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Amides and Amino Acid Derivatives of Biotin

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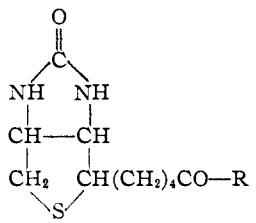
Amides of biotin, DL-biotin, DL-desthiobiotin, and substituted amides of biotin from aniline, *p*-aminobenzoic acid and sulfanilamide have been prepared. N-Biotinyl derivatives of glycine, β-alanine, and the ethyl esters of L-glutamic acid, L-leucine, L- and DL-aspartic acid were characterized. Biotin acid chloride generally, or in some reactions the methyl ester, is allowed to react with the amino compound under appropriate conditions.

A series of amides and amino acid derivatives of biotin have been prepared to permit an extension of the knowledge of the biological properties of biotin derivatives. The amino acid representatives were of particular interest in connection with the studies on biocytin.[1] They were examined microbiologically, and the results are summarized in an accompanying paper.[2]

As a rule, it was found most satisfactory to start with biotin acid chloride, although biotin methyl ester can be used in some reactions with amino compounds. Biotin acid chloride is easily prepared by treating biotin with an excess of thionyl chloride at room temperature. The biotin quickly dissolves, and the excess thionyl chloride may then be evaporated at reduced pressure, leaving biotin acid chloride as a crystalline residue.

The preparation of N-biotinyl derivatives of amino acids was accomplished by various methods. To prepare biotinyl derivatives of amino acids such as glycine and *p*-aminobenzoic acid, it was possible to treat biotin acid chloride with a solution of the amino acid in cold aqueous alkali; N-biotinylglycine (I) and *p*-(N-biotinyl)-aminobenzoic acid (II) were obtained. This procedure was not always applicable, and it was found advantageous to avoid the use of an aqueous medium since biotin acid chloride hydrolyzes readily.



I, R = $-NHCH_2CO_2H$
II, R = $-NHC_6H_4CO_2H$
III, R = $-NHCH(CH_2)_2CO_2C_2H_5$ with $CO_2C_2H_5$
IV, R = $-NHCH(CH_2)_3CH_3$ with $CO_2C_2H_5$
V, R = $-NHCHCH_2CO_2C_2H_5$ with $CO_2C_2H_5$
VI, R = $-NHCH_2CH_2CO_2H$
VII, R = $-NH_2$

By the preferred method, the amino acid ester hydrochloride was dissolved in pyridine, and this solution was added to the biotin acid chloride. The reaction was complete in a few hours at room temperature. The pyridine was removed by evaporation at reduced pressure, and the biotinyl amino acid ester was dissolved in chloroform and impurities were removed by washing with dilute acid and sodium bicarbonate solutions. In this way, N-biotinyl-L-glutamic acid ethyl ester (III), N-biotinyl-L-leucine ethyl ester (IV) and N-biotinyl-L- and DL-aspartic acid ethyl ester (V) were obtained.

Some difficulty was encountered in preparing N-biotinyl-β-alanine (VI). Numerous efforts to combine β-alanine directly with biotin acid chloride in various media were unsatisfactory. When freshly distilled β-alanine ethyl ester was allowed to react directly with biotin acid chloride, using the excess β-alanine ester to combine with liberated hydrogen chloride, N-biotinyl-β-alanine ethyl ester was obtained. Alkaline hydrolysis of the ester yielded the N-biotinyl-β-alanine (VI).

Biotin amide (VII) was made by reaction of the biotin acid chloride with liquid ammonia or with concentrated ammonium hydroxide; it was also made by reaction of biotin methyl ester with concentrated ammonium hydroxide. DL-Biotin amide and DL-desthiobiotin amide were also characterized. Biotin anilide was made by reaction of the acid chloride with an excess of aniline, and N^4-biotinylsulfanilamide was formed by reaction of the acid chloride with sulfanilamide in pyridine.

In purifying the N-biotinyl derivatives which did not contain an acidic group, it was found advantageous to remove unreacted biotin by passing a solution of the reaction mixture over an anion exchange resin.

Experimental

Biotin Amide.—Fifty-seven milligrams of biotin was treated with about 2 ml. of thionyl chloride at room temperature in the absence of moisture. The biotin dissolved readily and when all was in solution, the excess thionyl chloride was evaporated at reduced pressure. Biotin acid chloride crystallized as the thionyl chloride was removed. Excess liquid ammonia was added to the acid chloride. When the ammonia had evaporated, the residue was washed with water leaving 35 mg. of biotin amide. The reaction was carried out equally well by treating the acid chloride with cold concentrated ammonia hydroxide. The biotin amide was obtained as a white crystalline precipitate. It was recrystallized from methanol or water in rosettes which melted at 243–244°, $[\alpha]^{23}D$ +80°; *c* 0.25 g./100 ml., ethanol.

Anal. Calcd. for $C_{10}H_{17}N_3O_2S$: C, 49.36; H, 7.04; N, 17.27. Found: C, 49.53; H, 7.12; N, 17.12.

Biotin amide was also obtained by ammoniation of biotin methyl ester. Sixty-five milligrams of biotin methyl ester was treated with 10 ml. of concentrated ammonium hydroxide and the mixture was stirred at room temperature for six hours. The ester dissolved slowly and the amide crystallized from solution. It was collected on a filter and washed with water; m.p. 243–244°; yield 20 mg.

DL-Biotin Amide.—One hundred and two milligrams of DL-biotin was treated with about 2 ml. of thionyl chloride in the absence of moisture. The solid dissolved rapidly at room temperature and the excess reagent was evaporated at reduced pressure leaving DL-biotin acid chloride as a clear oil. This product was treated with 20 ml. of cold concentrated ammonium hydroxide. The DL-biotin amide formed a precipitate which was collected on a filter and washed with water; yield 72 mg. It was recrystallized from methanol, and found to melt at 265–267°.

Anal. Calcd. for $C_{10}H_{17}N_3O_2S$: N, 17.27. Found: N, 16.94.

DL-Desthiobiotin Amide.—One hundred and six milligrams of DL-desthiobiotin was treated with about 2 ml. of

(1) Wright, Cressen, Skeggs, Wood, Peck, Wolf and Folkers, THIS JOURNAL, **72**, 1048 (1950).

(2) Wright, Skeggs and Cresson, *ibid.*, **73**, 4144 (1951).

thionyl chloride in the absence of moisture. As soon as the solid had dissolved, the excess thionyl chloride was evaporated at reduced pressure leaving DL-desthiobiotin acid chloride as a clear oil. This product was treated with about 20 ml. of cold concentrated ammonium hydroxide. The DL-desthiobiotin amide precipitated as a white solid. It was collected on a filter, washed with cold water, and recrystallized from methanol; m.p. 187–188°.

Anal. Calcd. for $C_{10}H_{19}N_3O_2$: C, 56.31; H, 8.98; N, 19.70. Found: C, 56.40; H, 9.02; N, 19.67.

N-Biotinylglycine.—Ninety-seven milligrams of biotin was converted to biotin acid chloride. The crystalline biotin acid chloride was treated with a cold solution which consisted of 250 mg. of glycine in about 1 ml. of 2.5 *N* aqueous sodium hydroxide. The reaction mixture was shaken until all of the solid material had dissolved. The solution was then acidified with concentrated hydrochloric acid, which caused precipitation of biotinyl glycine. The product was collected on a filter and washed with water. It was recrystallized from hot water; m.p. 213.5–214.5°; $[\alpha]^{26}D$ + 83.5°; *c* 0.503 g./100 ml., 0.1 *N* sodium hydroxide.

Anal. Calcd. for $C_{12}H_{19}N_3O_4S$: C, 47.82; H, 6.35. Found: C, 47.95; H, 6.90.

***p*-(N-Biotinyl)-aminobenzoic Acid.**—One hundred milligrams of biotin was converted to biotin acid chloride. A cold solution consisting of 500 mg. of *p*-aminobenzoic acid in about 2 ml. of 2.5 *N* sodium hydroxide solution was added. The reaction mixture was shaken in an ice-bath. After all of the solid had dissolved, the solution was acidified with concentrated hydrochloric acid. The *p*-(N-biotinyl)-aminobenzoic acid precipitated as a white solid. It was collected on a filter, washed with cold water and recrystallized from hot methanol. After three recrystallizations, the product weighed 39 mg. and decomposed over a range of 295–300°, $[\alpha]^{23}D$ +56.5°; *c*, 0.46 g./100 ml., 0.1 *N* sodium hydroxide. The same was dried in a weighing-pig at 140° for analysis.

Anal. Calcd. for $C_{17}H_{21}N_3O_4S$: C, 56.18; H, 5.83; N, 11.56. Found: C, 55.63; H, 5.88; N, 11.44, 11.63.

N-Biotinyl-L-glutamic Acid Ethyl Ester.—Ninety-four milligrams of biotin was converted to the acid chloride by treatment with 2 ml. of thionyl chloride. The crystalline biotin acid chloride was treated with a solution which consisted of 109 mg. of ethyl glutamate hydrochloride in 2 ml. of pyridine. The reaction mixture was allowed to stand at room temperature about 1.5 hours. It was then concentrated at reduced pressure, and the residue was dissolved in chloroform. The chloroform solution was washed with 1 *N* hydrochloric acid, water, 2% sodium bicarbonate solution, water, and then it was dried over magnesium sulfate and concentrated at reduced pressure to a clear oil. The ethyl N-biotinyl-L-glutamate failed to crystallize from any of the common organic solvents; it was obtained as a flocculent precipitate by diluting a chloroform solution with ether; yield 62 mg.

Anal. Calcd. for $C_{19}H_{31}N_3O_6S$: C, 53.13; H, 7.28; N, 9.78. Found: C, 52.97; H, 7.22; N, 9.82.

N-Biotinyl-L-leucine Ethyl Ester.—Ninety-eight milligrams of biotin was converted to the acid chloride. To it, was added a solution consisting of 126 mg. of L-leucine ethyl ester hydrochloride in about 1 ml. of pyridine. The reaction mixture was allowed to stand overnight at room temperature. The pyridine was evaporated at reduced pressure. The residue was dissolved in chloroform and the solution was washed with 1 *N* hydrochloric acid, 2% sodium bicarbonate solution, and with water. The chloroform solution was dried over magnesium sulfate, and then evaporated at reduced pressure. The N-biotinyl-L-leucine ethyl ester was an oil which crystallized from aqueous alcohol; m.p. 116°.

Anal. Calcd. for $C_{18}H_{31}N_3O_4S$: C, 56.08; H, 8.11; N, 10.90. Found: C, 56.09; H, 8.16; N, 10.74.

N-Biotinyl-L-Aspartic Acid Ethyl Ester.—To the biotin acid chloride from 100 mg. of biotin, was added 200 mg. of L-aspartic acid ethyl ester hydrochloride dissolved in 2 ml. of dry pyridine. The mixture was agitated, and allowed to stand at room temperature for 1.5 hours. The pyridine was removed at reduced pressure. The oily residue was dissolved in chloroform, and the solution was washed successively with 1 *N* hydrochloric acid, water, dilute sodium bicarbonate solution and water. The chloroform solution was dried over magnesium sulfate, filtered, and then the chloroform was removed at reduced pressure. The crude product weighed 74 mg. It was recrystallized from a mixture of ethanol and acetone; m.p. 152–153°; $[\alpha]^{23}D$ + 38°; *c*, 0.69 g./100 ml. ethanol.

Anal. Calcd. for $C_{18}H_{29}N_3O_6S$: C, 52.03; H, 7.04; N, 10.11. Found: C, 52.27; H, 7.04; N, 10.51.

N-Biotinyl-DL-Aspartic Acid Ethyl Ester.—One hundred and three milligrams of biotin was converted to the acid chloride. A solution of 163 mg. of DL-aspartic acid diethyl ester hydrochloride in 1 ml. of pyridine was added to the acid chloride, and the mixture was allowed to stand at room temperature with occasional shaking for 1.5 hours. The pyridine was then removed under reduced pressure leaving the N-biotinyl-DL-aspartic acid ethyl ester as an oil. The ester was obtained as an amorphous solid by diluting an alcoholic solution of the product with water; yield 53 mg.

Anal. Calcd. for $C_{18}H_{29}N_3O_6S$: C, 52.03; H, 7.04; N, 10.11. Found: C, 52.10; H, 7.12; N, 10.14.

N-Biotinyl-β-alanine Ethyl Ester.—Two hundred milligrams of biotin was converted to biotin acid chloride. To the acid chloride, was added 2 ml. of freshly distilled β-alanine ethyl ester. The mixture was allowed to stand at room temperature for 18 hours and then it was dissolved in about 20 ml. of 0.5 *N* hydrochloric acid solution. The aqueous solution was extracted continuously with chloroform for two hours, and the chloroform extract was dried over magnesium sulfate and concentrated under reduced pressure to give a white amorphous residue of N-biotinyl-β-alanine ethyl ester. This product was dissolved in a minimum of methanol, and the solution was diluted with one volume of water and passed over a small column of Amberlite IR-4B to remove the last traces of biotin. The eluate was concentrated to dryness at reduced pressure. The residue of N-biotinyl-β-alanine ethyl ester was a white solid which melted at 132–135°.

Anal. Calcd. for $C_{15}H_{25}N_3O_4S$: C, 52.47; H, 7.34; S, 9.34. Found: C, 52.40; H, 7.29; S, 8.78.

N-Biotinyl-β-alanine.—The N-biotinyl-β-alanine ethyl ester was hydrolyzed to the acid by treating it with 10 ml. of 0.5 *N* sodium hydroxide solution at 60° for 20 minutes. The hydrolysate was cooled and acidified with concentrated hydrochloric acid which caused precipitation of biotinyl-β-alanine as a white precipitate. The product was collected on a filter and washed with water. It was reprecipitated from 1 *N* ammonium hydroxide solution after filtration by treatment with concentrated hydrochloric acid. The biotinyl-β-alanine was collected on a filter and washed with water; m.p. 229–232°; $[\alpha]^{23}D$ + 69°; *c*, 0.49 g./100 ml., 0.1 *N* sodium hydroxide. Before analysis the sample was dried at 140° in a weighing pig.

Anal. Calcd. for $C_{13}H_{21}N_3O_4S$: C, 49.51; H, 6.71; equiv. wt., 315.4. Found: C, 49.26; H, 6.43; equiv. wt., 312.

Biotin Anilide.—One hundred milligrams of biotin was converted to the acid chloride. To the biotin acid chloride was added 2 ml. of freshly distilled aniline and the reaction was allowed to proceed overnight. The excess aniline was evaporated at reduced pressure, and the biotin anilide which remained was washed with 1 *N* hydrochloric acid and water. The product was dissolved in 80% aqueous methanol and the solution was passed over a column of Amberlite IR-4B to remove traces of biotin. The eluate was concentrated to a small volume, and the biotin anilide allowed to crystallize. The crystals were collected on a filter, washed with 10% methanol and dried. The product weighed 58 mg. and melted at 206–210°; $[\alpha]^{26}D$ + 71.5°; *c*, 0.52 g./100 ml., ethanol.

Anal. Calcd. for $C_{16}H_{21}N_3O_2S$: C, 60.16; H, 6.63. Found: C, 60.22; H, 6.60.

N^4-Biotinylsulfanilamide.—The biotin acid chloride from 100 mg. of biotin and 350 mg. of sulfanilamide were dissolved in about 1 ml. of pyridine. The pyridine was evaporated at reduced pressure after 24 hours. The residue was extracted with 1 *N* hydrochloric acid to remove excess sulfanilamide, then with water. No suitable solvent for recrystallization of the product was found; N^4-biotinylsulfanilamide was purified by dissolving it in 0.1 *N* sodium

hydroxide solution, filtering the solution and acidifying with dilute hydrochloric acid. The product separated in fine white crystals which melted at 276–278°; $[\alpha]^{25}D + 56°$; *c*, 0.47 g./100 ml., 0.1 *N* sodium hydroxide.

Anal. Calcd. for $C_{16}H_{22}N_4O_4S_2$: C, 48.22; H, 5.57; N, 14.06. Found: C, 48.27; H, 5.45; N, 14.04.

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Amides and Amino Acid Derivatives of Biotin: Microbiological Studies

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Biotinamide and N-biotinyl derivatives of glycine, β-alanine, L-aspartic acid, L-glutamic acid, L-leucine, *p*-aminobenzoic acid and the diethyl esters of N-biotinyl-L-aspartic and N-biotinyl-L-glutamic acids were examined microbiologically for biotin activity. Biotinamide, N-biotinyl-*p*-aminobenzoic acid and N-biotinyl-β-alanine have a spectrum of microbiological activity quite similar to that of biocytin, a form of biotin occurring in some natural products, but may be distinguished from biocytin by differences in solubility or acid stability. Representative compounds from the group synthesized readily combine with avidin.

The chemical properties of biocytin that became apparent during the development of fractionation procedures for the isolation of this naturally-occurring complex of biotin[1] were such as to suggest that biocytin is a biotinyl derivative which might be linked through nitrogen to an amino acid-like moiety. A series of amides and amino acid derivatives of biotin have been synthesized[2] and made available for microbiological examination. The activities of these compounds in promoting growth of *Lactobacillus arabinosus* and *Lactobacillus casei* in basal media free of biotin are summarized in Table I. Biotinamide, N-biotinyl-*p*-aminobenzoic acid and N-biotinyl-β-alanine were found to have differential activity with the two strains corresponding to that of biocytin and were examined with additional organisms. The data of Table II demonstrate that, with a variety of microörganisms that depend for growth on an exogenous source of biotin, biocytin, biotinamide, N-biotinyl-*p*-aminobenzoic acid and N-biotinyl-β-alanine have an essentially similar spectrum of activity. It may be noted that two artificially-induced biotin-deficient mutants *Escherichia coli* M81-78 and *Penicillium chrysogenum* 62078 are unable to utilize biocytin as a source of biotin.

TABLE I

MICROBIOLOGICAL ACTIVITIES OF BIOTIN DERIVATIVES

Compound	Biotin activity as determined by *Lactobacillus arabinosus*	Biotin activity as determined by *Lactobacillus casei*
Biocytin	−	+
Biotinamide	−	+
N-Biotinylglycine	+	+
N-Biotinyl-β-alanine	−	+
Diethyl ester of N-biotinyl-L-aspartic acid	−	±
N-Biotinyl-L-aspartic acid	−	−
Diethyl ester of N-biotinyl-L-glutamic acid	−	±
N-Biotinyl-L-glutamic acid	−	−
Ethyl ester of N-biotinyl-L-leucine	−	−
N-Biotinyl-L-leucine	−	−
N-Biotinyl-*p*-aminobenzoic acid	−	+

+, activity essentially that of an equivalent of biotin; ±, activity in the order of 25–50% that of an equivalent of biotin; −, no activity.

TABLE II

SPECTRA OF ACTIVITY OF BIOTIN DERIVATIVES

	Biocytin	Biotinamide	N-Biotinyl-*p*-aminobenzoic acid	N-Biotinyl-β-alanine
Lactobacillus arabinosus 17-5 (8014)	−	−	−	−
Lactobacillus pentosus (8041)	−(b)	−(a)	−	−
Leuconostoc mesenteroides (8042)	−	−	−	−
Escherichia coli M81-78	−			
Penicillium chrysogenum 62078	−	−(a)	−	−(b)
Lactobacillus acidophilus (4646)	+	+	+	+
Lactobacillus acidophilus 05	±			
Lactobacillus acidophilus (314)	+	+	−	−(b)
Lactobacillus acidophilus (8530)	+	+	+	+
Lactobacillus acidophilus (4357)	+	+	−	−(b)
Lactobacillus acidophilus K	±	+	+	+
Lactobacillus brevis (8287)	±	+	−	−(b)
Lactobacillus casei (7469)	+	+	+	±
Lactobacillus delbrückii LD5 (9595)	+	+	+	+
Lactobacillus plantarum (4943)	±	±	−(a)	−(a)
Streptococcus fecalis R (8043)	+	+	−(b)	±
Neurospora crassa (9278)	+	+	−	±
Propionibacterium shermanii (8262)	+	±	±	±

+, activity essentially that of an equivalent of biotin; −(a), activity about 50% that of an equivalent of biotin; −(b), activity about 10% that of an equivalent of biotin; −, no activity.

Biotinamide and N-biotinyl-*p*-aminobenzoic acid as demonstrated by the data of Table III differ from biocytin in being much more labile to acid hydrolysis. N-Biotinyl-β-alanine differs from biocytin as demonstrated by the data of Table IV in being much more readily extractable from aqueous solution with butanol.

Avidin combinability studies on biotinamide, N-biotinyl-*p*-aminobenzoic acid, N-biotinyl-L-glutamic acid, N-biotinyl-L-leucine and N-biotinyl-β-alanine as representative derivatives have given affinity ratios of 3–6 similar to that reported previously for biocytin.[3] Until the role of avidin is more clearly defined, the significance, if any, of these affinity ratios is obscure.

(1) L. D. Wright, E. L. Cresson, H. R. Skeggs, T. R. Wood, R. L. Peck, D. E. Wolf and K. Folkers, THIS JOURNAL, **72**, 1048 (1950).

(2) D. E. Wolf, J. Valiant and K. Folkers, *ibid.*, **73**, 4142 (1951).

(3) L. D. Wright, K. A. Valentik, H. M. Nepple, E. L. Cresson and H. R. Skeggs, *Proc. Soc. Exptl. Biol. Med.*, **74**, 273 (1950).