thiourea in *t*-butyl alcohol (5) with precipitation of the crystalline thiouronium salt (XIXa) in 80% yield. When XIXa was treated with 1 N sodium hydroxide for two hours at ambient temperature, cleavage of the N-acetyl and thiouronium groups occurred, but not hydrolysis of the nitrile group, giving the 2-amino-4-pyrimidinethiol (XXIa) in 89% yield. Treatment of XXIa with boiling 6 N hydrochloric acid for two hours caused hydrolysis of the nitrile group to 5-pyrimidylpropionic acid (XXIIIa) in 82% yield.

Attempts to convert XXIIIa to the corresponding acid chloride with thionyl chloride with or without pyridine catalysis under a variety of conditions led to tars or unchanged XXIIIa. Attempts to form the thiolactone (XXIVa) for activation of the carboxyl led to partial loss of the sulfur atom. Successful activation of the carboxyl group of XXIIIa was accomplished by the mixed anhydride method (7, 8). When XXIIIa in N,N-dimethylformamide was treated with ethyl chloroformate at 0° in the presence of triethylamine, a mixed anhydride formed which was characterized by conversion to the anilide (XXVIIa) in 71% yield with aniline at room temperature. When the mixed anhydride from XXIIIa was coupled with *p*-aminobenzoyl-L-glutamic acid, the final compound (XXVIIIa) was obtained in an analytically pure state in 24% yield.

The 4-mercapto-6-phenyl analog (XXVIIIb) was prepared by a generally similar route from VIIIb, but with some strikingly different chemical reactivities which required proper modification to be utilizable. Cyanoethylation of ethyl benzoylacetate with acrylonitrile in the presence of 40 mole percent of sodium methoxide afforded a 59% yield of VIIb. Condensation of VIIb with guanidine carbonate in *t*-butyl alcohol (4) afforded the desired 6-phenylpyrimidine (VIIIb) in 42% yield (9). Hydrolysis of the nitrile

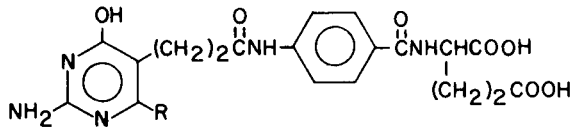
group of VIIIb with boiling 6 N hydrochloric acid resulted in the formation of the 5-pyrimidylpropionic acid (IXb) in 71% yield. Conversion of the acid (IXb) to the acid chloride Xb proceeded smoothly with boiling thionyl chloride without catalysis; the acid chloride (Xb) was further characterized by conversion to the anilide (XIb). Condensation of the acid chloride with *p*-aminobenzoyl-L-glutamic acid gave the tetrahydrofolate analog (Ib) in 57% yield. Although the carboxyl group of IXb could also be activated by the mixed anhydride method, and gave a 96% yield of anilide, XIb, condensation of the mixed anhydride with the less reactive *p*-aminobenzoyl-L-glutamic acid gave Ib in considerably lower yield than via the acid chloride (Xb).

Acetylation of the 2-amino-6-phenylpyrimidine (VIIIb) with acetic anhydride and pyridine gave XIIIb in 92% yield. Although considerable study was devoted to the conversion of the 6-phenyl-4-pyrimidinol (XIIIb) to the 4-chloropyrimidine (XVIIb), phosphorus oxychloride gave 0-14% yields, in contrast to the smooth conversion of the 6-methyl-4-pyrimidinol (XIIIa) to XVIIa. Direct conversion of the 2-amino-4-pyrimidinol (VIIIb) to the 2-amino-4-chloropyrimidine (XVIIIb) proceeded in 39% yield under the optimum conditions of 45 minutes reflux with phosphorus oxychloride without a diluent.

The 4-chloro-6-phenylpyrimidine (XVIIIb) reacted smoothly with thiourea in boiling *t*-butyl alcohol to give the thiouronium salt (XXb) in good yield, which was further converted with 1 N sodium hydroxide to the 2-amino-4-pyrimidinethiol (XXIb). The reactivity of the chloro group of the 6-phenylpyrimidine, XVIIIb, towards thiourea contrasts sharply with the failure of this same reaction with the analogous 6-methylpyrimidine (XVIIIa) (5); apparently the 6-phenyl group activates the 4-position of the pyrimidine ring. Another example of this activation occurred when the pyrimidinethiol (XXIb) was treated with hot 6 N hydrochloric acid; the product was not the expected 4-mercapto-5-pyrimidinepropionic acid (XXIIIb), but the 4-hydroxy analog (IXb) resulting by hydrolytic cleavage of the thiol. Note that in the 6-methyl series the conversion of 4-mercapto nitrile (XXIa) to the 4-mercapto-5-pyrimidylpropionic acid (XXIIIa) under the same conditions did not result in hydrolytic cleavage of the thiol group.

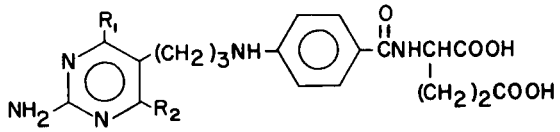
Fortunately the thiol group of XXIIIb was stable to base and thiouronium salt could be hydrolyzed with boiling 15% sodium hydroxide to the 4-mercaptopyrimidine-5-propionic acid (XXIIIb) in 55% overall yield from crude chloropyrimidine (XVIIIb).

Attempts to convert the 6-phenylpyrimidine-5-propionic acid (XXIIIb) to an acid chloride with thionyl chloride, thionyl chloride and pyridine, phosphorus pentachloride in acetyl chloride (10), or phosphorus pentachloride or phosphorus oxychloride without solvent were unsuccessful. Although the carboxyl group of XXIIIb could be activated by conversion to a mixed anhydride (7, 8) - as shown by conversion to the anilide (XVIIb) in 63% yield - the mixed anhydride was not sufficiently reactive to form XXVIIIb with the less reactive *p*-aminobenzoyl-L-glutamic acid. An attempt to condense XXIIIb with diethyl *p*-aminobenzoyl-L-glutamate using dicyclohexylcarboimide was also unsuccessful. Another means of activating carboxyl groups for conversion to amides is via the cyanomethyl ester (11). When the triethylammonium salt of the 4-mercapto-5-pyrimidylpropionic acid was condensed with chloro-



Ia, R = CH_3

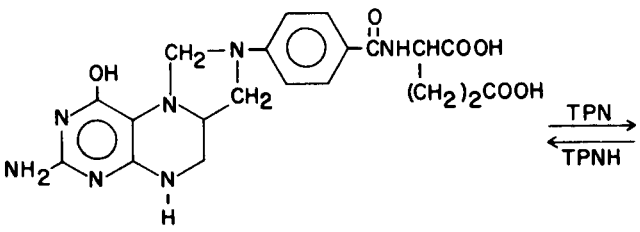
Ib, R = C_6H_5



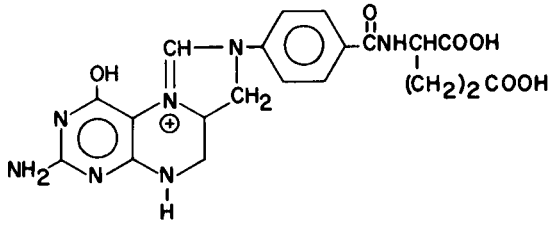
II, R_1 = OH, R_2 = CH_3

III, R_1 = OH, R_2 = C_6H_5

IV, R_1 = SH, R_2 = CH_3



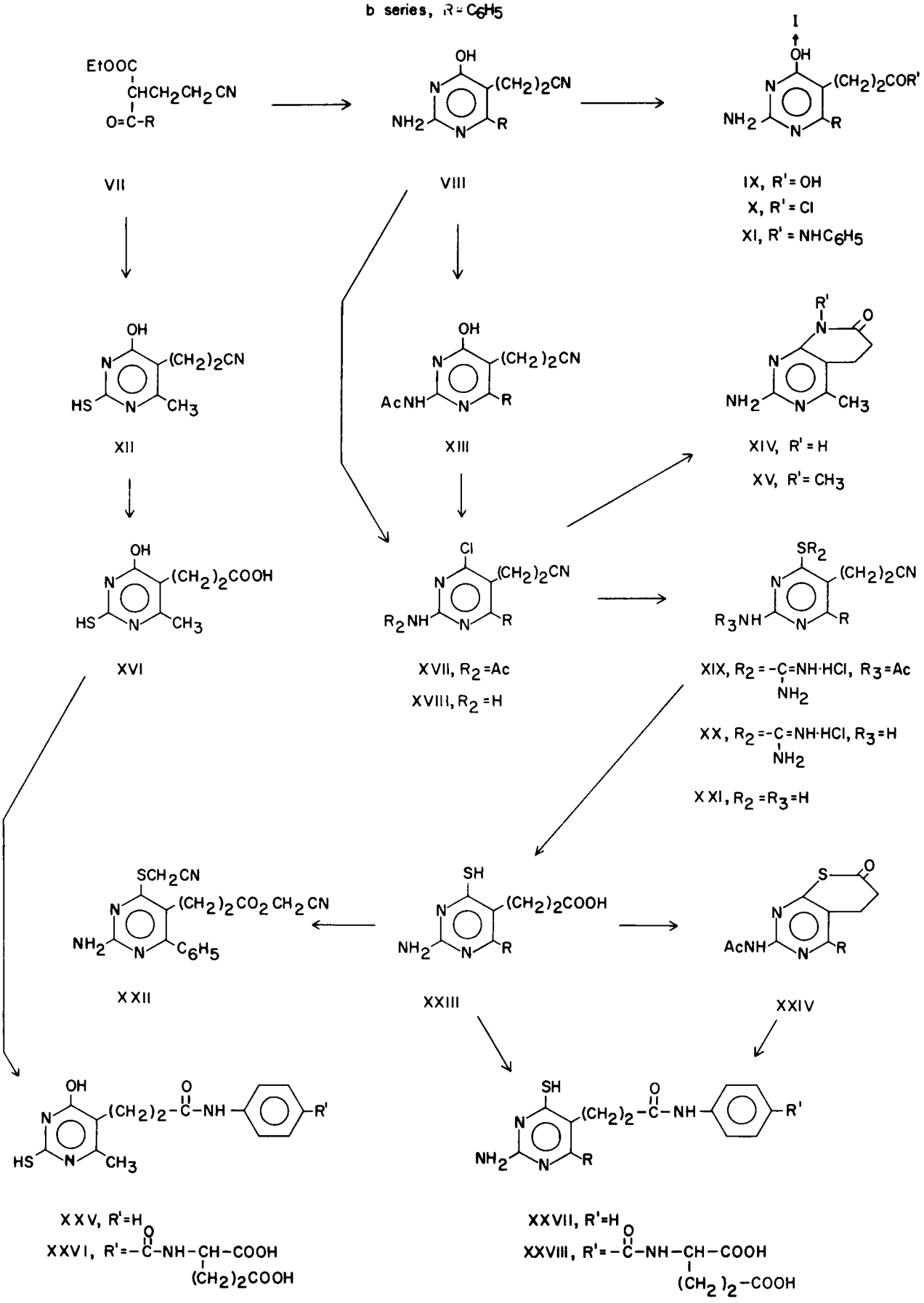
V



VI

a series, R = CH_3

b series, R = C_6H_5



acetonitrile, not only did formation of an ester group occur, but attack of the thiol function to give XXII also occurred. When XXII was treated with *p*-aminobenzoyl-L-glutamic acid in *t*-butanol or 2-methoxyethanol, XXII was recovered unchanged, showing that the cyanomethyl ester does not have a sufficiently activated carbonyl group to condense with such a weak nucleophile.

A successful activation of the carboxyl of XXIIIb was finally accomplished by taking advantage of the presence of the 4-thiol group; treatment of XXIIIb with acetic anhydride gave the crystalline acetamido thiolactone (XXIVb) in 43% yield. When the thiolactone (XXIVb) was condensed with aniline or *p*-aminobenzoyl-L-glutamic acid in boiling ethanol, the amides XXVIIb and XXVIIIb, were formed in 43 and 19% yields, respectively; surprisingly, the reaction did not proceed well in pyridine, dark and tarry products being obtained.

In order to synthesize a 2,4-diaminopyrimidine analog of Ia, one would need to be able to convert the 2-amino-4-chloropyrimidine (XVIIIa) or the 2-acetamido-4-chloropyrimidine (XVIIa) to 2,4-diamino-6-methyl-5-pyrimidinepropionitrile. Previous experience (12) with the synthesis of 2,4-diamino-6-methyl-5-alkylpyrimidines from the corresponding 2-amino- or 2-acetamido-4-chloropyrimdines showed that the use of fairly pure starting material was essential. Purification of the 2-acetamido-4-chloropyrimidine (XVIIa) led to large losses; this difficulty was previously circumvented (12) by methanolysis of the acetyl group to a 2-amino-4-chloropyrimidine which was readily obtained pure. Treatment of the crude, but crystalline 2-acetamido-4-chloropyrimidine (XVIIa) with methanolic di*iso*propylamine (12) gave a 60% yield of the 2-amino-4-chloropyrimidine (XVIIIa) sufficiently pure for further reaction. Since the overall yield for the three steps from VIIIa was about 40%, the direct replacement of the hydroxyl group of the 2-amino-4-pyrimidinol (VIIIa) by chlorine using phosphorus oxychloride was investigated; a 38% yield of quite pure 2-amino-4-chloropyrimidine (XVIIIa) was obtained. Thus the method of choice was obviously the direct replacement reaction since only one step was required and since the overall yields of XVIIIa were about the same for both sequences.

When the 2-amino-4-chloropyrimidine (XVIIIa) was reacted with methanolic ammonia at 160° for 24 hours - the usual conditions (12) - the desired 2,4-diamino-6-methyl-5-pyrimidylpropionitrile was not obtained; the latter had further cyclized by addition of the 4-amino group to the nitrile, followed by hydrolysis to a cyclic lactam (XIV) in 90% yield. That the rate-limiting step of the three was the amination step was indicated by reducing the severity of the conditions; at no time could a diamino nitrile be isolated, but less severe conditions gave a mixture of lactam (XIV) and starting 4-chloropyrimidine (XVIIIa).

Acid hydrolysis of the lactam (XIV) with boiling concentrated hydrochloric acid was slow; the 305 mμ peak of XIV did not disappear until 100 hours of reflux. At this time the solution showed a peak at 280 mμ in acid and 290 mμ in base - in reasonable agreement with that expected for a 2,4-diamino-5,6-dialkylpyrimidine (12,13); it is surprising that the 4-amino group did not undergo hydrolysis to a 4-hydroxyl group, as previously observed (12,14,15) with other related 2,4-diaminopyrimidines. Attempted purification of 2,4-diamino-6-methyl-5-pyrimidylpropionic acid, which had supposedly formed, met with failure.

It is interesting to note that reaction of the 2-amino-4-chloropyrimidine (XVIIIa) with methanolic methylamine also gave a lactam (XV) in 95% yield.

The final tetrahydrofolate analog with a 2-mercapto-4-hydroxy-6-methyl pyrimidyl moiety (XXVI) was synthesized in a relatively straight-forward manner. Condensation of the keto ester (VIIa) (16) with thiourea in the presence of excess methanolic sodium methoxide afforded the 2-mercapto-4-pyrimidinol (XII) in 60% yield. Hydrolysis of the nitrile group of XII proceeded smoothly with boiling 15% sodium hydroxide to give a 92% yield of the 5-pyrimidylpropionic acid (XVI).

Activation of the carboxyl group of XVI by the mixed anhydride method (7,8) was accomplished. However, reaction with aniline at room temperature was slow; in addition to a 42% yield of the anilide (XXV), a 50% yield of the ethyl ester of XVI was obtained by disproportionation of the mixed anhydride (8). Although suppression of the disproportionation reaction is possible by reducing the temperature to 0°, such conditions also suppress the rate of reaction of the mixed anhydride with the weakly nucleophilic *p*-aminobenzoyl-L-glutamic acid to a negligable rate. Even though the 2-amino-4-mercapto-5-pyrimidylpropionic acid (XXIIIa) could not be converted to an acid chloride, the reaction was successful when the 2-mercapto-5-pyrimidylpropionic acid (XVI) was reacted at the boiling point of thionyl chloride for a short time. The resultant acid chloride (74%) was characterized by conversion to anilide (XXV) in 66% yield. Condensation of the acid chloride of XVI with *p*-aminobenzoyl-L-glutamic acid in pyridine gave the final product (XXVI) in 35% yield.

ENZYME EVALUATION

The compounds in Table I were tested as inhibitors of 5,10-methylenetetrahydrofolate dehydrogenase from pigeon liver (17) by a modification of the procedure previously described (3). Since a somewhat different inhibitor-substrate ratio for 50% inhibition was obtained for compound Ia with the new assay method than previously reported (3), the result in Table I with compound Ia is by the new assay method, as are all other results in Table I.

As one could anticipate from the previous studies with II (3), III (4) and IV (5), conversion of the 4-hydroxyl of Ia to 4-mercapto (XXVIIIa) or the conversion of the 6-methyl of Ia to 6-phenyl (Ib) should give better inhibitors. However, in order to answer

the question whether or not both of these structural changes in the same molecule (XXVIIIb) would give an additively better inhibitor than either structural change alone, it was considered essential to re-establish first a base-line with compounds Ia, Ib, and XXVIIIa.

Replacement of the hydroxyl group of Ia by 4-mercapto (XXVIIIa) enhanced binding to the enzyme about ten-fold; replacement of the 6-methyl group of Ia by 6-phenyl (Ib) enhanced binding by about four-fold (Table I). However, replacement of the 6-methyl group of the 2-amino-4-mercapto analog (XXVIIIa) by 6-phenyl (XXVIIIb) gave no further enhancement in binding. The failure to enhance the binding additively to folic reductase by two such structural changes on 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinol has also been observed recently (2).

Replacement of the 2-amino group of Ia by 2-thiol gave a compound (XXVI) that was bound to 5,10-methylenetetrahydrofolate dehydrogenase as good or slightly better than Ia (Table I); either the 2-amino group in Ia is not necessary for binding to the enzyme or the 2-thiol group of XXVI gives the same contribution to binding as the 2-amino of Ia.

TABLE I

Inhibition of 5,10-Methylenetetrahydrofolate Dehydrogenase By N-(2-R_1-4-R_2-6-R_3-5-pyrimidylpropionyl)-*p*-aminobenzoyl-L-glutamic Acid

Compound Number	R_1	R_2	R_3	mM Conc. Inhibitor	% Inhibition	Estimated mM Conc. for 50% Inhibition (a)
Ia	NH_2	OH	CH_3	2.1	50	2.1
XXVIIIa	NH_2	SH	CH_3	0.10 (b)	32	0.22
Ib	NH_2	OH	C_6H_5	0.40 (b)	42	0.56
XXVIIIb	NH_2	SH	C_6H_5	0.10 (b)	26	0.30
XXVI	SH	OH	CH_3	1.1	50	1.1

A 100 mg. ampoule of tetrahydrofolic acid diacetate (General Biochemicals Co.) was opened and added to 20 ml. of 1 M mercaptoethanol previously adjusted to *p*H 7.4. This master solution was divided into 0.15 ml. aliquots and frozen at -20°; the solution is stable at least several months in the frozen state (18). An assay mix was prepared by adding to a 0.15 ml. frozen aliquot of tetrahydrofolate solution, 0.42 ml. of 0.5 M magnesium chloride, 0.42 ml. of 0.5 M magnesium chloride, 0.42 ml. of 0.3 M formaldehyde (*p*H 7.4), 0.83 ml. of 1 M mercaptoethanol (*p*H 7.4) and 0.95 ml. of 0.05 M Tris buffer (*p*H 7.4) containing 10 mM mercaptoethanol and 1 mM Versene (Buffer B) (18). In the upper and lower cuvettes of a Cary 11 spectrophotometer were placed 0.20 ml. of assay mix, 200 λ of enzyme solution prepared from water extraction of pigeon liver acetone powder (General Biochemical Co.) (3), and 2.6 ml. of Buffer B. When the system had balanced, the enzyme reaction was started by the addition of 100 λ of 3.1 mM TPN; the initial slope of optical density change on a 0-0.1 O.D. slide-wire was taken as the velocity. Inhibitors were dissolved in Buffer B and readjusted to *p*H 7.4. The final cuvette concentration of 5,10-methylene-*dl*-tetrahydrofolate was 31 μM and TPN was 100 μM.

(a) A plot of V_O/V_I against several concentrations of I was made in order to determine the 50% inhibition point ($V_O/V_I = 2$), where V_O = velocity of the enzyme reaction without inhibitor, V_I = velocity in presence of inhibitor, and I = concentration of inhibitor (19); in those cases where the 50% inhibition concentration could not be reached due to lack of full light transmission, the line was extended to $V_O/V_I = 2$. (b) Maximum concentration allowing full light transmission.

EXPERIMENTAL (20)

2-Amino-4-hydroxy-6-phenyl-5-pyrimidylpropionitrile (VIIIb).

To the vortex of a rapidly stirred solution of 0.50 g. (0.022 mole) of sodium in 50 ml. of absolute ethanol to which had been added 10 g. (0.052 mole) of ethyl benzoylacetate was added dropwise 2.7 g. (0.05 mole) of acrylonitrile over a period of 30 minutes. The solution was spin-evaporated *in vacuo*. The residual oil was dissolved in a mixture of 25 ml. of toluene and 50 ml. of benzene, cooled to 5°, then washed with three 50 ml. portions of ice-cold 3% sodium hydroxide to remove unchanged ethyl benzoylacetate. The organic layer was washed with water, dried with magnesium sulfate, then spin-evaporated *in vacuo*; yield, 7.4 g. (59%) of crude VIIb as a colorless oil with ν max (film) 2250 (C≡N); 1740 (ester C=O); 1600 (C=C); 1265 (ester C-O-C); 755, 660 cm^{-1} (C_6H_5).

A mixture of 3.0 g. (12.2 mmoles) of crude VIIb, 30 ml. of *t*-butyl alcohol and 1.17 g. (6.5 mmoles) of guanidine carbonate was gently refluxed with magnetic stirring for 48 hours during which time the product separated. The mixture was cooled and the product was collected on a filter and washed with *t*-butyl alcohol, then water; yield, 0.80 g., m.p. 305-310° dec. Concentration of the filtrate gave an additional 0.35 g. (total 42%) with the same m.p. that was suitable for further transformations.

In a pilot run (9), the product was recrystallized from 2-methoxyethanol to give white crystals, m.p. 315-317° dec.; ν max 3400, 3150 (OH, NH); 2250 (C≡N); 1650, 1555 (NH, pyrimidine); 750, 700 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{13}H_{12}N_4O$: C, 65.0; H, 5.00; N, 23.4. Found: C, 64.9; H, 5.16; N, 23.1.

2-Mercapto-4-hydroxy-6-methyl-5-pyrimidylpropionitrile (XII).

To a stirred mixture of 4.19 g. (0.055 mole) of thiourea, and 6.50 g. (0.121 mole) of sodium methoxide in 50 ml. of reagent methanol was added 10.0 g. (0.055 mole) of ethyl 2-acetyl-4-cyanobutyrate (3,16). After being refluxed with stirring for 48 hours, the solution was spin-evaporated *in vacuo*. The residue was dissolved in 20 ml. of water, clarified by filtration and acidified to about *p*H 4 with acetic acid. The product was collected on a filter and washed with water; yield, 5.1 g., m.p. 250-255°. By concentration of the filtrate, an additional 1.2 g. (total 59%) was obtained that was suitable for further transformations. Two recrystallizations from methanol afforded white crystals, m.p. 258-260°; λ max (*p*H 1), 280 mμ (ε, 21,200); λ max (*p*H 8.4) 280 mμ (ε, 22,700); λ max (*p*H 13), 262 (ε, 16,100), 310 mμ (shoulder, ε, 7,800); ν max 3200 (NH); 2250 (C≡N); 1650, 1550 cm^{-1} (NH, pyrimidine).

Anal. Calcd. for $C_8H_9N_3OS$: C, 49.2; H, 4.64; N, 21.5. Found: C, 49.3; H, 4.80; N, 21.6.

2-Acetamido-4-hydroxy-6-methyl-5-pyrimidylpropionitrile (XIIIa).

A solution of 2.0 g. (12 mmoles) of VIIIa in 3.8 ml. of acetic anhydride and 7.5 ml. of reagent pyridine protected from moisture was heated in a bath at 85-90° for 4 hours, then spin-evaporated *in vacuo*; the evaporation was repeated after the addition of 10 ml. of toluene in order to remove traces of pyridine. Recrystallization of the residue from ethyl acetate gave 2.2 g. (83%) of white crystals, m.p. 202-203°; ν max 3450, 3150 (NH, OH); 2250 (C≡N); 1670 (amide C=O); 1640, 1600, 1550 cm^{-1} (NH, pyrimidine).

Anal. Calcd. for $C_{10}H_{12}N_4O_2$: C, 54.5; H, 5.50; N, 25.4. Found: C, 54.4; H, 5.65; N, 25.2.

2-Acetamido-4-hydroxy-6-phenyl-5-pyrimidylpropionitrile (XIIIb).

Compound XIIIb was prepared as described for XIIIa, but the reaction time was 2 hours. Recrystallization from ethyl acetate gave 2.3 g. (92%) of product, m.p. 228-230°. Two more recrystallizations of a sample from ethyl acetate afforded white crystals, m.p. 230-232°; ν max 3200 (NH); 2250 (C≡N); 1650 (amide C=O); 1600, 1550 (NH, pyrimidine); 765, 700 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{15}H_{14}N_4O_2$: C, 63.8; H, 4.99; N, 19.9. Found: C, 63.8; H, 5.20; N, 19.7.

2-Acetamido-4-chloro-6-methyl-5-pyrimidylpropionitrile (XVIIa).

A stirred mixture of 5.0 g. (0.022 mole) of XIIIa, 50 ml. of reagent benzene and 5.0 g. (0.032 mole) of phosphorus oxychloride was surrounded by an oil bath preheated and maintained at 85°. Solution was complete in 5 minutes; as soon as turbidity appeared (an additional 5 minutes), the hot mixture was immediately poured into a stirred mixture of 32 g. of anhydrous sodium acetate in 100 ml. of water and 50 g. of ice. The mixture was stirred exactly 5 minutes, then the organic layer was separated. The aqueous layer was extracted with dichloromethane (3 x 20 ml.). The combined organic solutions, dried with magnesium sulfate, were spin-evaporated *in vacuo*; yield, 4.0 g. (77%) of a greenish-white solid, m.p. 130-140°, that was suitable for further transformations. Two recrystallizations from dichloromethane-hexane gave white crystals, m.p. 140-145°; ν max 3200 (NH); 2250 (C≡N); 1670 (amide C=O); 1600, 1550 cm^{-1} (NH, pyrimidine).

Anal. Calcd. for $C_{10}H_{11}ClN_4O$: C, 50.3; H, 4.62; N, 23.5; Cl, 14.9. Found: C, 50.4; H, 4.77; N, 23.3; Cl, 14.6.

2-Amino-4-chloro-6-methyl-5-pyrimidylpropionitrile (XVIIIa).

(A). A solution of 1.00 g. (4.2 mmoles) of crude XVIIa and 1 ml. of di*iso*propylamine in 70 ml. of methanol was refluxed for 21 hours (12). The solution was spin-evaporated *in vacuo* to about 20 ml. when the product began to separate. After being stored at 0° for 2 hours, the mixture was filtered and the white crystals were washed with cold methanol; yield, 0.47 g. (61%), m.p. 212-215°. Recrystallization from absolute ethanol gave white needles, m.p. 215-216°; λ max (*p*H 1) 314 mμ (ε, 8,100); λ max (*p*H 8.4) 235 (ε, 19,600), 300 mμ (ε, 5,800); λ max (*p*H 13) 300 mμ (ε, 7,300); ν max 3300 (NH); 2250 (C≡N); 1620, 1525 cm^{-1} (NH, pyrimidine).

Anal. Calcd. for $C_8H_9ClN_4$: C, 48.9; H, 4.59; N, 28.5. Found: C, 48.7; H, 4.75; N, 28.8.

(B). A solution of 500 mg. (2.5 mmoles) of VIIIa in 2.5 ml. of phosphorus oxychloride was refluxed for 30 minutes. The cooled solution was poured with stirring into 30 ml. of 1 N sodium hydroxide with ice cooling. After the excess phosphorus oxychloride has dissolved, the product was collected on a filter and washed well with water, then ether; yield, 230 mg. (38%), m.p. 212-215°, that was identical with preparation A.

2-Amino-4-chloro-6-phenyl-5-pyrimidylpropionitrile (XVIIIb).

A mixture of 200 mg. (0.84 mmole) of VIIIb and 1 ml. of phosphorus oxychloride was refluxed for 45 minutes, then cooled and poured into 25 ml. of ice cooled 4 N sodium hydroxide; yield, 80 mg. (39%), m.p. 195-205° dec., that was satisfactory for further transformations. Two recrystallizations from ethanol gave white crystals, m.p. 238-239°; λ max (*p*H 1) 235 (ε, 16,400), 325 mμ (ε, 10,100); λ max (*p*H 8.4) 237 (ε, 27,400), 310 mμ (ε, 9,400); λ max (*p*H 13) 237 (ε, 27,800), 310 mμ (ε, 8,100); ν max 3400, 3200 (NH); 2250 (C≡N); 1620, 1550, 1500 (NH, pyrimidine); 760, 680 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{13}H_{11}ClN_4$: C, 60.3; H, 4.27; N, 21.7. Found: C, 60.7; H, 4.18; N, 21.7.

2-Amino-4-methyl-7-oxopiperidino[2,3-d]pyrimidine (XIV).

A mixture of 500 mg. (2.15 mmoles) of XVIIIa and 20 ml. of methanol previously saturated with ammonia at 0° was heated in a steel bomb at 155° for 23 hours. The resulting mixture was spin-evaporated *in vacuo*; the evaporation was repeated after addition of 10 ml. of methanol leaving 335 mg. (90%) of product, m.p. above 300°. Two recrystallizations from absolute ethanol gave the analytical sample, m.p. above 300°; λ max (*p*H 1 or 8.4) 305 mμ (ε, 6,800); λ max (*p*H 13) 310 mμ (ε, 11,600); ν max 3350 (NH); 1690 (cyclic amide C=O); 1610, 1560 (NH, pyrimidine); no C≡N near 2250 cm^{-1}.

Anal. Calcd. for $C_8H_{10}N_4O$: C, 54.0; H, 5.65; N, 31.4. Found: C, 54.0; H, 5.72; N, 30.9.

2-Amino-4,8-dimethyl-7-oxopiperidino[2,3-d]pyrimidine (XV).

A mixture of 464 mg. (2 mmoles) of XVIIIa, 1.1 ml. of 40% aqueous methylamine and 5 ml. of ethanol was heated in a steel bomb at 145° for 24 hours, then processed as in the preparation of XIV. Trituration of the residue with 10 ml. of water gave 365 mg. (95%) of insoluble product, m.p. 220-235° dec. Two recrystallizations from absolute ethanol gave the analytical sample as nearly white crystals, m.p. 235-236° dec.; λ max (*p*H 1) 229 (ε, 23,100), 255 (ε, 10,600), 302 mμ (ε, 8,700); λ max (*p*H 8.4) 224 (ε, 31,600), 302 mμ (ε, 10,500); λ max (*p*H 13) 290 mμ (ε, 9,400); ν max 3400, 3200 (NH); 1660, 1560 (NH, pyrimidine), and no C≡N near 2250 cm^{-1}.

Anal. Calcd. for $C_9H_{12}N_4O$: C, 56.3; H, 6.31; N, 29.1. Found: C, 56.1; H, 6.25; N, 29.1.

2-Acetamido-5-(2-cyanoethyl)-6-methyl-4-pyrimidyl *iso*thiouronium chloride (XIXa).

A mixture of 5.0 g. (0.020 mole) of crude XVIIa, 2.5 g. of thiourea, and 40 ml. of *t*-butyl alcohol was refluxed with magnetic stirring for 1 hour. Solution was nearly complete in 5 minutes, then the product began to separate. The mixture was diluted with 10 ml. of acetone and cooled. The product was collected on a filter and washed with acetone; yield, 5.0 g. (80%), m.p. 155-165° dec., that was suitable for further transformation. No suitable solvent for recrystallization could be found. An analytical sample, m.p. 155-159° dec., separated from the reaction mixture when analytically pure XVIIa was employed; this compound had λ max (*p*H 1, 8.4) 242 (ε, 19,100), 292 mμ (ε, 8,200); λ max (*p*H 13) 309 mμ (ε, 12,000); ν max 3200 (NH); 2250 (C≡N); 1700 cm^{-1} (C=NH^+).

Anal. Calcd. for $C_{11}H_{15}ClN_6OS \cdot H_2O$: C, 39.7; H, 5.13; N, 25.2. Found: C, 39.8; H, 5.10; N, 24.9.

2-Amino-4-mercapto-6-methyl-5-pyrimidylpropionitrile (XXIIa).

A solution of 200 mg. (0.636 mmole) of XIXa in 4 ml. of 1 N sodium hydroxide was allowed to stand at room temperature for two hours when the change in ultraviolet spectrum was complete. The solution was clarified by filtration, then acidified to about *p*H 5 with dilute acetic acid. The product was collected on a filter and washed with water; yield, 110 mg. (90%) m.p. 240-245° dec. Two recrystallizations from absolute alcohol gave pale yellow crystals, m.p. 242-244° dec.; λ max (*p*H 1) 265 (shoulder, ε, 6,900), 347 mμ (ε, 18,700); λ max (*p*H 13) 265 (shoulder, ε, 9,300), 317 mμ (ε, 16,800); ν max 3400 (NH); 2250 (C≡N); 1650, 1600, 1560 cm^{-1} (NH, pyrimidine).

Anal. Calcd. for $C_8H_{10}N_4S$: C, 49.5; H, 5.16; N, 28.9. Found: C, 49.8; H, 5.31; N, 28.8.

2-Amino-4-mercapto-6-methyl-5-pyrimidylpropionic acid (XXIIIa).

A solution of 3.5 g. (18 mmoles) of XXIa in 30 ml. of 6 N hydrochloric acid was refluxed for 2 hours, then concentrated to about half volume by spin-evaporation. After neutralization to about *p*H 4 with 1 N sodium hydroxide, the mixture was filtered and the product washed with water. This solid was dissolved in 20 ml. of 5% aqueous sodium bicarbonate; after clarification of the solution by filtration, it was acidified to *p*H 5. The product was collected on a filter and washed with water; yield, 2.7 g. (71%), m.p. 220-225° dec., that was suitable for further transformations. Recrystallization from water gave the analytical sample, m.p. 222-225° dec.; the ultraviolet spectrum was similar to XXIa; ν max 3500, 3400 (NH, OH); 1700 (carboxyl C=O); 1625, 1550 (NH, pyrimidine); no C≡N near 2250 cm^{-1}.

Anal. Calcd. for $C_8H_{11}N_3O_2S$: C, 45.0; H, 5.21; N, 19.7. Found: C, 44.9; H, 5.24; N, 19.8.

2-Amino-4-hydroxy-6-phenyl-5-pyrimidylpropionic acid (IXb).

A mixture of 2.6 g. (10.7 mmoles) of VIIIb and 20 ml. of 6 N hydrochloric acid was refluxed for 3 hours, then cooled and neutralized to *p*H 5 with 1 N base. The product was collected on a filter and washed with water; yield, 1.9 g. (71%), m.p. 255-261° dec. that was suitable for further transformation. For analysis a sample was dissolved in 5% aqueous sodium bicarbonate and the filtered solution was acidified with dilute acetic acid. The product was collected on a filter and washed well with water; this reprecipitation was repeated twice more to give a white powder, m.p. 259-261° dec.; λ max (*p*H 1) 232 (ε, 17,600), 276 mμ (ε, 11,900); λ max (*p*H 8.4) 232 (ε, 20,000), 290 mμ (ε, 9,100); λ max (*p*H 13) 289 mμ (ε, 8,900); ν max 3400, 3200 (OH, NH); 1700 (carboxyl C=O); 1640, 1550 (NH, pyrimidine); no C≡N near 2250 cm^{-1}.

Anal. Calcd. for $C_{13}H_{13}N_3O_3$: C, 60.2; H, 5.06; N, 16.2. Found: C, 60.2; H, 5.08; N, 16.2.

2-Amino-4-mercapto-6-phenyl-5-pyrimidylpropionic acid (XXIIIb).

A mixture of 1.00 g. (3.88 mmoles) of crude XVIIIb, 297 mg. (3.9 mmoles) of thiourea and 10 ml. of *t*-butyl alcohol was refluxed with magnetic stirring for 45 minutes; in about 5 minutes the solution was almost clear, then the product (XXb) began to separate. The mixture was diluted with 10 ml. of acetone and chilled. The thiouronium salt (XXb) was collected on a filter and washed with acetone.

This salt was dissolved in 10 ml. of 15% aqueous sodium hydroxide and the solution was refluxed for 1 hour. The cooled solution was acidified to *p*H 4, then the product was collected on a filter and washed with water; yield, 0.55 g. (55%), m.p. 235-237° dec., that was suitable for further transformations. Two recrystallizations of a sample from water gave yellow leaflets, m.p. 253-254° dec.; λ max (*p*H 1) 262 (ε, 8,400), 347 mμ (ε, 18,500); λ max (*p*H 8.4) 260 (ε, 12,400), 360 mμ (ε, 18,700); λ max (*p*H 13) 327 mμ (ε, 15,400); ν max 3350, 3100 (NH, OH); 1695 (carboxyl C=O); 1650, 1590, 1550 (NH, pyrimidine); 750, 700 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{13}H_{13}N_3O_2S$: C, 56.8; H, 4.76; N, 15.3. Found: C, 57.0; H, 4.86; N, 15.0.

2-Mercapto-4-hydroxy-6-methyl-5-pyrimidylpropionic acid (XVI).

A solution of 200 mg. (1.02 mmoles) of XII in 3 ml. of 15% aqueous sodium hydroxide was refluxed for 75 minutes, then processed as described for XXIIIb; yield, 200 mg. (92%), m.p. 295-300° dec. that was suitable for further transformations. Two recrystallizations from water gave white crystals, m.p. 305-306° dec.; λ max (*p*H 1) 280 mμ (ε, 22,700); λ max (*p*H 13) 262 (ε, 18,600), 310 mμ (shoulder, ε, 10,300); ν max 3500, 3150-3000 (OH, NH); 1710 (carboxyl C=O); 1650, 1560 cm^{-1} (NH, pyrimidine).

Anal. Calcd. for $C_8H_{10}N_2O_3S$: C, 44.9; H, 4.67; N, 13.1. Found: C, 45.0; H, 4.86; N, 13.1.

2-Acetamido-5,6-dihydro-7-oxo-4-phenyl-7H-thiopyrano[2,3-d]pyrimidine (XXIVb).

A mixture of 137 mg. (0.50 mmole) of XXIIIb and 2 ml. of acetic anhydride was refluxed for 15 minutes. The solution was spin-evaporated *in vacuo* and the residue further spin-evaporated with 5 ml. of toluene. Recrystallization from ethyl acetate gave 60 mg. (40%) of product, m.p. 188-189° dec., which was unchanged on further recrystallization from the same solvent. The compound had λ max (*p*H 1) 238 (ε, 19,700), 297 (shoulder, ε, 8,700), 347 mμ (shoulder, ε, 5,200); λ max (*p*H 8.4) 242 (ε, 26,800), 302 mμ (ε, 9,400); λ max (*p*H 13) 320 mμ (ε, 10,800); ν max 3250 (NH); 1790, 1740 (ester C=O); 1700 (amide C=O); 1670, 1555 (NH, pyrimidine); 760, 700 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{15}H_{13}N_3O_2S$: C, 60.2; H, 4.38; N, 14.0. Found: C, 60.2; H, 4.53; N, 13.8.

2-Amino-4-hydroxy-6-phenyl-5-pyrimidylpropionyl chloride (Xb) hydrochloride.

To 0.648 g. (2.5 mmoles) of IXb was added dropwise 8.2 g. of thionyl chloride (Eastman White Label) over a period of 10 minutes. The solution was refluxed for 45 minutes during which time the product began to separate. The mixture was spin-evaporated to residue *in vacuo* with a calcium chloride trap in between the spin-evaporator and the water pump. The residue was triturated with 20 ml. of anhydrous reagent ether, then collected on a filter and washed with ether. The still moist product was transferred to a desiccator containing phosphorus pentoxide; yield, 0.650 g. (83%), m.p. 130-165° dec.; ν max 3200 (NH, OH); 1790 (acid chloride C=O); 1650, 1525 (NH, pyrimidine); 765, 690 cm^{-1} (C_6H_5). This compound was characterized as the anilide, XIb, as described later.

2-Mercapto-4-hydroxy-6-methyl-5-pyrimidylpropionyl chloride.

A mixture of 214 mg. (1 mmole) of XVI and 4.97 g. of thionyl chloride (Eastman White Label) was refluxed for 7 minutes when solution was complete. The reaction was processed as described above for Xb; yield, 170 mg. (74%), m.p. 130-145° dec.; ν max 3500-3050 (broad NH, OH); 1790 (acid chloride C=O); 1560 cm^{-1} (pyrimidine). This compound was characterized as the anilide, XXV, as follows:

2-Mercapto-4-hydroxy-6-methyl-5-pyrimidylpropionanilide (XXV).

To a magnetically stirred solution of 93 mg. (1 mmole) of aniline in 1 ml. of reagent pyridine was added, in one portion, 116 mg. (0.5 mmole) of the preceding acid chloride of XVI. After 45 minutes at ambient temperature protected from moisture, the solution was poured into 10 ml. of iced water and acidified to about *p*H 2 with 1 N hydrochloric acid. The red solid was collected on a filter and washed well with water. The wet solid was dissolved in 5 ml. of 0.1 N aqueous sodium hydroxide; the solution was clarified by filtration, then acidified to about *p*H 2 with 0.1 N hydrochloric acid. The product was removed by filtration and washed with water; yield, 85 mg. (59%), m.p. 215-216°. Recrystallization from methanol gave yellow crystals, m.p. 215-216°; λ max (*p*H 1, 8.4) 280 mμ (ε, 17,700); λ max (*p*H 13) 240 (ε, 20,600), 260 (shoulder, ε, 17,300), 310 mμ (shoulder, ε, 6,700); ν max 3450, 3100 (NH, OH); 1660, 1550 (amide C=O, NH, pyrimidine); 750, 675 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{14}H_{15}N_3O_2S$: C, 58.1; H, 5.23; N, 14.5. Found: C, 57.7; H, 5.50; N, 14.3.

2-Amino-4-hydroxy-6-phenyl-5-pyrimidylpropionanilide (XIb).

(A). Reaction of 156 mg. (0.5 mmole) of the acid chloride (Xb) hydrochloride with aniline as described for the preparation of XXV gave on addition to water, 110 mg. (66%) of product, m.p. 295-301° dec. Two recrystallizations from methanol gave the analytical sample as white crystals, m.p. 305-306° dec.; ν max 3400, 3300 (OH, NH); 1650, 1560 (amide C=O, NH, pyrimidine); 750, 700 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{19}H_{18}N_4O_2$: C, 68.2; H, 5.42; N, 16.7. Found: C, 68.4; H, 5.60; N, 16.6.

(B). To a magnetically stirred mixture of 129 mg. (0.5 mmole) of IXb, 102 mg. (1 mmole) of triethylamine, and 3 ml. of N,N-dimethylformamide cooled in an ice-bath and protected from moisture was added 55 mg. (0.5 mmole) of ethyl chloroformate in one portion. After being stirred for 20 minutes at 0°, the mixture was treated with 93 mg. (1 mmole) of aniline, then stirred at room temperature for 12 hours. The suspension was poured into 10 g. of iced water. The product was collected on a filter and washed with water; yield, 161 mg. (96%), m.p. 305-306° dec., that was identical with preparation A.

2-Amino-4-mercapto-6-methyl-5-pyrimidylpropionanilide (XXVIIa).

Conversion of 100 mg. (0.47 mmole) of XXIIIa to the mixed anhydride and coupling with aniline, as described for the preparation of XIb (method B), gave 102 mg. (71%) of product m.p. 255-260° dec. Recrystallization from absolute ethanol gave yellow crystals, m.p. 259-260° dec.; λ max (*p*H 1) 247 (ε, 17,600), 337 mμ (ε, 17,800); λ max (*p*H 13) 240 (inflection, ε, 22,800), 317 mμ (ε, 13,600); ν max 3300 (NH); 1660 (amide C=O); 1600, 1500 (NH, pyrimidine); 755, 680 cm^{-1} (C_6H_5).

Anal. Calcd. for $C_{14}H_{16}N_4OS$: C, 58.3; H, 5.55; N, 19.4. Found: C, 58.3; H, 5.74; N, 19.2.

2-Amino-4-mercapto-6-phenyl-5-pyrimidylpropionanilide (XXVIIb).

(A). Conversion of 138 mg. (0.5 mmole) of XXIIIb to the mixed anhydride and coupling with aniline as described for the preparation of XIb gave 110 mg. (63%) of product, m.p. 285-287° dec. Two recrystallizations from methanol afforded yellow crystals, m.p. 295° dec., with the proper spectral characteristics.

Anal. Calcd. for $C_{19}H_{18}N_4OS$: C, 65.2; H, 5.15; N, 16.0. Found: C, 65.4; H, 5.28; N, 15.8.

(B). A mixture of 75 mg. (0.25 mmole) of the thiolactone (XXIVb), 186 mg. (2 mmoles) of aniline and 2 ml. of absolute ethanol was refluxed for 10 hours. The solution was spin-evaporated *in vacuo*; the residue was dissolved in 3 ml. of 1 N sodium hydroxide, allowed to stand 1 hour, then the solution was filtered and acidified to *p*H 4 with dilute acetic acid. The crude product was collected on a filter and washed with water; yield, 40 mg. (45%), m.p. 250-255° dec. Recrystallization from methanol gave yellow crystals, m.p. 295° dec., that were identical with preparation A.

N-(2-Amino-4-hydroxy-6-phenyl-5-pyrimidylpropionyl)-*p*-aminobenzoyl-L-glutamic acid (Ib).

Reaction of 266 mg. (1 mmole) of freshly prepared Xb with 314 mg. of *p*-aminobenzoyl-L-glutamic acid in 2 ml. of reagent pyridine for 24 hours at room temperature gave, after dilution with 10 g. of iced water and acidification to *p*H 4 with dilute acetic acid, 315 mg. (58%) of insoluble product, m.p. 180-215° dec.; this material contained only 0.4% of *p*-aminobenzoyl-L-glutamic acid by the Bratton and Marshall assay (21). Two recrystallizations from methanol afforded the analytical sample, m.p. 195-217° dec., that gave negative Bratton and Marshall assay and had λ max (*p*H 1) 272 mμ (ε, 26,800); λ max (*p*H 8.4) 272 mμ (ε, 25,700); λ max (*p*H 13) 272 mμ (ε, 22,500).

Anal. Calcd. for $C_{25}H_{25}N_5O_7$: C, 59.2; H, 4.96; N, 13.8. Found: C, 59.2; H, 5.04; N, 13.7.

N-(2-Mercapto-4-hydroxy-6-methyl-5-pyrimidylpropionyl)-*p*-aminobenzoyl-L-glutamic acid (XXVI).

Compound XXVI was prepared from the acid chloride of XVI as described for the preparation of Ib; yield, 160 mg. (35%) which gave a negative Bratton and Marshall test (21). Two recrystallizations from water gave a pale yellow powder with λ max (*p*H 1) 275 mμ (ε, 37,000); λ max (*p*H 8.4) 275 mμ (ε, 42,800); λ max (*p*H 13) 267 (ε, 43,300), 315 mμ (shoulder, ε, 12,100).

Anal. Calcd. for $C_{20}H_{22}N_4O_7S$: C, 52.0; H, 4.76; N, 12.1. Found: C, 51.8; H, 4.90; N, 12.0.

N-(2-Amino-4-mercapto-6-methyl-5-pyrimidylpropionyl)-*p*-aminobenzoyl-L-glutamic acid (XXVIIIa).

To a solution of the mixed anhydride prepared at 0° from 200 mg. (0.94 mmoles) of XXIIIa, 100 mg. ethyl chloroformate and 152 mg. of triethylamine in 2 ml. of N,N-dimethylformamide, as described under the preparation of XIb, was added 266 mg. (1 mmole) of *p*-aminobenzoyl-L-glutamic acid. After being magnetically stirred at 5-6° for 24 hours protected from moisture, the mixture was poured into 20 g. of iced water and acidified to *p*H 3.5 with 1 N hydrochloric acid. After 48 hours at 5° to complete crystallization, the mixture was filtered and the product washed with water; yield, 110 mg. (24%), m.p. 180-215° dec., that gave a negative Bratton and Marshall test (21). Recrystallization from water afforded the analytical sample, m.p. 180-215° dec.; λ max (*p*H 1) 267 (ε, 27,600), 337 mμ (ε, 15,100); λ max (*p*H 8.4) 267 (ε, 29,400), 347 mμ (ε, 17,100); λ max (*p*H 13) 267 (ε, 31,100), 317 mμ (ε, 15,100).

Anal. Calcd. for $C_{20}H_{23}N_5O_6S$: C, 52.0; H, 5.02; N, 15.2. Found: C, 51.8; H, 5.02; N, 15.0.

N-(2-Amino-4-mercapto-6-phenyl-5-pyrimidylpropionyl)-*p*-aminobenzoyl-L-glutamic acid (XXVIIIb).

A mixture of 150 mg. (0.5 mmole) of XXIVb and 133 mg. (0.5 mmole) of *p*-aminobenzoyl-L-glutamic acid in 3 ml. of absolute ethanol was refluxed for 12 hours, then spin-evaporated *in vacuo*. The residue was dissolved in 5 ml. of 1 N aqueous sodium hydroxide; the solution was allowed to stand for 2 hours, then filtered. The filtrate was acidified to *p*H 2.5 with 3 N hydrochloric acid and the product was collected on a filter, then washed with water; yield, 50 mg. (19%), m.p. 195-215° dec., that gave a negative Bratton and Marshall test (21). One recrystallization from water gave a yellow powder, m.p. 195-215° dec.; λ max (*p*H 1) 272 (ε, 28,000), 347 mμ (ε, 17,400); λ max (*p*H 8.4) 270 (ε, 31,000), 360 mμ (ε, 17,800); λ max (*p*H 13) 269 (ε, 29,400), 327 mμ (ε, 15,900).

Anal. Calcd. for $C_{25}H_{25}N_5O_6S \cdot 1.4\ H_2O$: C, 54.7; H, 5.10; N, 12.8; O, 21.5. Found: C, 54.4; H, 4.95; N, 12.7; O, 21.0.

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