Vitamin B_6 Derivatives. 13.[1] Synthesis of Tetrahydrothiazine Derivatives of Vitamin B_6 and Their Biological Properties

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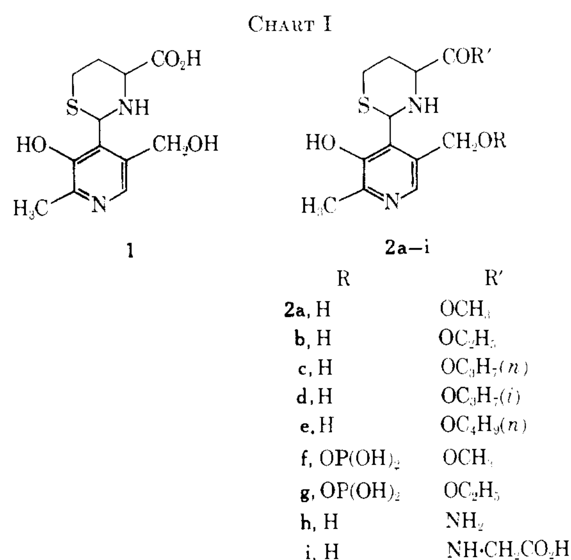
The synthesis and the biological properties of the tetrahydrothiazine derivatives **2a-i**, the esters and the amides of 2-(3-hydroxy-5-hydroxymethyl-2-methyl-4-pyridyl)tetrahydro-1,3-thiazine-4-carboxylic acid (**1**) and their phosphates, were investigated. The esters **2a-g** were obtained by the reaction of pyridoxal or pyridoxal 5-phosphate with alkyl DL-homocysteinates, and the amides **2h,i** were prepared by the condensation of pyridoxal with DL-homocysteinamide and DL-homocysteinylglycine. Also, *N*-(4-pyridoxyl)dehydrohomocysteine thiolactone·HCl (**5b**·HCl) was obtained by the reaction of DL-homocysteine thiolactone·HCl with pyridoxal. These compounds were shown to have the microbiological potencies as vitamin B_6 for the growth of *Saccharomyces carlsbergensis*. Measurement of the total vitamin B_6 concentrations in whole blood after oral administrations of **5b**·HCl for rabbits indicated an interesting maintenance of high concentration for longer period. Effective doses (ED_{50}, po) of **2i**·HCl and **5b**·HCl as vitamin B_6, which were tested by the antidotal effect on death of mice with convulsion induced by the lethal dose of OMP (500 mg/kg, sc), were 7.64 and 10.9 mg/kg, respectively.

Since pyridoxal 5-phosphate can function as a cofactor of many enzymes involved in the metabolism of amino acid, studies of the Schiff bases derived from pyridoxal and pyridoxal 5-phosphate, and various amino acids have been undertaken widely.[2a-j]

Pyridoxal and pyridoxal 5-phosphate react spontaneously with various amino acids to afford the corresponding Schiff bases. However, very few of these Schiff bases have been isolated in a crystalline form because of their instability.[3,4a] Support for the formation of the Schiff bases has been mainly spectrophotometry.[2a-j] Histidine, dopa, tryptophan, and cysteine react with pyridoxal and with pyridoxal 5-phosphate to form the respective isolable cyclic compounds.[4a-e] The first three amino acids yield cyclic compounds[5] which have no vitamin B_6 activity. The thiazolidine compound derived from cysteine, however, is active, its potency being that of pyridoxal for all organisms.[5] The formation of the tetrahydrothiazine **1**, 2-(3-hydroxy-5-hydroxymethyl-2-methyl-4-pyridyl)tetrahydro-1,3-thiazine-4-carboxylic acid, in the reaction of homocysteine with pyridoxal has been anticipated from observations of the uv spectra of the reaction mixture of these two substances.[2f] The tetrahydrothiazine **1** may be expected to have the same order of vitamin B_6 activity because of its similarity to the thiazolidine derivative, and also to have a particular biological significance in view of the role of vitamin B_6 on the metabolism of S-containing amino acids.

CHART I



	R	R′
2a,	H	OCH_3
b,	H	OC_2H_5
c,	H	$OC_3H_7(n)$
d,	H	$OC_3H_7(i)$
e,	H	$OC_4H_9(n)$
f,	$OP(OH)_2$	OCH_3
g,	$OP(OH)_2$	OC_2H_5
h,	H	NH_2
i,	H	$NH{\cdot}CH_2CO_2H$

With these expectations, the synthesis and isolation of **1** have been investigated together with studies of its physiological properties.[6a-d] As expected, **1** showed vitamin B_6 activity to the same extent as pyridoxal 5-phosphate and pyridoxine·HCl in experiments involving the growth of vitamin B_6-deficient rat and also in suppressing convulsions caused by an antivitamin B_6 agent, 4-amino-5-hydroxymethyl-2-methylpyrimidine (OMP). In the present paper, we report the synthesis and the biological properties of the tetrahydrothiazine derivatives **2a–i**, in which CO_2H is replaced by an amide or ester. The synthesis of **2a–i** was first attempted *via* a presumed Schiff base **5a**.[7]

The reaction of DL-homocysteine thiolactone·HCl (**4**·HCl) with pyridoxal in MeOH afforded a new compound in 57% yield. Its elementary analysis agreed

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(2) (a) D. Heinert and A. E. Martell, *J. Amer. Chem. Soc.*, **84**, 3257 (1962). (b) *Ibid.*, **85**, 183 (1963). (c) *Ibid.*, **85**, 188 (1963). (d) Y. Matsushima and A. E. Martell, *J. Amer. Chem. Soc.*, **89**, 1322 (1967). (e) B. Witkop and T. W. Beiler, *ibid.*, **76**, 5589 (1954). (f) Y. Matsuo, *ibid.*, **79**, 2011 (1957). (g) L. Schirch and R. A. Slotter, *Biochemistry*, **5**, 3175 (1966). (h) H. N. Christensen, *J. Amer. Chem. Soc.*, **80**, 99 (1958). (i) D. E. Metzler, *ibid.*, **79**, 485 (1957). (j) K. Nagano and D. E. Metzler, *ibid.*, **89**, 2891 (1967).

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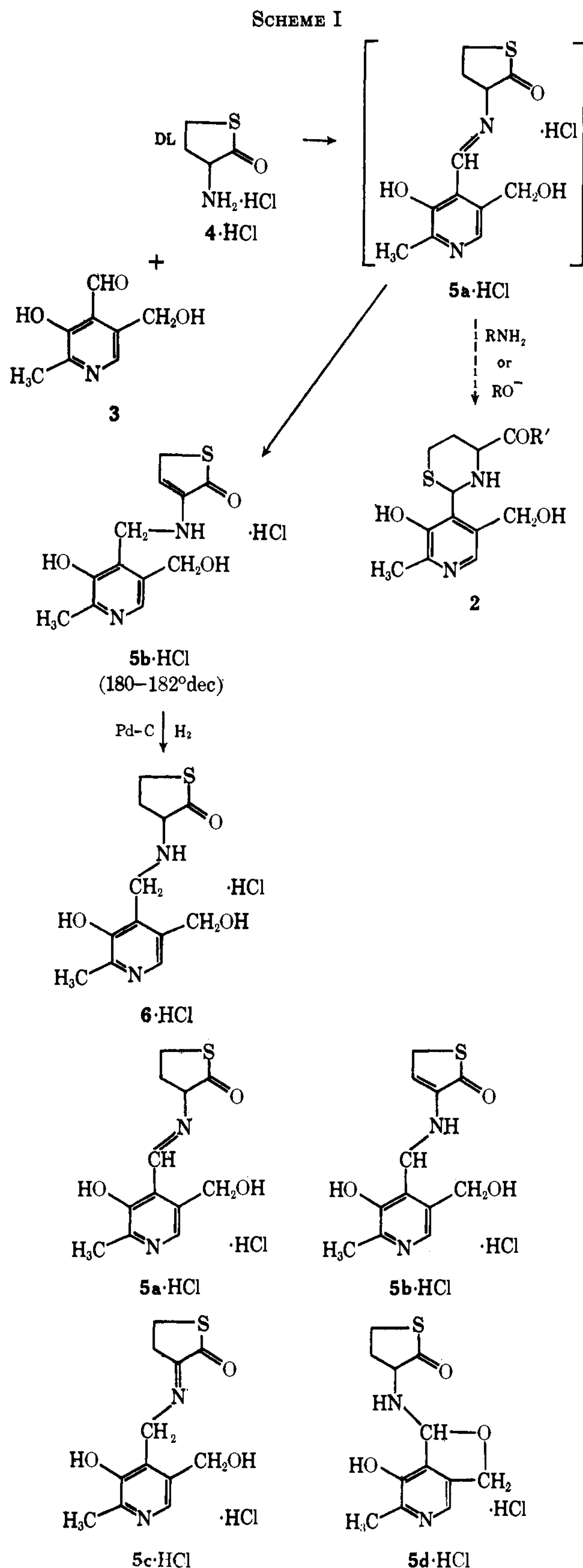
(7) While we were preparing this manuscript, an interesting investigation in which the formation of the Schiff base **5a** in the reaction of homocysteine thiolactone with pyridoxal in neutral D_2O soln was elucidated by nmr spectroscopy, has been reported by A. E. Martell and E. H. Abbott, *J. Amer. Chem. Soc.*, **92**, 1754 (1970).



well with that expected for **5a**·HCl. However, the nmr spectrum showed no azomethine proton signal at 8–9 ppm characteristic[8a–b] of an amino acid Schiff base of pyridoxal and suggested instead an olefinic proton signal at 6.10 ppm (1 H, triplet, J = 3.5 Hz) and the benzylic protons at 4.40 ppm (2 H, singlet). The uv spectrum of this compound in MeOH at pH 10.9 indicated an absorption maximum at 311 nm comparative to the additive curve of the spectra of pyridoxamine and the dehydrothiolactone.[9] This compound could not be reduced with $NaBH_4$ and unchanged starting material was recovered. Catalytic hydrogenation of the compound in the presence of 10% Pd/C in acid medium furnished the dihydro compound hydrochloride (**6**·HCl). These spectral data and the chemical properties suggested the enamine structure **5b** among the possible structures **5a–d**. The formation of **5b** could be explained by the migration of the double bond into the most stable endocyclic position. This unusual behavior on the tautomerism may be attributed to the strain of the 5-membered ring containing an S atom or to the transannular resonance[10] between the double bond and the d orbital of S. As the equilibrium of the tautomerism of the Schiff base was assumed to favor the aldimine structure **5a** under the conditions employed, it is quite interesting that the rearrangement of the Schiff base initially formed to **5b** has occurred preferentially. An attempt to prepare **5a** was unsuccessful and was abandoned. Therefore, the condensations of pyridoxal and of pyridoxal 5-phosphate with alkyl DL-homocysteinates (**7**) obtained by the ring cleavage of **4**·HCl with alkoxides were carried out; the results are given in Table I.

The synthesis of homocysteinyl peptides by the acylation of amino acids with DL-homocysteine thiolactone (**4**) was undertaken according to the procedure of Laliberte.[11] The reaction of *N*-benzyloxycarbonyl-DL-homocysteine thiolactone (**8**) with NH_3, or with Na glycinate in MeOH under N_2, furnished the corresponding amides **9a** and **9b**, respectively, in good yield. The treatment of these amides with Na in liquid NH_3, followed by isolation of the product through the lead mercaptide gave crude DL-homocysteinamide·HCl (**10a**·HCl) and DL-homocysteinylglycine·HCl (**10b**·HCl), resp, in low yields. These amides could not be purified owing to the ready oxidation of SH groups and were therefore used in the crude form.

The *tert*-butoxycarbonyl group was also employed as an amino protecting group. The treatment of **4**·HCl with *tert*-BuOCOCl[12] in the presence of Et_3N afforded *N*-*tert*-butoxycarbonyl-DL-homocysteine thiolactone (**11**) in 25% yield. Ammonolysis of **11** in MeOH gave *N*-*tert*-butoxycarbonyl-DL-homocysteinamide (**12**) in high yield and subsequent removal of the protective group proceeded smoothly with HCl in moist Et_2O (1% H_2O); it afforded **10a** in quant yield. Further attempts to transform **11** into homocysteinyl peptides were unsuccessful.

The reactions of **10a**·HCl and of **10b**·HCl with pyridoxal furnished **2h**·HCl and **2i**·HCl in yields of 30.3 and 50.8%, respectively. Their structures were elucidated by elementary analyses and the presence of a 4′-methine

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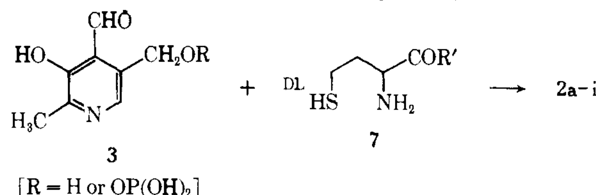
Table I

The Tetrahydrothiazine Derivatives of Pyridoxal and Pyridoxal 5-Phosphate



2	R	R′	Mp, °C	Yield, %	Recrystn solvent	Formula
a	H	OCH_3	86–89	65.0	H_2O	$C_{13}H_{18}N_2O_4S$
b	H	OC_2H_5	91–94	53.0	EtOH–H_2O	$C_{14}H_{20}N_2O_4S \cdot H_2O$
c	H	$OC_3H_7(n)$	127	48.0	PrOH–H_2O	$C_{15}H_{22}N_2O_4S$
d	H	$OC_3H_7(i)$	117–119	36.0	*i*-PrOH–H_2O	$C_{15}H_{22}N_2O_4S$
e	H	$OC_4H_9(n)$	118–119	12.0	MeOH–H_2O	$C_{16}H_{24}N_2O_4S$
f	$OP(OH)_2$	OCH_3	187	70.3	H_2O	$C_{13}H_{19}N_2O_7SP$
g	$OP(OH)_2$	OC_2H_5	169–172	19.5	H_2O	$C_{14}H_{21}N_2O_7SP \cdot 2H_2O$
h	H	NH_2	163–165	30.3	MeOH	$C_{12}H_{17}N_3O_3S \cdot HCl$
i	H	$NHCH_2CO_2H$	177–179	50.8	H_2O	$C_{14}H_{19}N_3O_5S \cdot HCl$

Scheme II

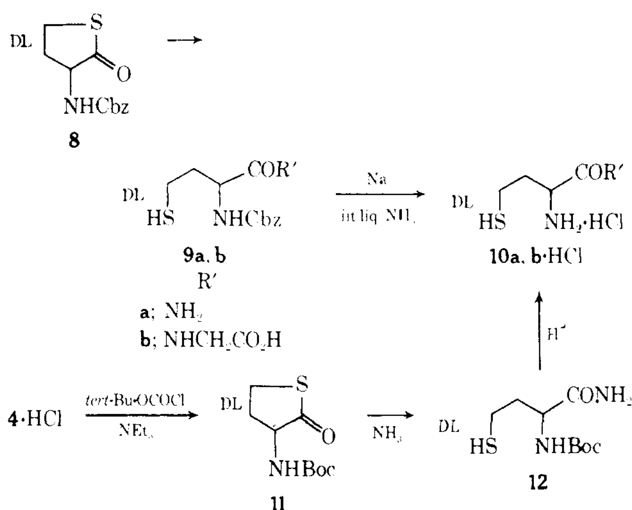


Table II

Incorporation of the Tetrahydrothiazine Derivatives into the Erythrocyte *in Vitro*[a]

Compd	Substituents in 2: R	Substituents in 2: R′	Total vitamin B_6 concn in erythrocyte after 1 hr, μg/ml
1	H	OH	0.72
2a	H	OCH_3	0.55
2b	H	OC_2H_5	0.72
2c	H	$OC_3H_7(n)$	0.87
2d	H	$OC_3H_7(i)$	0.82
2e	H	$OC_4H_9(n)$	0.97
2f	$OP(OH)_2$	OCH_3	0.34
2g	$OP(OH)_2$	OC_2H_5	0.36
2h·HCl	H	NH_2	0.70
2i·HCl	H	$NH \cdot CH_2CO_2H$	0.94
5b·HCl			0.92
PIN			0.59
No addition			0.12

[a] A mixture of the sample (5 μg), erythrocyte (1 ml), and 10% glucose soln (0.05 ml) was dild with 0.89 *M* phosphate buffer (pH 7.4) to a vol of 5 ml. The mixt was incubated at 37° for 60 min and then submitted to the microbiological assay (L. Atkin, A. S. Schultz, W. L. William, and C. N. Frey, *Ind. Eng. Chem., Anal. Ed.*, **15**, 141 (1943).

proton at 5.85 ppm (singlet) which is characteristic[4e,6a] of the proton adjacent to both N and S, and by their uv spectra; uv max nm (log ε), pH 2.0, 303 (4.10) 333 (4.10); pH 7.0, 252 (3.94) 333 (4.19); pH 12.0, 239 (4.43) 307 (4.10).

Biological Properties.—The microbiological potencies of **2i**·HCl and **5b**·HCl as vitamin B_6 were measured in the assay medium free of vitamin B_6 by the method of Atkin[13] for the growth of *Saccharomyces carlsbergensis*, and were shown to be 100 and 72% the activity of pyridoxine·HCl, resp. However, both compounds were ineffective on the growth of *Lactobacillus casei* and *Leuconostoc mesenteroides* P-60.

To determine whether the tetrahydrothiazines **2a–i** and **5b**·HCl incorporate into mammalian erythrocytes, the total vitamin B_6 concentration in the rabbit erythrocyte was measured *in vitro* (see the footnote to Table II). The amount of the sample incorporated into the erythrocytes after incubation was determined by the Atkin's[13] method using *S. carlsbergensis* as the assay organism. The data in Table II indicate that there is a relationship between the length of the alkyl group and the concentration of total vitamin B_6 in the erythrocyte. This suggests that the membrane permeability of the compound depends partly on the fat solubility of the compound. Further, the vitamin B_6 activities of **2i**·HCl and **5b**·HCl were tested by the antidotal effect on death of mice by convulsions induced by OMP. Doses of **2i**·HCl and **5b**·HCl were administered to the animals orally, and after 30 min the lethal dose of OMP (500 mg/kg) was injected sc. The 50% effective doses of the compounds, calcd from survival ratio of the test mice after 24 hr by the method of Weil,[14] were 7.64 mg/kg for **2i**·HCl and 10.9 mg/kg for **5b**·HCl. Also, the total vitamin B_6 concentrations in whole blood after oral administrations of **2i**·HCl and **5b**·HCl for rabbits were measured at intervals. The data in Table III indicate an interesting maintenance of high concentration of total vitamin B_6 for longer periods in the case of **5b**·HCl. This phenomenon can be explained by gradual release of vitamin B_6 by the hydro-

(13) L. Atkin, A. S. Schultz, W. L. William, and C. N. Frey, *Ind. Eng. Chem., Anal. Ed.*, **15**, 141 (1943).

(14) C. S. Weil, *J. Biometric Soc.*, **8**, 249 (1952).

TABLE III
TOTAL VITAMIN B_6 CONCENTRATION IN WHOLE BLOOD AFTER ORAL ADMINISTRATION OF EQUIMOLAR AMOUNTS OF THE COMPOUND TO PIN·HCl[a] (10 mg/kg) FOR THE RABBIT

Compd	Total vitamin B_6 concentration, μg/dl[b]				
	0 min	30 min	60 min	120 min	240 min
5b·HCl	18	112	224	348	356
2i·HCl	18	132	126	76	48
PAL·HCl[c]	18	148	90	48	52
1	18	152	305	159	110

[a] Pyridoxine·HCl. [b] The blood was collected in a syringe by heart puncture in each time after oral administration of the sample and submitted to the microbiological assay (see footnote *a*, Table II). [c] Pyridoxal·HCl.

lytic cleavage of the enamine bond. However, there remains the question as to what the active form of **5b**·HCl is; studies on this problem will be reported later.

Experimental Section

Biological Methods. Microbiological Assay.—The total vitamin B_6 concn was measured microbiologically using *S. carlsbergensis* as the assay organism according to Atkin's method.[13] The microbiological potency of the tetrahydrothiazine derivatives as pyridoxal was measured by the Rabinowitz-Snell's method[15a,b] using *Lactobacillus casei* and *Leuconostoc mesenteroides* P-60.

Chemical Methods.—Melting points were uncorrected and were determined with a Yamato apparatus MP-1. Uv spectra were determined with a Hitachi EPS-2U recording spectrophotometer for solns. Ir spectra were taken with a Hitachi EPI-S2 spectrophotometer for Nujol mulls. Nmr spectra were measured with JEOL C-60 at 60 MHz (Me_4Si). Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

General Procedure of the Tetrahydrothiazine Derivatives (2a-g).—The prepn of the tetrahydrothiazine derivatives **2a-g** has been reported in a previous paper.[6c]

To a soln of 2 molar equiv of the Na alkoxide in 30 parts of the corresponding alcohol was added **4**·HCl, and the mixt was stirred at room temp for 30 min. Then, the equiv amount of pyridoxal·HCl or pyridoxal 5-phosphate was added to the reaction mixt and stirred for 1 hr. Evapn of the solvent left a cryst residue, which gave **2a-g** on recrystn from H_2O or aq MeOH in 12-70% yield (Table I).

Condensation of Pyridoxal with 4·HCl.—A soln of pyridoxal (0.2 g, 1.2 mmoles) and **4**·HCl (0.19 g, 1.2 mmoles) in EtOH (2 ml) was stirred at room temp overnight. The reaction mixt was concd under reduced pressure and the residue was recrystd from EtOH to give 140 mg (41.2%) of **5b**·HCl as yellow prisms: mp 180-182° dec; nmr (in DMSO-d_6) δ 7.94 ppm (singlet, 1 H), 6.10 (triplet, J = 3.5 Hz, 1 H), 4.40 (2 H, singlet), 3.93 (doublet, J = 3.5 Hz, 2 H), 2.4 (singlet, 3 H). *Anal.* Calcd for $C_{12}H_{14}N_2O_3S$·HCl: C, 47.61; H, 4.99; N, 9.26. Found: C, 47.42; H, 5.17; N, 9.08. The free base of the condensation product was obtained as pale yellow prisms (MeOH), mp 172-175°. *Anal.* ($C_{12}H_{14}N_2O_3S$) C, H, N, S.

Hydrogenation of 5b·HCl.—A suspension of **5b**·HCl (2.5 g, 8.25 mmoles) and 10% Pd/C (5.0 g) in MeOH (500 ml) was hydrogenated at room temp under 3.73 kg/cm^2 of H_2 for 3 hr. After removal of the catalyst by filtration, the filtrate was concd under reduced pressure and the residual amorphous solid was recrystd from EtOH to give 1.33 g (52.8%) of **6**·HCl as colorless needles, mp 198-199°. *Anal.* ($C_{12}H_{16}N_2O_3S$·HCl) C, H, N, S.

***N-tert*-Butoxycarbonyl-DL-homocysteine Thiolactone (11).**—To a suspension of **4**·HCl (10 g, 0.064 mole) in Me_2CO (220 ml) were added dropwise a soln of an equiv amount of *tert*-BuOCOCl[12] in anhyd Et_2O (90 ml) and a soln of $NaHCO_3$ (11 g) in H_2O (80 ml) simultaneously with stirring and cooling at −10°. After standing overnight, the mixt was extd with $CHCl_3$ (200 ml). The ext was washed with 0.1 *N* HCl and then with H_2O and dried (Na_2SO_4). The dried org phase was concd, leaving a solid (6.5 g), which was recrystd from MeOH to furnish 3.3 g (25.3%) of **11** as colorless needles, mp 124-134°. *Anal.* ($C_9H_{15}NO_3S$) C, H, N, S.

***N-tert*-Butoxycarbonyl-DL-homocysteinamide (12).**—A soln of **11** (1.0 g, 4.3 mmoles) in 10% methanolic NH_3 (20 ml) was allowed to stand at room temp under N_2 for 3 hr. The mixt was concd under reduced pressure below 30° to an syrupy oil (0.75 g, 70%) which on standing crystd as colorless needles, mp 85-90°. *Anal.* ($C_9H_{18}N_2O_3S$) C, H, N, S.

***N*-Benzyloxycarbonyl-DL-homocysteinamide (9a).**—A soln of **8** (18.0 g, 0.0715 mole) in MeOH (220 ml) satd with NH_3 was allowed to stand at room temp under N_2 for 20 hr. The reaction mixt was concd under reduced pressure, leaving crystals (19.0 g, 98.8%), mp 114-116°. *Anal.* Calcd for ($C_{12}H_{16}N_2O_3S$) C, S; H, 6.01; N, 10.44. Found: H, 5.47; N, 10.86.

DL-Homocysteinamide·HCl (10a·HCl). (a) From 12.—12 (200 mg, 0.8 mmole) was dissolved in Et_2O (16 ml) contg H_2O (100 mg) and dry HCl (100 mg). Soon after, colorless crystals deposited and were filtered, weighing 110 mg (73.7%), mp 142-146° dec. *Anal.* ($C_4H_{11}N_2OS$·HCl) C, H, N.

(b) From 9a.—To a soln of **9a** (10.0 g, 0.0373 mole) in liq NH_3 (300 ml) was added Na (1.95 g, 0.085 g-atom) portionwise with stirring. Excess Na in the reaction mixt was decompd with NH_4Cl (0.5 g). The NH_3 was evapd and the residue was dissolved in degassed H_2O. The aq soln was adjusted to pH 5 with concd HCl and treated with $Pb(OAc)_2$ (7.1 g, 0.0187 mole).

The ppt (3.6 g) was filtered and washed with H_2O. A suspension of the ppt in H_2O was stirred with a stream of H_2S. After removal of PbS by filtration, the filtrate was lyophylized to give **10a**·HCl (0.9 g, 12.4%): mp 144-146°; ir (Nujol) cm^{-1}, 3400, 3200, 1680, 1620, 1585, 1497. This was used in the next step without further purification.

Condensation of 10a·HCl with Pyridoxal.—A mixt of **10a**·HCl (100 mg, 0.54 mmole) and pyridoxal (90 mg, 0.55 mmole) in abs MeOH (1.3 ml) was warmed on a water bath, till the mixt became a clear soln. The mixt was allowed to stand at room temp overnight and the deposited crystals were filtered and washed with small portions of MeOH, giving 55 mg (30.3%) of crude **2h**·HCl, mp 154-159°. Recrystn from MeOH afforded an analytically pure sample as colorless needles, mp 163-165° dec. *Anal.* ($C_{12}H_{17}N_3O_3S$·HCl) C, N; H: calcd, 5.36; found, 5.78.

***N*-Benzyloxycarbonyl-DL-homocysteinylglycine (9b).**—To a soln of glycine (2.4 g, 0.032 mole) and NaOEt [from Na (0.75 g, 0.033 g-atom) in EtOH (600 ml)] was added **8** (5.0 g, 0.02 mole) at room temp. The mixt was allowed to reflux under N_2 for 6 hr. After cooling, it was made acidic to congo red with HCl and extd with EtOAc. The ext was dried (Na_2SO_4) and evapd, leaving a crude product (6.0 g). Recrystn of the crude product from Me_2CO-ligroin and twice from $MeNO_2$ afforded **9b** (2.7 g, 41.5%) as colorless needles, mp 128-132°. *Anal.* ($C_{14}H_{18}N_2O_5S$) C, H, S; N: calcd, 8.59; found, 9.17.

DL-Homocysteinylglycine·HCl (10b·HCl).—To a soln of **9b** (24.5 g, 0.075 mole) in liquid NH_3 (700 ml) was added Na (4.4 g, 0.191 g-atom) portionwise with stirring. Excess Na was decompd with NH_4Cl (0.2 g). Then NH_3 was evapd from the mixt and the residue was dissolved in H_2O. The aq soln was adjusted to pH 5 with concd HCl and treated with $Pb(OAc)_2$ (7.1 g) in H_2O (10 ml). The ppt (14.0 g) were filtered and washed with H_2O. A suspension of the ppt in H_2O was stirred with a stream of H_2S. After removal of PbS by filtration, the filtrate was lyophylized to give colorless amorphous **10b**·HCl (4.8 g, 28.0%): ir (Nujol) cm^{-1}, 3700, 2300 (broad band), 1720, 1680. This was used in the next reaction without further purification.

Condensation of 10b·HCl with Pyridoxal.—A suspension of **10b**·HCl (4.0 g, 0.0156 mole) and pyridoxal (2.7 g, 0.0161 mole) was warmed with stirring to make a clear soln. This was allowed to stand overnight in a refrigerator and the deposited crystals were filtered to afford crude **2i**·HCl (3.0 g, 50.8%) which on recrystn from H_2O gave anal. sample as colorless needles, mp 177-179° dec. *Anal.* ($C_{14}H_{19}N_3O_5S$·HCl) C, H, N.

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