Synthesis of Biocytin-Containing Peptides

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Abstract: The reaction of biotin with *p*-nitrophenyl trifluoroacetate afforded a trifluoroacetyl derivative of the *p*-nitrophenyl ester of biotin. Alcoholysis of this intermediate yielded the desired biotin active ester which in turn was applied for the acylation of α-*tert*-butyloxycarbonyl-L-lysine. The product, *tert*-butyloxycarbonylbiocytin, was deprotected to give biocytin and was also coupled with L-threonine to produce *tert*-butyloxycarbonylbiocytinyl-L-threonine from which, by deprotection, the free dipeptide biocytinyl-L-threonine was obtained. Coupling of *tert*-butyloxycarbonylbiocytin to L-threonine methyl ester afforded the protected dipeptide ester, but the weak nucleophilic character of the urea group in the biotin moiety interfered with the activation of the carboxyl of biocytin and the formation of by-products was observed. Better results were achieved by the alternative approach, the preparation of a protected, lysine-containing dipeptide, N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-L-threonine methyl ester, selective deprotection of the ϵ-amino group of the lysine residue, and acylation with biotin *p*-nitrophenyl ester. The chain could then be lengthened by the use of mild acylating agents (active esters), and protected derivatives of L-methionylbiocytinyl-L-threonine and L-alanyl-L-methionylbiocytinyl-L-threonine could be secured in high yield and purity. The corresponding tripeptide and tetrapeptide amides were also prepared and deprotected for studies of their potential role in the enzymic activities of transcarboxylases from propionic acid bacteria. They were insufficient for the restoration of catalytic activity to trypsinized transcarboxylase.

Biotin[1] is an essential part of the active site of several enzymes.[2] In proteins it is attached to the ϵ-amino group of lysine residues: controlled hydrolysis produced biocytin,[3] ϵ-biotinyl-L-lysine. For studies of the mechanism of reactions catalyzed by biotin-containing enzymes, synthesis of model peptides with biocytin in their sequence is obviously desirable. The amino acid sequence of the biotin-containing carboxyl-carrier protein (BCP) of the transcarboxylase from *Propionibacterium shermanii* was recently determined.[4] The partial reactions of transcarboxylation are catalyzed[5] specifically by two subunits of the transcarboxylase:

$$\begin{array}{l} \mathrm{CH_3{-}\overset{\displaystyle COO^-}{\overset{|}{C}}H{-}COSCoA + BCP} \\ \qquad\qquad \overset{12S_H\ \text{subunit}}{\rightleftharpoons} \mathrm{CH_3{-}CH_2{-}COSCoA + {}^-OOC{-}BCP} \\ \mathrm{{}^-OOC{-}BCP + CH_3{-}CO{-}COO^-} \\ \qquad\qquad \overset{5S_E\ \text{subunit}}{\rightleftharpoons} \mathrm{BCP + {}^-OOC{-}CH_2{-}CO{-}COO^-} \end{array}$$

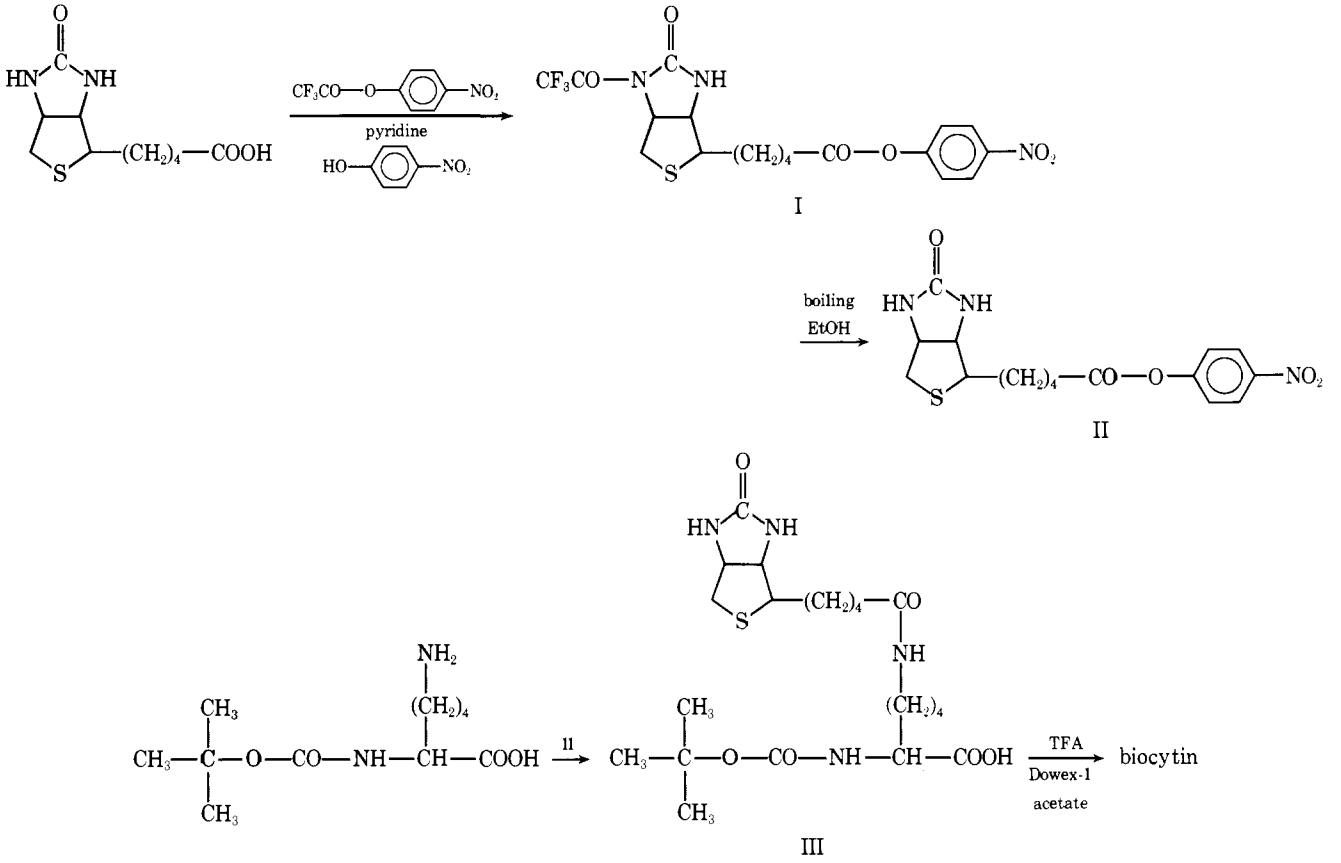
Biotinyl peptides derived from BCP were also active in these partial reactions, while biotin and biocytin were not. The question of what portion of the amino acid sequence of BCP is required from the environment of biocytin for the restoration of catalytic activity can best be answered by experiments with synthetic peptides. Our first objective, the synthesis of L-alanyl-L-methionylbiocytinyl-L-threonine amide, is reported in this paper.

For the synthesis of biocytin-containing peptides, two alternative approaches can be visualized: (a) the preparation of biocytin and its incorporation into a peptide chain, and (b) synthesis of lysine-containing peptides, followed by acylation of the selectively deprotected ϵ-amino group of a lysine side chain with biotin. It was known that the NH groups in the imidazolidinone ring of biotin are sufficiently reactive to be acylated with acid chlorides, e.g., with methyl chloroformate.[6] On the other hand, we assumed that the nucleophilicity of the NH groups in a (cyclic) urea derivative is sufficiently weak to render these groups inert toward moderately active acylating agents such as *p*-nitrophenyl esters.[7] Yet, an observation made during the preparation of biotin *p*-nitrophenyl ester cautioned us about the general validity of this assumption.

Biotin *p*-nitrophenyl ester was prepared in our laboratory several years ago and was used for the acylation of insulin[8] and dodecamethylenediamine.[9] We did not describe the preparation of the active ester because it was repeatedly obtained in low yield and could not be secured in analytically pure form. Subsequent reports in the literature[10] showed that similar difficulties were encountered by other investigators as well. Since, for the synthesis of biocytin and biocytin peptides, this active ester became once again a desired intermediate,[11] new attempts were made for its preparation. After experiments with tri-*p*-nitrophenyl phosphite[12] gave unsatisfactory results, esterification with Sakakibara's reagent, *p*-nitrophenyl trifluoroacetate,[13] was attempted.[14] With equimolar amounts of the reactants, only incomplete esterification of biotin could be achieved, therefore the reagent was applied in considerable excess and in the presence of added *p*-nitrophenol.[15] A crystalline product with unusually high specific rotation was isolated. It turned out to be different from the expected active ester. Elemental analysis, IR and UV spectra indicated a trifluoroacetyl derivative of biotin *p*-nitrophenyl ester. Tentatively, structure I (Scheme I) was assigned to this intermediate. This assignment is based on IR spectra and on the conditions of alcoholysis (boiling with 95% ethanol) necessary to convert compound I to the active ester II. An *O*-trifluoroacetyl derivative[16] should have a more reactive carbonyl group than found in I. The N-1′ rather than N-3′ was selected as the more likely point of acylation because of its lesser hindrance and on account of analogy with other *N*-acyl derivatives of biotin.[6] The originally expected *p*-nitrophenyl ester II was secured, by alcoholysis of I, in pure form and satisfactory yield.

While the formation of compound I demonstrated that in biotin at least one of the urea-amide groups can be acylated by an active ester of trifluoroacetic acid, we did not consider this observation as reason to exclude the possibility of chain lengthening of the biocytin peptides with active esters of protected amino acids. Acylation of N^{α}-*tert*-butyloxycarbonyl-L-lysine with active ester II afforded *tert*-butyloxycarbonylbiocytin III in excellent yield, and the product was obtained in chromatographically and analytically pure form after a single recrystallization from water. Removal of the protecting group yielded biocytin in high yield. Several attempts were made for the use of III in the preparation of biocytinyl-L-threonine. Conversion of *tert*-butyloxycarbonylbiocytin to its *p*-nitrophenyl ester yielded only a small amount of impure material. Probably the urea group in biotin interfered with the dicyclohexylcarbodiimide mediated esterification. Subsequently, *tert*-butyloxycarbonylbiocytinyl-L-threonine (IV)

Scheme I. Synthesis of Biotin *n*-Nitrophenyl Ester (II) and Biocytin (Bct)



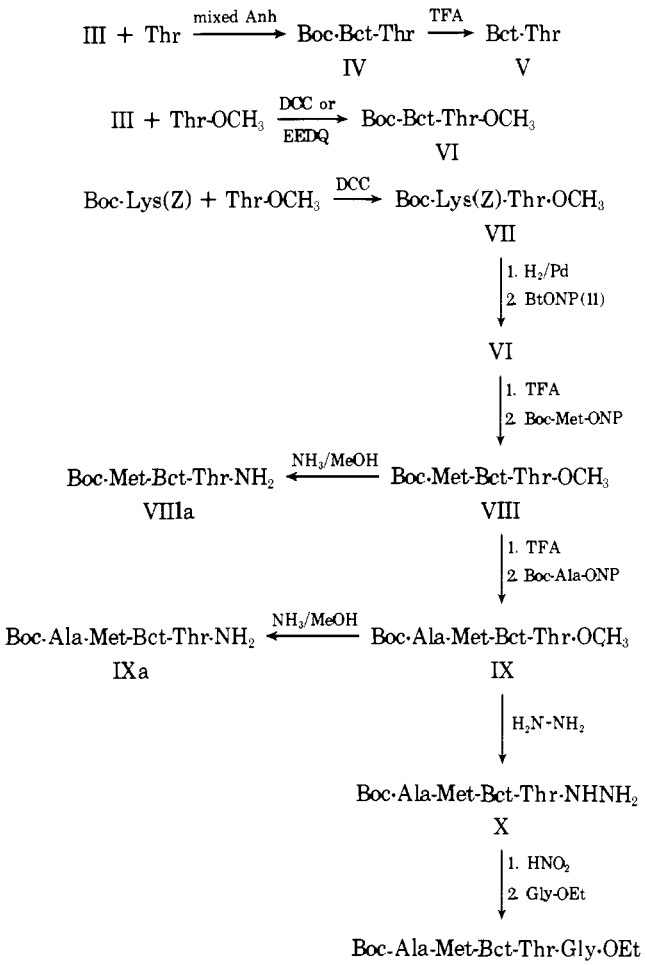
was synthesized via a mixed anhydride prepared with the aid of isobutyl chloroformate.[17] The protected dipeptide IV was obtained in fair yield, but not analytically pure, and on deprotection afforded biocytinyl-L-threonine (V), again in unsatisfactory purity. Coupling of III with L-threonine methyl ester by 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)[18] produced the protected dipeptide methyl ester VI in fair yield, but in impure form. Similar results were obtained with dicyclohexylcarbodiimide[19] as coupling reagent. It seemed to us that in all these reactions the reagents used for the activation of the carboxyl group of *tert*-butyloxycarbonylbiocytin were sufficiently reactive to produce side products formed by the interaction of the biotin moiety. The conclusion was drawn that it is preferable to start the synthesis with a lysine-containing peptide and to incorporate biotin subsequently.

Preparation of N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-L-threonine methyl ester by the Sheehan–Hess method[19] produced the desired dipeptide VII in excellent yield and—after chromatography on a column of silica gel—in homogeneous form. Selective deprotection of the ϵ-amino group by catalytic hydrogenation was followed by acylation with biotin *p*-nitrophenyl ester (II) to afford the chromatographically homogeneous protected dipeptide ester VI, in good yield. Since stepwise lengthening[20] of the chain of this peptide requires the application of only moderately activated derivatives of protected amino acids, we expected less interference by the cyclic urea part in the side chain of the biocytin residue. Therefore, the amino protecting group was removed from VI by acidolysis and the partially protected dipeptide derivative, biocytinyl-L-threonine methyl ester,[21] was allowed to react with the *p*-nitrophenyl ester of *tert*-butyloxycarbonyl-L-methionine. The protected tripeptide, *tert*-butyloxycarbonyl-L-methionylbiocytinyl-L-threonine methyl ester (VIII), was isolated in good yield in homogeneous, crystalline form. The next amino acid, alanine, was incorporated similarly, and the protected tetrapeptide derivative, *tert*-butyloxycarbonyl-L-alanyl-L-methionylbiocytinyl-L-threonine methyl ester (IX), was secured in high yield and purity. For testing of their possible catalytic activity,[22] after combination with the larger subunits of transcarboxylase, samples of the protected tripeptide and tetrapeptide methyl esters (VIII and IX) were ammonolyzed and the resulting amides deprotected with trifluoroacetic acid. The synthetic procedures are summarized in Scheme II.

The results of these experiments revealed that activation of the carboxyl group of protected biocytin requires reactants toward which the weakly nucleophilic NH group(s) of biotin are sensitive; the reactions take an ambiguous course with the formation of by-products. It was found preferable to prepare lysine-containing peptides and acylate these selectively with biotin active esters. On the other hand, once biotin is part of a peptide, the chain can be lengthened by the application of mild acylating agents such as *p*-nitrophenyl esters. Also, the protected tetrapeptide ester IX could be converted to the corresponding hydrazide and, in an exploratory experiment, the latter was coupled via the azide to glycine ethyl ester. Thus, the here described approach can probably be extended to the preparation of longer biocytin-containing peptide chains as well.

As mentioned before, the addition of the carboxyl carrier subunit (BCP) or its biotin-containing tryptic fragments to trypsinized transcarboxylase, from which the biotin-containing peptides were removed by gel filtration and affinity chromatography, can restore, in part, the enzymic activity. Biotin or biocytin cannot replace the biotin-containing tryptic fragments in such experiments. The di-, tri-, and tetrapeptide amides, Bct-Thr-NH_2, Met-Bct-Thr-NH_2, and Ala-Met-Bct-Thr-NH_2, applied as trifluoroacetate salts, were similarly inactive, even when added at a molar concentration about 300 times higher than that of the tryptic fragments. This indicates that,

Scheme II. Synthetic Routes to Biocytin-Containing Peptides



for the restoration of the characteristic catalytic activity of transcarboxylase, longer peptides are necessary. The active tryptic fragments probably encompass sequences, on one or both sides of biocytin, that extend beyond the four residues in Ala-Met-Bct-Thr.[23]

Experimental Section

Capillary melting points are uncorrected. Thin-layer chromatograms (TLC's) were run on glass plates precoated with silica gel (Brinkman) in the solvent systems: A, chloroform–methanol (9:1); B, chloroform–methanol (19:1); C, 1-butanol–AcOH–water (4:1:1); D, EtOAc–pyridine–H_2O–AcOH (60:20:11:6). Spots were revealed by UV absorption and charring or, in the case of *p*-nitrophenyl esters, by exposure to NH_3. For amino acid analysis, samples were hydrolyzed with constant boiling hydrochloric acid in evacuated sealed ampules at 110 °C for 16 h and analyzed by the Spackman–Stein–Moore method[24] on a Beckman Spinco 120C amino acid analyzer.

***N*-Trifluoroacetylbiotin *p*-Nitrophenyl Ester (I).** Finely powdered d-biotin (Calbiochem, 2.44 g, 10 mmol) was suspended in dry pyridine (60 ml), and *p*-nitrophenol (1.4 g, 10 mmol) and *p*-nitrophenyl trifluoroacetate (Aldrich, 10 g, 42.5 mmol) were added. The mixture was stirred and heated to 50 °C for 1 h. The clear solution was allowed to cool to room temperature and the solvent removed in vacuo. The residue was transferred into a large crystallizing dish and kept in vacuo over P_2O_5 overnight when a semisolid mass formed. This was triturated with ether (20 ml), filtered, and washed with ether (10 ml), water (20 ml), and once again with ether (20 ml). The product (I), dried in vacuo over NaOH and P_2O_5, weighed 3.4 g (74%): mp 160–164 °C; $[\alpha]^{25}D$ +98.5° (*c* 2, DMF containing 1% AcOH); R_fB 0.19; IR, CO bands at 1760 cm^{-1} and 1725 cm^{-1}; λ_{max}^{alc} 270 nm (ϵ 9500).

Anal. ($C_{18}H_{18}N_3O_6F_3S$) C, H, N, F.

Biotin *p*-Nitrophenyl Ester (II). Compound I (2.9 g, 6.3 mmol) was suspended in 95% ethanol (29 ml), heated to a boil, and, after all the material dissolved, heated for a few minutes more. The solution was cooled in an ice–water bath. The crystals that separated were collected, washed with ice cold 95% ethanol (5 ml), and dried in vacuo over P_2O_5: 2.27 g (99%); mp 160–163 °C (lit.[10] 156–158, 198–202 °C); $[\alpha]^{25}D$ +47° (*c* 2, DMF containing 1% AcOH); R_fB 0.19.

Anal. ($C_{16}H_{19}N_3O_5S$) C, H, N.

***tert*-Butyloxycarbonylbiocytin (III).** To a solution of N^{α}-*t*-Boc-L-Lys (Bachem, 0.89 g, 3.6 mmol) in H_2O (9 ml) was added pyridine (12 ml), followed by the addition of the active ester II (1.10 g, 3.0 mmol). The pH of the stirred mixture was kept between 9 and 10 by the addition of N NaOH: a total of 5.8 ml of alkali was consumed in about 0.5 h. Stirring was continued for an additional 0.5 h, then the mixture was transferred into a round-bottom flask, and concentrated in vacuo to a small volume. Water (20 ml) was added and the evaporation repeated. The concentrated solution was acidified to pH 2 with 0.1 N HCl; a white solid formed. After overnight storage in the refrigerator, the product was collected on a filter, washed with H_2O (10 ml), and dried in vacuo over P_2O_5. 1.3 g (92%); mp 133–136 °C dec; R_fC 0.6. A sample (70 mg) was recrystallized from H_2O (4 ml): 43 mg, mp 139–141 °C.

Anal. ($C_{21}H_{36}N_4O_6S$) C, H, N.

Biocytin. Compound III (283 mg, 0.6 mmol) was dissolved in 98% trifluoroacetic acid (TFA) containing 10% anisole (2 ml) and allowed to stand at room temperature for 15 min. The solution was evaporated in vacuo and the residue triturated with ether (20 ml), the solid trifluoroacetate secured by centrifugation and dried in vacuo (310 mg). It was dissolved in H_2O (4 ml) and the solution passed through a column (1.5 × 15 cm) of Dowex-1 ion exchange resin in acetate cycle. Evaporation of the ninhydrin positive fractions yielded crude biocytin (191 mg) that was dissolved in H_2O (2 ml) and crystallized by the addition of acetone (8 ml). The purified material, 163 mg (73%), melted at 243–246 °C (lit.[11] 241–243 °C); $[\alpha]^{25}D$ +55° (*c* 1, 0.1 N NaOH) (lit.[11] $[\alpha]^{25}D$ +53°); R_fC 0.19.

Anal. ($C_{16}H_{28}N_4O_4S$) C, H, N.

Biocytinyl-L-threonine (V). Compound III (0.47 g, 1 mmol) was suspended in peroxide-free tetrahydrofuran (THF) (15 ml); triethylamine (TEA) (0.14 ml, 1 mmol) was added and the mixture cooled to −20 °C. Isobutylchloroformate (0.14 g, 1 mmol) was added to the stirred mixture which was then kept at about −5 °C for 0.5 h. A solution containing L-threonine (0.24 g, 2 mmol) and TEA (0.28 ml, 2 mmol) in water (2 ml) was added to the solution of the mixed anhydride. The mixture was allowed to come to room temperature; stirring was continued overnight. After dilution with H_2O (10 ml), the mixture was evaporated to dryness with a stream of N_2. The residue was taken up in H_2O (10 ml) and acidified to pH 2 with 0.2 N HCl (12 ml). Concentration of the solution led to the separation of a gummy product. After decantation of the aqueous solution, this material was washed with H_2O (2 ml) and triturated with ether (20 ml in two portions); a solid formed. The aqueous solution was further concentrated to yield additional dipeptide (IV): a total of 406 mg (71%), R_fC 0.46. A part (287 mg) of the product was dissolved in 98% TFA containing 10% anisole (0.5 ml). After 15 min, the solution was evaporated in vacuo. The trifluoroacetate salt was triturated, washed with ether (10 ml in two portions), and dried. An aliquot (243 mg) of this material was dissolved in H_2O (3 ml) and passed through a Dowex-1 acetate column (25 × 1.5 cm) as described in the preparation of biocytin. A total of 12 ml of H_2O was used for elution. Evaporation and trituration of the residue with absolute ethanol (5 ml) yielded compound V: 110 mg, mp 145–149 °C dec, and a second crop, 50 mg, mp 194–197 °C dec. On TLC, a major spot with R_fC 0.14 and a weak spot with R_f 0.05 were found in both crops. For detection, ninhydrin and a platinic iodide spray[25] were used. Amino acid analysis (first crop): Thr 1.05, Lys 1.00. No free Thr or Lys were detected when the unhydrolyzed material was applied on the amino acid analyzer. This dipeptide could not be secured in analytically pure form.

***tert*-Butyloxycarbonylbiocytinyl-L-threonine Methyl Ester (VI).** a. A sample of compound III (0.473 g, 1 mmol) and freshly prepared L-threonine methyl ester[26] (0.133 g, 1 mmol) were dissolved in dimethylformamide (DMF) (5 ml), and EEDQ (Aldrich, 0.272 g, 1 mmol) was added. The mixture was kept at room temperature overnight. The solvent was removed in vacuo, and the residue was triturated and washed with EtOAc (30 ml), collected on a filter, washed with more EtOAc (20 ml), and dried. The product (0.437 g) was heterogeneous on TLC: in addition to the major component with R_fC 0.58, one minor spot could be detected.

b. Compound III (0.615 g, 1.3 mmol) and Thr-OMe (0.146 g, 1.1

mmol) were dissolved in DMF (5 ml), and dicyclohexylcarbodiimide (DCC) (0.206 g 1 mmol) was added. Two days later, the precipitate (*N,N'*-dicyclohexylurea) was removed by filtration, the solvent evaporated, and the residue triturated with ether (40 ml). The solid which formed was filtered, washed with ether (10 ml), and dried. The product (0.548 g) showed on TLC (system A) two spots of about equal intensity with R_f 0.11 and 0.20.

c. Freshly prepared L-threonine methyl ester[26] (1.4 g, 10.5 mmol) was dissolved in ethyl acetate (40 ml), along with N^{α}-Boc-N^{ϵ}-Z-L-lysine (Bachem, 3.8 g, 10 mmol). The solution was cooled in an ice bath, and molten dicyclohexylcarbodiimide (2.06 g, 10 mmol) was added dropwise. The reaction mixture was allowed to warm to room temperature. After 1.5 h, the dicyclohexylurea was filtered off and washed with EtOAc. The filtrate was diluted with EtOAc and washed with 0.1% $KHSO_4$ (2 × 100 ml), then with H_2O (100 ml), 0.5 N $KHCO_3$ (2 × 100 ml), and H_2O (1 × 100 ml). Two-thirds of the solution was evaporated in vacuo, and the oily residue (3.3 g) was dissolved in $CHCl_3$ (20 ml) and applied to a column (4.5 × 60 cm) of silica gel (120 g, 60–200 mesh, Baker analyzed). Fractions of 40 ml were collected. Fraction 1–10 were eluted with a mixture of 1% MeOH in $CHCl_3$; fractions no. 11–25 with a 3% MeOH in $CHCl_3$. Fractions 19–24 were combined and concentrated in vacuo to a glassy solid. The product, N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-benzyloxycarbonyl-L-lysyl-L-threonine methyl ester (VII), (3.14 g, 95%) was homogeneous on TLC (R_fA 0.56, R_fC 0.77), but could not be obtained in filterable crystals and was, therefore, not further characterized.

Compound VII (2.18 g, 4.4 mmol) was dissolved in 95% EtOH (60 ml) containing glacial acetic acid (0.4 ml) and hydrogenated for 2 h in the presence of a 10% palladium-on-charcoal catalyst (400 mg). After removal of the catalyst by filtration, the solution was concentrated in vacuo, and the oily residue was dissolved in 95% ethanol (20 ml) and evaporated in vacuo to leave a glassy solid: 1.89 g. The dipeptide acetate salt (1.69 g, 4 mmol) was dissolved in DMF (20 ml) along with II (1.75 g, 4.8 mmol) and diisopropylethylamine (DIEA) (0.64 ml, 4 mmol). After standing overnight at room temperature, the reaction mixture was fluorescamine negative; the solvent was removed in vacuo, and the residue was triturated with ether (2 × 50 ml), when a solid formed. The crude product (3.8 g) was dissolved in chloroform (50 ml) and applied to a column (4.5 × 60 cm) of silica gel (Baker, 120 g, 60–200 mesh). A total of 42 fractions (40-ml each) were collected. Fractions 1–14 were eluted with 1% MeOH in $HCCl_3$, fractions 15–21 with 3% MeOH in $CHCl_3$, fractions 22–35 with 10% MeOH in $CHCl_3$, and fractions 36–42 with 20% MeOH in $CHCl_3$. The product was secured from fractions 32–42; it solidified on trituration with ether (2 × 20 ml) and was dried in vacuo over NaOH: 1.96 g (83%); mp 88–92 °C dec; $[\alpha]^{25}D$ +20° (*c* 1, MeOH); TLC R_fA 0.13, R_fC 0.57, R_fD 0.49.

Anal. ($C_{26}H_{45}N_5O_8S$) C, H, N.

***tert*-Butyloxycarbonyl-L-methionyl-L-biocytinyl-L-threonine Methyl Ester (VIII).** *tert*-Butyloxycarbonylbiocytinyl-L-threonine methyl ester (VI, 1.98 g, 3.37 mmol) was dissolved in 98% TFA containing 10% anisole (6 ml) in a 40-ml centrifuge tube provided with a 24/40 glass joint.[27] The solution was allowed to stand at room temperature for 15 min and then concentrated in vacuo to a small volume. Ether was added, and the solid that formed was collected by centrifugation and dried in vacuo over NaOH: 2.21 g. The major part of the bistrifluoroacetate salt (2.07 g, 2.9 mmol) and Boc-Met-ONP (Bachem, 1.63 g, 4.4 mmol) were dissolved in DMF (16 ml) in a 40-ml centrifuge tube (cf. above), and DIEA (0.93 ml, 5.8 mmol) was added. After 1 h, the mixture was fluorescamine negative; it was concentrated in vacuo to a semisolid mass. After the semisolid was triturated with EtOAc (50 ml), a crystalline product separated which was then collected and washed with EtOAc (3 × 10 ml). On TLC, the product showed minor impurities, the active ester and *p*-nitrophenol. The crude material (20 g) was extracted with ether (2 × 15 ml) and ethyl acetate (2 × 25 ml), collected by centrifugation and dried in vacuo over NaOH: 1.8 g (87%); mp 135–142 °C dec; $[\alpha]^{25}D$ +9° (*c* 1, DMF containing 1% AcOH); TLC R_fA 0.13, R_fC 0.46, R_fD 0.51.

Anal. ($C_{31}H_{54}N_6O_9S_2$) C, H, N.

***tert*-Butyloxycarbonyl-L-methionyl-L-biocytinyl-L-threonine Amide (VIIIa).** The protected tripeptide ester VI (153 mg, 0.21 mmol) was dissolved in warm MeOH (10 ml) in a 100-ml round-bottomed flask and cooled in an ice bath while it was saturated with NH_3 for 1 h. The reaction mixture was allowed to stand at room temperature overnight. The solvent was removed in vacuo, the semisolid material solidified on trituration with 95% EtOH (2 × 10 ml). Aftery drying in vacuo overnight: 144 mg (97%); mp 197–199 °C dec; TLC R_fA 0.0, R_fD 0.45. For analysis, a sample (23 mg) was recrystallized from 95% EtOH (2 ml); 15 mg were recovered with melting point unchanged.

Anal. ($C_{30}H_{53}N_7O_8S_2$) C, H, N.

A sample of the protected tripeptide amide was deprotected, as described for biocytin. The trifluoroacetate salt was isolated by trituration with ether in quantitative yield. The product has no well-defined melting point; TLC R_fA 0.0, R_fD 0.1. Amino acid analysis: Thr 1.1, Met 1.0, Lys 1.0.

***tert*-Butyloxycarbonyl-L-alanyl-L-methionylbiocytinyl-L-threonine Methyl Ester (IX).** Compound VIII (1.09 g, 1.5 mmol) was dissolved in 98% TFA containing 10% anisole (3 ml) and allowed to stand at room temperature for 15 min. Most of the TFA was removed in vacuo and the residue triturated and washed with ether (3 × 15 ml). The solid was separated by centrifugation and dried in vacuo over NaOH: 1.24 g (98%); TLC R_fA 0.0, R_fD 0.15. Amino acid analysis: Thr 1.2, Met 1.1, Lys 1.0.

Anal. ($C_{30}H_{48}N_6O_{11}S_2F_6$) C, H, N, F.

The trifluoroacetate salt (1.2 g, 1.4 mmol) and Boc-Ala-ONP (0.6 g, 1.9 mmol) were dissolved in DMF (14 ml), and DIEA (0.48 ml, 3 mmol) was added to the solution. After 3 h at room temperature, the mixture gave no reaction with fluorescamine. The solvent was removed in vacuo, and the residue was triturated and washed with EtOAc (90 ml), collected by centrifugation, and dried in vacuo: 0.95 g (86%): mp 189–192 °C dec; $[\alpha]^{25}D$ +5° (*c* 1, DMF containing 1% AcOH); TLC R_fA 0.07, R_fC 0.62. Amino acid analysis: Thr 1.0, Ala 1.0, Met 0.9, Lys 0.95.

Anal. ($C_{34}H_{59}N_7O_{10}S_2$) C, H, N.

***tert*-Butyloxycarbonyl-L-alanyl-L-methionylbiocytinyl-L-threonine Amide (IXa).** A sample of compound IX was ammonolyzed as described for VIII. The product was obtained in quantitative yield: mp 194–196 °C dec; TLC R_fA 0.0, R_fD 0.38.

Anal. ($C_{33}H_{58}N_8O_9S_2$) C, H, N.

A sample of the protected tetrapeptide amide was treated with trifluoroacetic acid, and the salt was isolated by evaporation and trituration of the residue with ether in 98% yield. The product has no well-defined melting point: R_fA 0.0, R_fD 0.06. Amino acid analysis: Thr 0.95, Ala 1.0, Met 0.9, Lys 1.0.

***tert*-Butyloxycarbonyl-L-alanyl-L-methionylbiocytinyl-L-threonine Hydrazide (X).** A sample (0.158 g, 0.2 mmol) of the protected tetrapeptide ester IX was dissolved in warm MeOH (10 ml). Hydrazine (98%, 1 ml) was added, and the mixture was allowed to stand at room temperature overnight. Removal of the solvent and the excess hydrazine was followed by trituration of the residue with 95% EtOH (20 ml) to yield the hydrazide: 0.157 g (98%); mp 195–198 °C dec; TLC R_fA 0.0, R_fD 0.27. A sample (30 mg) was recrystallized from hot 95% EtOH (2 ml); the purified material (22 mg) melted at 202–205 °C dec.

Anal. ($C_{33}H_{59}N_9O_9S_2$) C, H, N.

A sample of the hydrazide X (80 mg, 0.1 mmol) was suspended in DMF (0.5 ml), and the mixture was cooled to −20 °C and treated with 0.5 ml of a solution prepared from concentrated HCl (0.9 ml) and DMF (8.1 ml). The solution was allowed to warm to −15 °C, and a M solution of $NaNO_2$ (0.15 ml) was added. After 5 min, the temperature was lowered to −25 °C, and DIEA (0.1 ml) was added, followed by glycine ethyl ester hydrochloride (28 mg, 0.2 mmol). After storage at 5 °C for 2 h, the mixture was allowed to stand overnight at room temperature. The solvent was removed, and the residue was triturated and washed with water (4 ml) and ether (5 ml). On TLC, the crude product (24 mg) showed a major spot at R_fD 0.3 and three minor spots. Evaporation of the aqueous washes and trituration of the residue with chloroform (4 ml) and water (1 ml) afforded more material (47 mg) of similar quality. Amino acid analysis: Thr 1.0, Gly 1.0, Ala 1.1, Met 1.0, Lys 1.0.

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References and Notes

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(15) Although *p*-nitrophenol is liberated in the reaction of *p*-nitrophenyl trifluoroacetate with carboxylic acids, additional *p*-nitrophenol was thought to enhance the probability of the attack of *p*-nitrophenol, rather than the urea group, on the intermediate mixed anhydride.
(16) The problem of alternative positions of the CO_2 in biotin was studied in detail. Cf., e.g., ref 6 and also T. C. Bruice and A. F. Hegarty, *Proc. Natl. Acad. Sci. U.S.A.*, **65,** 805 (1970); A. F. Hegarty and T. C. Bruice, *J. Am. Chem. Soc.*, **92,** 6561 (1970); R. F. Pratt and T. C. Bruice, *Biochemistry*, **10,** 3178 (1971); R. B. Guchhait, S. E. Polakis, D. Hollis, C. Fenselau, and M. D. Lane, *J. Biol. Chem.*, **249,** 6646 (1974).
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(22) The results of studies concerning catalytic activity observed when the biotin-containing peptides were combined with the longer subunits of transcarboxylase will be reported separately.
(23) (Added during revision) The tetrapeptide sequence was extended by coupling Boc-Ala-Met-Bct-Thr via its azide to Glu-Ile-Asn-Ala-Pro-Thr-NH_2 and deprotection of the decapeptide derivative thus formed. No restoration of catalytic activity was observed when this compound was added to trypsinized transcarboxylase. This suggests that an extension toward the N-terminus is more likely to give positive results.
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