

Synthesis and Enzymatic Carboxylation of a Biotin-containing Peptide Representing the Coenzyme Binding Site of *E. coli* Acetyl-CoA Carboxylase†

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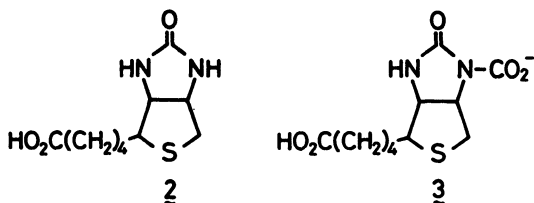
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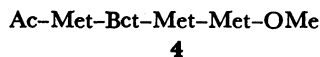
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A biotin-containing pentapeptide Boc-Glu-Ala-Met-Bct-Met (**1**) that corresponds to the coenzyme binding site of *E. coli* acetyl-CoA carboxylase has been prepared. Peptide **1** as well as free biotin served as a substrate for the carboxylation reaction catalyzed by the biotin carboxylase subunit dimer of *E. coli* acetyl-CoA carboxylase. The Michaelis constant, K_m , and the maximum velocity, V_{max} , for **1** were 18 mM and $2.8 \mu\text{M min}^{-1}$ ($1 \text{ M} = 1 \text{ mol dm}^{-3}$), respectively. The corresponding values for biotin were 214 mM and $28 \mu\text{M min}^{-1}$. Thus, the overall reactivity (V_{max}/K_m) of peptide **1** exceeded that of biotin by 20%.

The biotin (**2**)-dependent carboxylation reaction plays a pivotal role in the synthesis of long-chain fatty acids in biological systems.¹⁾ It is firmly established that the enzymatic carboxylation proceeds *via* the *N*-carboxybiotin intermediate (**3**), which in turn transfers the



carboxyl group to an acceptor substrate such as acetyl-CoA. By some enzymes biotin itself can be carboxylated, though the Michaelis constant of biotin carboxylation is enormously high compared to that for the natural substrate BCCP, biotin carboxyl carrier protein.²⁾ It is, hence, clear that the protein attaching to the coenzyme plays an important part in the recognition and binding of biotin to the enzyme active site.³⁾ In our previous communication we have prepared several biotin-containing peptides such as **4** that corresponds to a local biotin-binding sequence of *E. coli* acetyl-CoA carboxylase BCCP subunit.⁴⁾ Unfortunately, however, all



these peptides were sparingly soluble in most common solvents and were not feasible for kinetic studies of biotin carboxylation by the enzyme. To improve solubility the peptide sequence was extended towards the *N*-terminus to incorporate glutamic acid.⁵⁾ The presence of this extra carboxyl group as well as the *C*-terminal one should

increase the solubility of the resulting peptide in neutral aqueous media. This proved to be the case as described below. Furthermore, this peptide (**1**) was found to be carboxylated by the biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase. The preliminary results on the kinetics of this enzymatic carboxylation are also presented in this article.

Results

Synthesis. It was envisioned that the liquid phase synthesis of target peptide **1** may be achieved by either one of the following routes. In one route biotin is incorporated at the early stage of peptide synthesis by coupling Boc-Bct and methionine methyl ester.³⁾ The peptide chain is then elongated step by step up to the *N*-terminal glutamic acid. In the other the biotin-free pentapeptide is prepared first and the coenzyme is incorporated subsequently.⁴⁾ The latter strategy was actually adopted in order to be free from possible side reactions on the biotin skeleton and/or interference of the biotin's urea moiety with a coupling reaction.³⁾ In addition, expensive biotin may be saved in the

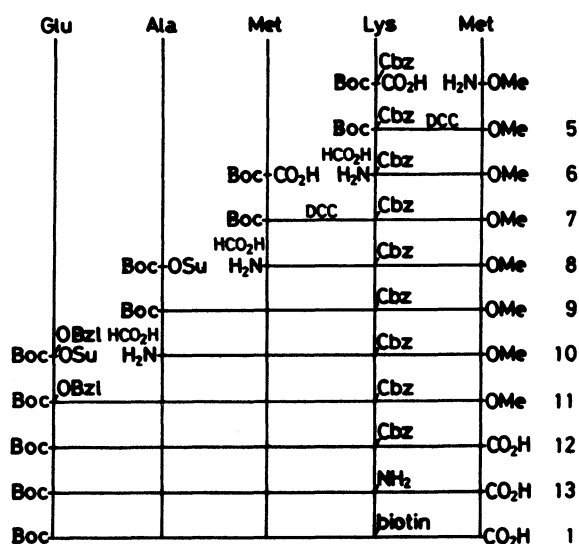


Fig. 1. Synthetic route to peptide **1**.

† The abbreviations used are: Ala, L-alanine; Glu, L-glutamic acid; Lys, L-lysine; Met, L-methionine; Bct, *N*-biotinyl-L-lysine; Boc, *t*-butoxycarbonyl; Cbz, benzyloxycarbonyl; CbzCl, benzyloxycarbonyl chloride; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; NEM, *N*-ethylmorpholine; ATP, adenosine 5'-triphosphate; NADH, nicotinamide adenine dinucleotide, reduced form; DEAE, diethylaminoethyl.

TABLE 1. PHYSICAL PROPERTIES AND ANALYTICAL DATA OF PEPTIDES

Compd	Yield %	Mp $\theta_m/^\circ\text{C}$	TLC, $R_f^{a)}$	$[\alpha]_D^{27}/^\circ$	Formula	Calcd (%)			Found (%)		
						C	H	N	C	H	N
5	72	101.5–103	0.89(A)	–7.6(c1, AcOEt)	$\text{C}_{25}\text{H}_{39}\text{N}_3\text{O}_7\text{S}$	57.12	7.48	7.99	56.74	7.58	8.06
7	44	137–138	0.81(B)	–14.5(c1, AcOEt)	$\text{C}_{30}\text{H}_{48}\text{N}_4\text{O}_8\text{S}_2$	54.86	7.37	8.53	54.95	7.52	8.48
9	67	155–156.5	0.81(C)	–28.6(c1, DMF)	$\text{C}_{33}\text{G}_{53}\text{N}_5\text{O}_9\text{S}_2 \cdot \text{H}_2\text{O}$	53.13	7.43	9.39	53.24	7.30	9.45
11	57	153–154.5	0.88(D)	–17.0(c1, MeOH)	$\text{C}_{45}\text{H}_{66}\text{N}_6\text{O}_{12}\text{S}_2 \cdot \text{H}_2\text{O}$	56.00	7.10	8.70	55.96	7.06	9.02
12	80	167–169	0.77(E)	ND ^{b)}	$\text{C}_{37}\text{H}_{58}\text{N}_6\text{O}_{12}\text{S}_2 \cdot \text{H}_2\text{O}$	51.61	7.02	9.76	51.52	6.97	9.74

a) TLC solvents: A, AcOEt: pet. ether=5 : 1; B, AcOEt: pet. ether=1 : 1; C, MeOH: AcOEt: ether=1 : 1 : 7; D, MeOH: AcOEt=1 : 4; E, MeOH. b) ND: Not determined.

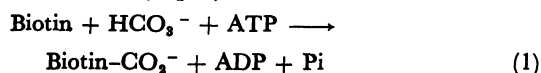
TABLE 2. KINETIC PARAMETERS FOR THE CARBOXYLATION OF BIOTIN AND DERIVATIVES BY THE BIOTIN CARBOXYLASE SUBUNIT OF *E. coli* ACETYL-CoA CARBOXYLASE AT 30.0 °C

Substrate	K_m/mM	$V_{\max}/\mu\text{M min}^{-1}$	$(V_{\max}/K_m)/10^5 \text{ min}^{-1}$	Ref.
Biotin	214	28	13	This work
1	18	2.8	16	This work
BCCP	0.0004	—	9.4×10^5	12, 13

latter route. Thus, the parent pentapeptide was prepared according to the scheme illustrated in Fig. 1. The DCC coupling method was employed for the synthesis of di- and tripeptide. The third and fourth couplings were accomplished by the active ester method of *N*-hydroxysuccinimide. Each coupling proceeded smoothly and the products obtained were analytically pure (Table 1).

The carboxyl groups of Glu side chain and C terminal Met were deprotected by saponification. The final deprotection of the Lys amino moiety with Na/NH_3 was followed by coupling of the resulting peptide with *p*-nitrophenyl biotinylate.⁴⁾ This led to the incorporation of coenzyme to the peptide, but in low yield (12%). The product was satisfactory by all criteria set forth previously for the purity and identity of biotin-containing peptides; TLC, ^1H NMR and a color test with *p*-dimethylaminocinnamaldehyde.⁴⁾ Peptide 1 is soluble considerably in neutral to alkaline aqueous solutions and feasible for kinetic studies on its enzymatic carboxylation.

Enzymatic Carboxylation. The carboxylation of peptide 1 and biotin mediated by the biotin carboxylase subunit dimer of *E. coli* acetyl-CoA carboxylase was carried out at 30 °C (Eq. 1).



The reaction was followed either by the incorporation of radioactive hydrogencarbonate into biotin or the formation of ADP.⁶⁾ Both methods gave virtually identical results. Peptide 1 as well as biotin was carboxylated at a reasonable rate by ≈ 8 milli-units of enzyme in the presence of 1 mM ATP and 8 mM sodium hydrogencarbonate. The initial rate data obtained by the coupled assay were analyzed in terms of a Lineweaver-Burk plot (Fig. 2). The kinetic parameters are summarized in Table 2. The Michaelis constant K_m for biotin (214 mM) is close to the literature value (170 mM).²⁾ The corresponding value for peptide 1 (18 mM) indicates that the binding of 1 to the enzyme is about 12 times tighter than that of biotin. On the other hand, the maximum rate, $V_{\max}$, is larger for

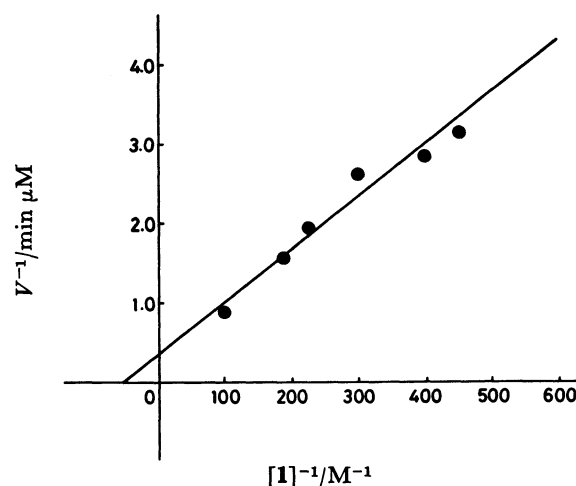


Fig. 2. Lineweaver-Burk plot for the carboxylation of peptide 1 catalyzed by the biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase in 100 mM triethanolamine buffer, pH 8.0 at 30.0 °C.

biotin by a factor of ten than for peptide 1. This makes the difference in the first-order rate constant ($V_{\max}/K_m$) between the two substrates smaller, only 20% greater in favor of peptide 1.

Discussion

Part of the primary structure adjacent to the coenzyme-binding site of several biotin-dependent enzymes has been elucidated.^{5,7,8)} These enzymes share a strikingly similar local sequence in the vicinity of biocytin, suggesting that these enzymes evolved from a common ancestor. An interesting feature of the amino acid sequence, as viewed from a chemical standpoint, is the fact that biocytin is flanked by methionines in all cases. In the case of *E. coli* acetyl-CoA carboxylase another methionine is placed at the C-terminal side of the sequence. Thus, the successive four amino acid residues contain a sulfide moiety each in their side chain. It seems likely that this sulfide cluster plays a certain

role in the carboxylation-transcarboxylation cycle of biotin catalysis. For example, the sulfide cluster might be required for the efficient binding of biotin to the enzyme active site during its carboxylation and subsequent carboxyl transfer reactions. In other words, the enzyme recognizes not only the coenzyme but also the neighboring amino acid residues. Also, the peptide sequence adjacent to biocytin could help biotin to assume a specific spatial orientation that may presumably be needed for biotin to bind to the enzyme active site tightly. A further possibility would be the protection by the acyclic sulfides of the biotin's cyclic sulfide from being oxidized. All these hypotheses could be proven only when model peptides representing the biotin binding site become available to test their interaction with the enzyme.^{3,4} In this article we have prepared a pentapeptide that represents the coenzyme binding site of *E. coli* acetyl-CoA carboxylase. The synthetic strategy was basically the same as that adopted previously for the synthesis of tetrapeptides.⁴ The target peptide (**1**) obtained gave correct analysis but the yield at the final biotinylation step was far from satisfactory (12%). This result is rather strange in view of the fact that biotinylation was successfully accomplished even with larger peptides such as glucagon and insulin by the same method.^{9,10} In our hands, the active ester method gave the best results, and other coupling methods tested such as the mixed anhydride method with isobutyl chloroformate and the DCC coupling method gave even poorer yields. This poses a serious problem to the synthesis of larger biotin-containing peptides by this method. An alternative route is the incorporation of biotin at the early stage of peptide synthesis by, for example, coupling of Boc-Bct with a growing peptide chain.³ In addition, the solid phase method may be worth a serious consideration to save time in accomplishing such a synthesis. Our preliminary attempt to prepare biotin-containing peptides by this method appears promising (unpublished results from this laboratory).

The biotin carboxylase subunit of *E. coli* acetyl-CoA carboxylase was capable of carboxylating the biotinyl moiety of peptide **1** as well as free biotin. In conformity with our expectation, peptide **1** was found to bind to the enzyme by one order of magnitude more tightly than biotin. This clearly indicates that the amino acid residues surrounding the coenzyme play an indispensable role in the interaction of biotin with the enzyme active site. All or at least two of the substrates involved in this carboxylation (Eq. 1) must encounter at some moment of the reaction in the enzyme active site. In light of the fact that magnesium(II) ion is the essential component of the reaction and that the metal ion is often found associated with ATP in many biochemical reactions involving ATP,¹¹ a ternary metal complex composed of magnesium(II), ATP and biotin or HCO_3^- may be the reactive species in this carboxylation reaction. We showed previously that amide groups of peptide **4** have potential to interact with magnesium ion.⁴ This could increase the affinity of the biotinyl moiety for the metal ion, and eventually for the enzyme active site.

In contrast to the binding process, the subsequent

catalytic process was less favorable for peptide **1** than for free biotin. This unfavorable catalysis offsets a large gain in binding, leading to an enhancement of the overall reactivity of **1** relative to biotin by a small margin (20%). Nevertheless, this figure is significant, since all synthetic derivatives of biotin reported to date such as *d*-biotin methyl ester and biocytin are poorer substrates than biotin for acetyl-CoA carboxylase.² The natural substrate of *E. coli* acetyl-CoA carboxylase (BCCP) shows not only tight binding but also high reactivity, but its overall reactivity is governed predominantly by the former term (Table 2).^{12,13} In this sense, an improvement of the binding properties of biotin leads automatically to the development of a good substrate for the enzyme. Such a substrate and its analogues are of vital importance in studying and understanding the molecular basis of enzyme-substrate interaction in this carboxylase system. A further synthetic approach to this goal is now in progress in this laboratory.

Experimental

Apparatus. Melting points were determined on a Yanagimoto hot-stage apparatus and are uncorrected. IR spectra were taken on a JASCO A-100 spectrophotometer, while UV spectra were on a Hitachi 200-10 spectrophotometer. ¹H NMR spectra were recorded either on a JEOL JNM-MH-100 or a JEOL JNM-FX-90Q spectrometer. Optical rotations were determined on a Jasco DIP-4. Radioactivity was determined on an Aloka liquid scintillation counter LSC-703.

Materials. Amino acids, *O*-*t*-butyl *S*-4,6-dimethyl-2-pyrimidinyl thiocarbonate, CbzCl, DCC, and HOBt were obtained from Protein Research Foundation. *d*-Biotin, *p*-dimethylaminocinnamaldehyde, *N*-hydroxysuccinimide, and NEM were the products of Wako Pure Chemical Ind. Co. Scintillation cocktail ACS II and sodium [¹⁴C] hydrogen-carbonate (0.1 mCi/mmol, 1 Ci = 3.7×10^{10} becquerels) were purchased from Amersham. DEAE-cellulose and phosphocellulose were the products of Brown Company. Lactate dehydrogenase and pyruvate kinase were obtained from Boehringer Mannheim, while bovine serum albumin (BSA), phosphoenol pyruvate (PEP), glutathione, NADH, and ATP were from Seikagaku Kogyo Co.

Enzyme Preparation and Assay. *Escherichia coli* B cells were grown in an enriched medium composed of 1 M potassium phosphate buffer (pH 7.4), yeast extracts and D-glucose. The harvested cells were broken on a French press. The cell-free extracts were subjected to enzyme purification according to the procedures of Guchhait *et al.*⁶ Enzyme activity was determined by following either the incorporation of sodium [¹⁴C] hydrogencarbonate into biotin or the formation of ADP by the "coupled assay".⁶ In the former method, the assay mixture (0.50 ml) consisting of 1 mM ATP, 8 mM MgCl₂, 8 mM NaH¹⁴CO₃ (0.1 mCi/mmol), 50 mM potassium *d*-biotinate, 3 mM glutathione, 0.6 mg/ml BSA, 10% ethanol and the enzyme in 100 mM triethanolamine buffer (pH 8.0) was incubated at 30 °C for a certain period of time. The reaction was initiated by the enzyme and was terminated by the addition of 2 drops of ice-cold 1-octanol. This was followed immediately by an introduction of gaseous carbon dioxide to the reaction mixture at 0 °C for 40 min. An aliquot (0.10 ml) of ice-cold 0.1 M NaOH was added and the resulting solution was transferred to a scintillation vial containing 6 ml of ACS II to determine radioactivity. In the latter method, the formation of ADP was coupled with the oxidation of NADH.

Thus, an assay mixture (1.0 ml) contained the same components as those described above except for unlabeled hydrogencarbonate, instead of radioactive one, plus 0.5 mM PEP, 5 units of lactate dehydrogenase, 3 units of pyruvate kinase and 0.2 mM NADH. The reaction was started by the addition of enzyme at 30 °C and the decrease of NADH absorption was determined at 340 nm. One unit of enzyme activity is defined by the amount of enzyme needed to form 1 μ mol of 1-carboxybiotinate per min.

Boc-Lys(Cbz)-Met-OMe (5). To a solution of 21.5 g (55 mmol) of Boc-Lys(Cbz), 11.5 g (55 mmol) of Met-OMe·HCl and 6.3 g (55 mmol) of NEM in 180 ml DMF was added 11.3 g (55 mmol) of DCC at 0 °C. The whole mixture was stirred for 8 h at room temperature. The precipitated DCUrea was filtered off. Evaporation of the solvent left an oil, which was taken up in 180 ml of ethyl acetate. The solution was washed with the following solutions: 4% NaHCO₃, saturated NaCl, 10% citric acid, saturated NaCl, H₂O. The organic layer was dried over Na₂SO₄, concentrated to ca. 50 ml and kept in a freezer. The product was obtained in 72% yield (21.3 g).

Lys(Cbz)-Met-OMe·HCO₂H (6). A sample of **5** (21.3 g, 41 mmol) was allowed to react with 100 ml of 99% HCO₂H for 5 h at room temperature. Methanol was added and the solvents were evaporated *in vacuo*. The product was crystallized from methanol-ether in 90% yield (19.5 g). Mp 203–204 °C. Complete removal of the Boc group was confirmed by the disappearance of the 1.35 ppm signal.

Boc-Met-Lys(Cbz)-Met-OMe (7). This peptide was synthesized by reaction of **6** (21.1 g, 46 mmol) with Boc-Met (11.5 g, 46 mmol) in the presence of NEM (5.3 g, 46 mmol), HOBt (6.23 g, 46 mmol), and DCC (9.52 g, 46 mmol). The reaction mixture was worked up analogously to that for **5**. The product was recrystallized from ethyl acetate-petroleum ether.

Met-Lys(Cbz)-Met-OMe·HCO₂H (8). Reaction of **7** (10.0 g, 51 mmol) with 100 ml of 99% HCO₂H in the way identical with that described for **6** gave 7.8 g of **8** in 87% yield.

Boc-Ala-Met-Lys(Cbz)-Met-OMe (9). Boc-Ala-OSu (3.23 g, 11 mmol) was allowed to react with **8** in 50 ml of DMF containing 1.27 g (11 mmol) of NEM for 8 h at room temperature. The solvent was removed *in vacuo* and the residue was taken up in 400 ml of ethyl acetate. The organic solution was washed with the solvents used for **5**. After drying over Na₂SO₄, the solution was concentrated to a small volume to provide 5.5 g of the product in 67% yield.

Ala-Met-Lys(Cbz)-Met-OMe·HCO₂H (10). Treatment of 4.6 g (6.3 mmol) of **9** with 100 ml of 99% HCO₂H and the subsequent work-up yielded 4.9 g of the product (94%).

Boc-Glu(OBzl)-Ala-Met-Lys(Cbz)-Met-OMe (11). Reaction of 3.5 g (5.0 mmol) of **10** with 3.38 g (7.5 mmol) of Boc-Glu(OBzl)-OSu in 100 ml of DMF containing 0.60 g (5.0 mmol) of NEM gave 2.7 g of the product.

Boc-Glu-Ala-Met-Lys(Cbz)-Met (12). A sample of **11** (1.04 g, 1.1 mmol) was dissolved in 27 ml of methanol and 14 ml of dioxane. To this was added 2.3 ml of 1 M sodium hydroxide and the whole mixture was stirred for 7 h at room temperature. After adding 11 ml of water, the organic solvents were removed *in vacuo*. The remaining aqueous solution was washed with ether and then brought to pH 3 with 10% citric acid at 0 °C. The product obtained was further purified by recrystallization from methanol-ether. The NMR spectrum of this peptide revealed a complete removal of *O*-Bzl and *O*-Me protecting groups.

Boc-Glu-Ala-Met-Lys-Met (13). A sample of **12** (1.2 g, 1.4 mmol) was dissolved in 80 ml of liquid ammonia. Sodium was added in small portions with stirring. When the blue color of the solution was retained for a few minutes, the

reaction was quenched by ammonium chloride. Ammonia was evaporated off. Methanol was added to the residue and the insoluble material was filtered off. The filtrate was concentrated to dryness and the solid left was taken up in 1% acetic acid. The insoluble starting material was removed by filtration. For desalting the filtrate was subjected to column chromatography on a Sephadex G-10 column (3.2 × 56 cm) with 1% acetic acid as an eluant. The peak fractions were pooled and lyophilized to yield 0.77 g (70%) of the product, mp 221–223 °C. TLC *R_f* 0.45 (E). Complete removal of the protecting group was ascertained on the basis of the lack of signals for the benzyloxycarbonyl group at 4.98 and 7.28 ppm in the NMR spectrum of this peptide.

Boc-Glu-Ala-Met-Bct-Met (1). A sample of **13** (0.26 g, 0.33 mmol) dissolved in 2 ml of water was mixed with *p*-nitrophenyl biotinate (0.14 g, 0.50 mmol)¹⁴ in 10 ml of DMF containing 0.15 g (1.32 mmol) of NEM. The whole mixture was stirred for a day at room temperature. The solvents were removed *in vacuo* and methanol (40 ml) was added to the residue. The precipitates formed were collected by filtration, 47 mg (12%), mp 199–203 °C. TLC *R_f* 0.59 (E). This peptide is ninhydrin-negative but develops a pink color upon spraying of *p*-dimethylaminocinnamaldehyde, confirming the presence of a biotinyl moiety.¹⁵ The characteristic ¹H NMR signals for the biotinyl protons are the following: (DMSO-*d*₆) 2.84 (d, *J* = 14 Hz, 5-exo), 2.56 (d, *J* = 14 Hz, 5-endo), 6.36 (s, NH) and 6.41 (s, NH).

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References

- 1) H. G. Wood and R. E. Barden, *Ann. Rev. Biochem.*, **46**, 385 (1977).
- 2) P. Dimroth, R. B. Guchhait, E. Stoll, and M. D. Lane, *Proc. Natl. Acad. Sci. U. S. A.*, **67**, 1353 (1970).
- 3) M. Bodansky and D. T. Fagan, *J. Am. Chem. Soc.*, **99**, 235 (1977).
- 4) H. Kondo, F. Moriuchi, and J. Sunamoto, *Bull. Chem. Soc. Jpn.*, **55**, 1579 (1982).
- 5) M. R. Sutton, R. R. Fall, A. M. Nervi, A. W. Alberts, P. R. Vagelos, and R. A. Bradshaw, *J. Biol. Chem.*, **252**, 3934 (1977).
- 6) R. B. Guchhait, S. E. Polakis, P. Dimroth, E. Stoll, J. Moss, and M. D. Lane, *J. Biol. Chem.*, **249**, 6633 (1974).
- 7) D. B. Rylatt, D. B. Keech, and J. C. Wallace, *Arch. Biochem. Biophys.*, **183**, 113 (1977).
- 8) W. L. Maloy, B. U. Bowien, G. K. Zwolinski, K. G. Kumar, H. G. Wood, L. H. Ericsson, and K. A. Walsh, *J. Biol. Chem.*, **254**, 11615 (1979).
- 9) K. C. Flanders, D. H. Mart, R. J. Folz, R. D. England, S. A. Codican, D. E. Harris, A. D. Floyd, and R. S. Gurd, *Biochemistry*, **21**, 4244 (1982).
- 10) K. Hofmann, F. M. Finn, H.-J. Friesen, C. Diaconescu, and H. Zahn, *Proc. Natl. Acad. Sci. U. S. A.*, **74**, 2697 (1977).

- 11) J. Moss and M. D. Lane, *Adv. Enzymol.*, **35**, 321 (1971).
 - 12) A. M. Nervi, A. W. Alberts, and P. R. Vagelos, *Arch. Biochem. Biophys.*, **143**, 401 (1971).
 - 13) S. E. Polakis, F. S. Guchhait, E. E. Zwergel, and M. D. Lane, *J. Biol. Chem.*, **249**, 6657 (1974).
 - 14) E. A. Bayer and M. Wilchek, *Methods Enzymol.*, **46**, 613 (1977).
 - 15) D. B. McCormick, *J. Heterocycl. Chem.*, **10**, 235 (1973).
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