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The Synthesis of *cis*- and *trans*-3-Hydroxy-L-proline, Two New Amino Acids from the Antibiotic Telomycin[1]

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The synthesis of *cis*- and *trans*-3-hydroxy-DL-proline is described. Removal of the D-isomer from *cis*- and *trans*-3-methoxy-DL-proline by reaction with D-amino acid oxidase followed by hydrolysis of the L-amino acid with hydrobromic acid afforded the corresponding *cis*- and *trans*-3-hydroxy-L-proline, which are identical in physical and chemical behavior with two amino acids from Telomycin. The two racemates of 3-methoxyproline and the corresponding racemates of 3-hydroxyproline were assigned to the *cis* and *trans* series on the basis of the classically rigorous conversion of *trans*-3-methoxy-L-proline to L-methoxysuccinamide.

The acid hydrolysis of the antibiotic Telomycin gave two amino acids which could not be identified by comparison with amino acids previously known synthetically or in nature.[2]

In accordance with the difference in their relative mobilities upon electrophoresis, the designations "fast moving" and "slow moving" hydroxyprolines were assigned to these two amino acids. Each amino acid has an analysis corresponding to $C_5H_9NO_3$, yields proline by reaction with hydriodic acid, and is not attacked by D-amino acid oxidase.[3]

Based on the above experimental evidence the two amino acids were tentatively assigned the *cis*- and *trans*-3-hydroxy-L-proline structure. This structural assignment has now been verified by synthesis, and the "slow moving" hydroxyproline has been shown to be the *trans* isomer by the evidence presented later in this paper.

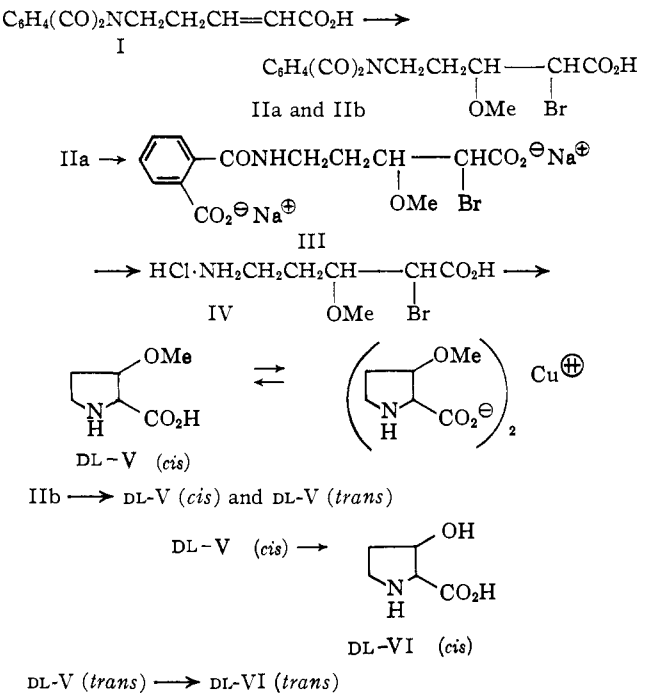
By a modification of the procedure of Carter and West[4] for the conversion of crotonic acid to 2-bromo-3-methoxybutyric acid, 5-phthalimido-2-pentenoic acid (I)[5] produced 2-bromo-3-methoxy-5-phthalimidopentanoic acid (II). The product II was isolated as a mixture of racemates IIa and IIb, which were separated by fractional crystallization from toluene. The effectiveness of this separation could be seen by comparison of the infrared spectra of IIa and IIb, each of which exhibits a single, sharp carboxyl peak in the carbonyl region but at different wave numbers.

Removal of the phthaloyl group from IIa was effected by titration with base to the phthalamic acid salt III, followed by hydrolysis with aqueous acid to afford 2-bromo-3-methoxy-5-aminopentanoic acid hydrochloride (IV). Methyl 2-bromo-3-methoxypropionate was saponified without loss of the bromine group by similar base treatment.[6]

After separation of phthalic acid by extraction into ether and concentration of the aqueous acidic solution, cyclization of the amine hydrochloride IV was achieved with base. 2-Chloro-5-aminopentanoic acid hydrochloride undergoes cyclization to proline by this same procedure.[7] Neutralization of the basic reaction media, removal of sodium chloride on a cation-exchange resin, and purification of the product *via* the copper salt gave *cis*-3-methoxy-DL-proline (V).

When IIb was treated according to the foregoing reaction sequence, a mixture of *cis*- and *trans*-3-methoxy-DL-proline (V) was obtained. The racemates V were separated by fractional crystallization of the copper salts. The identity of the two *cis* racemates V (from IIa and IIb) was established by an undepressed mixture melting point and comparison of their infrared spectra.

The *cis* and *trans* racemates V, by reaction with constant-boiling hydrobromic acid at reflux, yielded the corresponding *cis*- and *trans*-3-hydroxy-DL-proline (VI) without epimerization at either asymmetric center, in analogy to the conversion of O-methylthreonine to threonine.[4]



cis- and *trans*-3-Methoxy-L-proline (V) were obtained by the stereospecific action of hog kidney D-amino acid oxidase on the corresponding racemates V.[8] Removal of the D-isomer by reaction with the oxidase afforded, as initial products, hydrogen peroxide and 2-keto-3-methoxy-5-aminopentanoic acid (VII), the latter of which strongly inhibits the action of the oxidase as does the product from D-proline, 2-keto-5-aminopentanoic acid. As the enzyme preparation possessed high catalase activity, cyanide ion was added to the reaction solution to inhibit this peroxide-splitting catalyst and thereby permit the accumulated hydrogen peroxide to

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(2) (a) F. Irreverre, K. Morita, A. V. Robertson, and B. Witkop, *Biochem. Biophys. Res. Communications*, **8**, 453 (1962); (b) F. Irreverre, K. Morita, S. Ishii, and B. Witkop, *ibid.*, **9**, 69 (1962); (c) J. D. Ogle, R. B. Arlinghaus, and M. A. Logan, *J. Biol. Chem.*, **237**, 3667 (1962); (d) K. A. Piez, E. A. Eigner, and M. S. Lewis, *Biochem.*, **2**, 58 (1963). The detection of 3-hydroxyproline in collagen from bovine Achilles tendon, rat skin, rat tail tendon, skin of spiny dogfish (*Squalus acanthias*), and the swim bladder of the carp (ichthyocol) has been reported recently.

(3) J. C. Sheehan, P. E. Drummond, J. N. Garner, K. Maeda, D. Mania, S. Nakamura, A. K. Sen and J. A. Stock, in preparation.

(4) H. E. Carter and, H. D. West, "Organic Syntheses," Coll. Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1955, p. 813.

(5) B. R. Baker, R. E. Schaub, M. W. Querry, and J. H. Williams, *J. Org. Chem.*, **17**, 77 (1952).

(6) Reference 4, p. 774.

(7) N. F. Albertson and J. L. Fillman, *J. Am. Chem. Soc.*, **71**, 2818 (1949).

(8) J. R. Parikh, J. P. Greenstein, M. Winitz, and S. M. Birnbaum, *ibid.*, **80**, 953 (1958).

convert the toxic α-keto acid VII to the innocuous 2-methoxy-4-aminobutyric acid (VIII) and carbon dioxide. To ensure complete oxidation of the D-amino acid, the end point of the reaction was determined by the absence of carbon dioxide evolution, as shown by a constant pH, when fresh enzyme was added. Dialysis of the reaction mixture followed by purification of the product V on three different ion-exchange columns produced *cis*- and *trans*-3-methoxy-L-proline (V), which were electrophoretically pure.

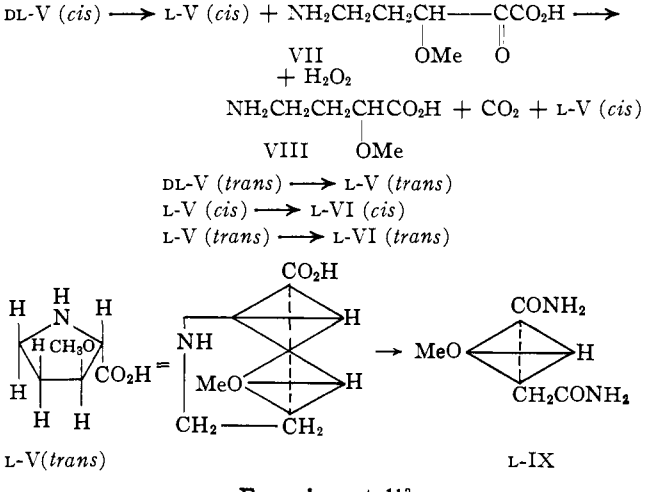
The *cis*- and *trans*-L-amino acid V gave the corresponding *cis*- and *trans*-3-hydroxy-L-proline (VI). The four resolution products of β-methoxynorleucine also yielded the corresponding optically-active forms of β-hydroxynorleucine without epimerization at either asymmetric center with hydrobromic acid.[9]

The L-configuration of the four optically-active amino acids V, VI was further confirmed by the observed positive shift in optical rotation in passing from water to aqueous hydrochloric acid (Clough–Lutz–Jirgensons rule).[10]

The synthetic *cis*- and *trans*-3-hydroxy-L-proline (VI) proved inseparable from the natural "fast moving" and "slow moving" hydroxyprolines, respectively, by paper chromatography in three solvent systems and by electrophoresis.

With ninhydrin spray, "fast moving" hydroxyproline, "slow moving" hydroxyproline, and *cis*- and *trans*-3-hydroxy-L-proline (VI) all gave a yellow color, but with isatin spray only "fast moving" hydroxyproline and the *cis*-L-amino acid VI gave a color (blue).

trans-3-Methoxy-L-proline (V) was converted to L-methoxysuccinamide (IX)[11] by oxidative ring cleavage with permanganate, esterification, and amidation, a series of reactions not involving cleavage of any bond at carbon atom 3.[12] The conclusion could thereby be drawn that the configuration at carbon atom 3 of the parent amino acid was L and that a *trans* relationship existed between the carboxyl and methoxyl groups. Thus, a *cis* or *trans* configurational designation can now be assigned rigorously to both racemates of 3-methoxyproline (V) and also to the corresponding racemates of 3-hydroxyproline (VI).



Experimental[13]

2-Bromo-3-methoxy-5-phthalimidopentanoic Acid (II).[4]—To a solution of 157 g. (0.64 mole) of 5-phthalimido-2-pentenoic acid (I)[5] in 6280 ml. of warm methanol was added 250 ml. (262 g., 4.37 moles) of glacial acetic acid and 138.7 g. (0.64 mole) of mercuric oxide. The solution was swirled and warmed until the bright orange color disappeared (approximately 20 min.) and then stirred for 48 hr. at room temperature. After concentration of the methanolic solution under reduced pressure to a 1400-ml. volume, a precipitate was collected by filtration, washed with two 90-ml. portions of methanol, and air dried to give 300 g. of a colorless solid.

The solid was dissolved in a solution of 114.4 g. (0.96 mole) of potassium bromide in 1120 ml. of water by warming. After removal of a small amount of insoluble material by filtration, a solution of 114.4 g. (0.96 mole) of potassium bromide and 102.5 g. (0.64 mole) of bromine in 170 ml. of water was added dropwise with stirring at such a rate that bromine did not accumulate (approximately 1.5 hr.) with ice-bath cooling and exposure to a No. 2 photoflood lamp fitted with a reflector. A small amount of an insoluble gum was removed by extraction into ether and excess bromine was destroyed with sodium bisulfite. The aqueous solution was acidified with 48% hydrobromic acid and the product extracted into ether, which was then washed with water and dried over sodium sulfate. Removal of the ether under reduced pressure yielded a colorless oil, which solidified on digestion with pentane to produce 137.5 g. (60%) of a colorless solid II. Trituration with three 280-ml. portions of boiling toluene afforded 47.4 g. (21%) of crystalline IIa, m.p. 149–152°, $\lambda_{max}^{dioxane}$ 1750 cm.$^{-1}$ (CO_2H). Three recrystallizations from benzene gave an analytical sample, m.p. 150–153°.

Anal. Calcd. for $C_{14}H_{14}NO_5Br$: C, 47.2; H, 3.93; N, 3.93; Br, 22.5. Found: C, 47.44; H, 3.99; N, 4.26; Br, 23.25.

Evaporation of the toluene yielded 58.6 g. (26%) of crystalline IIb, m.p. 108–111°, $\lambda_{max}^{dioxane}$ 1745 cm.$^{-1}$ (CO_2H). After three recrystallizations from toluene an analytical sample, m.p. 112–114°, was obtained.

Anal. Found: C, 47.46; H, 3.95; N, 3.96; Br, 22.16.

***cis*- and *trans*-3-Methoxy-DL-proline (V).**[6,7]—A stirred, ice-cooled suspension of 1 g. (2.81 mmoles) of IIa in 4.33 ml. of water was titrated with 2 *N* aqueous sodium hydroxide (approximately 2 hr.) using phenolphthalein as an indicator. The solution was then acidified to 1 *N* with concentrated hydrochloric acid, heated under reflux for 1 hr., cooled, and the product extracted into ether, which was then dried over sodium sulfate. Removal of the ether afforded 0.451 g. (97%) of crystalline product, the identity of which with 1,2-phthalic acid was established by comparison of the infrared spectra.

The aqueous layer was concentrated under reduced pressure to a colorless solid (purple-ninhydrin positive), which was dissolved in a solution of 0.674 g. (16.85 mmoles) of sodium hydroxide in 4.5 ml. of water and the 2.5 *N* solution stored at room temperature for 2 days. Neutralization with concentrated hydrochloric acid, separation of sodium chloride on a column of Dowex 50W-X8 ($H^{\oplus}$) cation-exchange resin (1 *N* ammonium hydroxide elution), and lyophilization of the ninhydrin-positive eluate gave a light yellow oil.

The oil in 10 ml. of water was decolorized with charcoal and the aqueous solution heated, under reflux, with 3 g. (24.3 mmoles) of cupric carbonate for 1 hr. Excess cupric carbonate was removed from the hot solution by filtration and the filtrate concentrated *in vacuo* to a blue solid, which was crystallized from ethanol to yield 170 mg. of a copper salt. Cupric ion was removed on a column of Dowex 50W-X8 ($NH_4^{\oplus}$) resin and the eluate lyophilized to a colorless oil, which was crystallized from methanol–ethyl acetate to produce 98.4 mg. (24%) of hygroscopic *cis*-3-methoxy-DL-proline (V), m.p. 196–198°, electrophoretically pure.[14] The product was recrystallized twice from methanol–ethyl acetate to afford an analytical sample, m.p. 205.5–206.5°.

Anal. Calcd. for $C_6H_{11}NO_3$: C, 49.6; H, 7.59; N, 9.66. Found: C, 49.47; H, 7.54; N, 9.16.

The foregoing procedure was repeated on 12 g. (33.7 mmoles) of IIb and again 1,2-phthalic acid (100%) was obtained, but, in contrast to the previous experiment, from the cold aqueous filtrate (after removal of excess cupric carbonate) was collected 1.552 g. of a copper salt, which upon removal of cupric ion gave from ethanol–ethyl acetate 851.2 mg. (17%) of large needles, *trans*-3-methoxy-DL-proline (V), m.p. 184–185°, electrophoretically pure.[14]

Anal. Found: C, 49.65; H, 7.56; N, 9.54.

The aqueous mother liquors were concentrated to dryness under reduced pressure and the residual blue solid crystallized from ethanol to yield 1.734 g. of a copper salt, which upon removal of cupric ion produced from methanol–ethyl acetate 1.014 g. (21%) of crystalline *cis*-3-methoxy-DL-proline (V), m.p. 196–198°, electrophoretically pure.[14] Two recrystallizations from methanol–ethyl acetate gave a sample, m.p. 205.5–206.5°, unchanged on admixture with *cis*-3-methoxy-DL-proline (V) (from IIa); the infrared spectra were identical.

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(10) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, Inc., New York, N. Y., 1961, p. 83.

(11) T. Purdie and G. B. Neave, *J. Chem. Soc.*, 1517 (1910).

(12) A. Neuberger, *ibid.*, 429 (1945).

(13) All melting points are uncorrected. Analyses were carried out by Dr. S. M. Nagy, Chemistry Department, M.I.T., Cambridge 39, Mass.

(14) pH 1.9, 3 kv., 3 hr.

***cis*- and *trans*-3-Hydroxy-DL-proline (VI).**[5]—A solution of 103.3 mg. (0.713 mmole) of the *cis* racemate V in 1.5 ml. of constant-boiling hydrobromic acid was heated under reflux for 2 hr. and then evaporated to dryness under reduced pressure. The residue in aqueous solution was passed through a column of Amberlite IR-4B ($OH^{\ominus}$) resin and the eluate lyophilized to a colorless oil, which was crystallized from methanol to yield 78.3 mg. (84%) of truncated prisms, *cis*-3-hydroxy-DL-proline (VI), electrophoretically pure.[14] After two recrystallizations from methanol–water, an analytical sample was obtained, m.p. 225–235° dec.

Anal. Calcd. for $C_5H_9NO_3$: C, 45.75; H, 6.86; N, 10.7. Found: C, 45.76; N, 6.75; N, 10.73.

By the foregoing procedure 94.7 mg. (0.654 mmole) of the *trans* racemate V produced from ethanol 72.1 mg. (84%) of small needles, *trans*-3-hydroxy-DL-proline (VI), electrophoretically pure.[14] Three recrystallizations from ethanol–water afforded an analytical sample, m.p. 224–230° dec.

Anal. Found: C, 45.77; H, 6.85; N, 10.39.

***cis*- and *trans*-3-Methoxy-L-proline (V).**[8]—To a solution of 500 mg. (3.45 mmoles) of *cis* racemate V in 27.6 ml. of 0.02 *N* aqueous potassium cyanide was added 6.9 ml. of a 0.1 *M* sodium pyrophosphate buffer containing 1.035 g. of D-amino acid oxidase (Nutritional Biochemicals Corporation). The solution was stirred open to the air at room temperature, a 1% lithium hydroxide solution being added periodically to maintain a pH of 8. After 24 hr. the addition of 500 mg. of fresh enzyme in 3.45 ml. of buffer failed to cause the pH to change over another 24-hr. period.

The reaction mixture was acidified to pH 5 with glacial acetic acid, concentrated under reduced pressure to a 10-ml. volume, and dialyzed against 750 ml. of water overnight. The dialyzate was lyophilized and the residue in aqueous solution adsorbed on a column of Dowex 50W-X8 ($H^{\oplus}$) resin. The column was washed to neutrality with water, eluted with 1 *N* ammonium hydroxide, and the ninhydrin-positive eluate fractions evaporated under reduced pressure to give a brown oil. The oil in aqueous solution was purified on a column of Dowex 1-X8 ($OH^{\ominus}$) resin (1 *N* glacial acetic acid elution) and by passage through a column of Amberlite IR-4B ($OH^{\ominus}$) resin. Lyophilization of the eluate yielded yellow crystals, which after recrystallization from methanol produced 93.9 mg. (38%) of rods, *cis*-3-methoxy-L-proline (V), m.p. 212–214°, electrophoretically pure,[14] $[\alpha]^{25}D$ −110.8° (*c* 1 in H_2O), $[\alpha]^{25}D$ −65.7° (*c* 1 in 5 *N* hydrochloric acid).

Anal. Calcd. for $C_6H_{11}NO_3$: C, 49.6; H, 7.59; N, 9.66. Found: C, 49.87; H, 7.54; N, 9.44.

By the foregoing procedure 500 mg. (3.45 mmoles) of the *trans* racemate V afforded from ethanol 105.2 mg. (42%) of crystalline *trans*-3-methoxy-L-proline V, m.p. 216.5–219°, electrophoretically pure,[14] $[\alpha]^{25}D$ −25.3° (*c* 1 in H_2O), $[\alpha]^{25}D$ + 7.8° (*c* 1 in 5 *N* hydrochloric acid).

Anal. Found: C, 49.76; H, 7.87; N, 10.26.

***cis*- and *trans*-3-Hydroxy-L-proline (VI).**[5,9]—By the procedure described for the preparation of the *cis* and *trans* racemates VI, 90.7 mg. (0.625 mmole) of the *cis*-L-amino acid V gave from methanol 64.7 mg. (79%) of crystalline *cis*-3-hydroxy-L-proline (VI), electrophoretically pure.[14] After three recrystallizations from ethanol–water an analytical sample was obtained, m.p. 245–255° dec., $[\alpha]^{25}D$ −90.3° (*c* 1 in H_2O), $[\alpha]^{25}D$ −47.9° (*c* 1 in 5 *N* hydrochloric acid).

Anal. Calcd. for $C_5H_9NO_3$: C, 45.75; H, 6.86; N, 10.7. Found: C, 45.26; H, 6.82; N, 10.7.

The *trans*-L-amino acid V (58.3 mg., 0.402 mmole) yielded from ethanol 40.6 mg. (77%) of crystalline *trans*-3-hydroxy-L-proline (VI), electrophoretically pure.[14] The product recrystallized twice from ethanol–water produced an analytical sample, m.p. 228–235° dec., $[\alpha]^{25}D$ −22.8° (*c* 1 in H_2O), $[\alpha]^{25}D$ +2.6° (*c* 1 in 5 *N* hydrochloric acid).

Anal. Found: C, 46.11; H, 7.03; N, 10.71.

Comparison of *cis*- and *trans*-L-Hydroxyproline (VI) with the Two Amino Acids from Telomycin.—The relative mobilities (taking 4-hydroxyproline as unity) were: electrophoresis[14]; 4-Hypro (1), *allo*-4-Hypro (1.15), *cis*-3-Hypro (1.15), *trans*-3 Hypro (0.83), "fast moving" Hypro (1.15), "slow moving" Hypro (0.83); paper chromatography [(a) *n*-butyl alcohol saturated with 10% aqueous diethylamine, 120 hr., Whatman No. 1 paper[15]; (b) *n*-butyl alcohol–water–acetone–concentrated ammonium hydroxide (8:6:1:1), 116 hr., Whatman No. 1 paper[16]; (c) *t*-amyl alcohol–2,4-lutidine–water (178:178:114), 165 hr., Whatman No. 1 paper.[17]]: 4-Hypro (1, 1, 1), *allo*-4-Hypro (1.35, 1, 0.72), *cis*-3-Hypro (1.80, 1.29, 0.69), *trans*-3-Hypro (1.16, 1.08, 1.37), "fast moving" Hypro (1.80, 1.29, 0.69), "slow moving" Hypro (1.16, 1.08, 1.37).

L-Methoxysuccinamide.[12]—A solution of 104.2 mg. (0.718 mmole) of *trans*-3-methoxy-L-proline (V) in 2 ml. of water was added dropwise to a stirred solution of 302.5 mg. (1.925 mmoles) of potassium permanganate in 7.2 ml. of water over a period of 20 min. The reaction mixture was then heated to 70° for 10 min., cooled, and manganese dioxide removed by filtration with Celite. The solid collected was washed well with boiling water, the combined filtrates acidified with 1.192 ml. (2.384 mmoles) of 2 *N* hydrochloric acid, and then concentrated to dryness *in vacuo*. The residue was repeatedly extracted with ether which was then dried over sodium sulfate. Removal of the ether afforded 41.9 mg. (39%) of a light yellow oil $\lambda_{max}^{CHCl_3}$ 1725 cm.$^{-1}$ (CO_2H).

The oil was taken up in 10 ml. of ether and treated at 0° with an ethereal solution of diazomethane prepared from 2.06 g. of N-nitrosomethylurea. The solution was stored at 0° for 22 hr. and then allowed to evaporate to dryness to give 70.4 mg. (100%) of a light yellow oil, $\lambda_{max}^{CHCl_3}$ 1750 cm.$^{-1}$ (CO_2CH_3).

The oil was dissolved in 5 ml. of methanol which had been saturated with ammonia and the solution stored at ambient temperature for 3 days. Removal of the solvent under reduced pressure yielded 52.1 mg. (100%) of a light yellow oil, λ_{max}^{KBr} 1665 cm.$^{-1}$ ($CONH_2$). A crystallization and recrystallization from ethanol produced a sample, L-methoxysuccinamide (IX), m.p. 180–181°, $[\alpha]^{25}D$ −55.4° (*c* 0.72 in methanol), $[\alpha]^{25}D$ −39.6° (*c* 0.72 in H_2O). Purdie and Neave[11] gave the following values for L-methoxysuccinamide: m.p. 179°, $[M]^{20}D$ −83° (methanol) and $[M]^{20}D$ −58.8° (H_2O), which convert to $[\alpha]^{20}D$ −56.8° (methanol) and $[\alpha]^{20}D$ −40.3° (H_2O), respectively.

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