stand at room temperature for 45 min. and then kept at 0° for 16 hr. The solution was then acidified with glacial acetic acid to pH ~3, giving a white gummy amorphous product which was collected, dried, and triturated with hot diisopropyl ether, leaving 2.17 g. (75%) of crystals, m.p. 148–151°. Three successive recrystallizations from chloroform–diisopropyl ether yielded 1.0 g. (42%), m.p. 161–162°, $[\alpha]^{25}D$ +11.9 ± 2.5 (*c* 2.03, ethanol). *Anal.* Calcd. for $C_{28}H_{31}N_3O_4 + {}^1/_4H_2O$: C, 70.30; H, 6.64; N, 8.78; H_2O, 0.9. Found: C, 70.09; H, 6.64; N, 8.57; H_2O, 0.6.

11. Benzyloxycarbonylglycyl-L-tryptophan.—L-Tryptophan, (2.04 g., 10 mmoles), 1.68 g. (20 mmoles) of sodium bicarbonate, and 25 ml. of water were mixed, giving partial solution and a pH about 8. Then a solution of 3.06 g. (10 mmoles) of the N-hydroxysuccinimide ester of benzyloxycarbonylglycine in 15 ml. of acetonitrile was added at room temperature. All materials were in solution in a short time. After half an hour, the solution was concentrated to about three-fourths of the original volume on a rotary evaporator under vacuum and with slight warming by a water bath. Acidification with concentrated hydrochloric acid to pH about 1 precipitated a gum. This was extracted into 25 ml. of ethyl acetate, and the resulting solution was dried briefly over sodium sulfate. The clear solution was then diluted to cloudiness with petroleum ether (about 35 ml.) and refrigerated. The resulting crystalline solid was collected; weight 2.38 g., m.p. 140–142°. Concentration of the filtrate yielded 0.90 g. with somewhat lower m.p.; recrystallization of this from about 100 ml. of ethanol–water (1:9) gave 0.77 g., m.p. 141–142°. The combined products were recrystallized from 115 ml. of ethanol–water (1:2) to yield 2.75 g. (70% yield) of pure product, m.p. 142–143°, $[\alpha]^{25}D$ + 32.9 ± 2.17° (*c* 2.3, absolute alcohol). Use of lesser amounts of sodium bicarbonate gave slightly lower yields. Weygand and Steglich[24] prepared the compound in 56% yield, m.p. 141–142°, $[\alpha]^{24}D$ +33.3° (*c* 2.34, ethanol) and Erlanger and Kokowsky[25] in 50% yield in a two-step process.

12. Benzyloxycarbonyl-L-isoglutaminyl-L-asparagine.—As paragine monohydrate (0.75 g., 0.005 mole) was dissolved in 10 ml. of water with heating. The solution was cooled to room temperature and 0.7 ml. (0.005 mole) of triethylamine was added. Then 1.89 g. (0.005 mole) of the N-hydroxysuccinimide ester of benzyloxycarbonyl-L-isoglutamine dissolved in 20 ml. of tetrahydrofuran was added with stirring at room temperature. After 30 min. the reaction was diluted with 20 ml. of water and acidified to pH 2 with hydrochloric acid. After overnight chilling, 0.87 g., m.p. 179–185°, was collected. Chilling the filtrate gave 0.5 g., m.p. 171–173°. The two fractions were recrystallized separately from DMF–acetonitrile (1:2) over a 6-day period. When gel-like material formed, it was redissolved by gentle warming; finally crystals formed on cooling. The two fractions yielded 0.95 g., m.p. 187–190°, and 0.3 g., m.p. 184–187° (total yield 63%). Ressler and du Vigneaud[26] report 41% yield, m.p. 187.5–188°, by a mixed anhydride procedure.

13. Benzyloxycarbonylglycyl-L-phenylalanylglycine.—L-Phenylalanylglycine hydrate[27] (0.775 g., 3.2 mmoles) and 0.252 g. (3 mmoles) of sodium bicarbonate were dissolved in 8 ml. of water. A solution of 0.6125 g. (2 mmoles) of the N-hydroxysuccinimide ester of benzyloxycarbonylglycine in 8 ml. of dimethoxyethane was added with stirring at room temperature. After 40 min., 10 ml. more water was added and the solution was acidified to pH 2 with hydrochloric acid. Crystals formed immediately. After overnight chilling, 0.75 g. (94%) of white crystals, m.p. 155–160°, was collected. The product was recrystallized from hot ethyl acetate to give, in two fractions, 0.74 g. (92%), m.p. 157–158°. Kenner and Stedman[28] obtained a glass in 60% yield by a mixed anhydride procedure; two recrystallizations gave a product, m.p. 155.5–157.9° (yield not given). We have obtained a yield of 85% by saponification of the ethyl ester, m.p. 157–158°.

14. Benzyloxycarbonyl-L-prolylglycyl-L-phenylalanylglycine.—A solution of 2.79 g. (10 mmoles) of glycyl-L-phenylalanylglycine[29] and 1.68 g. (20 mmoles) of sodium bicarbonate in 50 ml. of water plus 25 ml. of ethanol was made by warming, then cooling to room temperature. To this was added a solution of 3.46 g. (10 mmoles) of the N-hydroxysuccinimide ester of benzyloxycarbonyl-L-proline in 25 ml. of ethanol, also made by warming and cooling to room temperature; 5 ml. of wash ethanol was also used. The resulting solution was allowed to stand for 18 hr. Then it was acidified to pH about 1.5 by the addition of hydrochloric acid. Some of the ethanol was removed by vacuum distillation, and an oil precipitated from the remaining solution. This solidified on refrigeration, and it was filtered off; dry weight 4.53 g. (89%), m.p. 148–152°. Recrystallization from 105 ml. of water plus 35 ml. of alcohol gave the pure tetrapeptide derivative, wt. 3.87 g. (76% yield), m.p. 154–155°. Working up the filtrate gave 0.25 g. more, making 4.12 g. in all (80% yield), $[\alpha]^{25}D$ −27.6 ± 2.5° (*c* 2, dioxane). *Anal.* Calcd. for $C_{26}H_{30}N_4O_7$: C, 61.16; H, 5.92; N, 10.98. Found: C, 61.45; H, 6.11; N, 11.01.

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(24) F. Weygand and W. Steglich, *Ber.*, **93**, 2983 (1960); *p*-nitrophenyl ester method used.

(25) B. F. Erlanger and N. Kokowsky, *J. Org. Chem.*, **26**, 2534 (1961).

(26) C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **79**, 4511 (1957); mixed anhydride method used.

(27) D. Ben-Ishai, *J. Org. Chem.*, **19**, 62 (1954).

(28) G. W. Kenner and R. J. Stedman, *J. Chem. Soc.*, 2069 (1952).

(29) L. Zervas and D. M. Theodoropoulos, *J. Am. Chem. Soc.*, **78**, 1359 (1956).

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, AND THE U. S. NAVAL MEDICAL RESEARCH INSTITUTE, BETHESDA 14, MARYLAND]

The Betaines of 3-Hydroxyproline. Assignment of Configuration and Inhibition of Acetylcholinesterase

BY F. SAKIYAMA,[1] F. IRREVERRE, S. L. FRIESS,[2] AND B. WITKOP

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The conversion of *trans*-3-hydroxy-L-proline and of *cis*-3-hydroxy-L-proline to their betaines under nonepimerizing conditions leads to the so-called 3-hydroxystachydrine a and the optical antipode of 3-hydroxystachydrine b, respectively, from *Courbonia virgata*. Weak competitive inhibitory activities in the system acetylcholinesterase–acetylcholine (AChE–ACh) are shown by *cis*- as well as *trans*-hydroxystachydrines. The differences in activities are discussed in terms of two-point attachment of the inhibitors to the catalytic surface of the enzyme.

We have recently described the isolation of *trans*-3-hydroxy-L-proline from marine sponge[3] and *cis*-3-hydroxy-L-proline from the antibiotic telomycin.[4] The stereochemical assignments rest on the mode of synthesis, *i.e.*, stereoselective hydroboration of 3,4-dehydroproline,[3] and on the oxidative conversion of the

(1) Fellow in the Visiting Program of the U. S. Public Health Service.

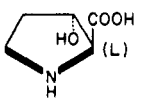
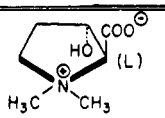
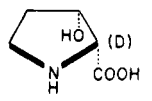
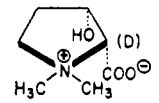
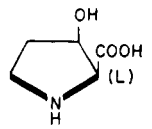
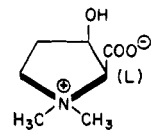
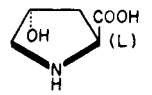
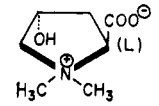
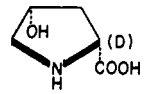
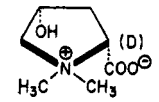
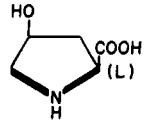
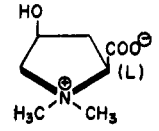
(2) U. S. Naval Medical Research Institute.

(3) F. Irreverre, K. Morita, A. V. Robertson, and B. Witkop, *Biochem. Biophys. Res. Commun.*, **8**, 453 (1962); *J. Am. Chem. Soc.*, **85**, 2824 (1963); *cf.* J. D. Ogle, R. B. Arlinghaus, and M. A. Logan, *J. Biol. Chem.*, **237**, 3667 (1962).

(4) F. Irreverre, K. Morita, S. Ishii, and B. Witkop, *Biochem. Biophys. Res. Commun.*, **9**, 69 (1962); *cf.* J. C. Sheehan and J. G. Whitney, *J. Am. Chem. Soc.*, **84**, 3980 (1962).

CHART I

BETAINES OF 3- AND 4-HYDROXYPROLINES

Parent Amino Acid	Betaine	M.P.	$[\alpha]_D^{20}$ in H_2O	$[\alpha]_D^{20}$ in NaOH	Effect on Cholinesterase	Literature
 trans-3-hydroxy-L-proline	 3-hydroxystachydrine a	-250° (natural) -245° (synthetic)	+10.0° (30°C.) +9.6°	+33.9° → +6.9° (24 hours)	K_I (competitive) $1.07 \pm 0.01 \times 10^{-4}$	J. W. Cornforth and A. J. Henry, J. Chem. Soc., (1952), 597. Natural: from Courbonia virgata
 cis-3-hydroxy-D-proline	 3-hydroxystachydrine b	209-210° (natural)	+53.0° (22°C.)	—	$8.84 \pm 0.57 \times 10^{-4}$	J. W. Cornforth and A. J. Henry, J. Chem. Soc., (1952), 597. Natural: from Courbonia virgata
 cis-3-hydroxy-L-proline	 cis-3-hydroxy-L-stachydrine (synthetic)	210-211° (synthetic)	-46.0°	-32.9° → -8.7° (24 hours)	16×10^{-4}	F. Sakiyama, F. Irreverre and B. Witkop, this paper.
 4-hydroxy-L-proline	 L-betonicine (natural)	252-253°	-34.2°	-36.0° → 0° (24 hours)	$8.5 \pm 0.4 \times 10^{-5}$	S. L. Friess, A. A. Patchett and B. Witkop, J. Am. Chem. Soc., 79, 459 (1957).
 4-allohydroxy-D-proline	 D-turicine (natural)	259-260°	+37.8°	+51.1° → 0° (24 hours)	—	A. A. Patchett and B. Witkop, J. Am. Chem. Soc., 79, 185 (1957).
 4-allohydroxy-L-proline	 L-turicine (synthetic)	252-254°	-39.0°	—	No inhibitor	cf. Choline: 4.5×10^{-4} Stachydrine: 5.4×10^{-4}

trans-3-methoxyproline to L-methoxysuccinimide of known configuration.[5]

Two betaines of 3-hydroxyproline have been isolated from the fruit of *Courbonia virgata*, namely, 3-hydroxystachydrine a, $[\alpha]_D$ +10°, and 3-hydroxystachydrine b, $[\alpha]_D$ +53°.[6] We have now been able to assign structures and absolute configurations to these 3-hydroxystachydrines by the conversion of *cis*- and *trans*-hydroxyproline to the respective betaines under nonepimerizing conditions.[7]

As summarized in Chart I, the betaine of *trans*-3-hydroxy-L-proline is identical with 3-hydroxy-L-stachydrine a, whereas the betaine of *cis*-3-hydroxy-L-proline is the antipode of 3-hydroxystachydrine b. These relations are reminiscent of the naturally occurring betaines of 4-hydroxyproline, *i.e.*, L-betonicine, the betaine of 4-hydroxy-L-proline, and D-turicine, the betaine of 4-allohydroxy-D-proline (Chart I).

(5) J. C. Sheehan and J. G. Whitney, *J. Am. Chem. Soc.*, **85**, 3863 (1963).
(6) J. W. Cornforth and A. J. Henry, *J. Chem. Soc.*, 597 (1952).
(7) *Cf.* A. A. Patchett and B. Witkop, *J. Am. Chem. Soc.*, **79**, 185 (1957).

Again, one may suspect that 3-hydroxystachydrine b arises in the plant from 3-hydroxystachydrine a by enzymatic or nonenzymatic epimerization at C-2. The hydrogen at C-2 is easily abstracted by base, and both betaines show mutarotation in alkaline solution reaching end values indicative of an equilibration mixture of *cis*- and *trans*-betaines. The rotational values alone permit no exact assignment of a possible preferred thermodynamic conformation for the *cis*- and *trans*-1,2-disubstituted pyrrolidine rings, respectively.

As with betonicine and turicine,[8] interference with surface binding and inhibition of acetylcholinesterase was studied. The respective enzyme-inhibitor dissociation constants, K_I (competitive), are tabulated in Chart I.

All 3-hydroxystachydrines are comparatively weak inhibitors in the system AChE–ACh. The following points of interest emerge.

(1) All members of the class fall within one order of magnitude of the potency in AChE–ACh inhibition

(8) S. L. Friess, A. A. Patchett, and B. Witkop, *ibid.*, **79**, 459 (1957).

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*.

(9) S. L. Friess, E. R. Whitcomb, B. T. Hogan, and P. A. French, *Arch. Biochem. Biophys.*, **74**, 451 (1958).

(10) P. W. Wilson, "Respiratory Enzymes," H. A. Lardy, Ed., Burgess, Minneapolis, Minn., 1949, p. 23.

[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-

(1) Associate in the Visiting Program of the USPHS, 1963–1964.

(2) H. C. Brown and G. Zweifel, *J. Am. Chem. Soc.*, **83**, 2544 (1961).