shown by choline, a weak inhibitor of $K_I = 4.5 \times 10^{-4}$ under the conditions used in this investigation.

(2) The pattern of structural discrimination shown by the enzyme fits that previously noted in the turicine–betonicine series,[8] even though the levels of discrimination are not quite as dramatic. The *trans*-3-hydroxy-L-stachydrine, in which the COO^- function would not interfere with two-point binding *via* the $\equiv\overset{+}{N}-$ and –OH functions, is a much better inhibitor of the enzyme than either of the *cis*-3-hydroxystachydrines, in which the "crutch effect" of the COO^- prevents the attaining of optimal interaction distances between the site surface and the $[-\overset{+}{N}\equiv, -OH]$ functional unit of the enzyme. Further, the crutch effect of the COO^- is so pronounced that it more than negates the additional binding force expected from the –OH function that should have made *cis*- and *trans*-3-hydroxystachydrine more potent than stachydrine itself. In effect, the crutch COO^- reduces the binding contributions of the $-\overset{+}{N}\equiv$ and –OH groups of 3-hydroxystachydrine a and b to values whose sum in each case is *less than* that of the $-\overset{+}{N}\equiv$ value in stachydrine.

(3) Even in the weak inhibitors 3-hydroxystachydrine a and b, the enzyme shows discrimination between D- and L-configuration of the ring α-carbon atom by a factor of about 2. This is reminiscent of the situation with the inhibitors D- and L-1-(N-piperidino)-2-dimethylaminopropane acting on AChE, in which the bulky methyl substituent bestows asymmetry on C-2 which evokes enzymatic discrimination between the optical antipodes by a factor of about 4.[9]

Experimental

***trans*-3-Hydroxy-L-stachydrine.**—To a solution of 76 mg. of *trans*-3-hydroxy-L-proline in 0.3 ml. of water was added 150 mg. of silver oxide. The copious precipitate which formed immediately was worked into a slurry by stirring and the addition of 0.2 ml. of water. This thick suspension was stirred at room temperature for 3.5 hr. Methanol (1 ml.) and methyl iodide (0.08 ml.) were added and stirring was continued for 3 hr. at room temperature. A further quantity of methyl iodide (0.05 ml.) was added and the reaction mixture was kept for 1 hr. Excess silver oxide and silver iodide then were removed by filtration, and the filtrate was evaporated under reduced pressure. By treatment of the oily residue with ethanol and acetone, crystallization was induced. The betaine crystallized in the form of fine colorless needles (97 mg.). The pure betaine was obtained by recrystallization from water–ethanol, m.p. ~245° dec., lit.[6] m.p. 250°, $[\alpha]^{20}D$ +9.6 ± 1.0° (*c* 0.88, H_2O), lit.[6] $[\alpha]^{30}D$ +10.0° (*c* 2.9, H_2O).

Anal. Calcd. for $C_7H_{13}NO_3$: C, 52.81; H, 8.23; N, 8.80. Found: C, 53.03; H, 8.12; N, 8.72.

Mutarotation, hr.	$[\alpha]^{20}D$ (*c* 0.65, 1.0 *N* NaOH)
0	+33.9 ± 1.5°
3	+25.5 ± 1.5°
5	+19.3 ± 1.5°
24	+ 6.9 ± 1.5°

***cis*-3-Hydroxy-L-stachydrine.**—*cis*-3-Hydroxy-L-proline (75.5 mg.) was methylated as the silver salt as described for the *trans* derivative. The yield of the crude betaine was 75 mg. Recrystallization from ethanol–ether afforded fine colorless crystals, m.p. 210–211° dec., $[\alpha]^{20}D$ −46.0 ± 3.0° (*c* 0.86, H_2O). For the naturally occurring *cis*-3-hydroxy-D-stachydrine the literature[6] reports: m.p. 209–210° dec., $[\alpha]^{22}D$ +53° (*c* 2.5, H_2O).

Anal. Calcd. for $C_7H_{13}NO_3 \cdot H_2O$: N, 7.91. Found: N, 8.06.

Mutarotation, hr.	$[\alpha]^{20}D$ (*c* 0.288, 1.0 *N* NaOH)
0	−32.9 ± 3.0°
3	−26.0 ± 2.0°
5	−26.0 ± 2.0°
24	− 8.7 ± 3.0°

Enzyme Inhibitor Studies.—The acetylcholinesterase preparation employed was highly purified from electric eel tissue, with a specific activity of 9.50×10^3 μmoles of acetylcholine chloride hydrolyzed per hr. per mg. of dry weight protein. All kinetic measurements involving enzyme substrate and inhibitors were carried out in the pH-stat as previously described[7] using a micro cell, an optimum ACh substrate level of 3.33×10^{-3} *M*, pH 7.40, and temperature of 25.20 ± 0.7°. The poorly poised phosphate buffer (containing 0.10 *M* NaCl) was identical with that of the previous studies on cyclic AChE–ACh inhibitors of moderate-to-weak power.

In view of the tiny amounts of the three betaines available for study, and their low order of inhibitory potency, it was necessary to use small reaction volumes (2.00 ml.) in the kinetic determinations. Amounts of the inhibitors limited these determinations of relative strength to a single inhibited run with I, runs at three inhibitor concentrations with II, and runs at four concentrations with III. As before, indices of inhibitory strength were calculated in the form of enzyme-inhibitor dissociation constants K_I, using the equation for competitive inhibition as given by Wilson.[10]

Acknowledgment.—We are greatly indebted to Dr. J. W. Cornforth for the donation of original samples of betaines isolated from *Courbonia virgata*.

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[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA 14, MARYLAND]

Conversion of Baikiain to *trans*-5- and *trans*-4-Hydroxypipecolic Acids by Hydroboration

BY Y. FUJITA,[1] F. IRREVERRE, AND B. WITKOP

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The hydroboration of DL-baikiain (4,5-dehydro-DL-pipecolic acid) *via* its N-carbobenzyloxy methyl ester derivative led, after removal of the protecting groups, to 72% of *trans*-5-hydroxy-DL-pipecolic acid and 28% of *trans*-4-hydroxy-DL-pipecolic acid, separable by preparative ion-exchange column chromatography.

The hydroboration of cyclic and bicyclic olefins is a stereoselective process.[2] Similarly, hydroboration of an olefinic heterocyclic compound, *viz.*, N-carbobenzyloxy-3,4-dehydro-DL-proline methyl ester (I), gave, after oxidation, saponification, and hydrogenolysis, 70% of *trans*-3-hydroxy-DL-proline (II) and 10% of *trans*-4-

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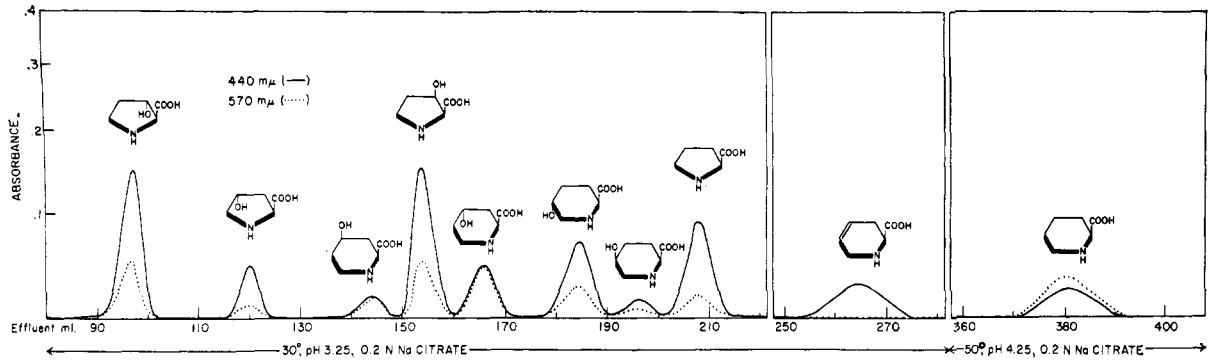
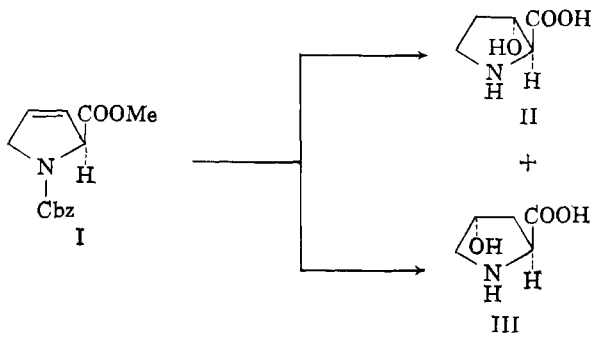
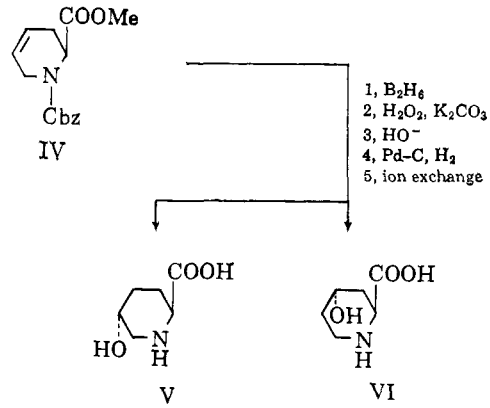


Fig. 1.—Chromatographic analysis of mixture of cyclic amino acids; 150-cm. column of Amberlite IR-120 at 30 and 50°. The curves represent absorbance at 440 mμ (———) and at 570 mμ (. . . . .) of the following compounds: *trans*-3-hydroxy-L-proline (1 μmole); *trans*-4-hydroxy-L-proline (1 μmole); *cis*-4-hydroxy-L-pipecolic acid (2.9 μmoles); *cis*-3-hydroxy-L-proline (1 μmole); *trans*-4-hydroxy-L-pipecolic acid (2 μmoles); *trans*-5-hydroxy-L-pipecolic acid (2 μmoles); *cis*-5-hydroxypipecolic acid (amt. ?); L-proline (1 μmole); DL-baikiain (2 μmoles); DL-pipecolic acid (4 μmoles).

hydroxy-DL-proline (III).[3,4] The *cis* isomer, 4-allohydroxyproline, was formed in negligible amount.



This interesting reaction has now been extended to a six-membered unsaturated cyclic amino acid in order to gain information on the effect of ring size and position of the nitrogen, relative to the double bond, on the location and stereochemistry of the hydroxyl in the resulting hydroxyamino acid. This paper describes the results of the hydroboration of DL-baikiain, easily accessible by total synthesis.[5]



The hydroboration and alkaline oxidation of IV was carried out under conditions mild enough to retain the ester function. This made possible an effective purification of the mixture of methyl N-carbobenzyloxy-4- and 5-hydroxypipecolates on a column of silicic acid. Saponification and hydrogenolysis of this purified mixture gave the free amino acids which were subjected to analytical and preparative column chromatography. The major product (~72%) of the hydroboration is *trans*-5-hydroxy-DL-pipecolic acid indistinguishable in its chromatographic behavior (Fig. 1) from the natural amino acid from dates.[6] The minor product (28%) is *trans*-4-hydroxy-DL-pipecolic acid indistinguishable on the amino acid analyzer (Fig. 1) from the natural amino acid from Acacia leaves.[7]

Experimental

N-Carbobenzyloxy-DL-baikiain Methyl Ester (IV).—DL-Baikiain hydrochloride[5] (1.6 g., 10 m*M*), dissolved in 8.5 ml. of 4 *N* NaOH, was treated at 0° with benzyloxycarbonyl chloride (1.62 ml.) under the usual Schotten–Baumann conditions, yielding an oily substance which resisted crystallization from a variety of organic solvents. Esterification of this substance with ethereal diazomethane yielded the methyl ester of carbobenzyloxy-DL-baikiain, a colorless oil weighing 2.3 g. (84%) which likewise resisted many attempts at crystallization. Thin layer chromatography (silica gel G, benzene–EtAc (4:1)) and gas chromatography (1% SE-30 on Gaschrome P, N_2, 203°) proved the substance to be homogeneous.

Hydroboration of N-Carbobenzyloxy-DL-baikiain Methyl Ester.—The above methyl ester (2.3 g., 8.4 m*M*) in 20 ml. of 1,2-dimethoxyethane was treated at 0° with diborane generated from lithium borohydride (500 mg. in 15 ml. of dimethoxyethane) and an excess of boron trifluoride (2.45 ml. of boron trifluoride dietherate/2.5 ml. of dimethoxyethane). After storage of the reaction mixture for 2 hr. at room temperature, excess hydride was decomposed, the organoborane oxidized with 16 ml. of 30% hydrogen peroxide and 16 g. of potassium carbonate, and the mixture allowed to stand at room temperature for 4 days with stirring. The dimethoxyethane was removed *in vacuo* and the mixture of N-benzyloxycarbonylhydroxypipecolic acid methyl esters was extracted with ethyl acetate (3 × 30 ml.) and the extract dried over anhydrous sodium sulfate. After removal of ethyl acetate, the oil was dissolved in a mixture of 10 ml. of ethyl acetate and 40 ml. of benzene and fractionated on a silicic acid column (Mallinckrodt, 100 mesh, 2.4 × 35 cm.). The column was eluted with ethyl acetate–benzene (1:4) and the eluate was collected in 5-ml. fractions. Fractions 218 to 322 which contained the benzyloxycarbonyl-4- and -5-hydroxypipecolic acid methyl esters, detected by thin layer chromatography and by gas chromatography, were pooled and the solvent removed. The colorless residual oil weighed 679 mg. (27% yield).

Saponification of the esters was performed at room temperature overnight with 5 ml. of methanol and 1.39 ml. of 2.0 *N* NaOH (2.78 m*M*). After removal of methanol *in vacuo* and acidification with 1.5 ml. of 2.0 *N* HCl at 0°, the oily N-carbobenzyloxy acids were extracted with ethyl acetate, then washed with water, and dried over anhydrous sodium sulfate. The

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TABLE I

R_f VALUES AND R_f RATIOS OF *trans*-5-HYDROXY- AND *trans*-4-HYDROXYPIPECOLIC ACIDS BY THIN-LAYER CHROMATOGRAPHY[a]

Compound,[b] pipecolic acids	R_f Solvent[d] 1	2	3	4	R_f ratio[c] Solvent[d] 5	6	7	8
trans-5-Hydroxy-L-, and DL-	0.14	0.26	0.53	0.54	0.67	0.68	0.43	1.06
trans-4-Hydroxy-L-, and DL-	0.11	0.22	0.46	0.47	0.29	0.50	0.24	0.81

[a] Plates coated with silica gel, Brinkmann GF 254. [b] The values of the synthetic and natural compounds are the same. [c] R_f ratio = R_f of cyclic amino acid/R_f of valine. [d] Solvents: 1, benzene–HAc–H_2O (20:20:2); 2, *n*-butyl alcohol–HAc–H_2O (40:10:10); 3, methyl alcohol–pyridine–H_2O (180:8:40); 4, *n*-propyl alcohol–0.2 *N* citrate buffer pH 3.25 (32:18); 5, ethyl alcohol–concentrated ammonium hydroxide (9:1); 6, ethyl acetate–pyridine–H_2O (70:30:10); 7, *t*-butyl alcohol–ethyl alcohol–concentrated ammonium hydroxide (45:45:10); 8, *sec*-butyl alcohol–diethylamine–H_2O (40:5:5).

solvent was removed *in vacuo* and the residue, in 8 ml. of 50% acetic acid, was hydrogenolyzed at room temperature in the presence of 300 mg. of 10% palladium-on-charcoal. At the end of the reaction, the catalyst was removed by filtration, the solution evaporated to dryness, and the oil dried in a desiccator over potassium hydroxide. It weighed 315 mg. (91% from N-carbobenzyloxy-4- and -5-hydroxypipecolic acid methyl ester). The entire sample was dissolved in 5 ml. of citrate buffer pH 2.2 and used for column chromatography.

By use of an automatic amino acid analyzer only two major ninhydrin-positive peaks were observed which corresponded to authentic samples:

Acid	Composition μmoles/5 ml.	%
trans-4-Hydroxypipecolic	500	28
trans-5-Hydroxypipecolic	1268	72

Neither before nor after silicic acid column chromatography could the *cis* isomers of 4- and 5-hydroxypipecolic acids be detected on the automatic amino acid analyzer.

Preparative Column Chromatography.—The solution of the reaction mixture in citrate buffer was divided into two equal portions and each portion was submitted to column chromatography on Amberlite IR-120 following the same procedure and apparatus used for the separation of 3-hydroxyprolines.[3,4] When the pump was set to deliver 11.3 ml. of eluate per tube, the *trans*-4-hydroxypipecolic acid was located in tubes 173–200 and the *trans*-5-hydroxypipecolic acid in tubes 213–255. The tubes containing each compound were pooled and the eluate desalted on a column of Dowex-50W in the acid form. The amino acid was eluted from the column with 7.0 *N* ammonium hydroxide and the latter was evaporated to dryness *in vacuo*.

Since neither free amino acid appeared to crystallize from aqueous ethanol, they were converted into their hydrochlorides.

The *trans*-4-hydroxy-DL-pipecolic acid hydrochloride crystallized from 80–90% ethanol. The crystals, dried over phosphorus pentoxide *in vacuo*, weighed 33 mg., m.p. 161–163°.

Anal. Calcd. for $C_6H_{12}NO_3Cl$: C, 39.67; H, 6.66; N, 7.71. Found: C, 38.96; H, 6.77; N, 8.09.

The *trans*-5-hydroxy-DL-pipecolic acid hydrochloride crystallized from 80–90% alcohol. The crystals, dried over phosphorus pentoxide *in vacuo*, weighed 105 mg., m.p. 192–194°.

Anal. Calcd. for $C_6H_{12}NO_3Cl$: C, 39.67; H, 6.66; N, 7.71. Found: C, 39.58; H, 6.84; N, 7.78.

The synthetic *trans*-4-hydroxy-DL-pipecolic acid behaved exactly as the known L-compound in color reactions. On paper both gave a yellow color with ninhydrin which turned to grayish brown on standing. When viewed under an ultraviolet light the ninhydrin spots exhibited a dull, brick red fluorescence. Both gave a blue color with isatin and a reddish orange color with sodium 1,2-naphthoquinone-4-sulfonate[8] characteristic of the cyclic amino acids. The synthetic and natural compounds cochromatographed on the automatic amino acid analyzer, exhibiting a unique and characteristic spectral ratio, *i.e.*, the yellow (440 mμ) and the red (570 mμ) curves were practically coincident (Fig. 1). The racemic and the natural 4-hydroxypipecolic acids cochromatographed in eight solvent systems (Table I).

The synthetic *trans*-5-hydroxy-DL-pipecolic acid and the natural amino acid likewise gave a blue color with isatin and a reddish orange with sodium 1,2-naphthoquinone-4-sulfonate. With ninhydrin on paper the 5-isomer gave a brilliant purple color which fluoresced under an ultraviolet light as bright red. The compounds cochromatographed on the amino acid analyzer giving a maximum absorption with ninhydrin at 440 mμ, following the spectral pattern of most cyclic amino acids. They also cochromatographed in eight solvent systems (Table I).

Acknowledgments.—We are greatly indebted to Dr. J. W. Clark-Lewis for samples of *cis*- and *trans*-4-hydroxy-L-pipecolic acid. The technical assistance of Mr. Edwin Wilson is gratefully acknowledged.

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Differential Oxidation of Protein-Bound Tryptophan and Tyrosine by N-Bromosuccinimide in Urea Solution

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Spectrophotometric evidence is provided for the existence of N-bromourea arising from the action of N-bromosuccinimide on 8.0 *M* solutions of urea in water. N-bromourea reacts with tyrosine or its analog at a rate >200 times more slowly than does N-bromosuccinimide in aqueous systems. This difference in reactivity permits the cleavage of tryptophan peptide bonds in protein without cleavage of tyrosine bonds.

N-Bromosuccinimide (NBS) has been widely used for cleavage of both tryptophyl and tyrosyl peptide bonds in proteins and for the approximate estimation of tryptophan by spectrophotometric titration.[3] The reactions have been carried out frequently in a solution of 8.0 *M* urea in order to increase the accessibility of the amino acid residues and to improve the yield of cleavage products. The effect of urea on the reaction of NBS with model peptides has not been studied, and the following experiments with tyrosine and tryptophan analogs demonstrate their differential reactivity under such conditions.

(1) Visiting Scientist of the U. S. Public Health Service, summer, 1963.
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(3) *Cf.* B. Witkop, *Advan. Protein Chem.*, **16**, 221 (1961).