Synthesis of Biotin-containing Peptides Representing the Biotin Binding Site of *E. coli* Acetyl-CoA Carboxylase†

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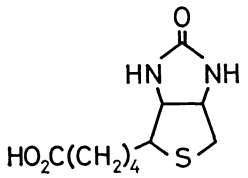
Two biotin-containing peptides, Ac–Met–Bct–Met–Met–OMe (**1**) and Boc–Met–Bct–Met–Met, that correspond to the local sequence of biotin carboxyl carrier protein subunit of *E. coli* acetyl-CoA carboxylase have been synthesized. The precursor tetrapeptide Boc–Met–Lys(Cbz)–Met–Met–OMe was prepared first by the stepwise elongation method and biotin was incorporated subsequently by the active ester method. Another biotin-containing peptide, HCO–Met–Bct–Met–Met–OMe, has also been prepared. Magnesium ion binding to peptide **1** in acetonitrile has been studied by means of ^{13}C NMR spectroscopy. The metal interacts principally with the amide carbonyls rather than sulfide moieties, as deduced from a significant downfield shift of the former carbon signals in ^{13}C NMR.

In biotin-requiring carboxylases the prosthetic group biotin (**4**) is attached to the ε-amino moiety of a certain lysine residue on the so-called BCCP (biotin carboxyl carrier protein) subunit.[1] The binding of BCCP to other subunits directly involved in the transformation of biotin and substrates is very tight. In some instances biotin itself can bind to the enzyme with a high K_m value relative to that for BCCP.[2,3] It is, hence, clear that the polypeptide part of BCCP contributes much to the binding of biotin, but what part of the amino acid sequence is required remained to be clarified. In light of the accumulating knowledge on the primary structure around biocytin (biotinyllysine, **5**) of several biotin enzymes,[4-6] Bodanszky



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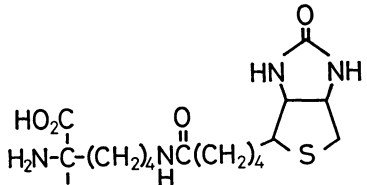

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and Fagan were the first to undertake this subject.[7] They prepared biotin-containing peptides such as **6**, that corresponds to the local structure adjacent to biocytin of transcarboxylase. Unfortunately, however, the peptides failed to restore enzymic activity. It was later reported that the amino acid sequence of transcarboxylase on which their model peptides based was in error.[8]

Boc-Ala-Met-Bct-Thr-Gly-OEt **6**

According to the known primary structure of biotin enzymes, they have a unique structure in that biocytin is flanked by methionines. In the case of acetyl-CoA carboxylase from *E. coli*, an additional Met is placed at the C terminal side of the sequence (**7**). Since

-Met-Bct-Met-Met- **7**

each amino acid residue in **7** carries a sulfide moiety, we have speculated that these four sulfide groups may be playing an important role in the binding of biotin to the enzyme. If one recalls that the enzyme requires metal ions such as Mn^{2+} and Mg^{2+} for activity, this might be performed through chelate formation. Hence, we have prepared model peptides **1**—**3**, analogous to **7**, and studied their interaction with metal ions by means of ^{13}C NMR spectroscopy.[9]

Results and Discussion

Synthesis. The liquid phase synthesis of **1** and **2** has been achieved according to the routes shown in Fig. 1 and Scheme 1. The basic strategy was to prepare the tetrapeptide Boc–Met–Lys(Cbz)–Met–Met–OMe **8** first and biotin was incorporated to the peptide subsequently. This should help avoid possible side reactions on the biotin moiety and interference of the biotin's urea group with amino acid coupling.[7] The α-amino function of both Met and Lys was protected with Boc, while the ε-amino moiety of Lys was with Cbz. An alternate combination of protecting groups may appear equally well as long as special care is taken for the removal of Cbz groups. One of the most popular methods of doing this, hydrogenolysis, did not work even in the presence of boron trifluoride etherate even at the dipeptide level.[10] So, this approach was abandoned and switched to the alternative one without testing extensively other deprotection methods. The Boc group was removed

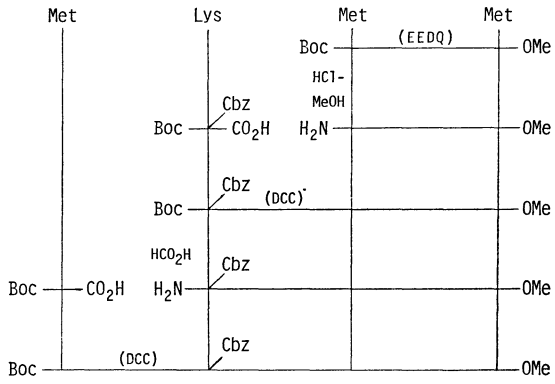


Fig. 1. Synthetic route to **8**.

† The abbreviations used in this article follow the nomenclature recommended by the IUPAC-IUB Commission of Biochemical Nomenclature: Met, L-methionine; Lys, L-lysine; Bct, biotinyl-L-lysine; Cbz, benzyloxycarbonyl; Boc, *t*-butoxycarbonyl; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; NEM, *N*-ethylmorpholine; TEA, triethylamine.

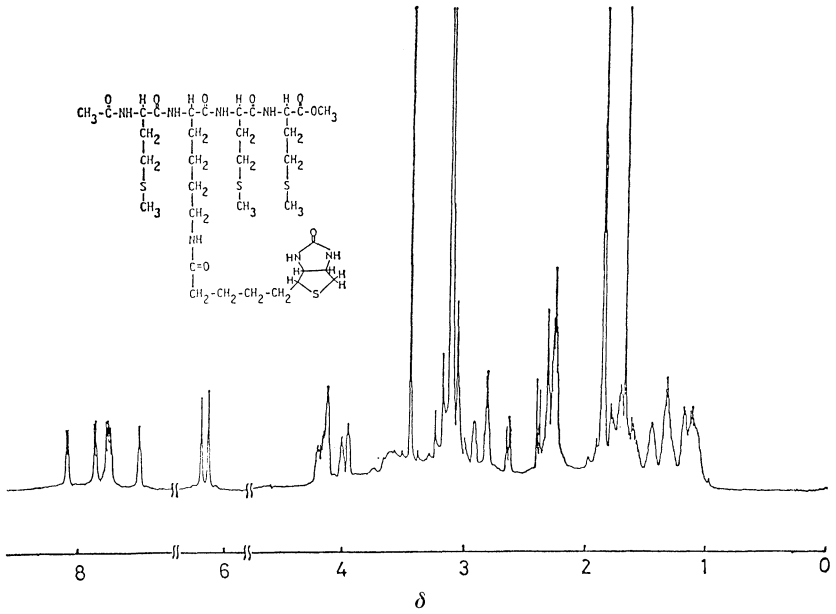
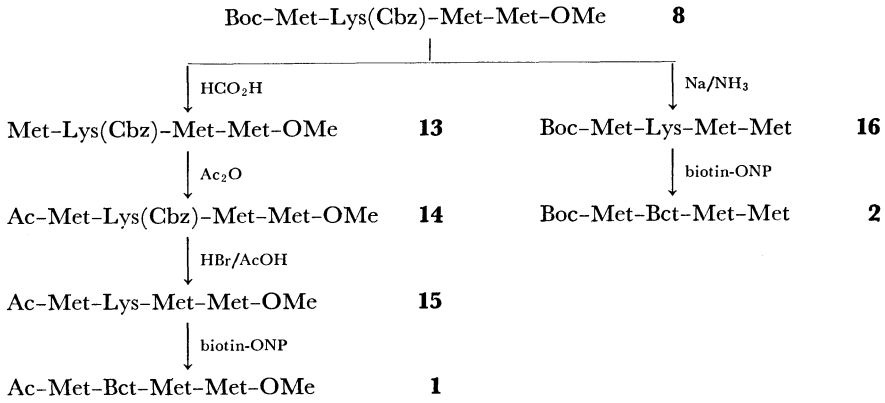
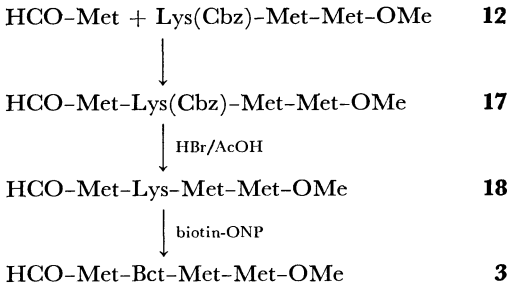


Fig. 2. 500 MHz NMR spectrum of **1** in DMSO-d_6.



Scheme 1.



Scheme 2.

from Boc–Met–Met–OMe with 10 M HCl in methanol and from Boc–Lys(Cbz)–Met–Met–OMe and Boc–Met–Lys(Cbz)–Met–Met–OMe with formic acid.

The tetrapeptide **8** was derivatized to the final products according to the synthetic routes shown in Scheme 1. Removal of Boc in **8** with formic acid followed by acetylation gave **14**, whose Cbz was removed with 25% HBr in acetic acid to give **15**. Treatment of **8** with sodium in liquid ammonia provided **16** smoothly. Introduction of biotin into **15** and **16** was accomplished by the active ester method. In view of the fact that *N*-hydroxysuccinimide ester of biotin is successfully employed for the covalent attachmen of biotin to the affinity adsorbents,[11] we have tested this method first. The reaction was carried out in DMF in the presence of TEA or NEM at temperatures ranging from room temperature to 50 °C with total failure. Subsequently, the *N*-hydroxysuccinimide ester was replaced by *p*-nitrophenyl ester of biotin,[7] and its reaction with **15** or **16** at ambient temperatures gave **1** or **2**, respectively, but in low yields (28—38%). Coupling of **15** with biotin acid chloride has also been tried,[12] but this method did not work either. The synthesis of **3** has also been done according to the route shown in Scheme 2.

The structure of biotin-containing peptides **1**—**3** has been ascertained by the following criteria. All the peptides gave a characteristic pink color upon reaction with *p*-dimethylaminocinnamaldehyde, indicating the presence of a biotinyl moiety.[13] In Fig. 2 is shown the 500 MHz NMR spectrum of **1** in DMSO-d_6. Two singlets are clearly observable for the biotin N–H protons at 6.14 and 6.20 ppm as well as five signals for the amide protons at 7.5—8.1 ppm. Other peaks are also reasonably assignable to individual protons of **1**. There are six amide carbonyl signals in

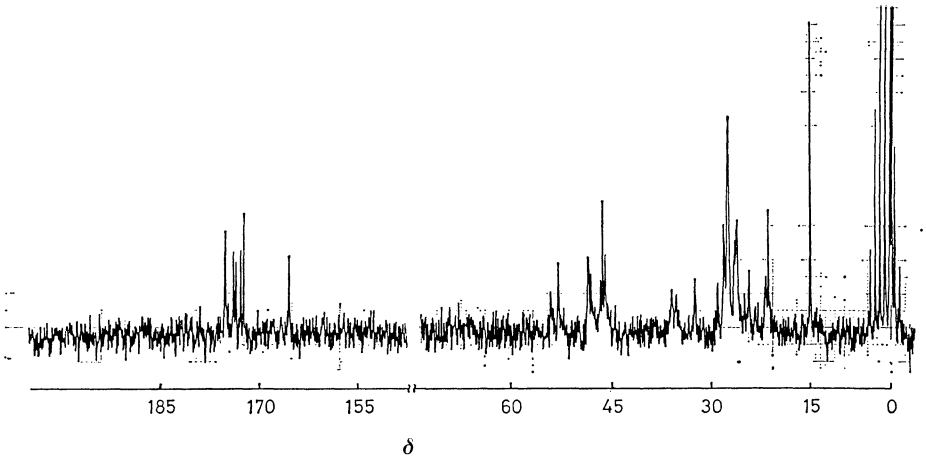


Fig. 3. ^{13}C NMR spectrum of **1** in CD_3CN in the presence of 4 mol of magnesium perchlorate.

Table 1. ^{13}C NMR chemical shifts of carbonyl carbons of **1** and **8** in CD_3CN in the absence or presence of 4 mol of $Mg(ClO_4)_2$

8	**8**+Mg^{2+}	$\Delta\delta$ [a]	**1**+Mg^{2+}	$\Delta\delta'$ [b]
174.6	176.5	1.9	176.4	1.8
173.8	176.4	2.6	176.2	2.4
172.5	174.4	1.9	174.7	2.2
172.1	173.6	1.5	174.2	2.1
172.0	173.0	1.0	173.4	1.4
157.4	158.5	1.1	172.8	—
164.3[c]			164.4	0.1

a) $\Delta\delta$=column 2 - column 1. b) $\Delta\delta'$=column 4 - column 1. c) Value for biotin *p*-nitrophenyl ester.

the 172—177 ppm region as well as one urea carbonyl signal at 164.4 ppm in the ^{13}C NMR spectrum of **1** in CD_3CN in the presence of magnesium perchlorate (Fig. 3). Results of elemental analysis are also satisfactory (see Experimental), establishing the purity and structure of these compounds.

Magnesium Ion Binding. All the biotin-containing peptides (**1**—**3**) are sparingly soluble in acetonitrile but they become considerably soluble in the presence of magnesium perchlorate, suggesting that the peptides interact with magnesium ion. In order to prove this, further studies were done by using ^{13}C NMR spectroscopy. Because of the poor solubility of **1**, **8** was chosen as a reference compound. As seen in Table 1, all five amide carbonyls of **8** show a considerable downfield shift (average 1.8 ppm) in the presence of 4 mol of magnesium perchlorate. This is true for **1**: five amide carbonyls show a downfield shift of 2.0 ppm on the average, compared to the amide carbonyls of **8** in the absence of magnesium perchlorate. On the other hand, the ureido carbonyl of biotin suffers little effect, in line with our previous finding that this moiety of 2-imidazolidinones is a poor ligand to magnesium.[9] In contrast to the carbonyl signals, other carbon signals including those of *S*-methyl carbons of methionines were not affected by magnesium perchlorate. This indicates that the metal coordinates to the carbonyls rather than the sulfides. It is plausible that the magnesium ion plays its role through association with ATP or its conjugate with hydrogencarbonate in enzymatic carboxylations. In order for biotin to be carboxylated the coenzyme must come into contact with other reactants at the enzyme active site. This might be made possible through coordination of peptide carbonyls adjacent to the biotin in BCCP to form a ternary complex composed of BCCP, Mg^{2+} and ATP or its conjugate with hydrogencarbonate.

Experimental

^1H NMR spectra were taken on a JEOL JNM-MH-100 spectrometer. ^{13}C NMR spectra were obtained on a JEOL JNM-FX-90Q spectrometer operating at 22.50 MHz. Parameters for these experiments were the following: spectral width 5000 Hz with acquisition of 8 K data points, 7 μs pulse, and a recovery time of 3 s. The numbers of spectral accumulations were in the range 2000—5000. Samples were dissolved in CD_3CN for measurements at 25.0 °C and chemical shifts are given in ppm downfield from internal TMS. Optical rotation was determined on a JASCO DIP-4 digital polarimeter.

TLC on silica gel was run in the following solvent systems: ethyl acetate (A), CH_2Cl_2–ethyl acetate (5:1, B), ethyl acetate–methanol (4:1, C), and ethyl acetate–methanol (5:1, D). Spots on the TLC plate were detected with either I_2 vapor or ninhydrin. Biotin-containing peptides gave a pink color upon spraying of *p*-dimethylaminocinnamaldehyde.[13]

L-Methionine, L-lysine, EEDQ, DCC, *N*-hydroxysuccinimide, 25% HBr/AcOH, CbzCl, and *O*-*t*-butyl *S*-4,6-dimethyl-2-pyrimidinyl thiocarbonate were obtained from Protein Research Foundation. Trimethylacetyl chloride was purchased from Aldrich Co. Ltd. *d*-Biotin and other reagents were obtained from Wako Pure Chemical Co.

*Boc–Met–Met–OMe (**9**).* *N*-(*t*-Butoxycarbonyl)methionine and Met–OMe were coupled by the EEDQ method.[14] After the usual work-up, a crude product was obtained as a syrup. This was purified by chromatography on silica gel with ethyl acetate as an eluent, followed by recrystallization from ether and petroleum ether. Yield 68%, mp 67—69 °C (lit,[15] 66—67 °C), $[\alpha]_D^{25}$ −30.5° (*c* 3.1, CH_3OH), R_f 0.90 (A). Found: C, 48.34; H, 7.67; N, 7.16%. Calcd for $C_{16}H_{30}N_2O_5S_2$: C, 48.71; H, 7.66; N, 7.10%.

*Met–Met–OMe·HCl (**10**·HCl).* A 2.0 g (5.0 mmol) sample of **9** was dissolved in 10 ml of 10 M HCl in methanol. The mixture was stirred at room temperature for 1 h. The solvent was removed *in vacuo* and then 100 ml of ether was added. After allowing the suspension to stand at −5 °C for 1 d, the supernatant was discarded. The same procedure was repeated twice more. The oily residue was dried *in vacuo* at room temperature to give a very hygroscopic product. Yield 1.3 g (87%). R_f 0.40 (A). Complete removal of the protecting group was confirmed by NMR.

*Boc–Lys(Cbz)–Met–Met–OMe (**11**).* A solution of N^{α}-Boc-N^{ϵ}-Cbz-Lys[16] (1.0 g, 2.6 mmol), DCC (0.54 g, 2.6 mmol), and HOBt (0.35 g, 2.6 mmol) in 15 ml of DMF was mixed with a solution of **10**·HCl (0.76 g, 2.6 mmol) and NEM (0.30 g, 2.6 mmol) in 15 ml of DMF. The reaction mixture was stirred at room temperature for 8 h and the precipitate formed was filtered off. The filtrate was concentrated *in vacuo* to about 5 ml, and then diluted with 100 ml of ethyl acetate. The solution was washed successively with the following solvents: 4% $NaHCO_3$, saturated NaCl, 10% citric acid, and saturated NaCl. After drying over sodium sulfate at −5 °C (to effect complete precipitation of DCUrea), the solution was concentrated to about 50 ml, and kept overnight in a freezer. The crystalline mass formed was collected by filtration. Yield 1.2 g (71%), mp 105—106 °C, $[\alpha]_D^{25}$ −18.7° (*c* 3.2, CH_2Cl_2), R_f 0.92 (A), 0.88 (B). Found: C, 54.94; H, 7.52; N, 8.60%. Calcd for $C_{30}H_{48}N_4O_8S_2$: C, 54.86; H, 7.37; N, 8.53%.

*Lys(Cbz)–Met–Met–OMe·HCO_2H (**12**·HCO_2H).* A 1.2 g (1.8 mmol) sample of **11** was dissolved in 10 ml of formic acid and stirred at room temperature. The reaction was complete in 3 h, as inspected by TLC. The mixture was diluted with 100 ml of methanol and the solvents were evaporated *in vacuo*. The product was precipitated by addition of ether. Yield 0.82 g (84%). R_f 0.25 (A). Complete removal of the protecting group was confirmed by NMR.

*Boc–Met–Lys(Cbz)–Met–Met–OMe (**8**).* In 30 ml of DMF were dissolved 5.0 g (92 mmol) of **12**·HCO_2H, 1.1 g (92 mmol) of NEM, 2.3 g (92 mmol) of Boc–Met, 1.9 g (92 mmol) of DCC, and 1.4 g (92 mmol) of HOBt. The mixture was stirred overnight at room temperature. The DCUrea formed was filtered off. After removal of the solvent *in vacuo*, the residue was taken up in 1 L of ethyl acetate. The solution was washed as for **11** and then dried over sodium sulfate. Concentration of the solution to about 300 ml gave precipitates of crude product. They were recrystallized from ethyl acetate. Yield 4.5 g (63%), mp 172—173 °C, $[\alpha]_D^{25}$ −22.7° (*c* 2.6, CH_2Cl_2). R_f 0.61 (B). Found: C, 53.22; H, 7.33; N, 8.84%. Calcd for $C_{35}H_{57}N_5O_9S_3$: C, 53.34; H, 7.29; N, 8.89%.

*Met–Lys(Cbz)–Met–Met–OMe·HCO_2H (**13**·HCO_2H).* A sample of **8** (4.8 g, 4.2 mmol) was dissolved in 50 ml of formic acid and the solution was stirred for 4 h at room temperature. After addition of 100 ml of methanol, the solvents were evaporated *in vacuo*. The same procedure was repeated twice more. Addition of ether to the syrupy residue gave precipitates of the product (4.2 g, 95%).

*Ac–Met–Lys(Cbz)–Met–Met–OMe (**14**).* To a DMF solution (10 ml) containing 0.90 g (1.2 mmol) of **13**·HCO_2H and 0.14 g (1.2 mmol) of NEM were added slowly 8 ml of acetic anhydride. The whole mixture was stirred for 3 h. The precipitates formed were collected by filtration and washed with ether. Yield 0.82 g (91%), mp 213—214 °C. R_f 0.26 (C), 0.86 (D). $[\alpha]_D^{25}$ −27.5° (*c* 2.0, DMF). Found: C, 52.22; H, 7.07; N, 9.48%. Calcd for $C_{32}H_{51}N_5O_8S_3$: C, 52.65; H, 7.04; N, 9.59%.

*Ac–Met–Lys–Met–Met–OMe·CH_3CO_2H (**15**·AcOH).* A sample of **14** (1.0 g, 1.4 mmol) was dissolved in 30 ml of 25% HBr/AcOH with 5 ml of dimethyl sulfide. The mixture was stirred for 3 h and then 50 ml of dichloromethane and 400 ml of ether were added. The precipitates formed were filtered off to give 0.85 g (92%) of the product.

*Ac–Met–Bct–Met–Met–OMe (**1**).* **15**·AcOH (0.17 g, 0.26 mmol), TEA (2.6 mg, 0.26 mmol), and biotin *p*-nitrophenyl ester[7,17] in 4 ml of DMF were allowed to react at room temperature for 15 h. The solution was first clear but precipitates gradually formed. They were filtered off and washed with water, methanol, and ether. Yield 60 mg (28%), mp 231 °C (dec). Found: C, 48.84; H, 7.22; N, 11.88%. Calcd for $C_{34}H_{59}N_7O_8S_4\cdot H_2O$: C, 48.61; H, 7.32; N, 11.67%.

*Boc–Met–Lys–Met–Met (**16**).* **8** (1.0 g, 1.3 mmol) was dissolved in 100 ml of liq. ammonia. Sodium was added in small portions until the blue color was retained for 5 min. Ammonium chloride was added until the blue color disappeared. Ammonia was evaporated off and 50 ml of methanol was added to the residue. The suspension was warmed at 40 °C for 30 min. The insoluble material was removed by filtration and evaporation of the filtrate *in vacuo* gave 0.60 g (71%) of the product. Removal of the Cbz and ester groups was confirmed by NMR.

*Boc–Met–Bct–Met–Met (**2**).* To a methanol solution (3 ml) of **16** (0.60 g, 0.92 mmol) and 92 mg (0.92 mmol) of TEA were added 80 mg (2 mmol) of biotin *p*-nitrophenyl ester.[7,17] The mixture was stirred at room temperature for 15 h, whereupon 80 ml of ether was added. The precipitates formed were filtered off and then washed with water. Yield 0.30 g (38%), mp 174—176 °C. R_f 0.45 (C). Found: C, 48.14; H, 7.43; N, 10.92%. Calcd for $C_{36}H_{63}N_7O_9S_4\cdot 2H_2O$: C, 47.92; H, 7.48; N, 10.86%.

*HCO–Met–Lys(Cbz)–Met–Met–OMe (**17**).* To a cold (−10 °C) solution of HCO–Met[18] (0.48 g, 2.7 mmol), pyridine (0.2 ml, 2.7 mmol), and *N*-ethylpiperidine (0.4 ml, 2.7 mmol) in CH_2Cl_2 (10 ml) was added a CH_2Cl_2 solution (2 ml) of trimethylacetyl chloride (0.33 g, 2.7 mmol). The mixture was allowed to react at −10 °C for 20 min, whereupon a CH_2Cl_2 solution of **12**·HCO_2H (1.5 g, 2.7 mmol) and NEM (0.3 g, 2.7 mmol) was added. The whole solution was stirred for 15 min at −10 °C and then for 1 h at room temperature. The solvent was removed *in vacuo* and the solid material left was filtered and washed with 10% citric acid, water, and 5% $NaHCO_3$. Yield 1.8 g (93%), mp 204—205 °C. R_f 0.35 (D). $[\alpha]_D^{26}$ −14.8° (*c* 1, CH_3OH). Found: C, 51.59; H, 6.98; N, 9.69%. Calcd for $C_{31}H_{49}N_5O_8S_3\cdot 0.5H_2O$: C, 51.36; H, 6.95; N, 9.66%.

*HCO–Met–Lys–Met–Met–OMe·HBr (**18**·HBr).* **17** (1.5 g, 2.1 mmol) was dissolved in 10 ml of 25% HBr/AcOH with 0.62 g (10 mmol) of dimethyl sulfide. The mixture was stirred for 3 h and then concentrated *in vacuo*. The residue was purified by reprecipitation from methanol–ether three times. Yield 1.3 g (94%). R_f 0.15 (C). The structure was confirmed by NMR.

*HCO–Met–Bct–Met–Met–OCH_3 (**3**).* **18**·HBr (1.3 g, 1.9 mmol), TEA (0.19 g, 1.9 mmol), and biotin *p*-nitrophenyl ester[7,17] (1 g, 2.8 mmol) were dissolved in 4 ml of DMF. Two ml of water were added and the solution was stirred at room temperature for 4 h. The precipitates formed were filtered and washed with water, methanol, and ether. Yield 0.5 g (35%), mp 205—206 °C. R_f 0.04 (D). Found: C, 48.27; H, 7.12; N, 11.85%. Calcd for $C_{32}H_{57}N_7O_8S_4$: C, 48.28; H, 7.22; N, 12.32%.

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