Total Synthesis of Camel β-Melanotropin by the Solid-Phase Method†

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ABSTRACT: Total synthesis of camel β-MSH has been accomplished by the solid-phase method. The synthetic product was shown to behave as the natural hormone in amino acid analysis, paper electrophoresis, disc electrophoresis, enzymic digests, optical rotation, and bioassays.

In the preceding paper (Li *et al.*, 1975) we described the isolation and amino acid sequences of four melanotropins from camel (*Camelus dromedarius*) pituitary glands. One of these is a β-MSH[1] with the following primary structure:

Asp-Gly-Gly-Pro-Tyr-Lys-Met-Gln-His-Phe-
1 5 10

Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
15

In comparison with the structure of bovine β-MSH (Geschwind *et al.*, 1957), it is designated as [Gly^{2}Gln^8]-β_b-MSH or β_{C1}-MSH.[2] Bovine (Schwyzer *et al.*, 1963), human (Yajima *et al.*, 1969), and macaque (Yajima *et al.*, 1970) β-melanotropins have been synthesized by solution methods. Recently, the human and monkey hormones have been synthesized (Wang *et al.*, 1973) using the solid-phase procedure (Merrifield, 1964). In this paper, we report the synthesis of β_{C1}-MSH with the formyl protecting group (Ohno *et al.*, 1973; Yamashiro and Li, 1973a,d) for tryptophan. It will be seen that the synthetic product is identical with the natural hormone in every criterion tested.

Reaction of the tetramethylammonium salt (Loffet, 1971) of N$^\alpha$-Boc-(β-Bzl)Asp with chloromethylated polymer gave N$^\alpha$-Boc-(β-Bzl)Asp resin which was then subjected to the procedure for solid-phase peptide synthesis (Merrifield, 1964). Removal of the N$^\alpha$-Boc protecting group was carried out by treatment for 15 min in 50% trifluoroacetic acid in dichloromethane (Gutte and Merrifield, 1969). Coupling of Boc-amino acid was achieved with dicyclohexylcarbodiimide (Sheehan and Hess, 1955) along with use of the following side-chain protecting groups: serine and aspartic acid, benzyl; lysine and tyrosine, *o*-bromobenzyloxycarbonyl (Yamashiro and Li, 1973c); arginine, tosyl; and histidine, Boc (Yamashiro *et al.*, 1972). The glutamine residue was introduced using its *p*-nitrophenyl ester (Bodanszky and du Vigneaud, 1959).

Since the Boc-protecting group has proven successful for protecting the imidazole moiety of histidine in solid-phase peptide synthesis (Yamashiro *et al.*, 1972), an improved method for preparing N$^\alpha$-Boc-(Im-Boc)His is described.

The finished peptide was cleaved from the resin and all protecting groups were removed with liquid HF (Sakakibara *et al.*, 1967; Lenard and Robinson, 1967; Mazur and Plum, 1968) with the exception of the formyl group (Ohno *et al.*, 1972). Preliminary purification was carried out by gel filtration on Sephadex G-10 and G-25. For deformylation (Yamashiro and Li, 1973d), the material recovered from Sephadex G-25 was treated with 1 M NH_4HCO_3 (pH 9.0) at 25° for 24 hr. The deformylated product was submitted to chromatography on CMC (Peterson and Sober, 1956) as shown in Figure 1A; the material from the main peak was further purified by partition chromatography (Yamashiro, 1964) on Sephadex G-25 (Figure 1B). The peptide recovered from the main peak in this step was shown to be the desired product. The overall yield[3] of synthetic β_{C1}-MSH was 25% based on the starting Boc-(β-Bzl)Asp resin.

When the synthetic product and the natural hormone were subjected to partition chromatography on the same column, they behaved identically, with an R_F value of 0.22 (Figure 2). Gel filtration on Sephadex G-25 of both natural and synthetic hormones gave a single peak with V_e/V_0 = 1.45. The behavior of the synthetic product on paper electrophoresis at pH 3.7 and 6.9 was identical with that of the natural hormone. Polyacrylamide gel electrophoresis at pH 4.5 of both natural and synthetic hormones showed a single band for each with the same mobility as shown in Figure 3. Amino acid analysis of acid and enzymic hydrolysates of the synthetic product gave values in agreement with the data obtained with the natural hormone (Table I). Electrophoretic patterns of tryptic and chymotryptic digests of natural and synthetic β_{C1}-MSH (Figure 4) were also identical. Values for optical rotation of synthetic and natural hormones did not differ significantly (Table II).

Table III presents the biological data for the synthetic and natural β_{C1}-MSH. It is evident that both the melanocyte-stimulating and lipolytic activities of the synthetic product are identical with those exhibited by natural β_{C1}-MSH.

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[1] Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (1972). Other abbreviations used are: MSH, melanocyte-stimulating hormone, melanotropin; β_b-MSH, bovine β-MSH; For, formyl; CMC, carboxymethylcellulose.

[2] Camel pituitary glands have two β-melanotropins, which are designated β_{C1}-MSH and β_{C2}-MSH (see preceding paper, Li *et al.*, 1975). The difference between the two is found in position 8; the former contains glutamine and the latter glutamic acid.

[3] β_{C1}-MSH was also synthesized by the same procedure without the formyl protecting group for tryptophan. The yield was only 10% based on starting Boc-(β-Bzl)Asp resin.

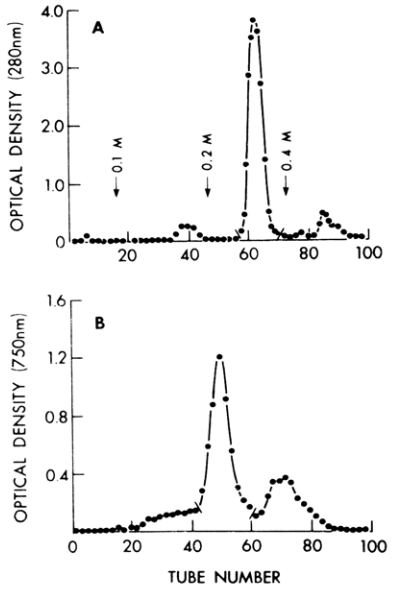

FIGURE 1: (A) Chromatography of a partially purified sample (90 mg) of synthetic β_{C1}-MSH on CMC, 8.3 ml/tube; and (B) partition chromatography on Sephadex G-25 of synthetic β_{C1}-MSH (55 mg) obtained from CMC [see A; hold-up volume, 55 ml; 4.7 ml/tube]. These chromatograms were effected as described in the Experimental Section.

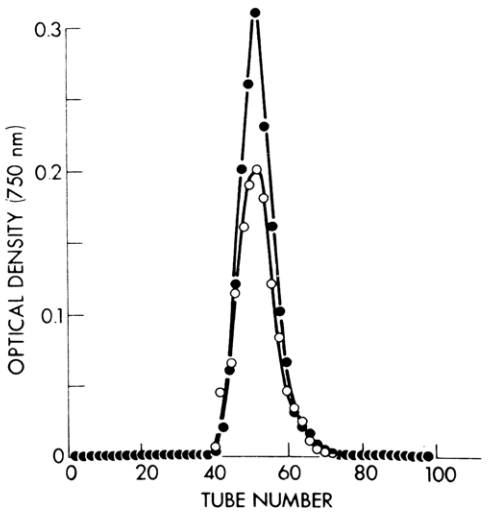

FIGURE 2: Partition chromatography on Sephadex G-25 of synthetic (●) and natural (○) β_{C1}-MSH (1.5 mg each); hold-up volume, 16 ml; 1.45 ml/tube.

Experimental Section

Melting points were determined on a Fisher-Johns block and were not corrected. Paper electrophoresis was performed on paper (Whatman No. 3MM) at 400 V for 5 hr with the following buffers: pyridine–acetate, pH 3.7; and collidine–acetate, pH 6.9. Electrophoresis on 7% polyacrylamide gel was carried out at pH 4.5 for 1 hr (Davis, 1964). Amino acid analyses were performed according to the procedure of Spackman *et al.* (1958). Tryptophan determinations were carried out by the methods of Liu and Chang (1971). Chromatography on CMC was performed in a 1 × 55 cm column with a starting buffer of 0.01 M ammonium acetate of pH 4.5. A gradient with respect to pH and salt concentrations was then effected by introducing, at appropriate times, solutions of 0.1, 0.2, and 0.4 M ammonium acetate of pH 6.5 through a 500-ml mixing chamber containing the starting buffer. Partition chromatography (Yamashiro, 1964) on Sephadex G-25 (medium) was run in a 1.92 × 56.0 cm column in the solvent system: 1-butanol–pyridine–0.1% acetic acid, 5:3:11.

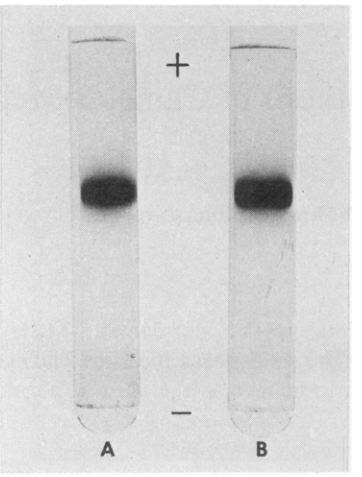

FIGURE 3: Electrophoresis of natural (A) and synthetic (B) β_{C1}-MSH on polyacrylamide gel at pH 4.5 (110-μg samples).

Table I: Amino Acid Analyses[a] of Natural and Synthetic β_{C1}-MSH.

Amino Acid	Theor.	Acid Hydrolysis: Natural[b]	Acid Hydrolysis: Synthetic	Enzyme Digest: Natural	Enzyme Digest: Synthetic
Lys	2	1.9	2.0	1.3	1.0
His	1	0.9	1.0	1.1	0.9
Arg	1	1.0	0.9	1.1	1.0
Asp	2	1.9	2.0	0.9	1.0
Ser	1	0.8	0.9	0.9	1.0
Gln	1				
Glu	0	0.9	1.0		
Pro	3	2.7	2.9	0.9	1.0
Gly	3	2.8	3.0	2.5	2.8
Met	1	1.0	1.0	1.0	1.1
Tyr	1	1.0	1.0	0.9	0.9
Phe	1	1.0	1.0	1.1	1.0
Trp	1	1.0	0.9	1.1	1.0

[a] Values in molar ratio. [b] Taken from Li *et al.* (1975).

Chloromethylated (0.69 mequiv/g) styrene–1% divinylbenzene (200–400 mesh) was obtained from Bio-Rad Labs. Carboxymethylcellulose (CMC) was purchased from Schleicher and Schuell.

Improved Procedure for the Preparation of Dicyclohexylamine Salt of N^{α}-Boc(Im-Boc)histidine. Histidine free base (6.6 g, 42.5 mmol) was suspended in 250 ml of dimethyl sulfoxide and 30 ml of triethylamine (214 mmol), and 30 ml of Boc-azide (214 mmol) was added. This mixture was gently stirred for 48 hr, by which time it became clear. The following work-up was performed at 4°. After the addition of 900 ml of water, the solution was washed with two 200-ml portions of ether. The aqueous phase was cooled; the pH was lowered to 2.5 with 2 N HCl and then extracted with two 200-ml portions of ethyl acetate. The ethyl acetate layer was washed with two 200-ml portions of water and 100 ml of saturated NaCl solution. The washed ethyl ace-

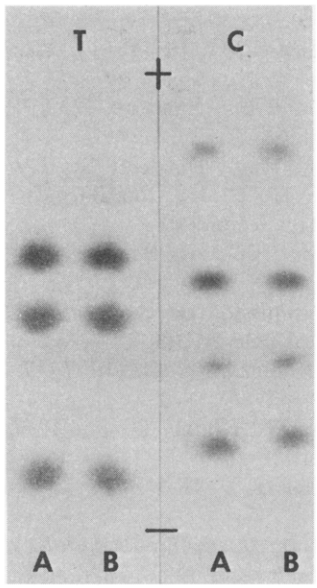

FIGURE 4: Paper electrophoresis of natural (A) and synthetic (B) β_{C1}-MSH after treatment with trypsin (T) and chymotrypsin (C). Electrophoresis of tryptic and chymotryptic digests was run at pH 3.7 and 6.9, respectively.

tate solution was then dried over anhydrous $MgSO_4$ at room temperature. Removal of the drying agent and solvent gave 10 g of oil which was dissolved in 60 ml of chloroform, cooled at 0°, mixed with dicyclohexylamine (12 ml), and diluted at 0° with 300 ml of petroleum ether. Crystallization at 4° gave two successive crops: 15 g of salt (67% yield); mp 155–157°; $[\alpha]^{24}D$ +18.8° (*c* 2.0, $CHCl_3$); lit. (Yamashiro *et al.*, 1972) mp 157–159°; $[\alpha]^{24}D$ +17.6° (*c* 2.0, $CHCl_3$).

N^{α}-Boc-β-Benzylaspartyl Polymer. The esterification of the first amino acid to the chloromethylated resin was carried out with the following modification (Yamashiro and Li, 1973b) of the Loffet procedure (Loffet, 1971). N^{α}-Boc-(β-Bzl)aspartic acid (3.23 g, 10 mmol) in 7.0 ml of methanol was mixed with 9.4 ml of 0.98 N tetramethylammonium hydroxide in methanol and evaporated *in vacuo*. The oil was reevaporated from dioxane and then from methanol and dried *in vacuo* over P_2O_5 for 5 hr. The salt was treated with 4 g of chloromethylated polymer in dimethylformamide (28 ml) for 17 hr at 24°. The resin was filtered off and washed with dimethylformamide, dimethylformamide–water (1:1), water, and EtOH; yield, 4.49 g. A sample of the resin was hydrolyzed in propionic acid–12 M HCl (Scotchler *et al.*, 1970) and amino acid analysis gave 0.36 mmol of Asp/g.

Protected β_{C1}-MSH Resin. Starting with N^{α}-Boc-(β-Bzl)Asp resin (1.0 g, 0.36 mmol), the following cycle was carried out: (1) wash with four 12-ml portions of dichloromethane (retention volume of the resin for dichloromethane was 5 ml after filtration); (2) removal of the Boc group with 50% trifluoroacetic acid in dichloromethane for 15 min; (3) wash with two 12-ml portions of dichloromethane; (4) wash with two 12-ml portions of 50% dioxane in dichloromethane; (5) wash with two 12-ml portions of dichloromethane; (6) 5 min of neutralization with 12 ml of 5% diisopropylethylamine in dichloromethane; (7) wash with six 12-ml portions of dichloromethane; (8) add 1.45 mmol of the appropriate Boc-amino acid in 7.5 ml of dichloromethane and shake for 5 min; (9) add 1.45 mmol of *N,N'*-dicyclohexylcarbodiimide in 2.4 ml of dichloromethane and shake for 2 hr; (10) wash with three 12-ml portions of dichloromethane; (11) wash with three 12-ml portions of absolute EtOH.

In order to introduce the glutamine residue, steps 8 and 9 were replaced by treatment with 3.6 mmol of the *p*-nitrophenyl ester of Boc-glutamine which was added in 15 ml of dimethylformamide followed by a coupling time of 13 hr. For the last three washings in step 7 and the washings in step 9, dimethylformamide was used. N^{α}-Boc-(N^{i}-For)Trp and N^{α}-Boc-(N^{G}-Tos)Arg were dissolved in 5 and 10% dimethylformamide in CH_2Cl_2, respectively. The yield of the protected octadecapeptide resin was 2.09 g.

β_{C1}-MSH. A sample of the protected peptide resin (1.38 g) was mixed with 15 ml of liquid HF containing 2.8 ml of anisole and stirred for 1 hr at 0°. After evaporation of HF with nitrogen the residue was dried *in vacuo* over NaOH and then stirred with 15 ml of ethyl acetate for 15 min and filtered. The resin was extracted with two 15-ml portions of CF_3COOH and filtered off. The combined filtrates were evaporated *in vacuo*, and the resulting oily residue was dissolved with 3 ml of 0.5 N acetic acid and submitted to gel filtration on Sephadex G-10 (2 × 25 cm column) in 0.5 N acetic acid. The material (430 mg) in the single peak (280-nm detection) was recovered by lyophilization and submitted to chromatography on Sephadex G-25 column (2.5 × 136 cm) in 0.5 N acetic acid. Fractions corresponding to the peak (elution volume of 480 ml) were pooled and lyophilized, yielding 330 mg. Ninety milligrams of the lyophilized

Table II: Optical Rotation of Natural and Synthetic β_{C1}-MSH.

Wavelength (nm)	$[\alpha]^{27}\lambda$ (deg)	
	Natural	Synthetic
589	−95.7	−91.8
500	−144.0	−128.4
400	−245.1	−233.5
350	−361.9	−346.3
300	−622.6	−596.1
250	−1404.7	−1375.5

Table III: Biological Activities of Synthetic β_{C1}-MSH.

Bioassay	Synthetic		Natural	
	Dose (ng)	Response	Dose (ng)	Response
Melanotropic[a]	3	52.8 ± 3.6	3	53.7 ± 5.4
activity	9	66.5 ± 0.6	9	67.0 ± 0.4
Lipolytic[b]	17	1.60 ± 0.2	17	1.75 ± 0.03
activity	51	3.98 ± 0.3	51	4.26 ± 0

[a] Melanotropic activity expressed as per cent decrease in reflectance. Five skins used for each assay. Values expressed in mean ± standard error. The potency of the synthetic β_{C1}-MSH is computed to be 95% of the natural hormone with 95% confidence limit of 51–160 and λ of 0.2. [b] Lipolytic activity expressed in micromoles of glycerol production per gram of cells per hour. Triplicate determinations with values expressed in mean ± standard error. The synthetic β_{C1}-MSH is computed to be 91% of the natural hormone with a confidence limit of 74–111 and λ of 0.04.

product was treated with 100 ml of 1.0 M NH_4HCO_3 at pH 9.0 for 24 hr at 25° to remove the formyl group from the tryptophan residue as previously described (Yamashiro and Li, 1973d). The reaction was terminated by three successive lyophilizations, and the lyophilized material submitted to chromatography on carboxymethylcellulose (Figure 1A). Isolation of the material from the main peak gave 55 mg. Further purification of this product was achieved by partition chromatography as shown in Figure 1B. This chromatography gave a main peak with an R_F value of 0.23 as detected at 750 nm by the Folin–Lowry reaction (Lowry *et al.*, 1951); isolation of the material represented by the main peak gave 38 mg. Rechromatography of this material on the same column gave a single peak with R_F 0.22 and a final yield of 35 mg of β_{C1}-MSH (25% yield based on starting N^{α}-Boc-(β-Bzl)Asp resin).

Partition chromatography of 1.5-mg samples of both natural (Li *et al.*, 1975) and synthetic β_{C1}-MSH on a Sephadex G-25 column (1.9 × 19.8 cm) gave a single peak for each sample with identical R_F values of 0.22 (Figure 2). Gel filtration of 0.55-mg samples of both natural and synthetic hormones on a Sephadex G-25 column (1.37 × 115 cm, V_0 = 65 ml) in 0.5 N acetic acid also gave a single peak eluting at 94.5 ml. On paper electrophoresis at pH 3.7 the synthetic and natural hormones each gave a single spot (ninhydrin detection) with identical R_F values of 0.55 relative to lysine; at pH 6.9, the two samples also gave a single spot with identical R_F values of 0.26. The behavior of the synthetic peptide on polyacrylamide gel electrophoresis at pH 4.5 (Figure 3) was exactly the same as that of the natural β_{C1}-MSH; a single homogeneous band was detected after coloration with Amido-Schwarz. Samples (0.15 mg) of the synthetic and natural hormones were treated separately with 3 μg each of trypsin and chymotrypsin in 35 μl of Tris buffer (pH 8.5, 0.01 M Mg^{2+}) at 37° for 5 hr. Paper electrophoresis of the tryptic and chymotryptic digests (10-μl aliquot) at pH 3.7 and 6.9, respectively, gave the peptide patterns (ninhydrin detection) shown in Figure 4. Amino acid analyses of enzymic (first with trypsin and chymotrypsin, and then LAP) and acid hydrolysates of synthetic and natural β_{C1}-MSH are summarized in Table I.

Optical rotatory dispersion of synthetic and natural β_{C1}-MSH was measured in 0.1 N acetic acid in a Cary 60 recording spectropolarimeter. The general procedures used in this laboratory for these measurements have been described (Bewley and Li, 1967). In all measurements, dynode voltages did not exceed 500 V. Peptide concentrations were determined from their absorption spectra taken at 360–240 nm on a Beckman DK-2A recording spectrophotometer.

The melanocyte-stimulating activity of the synthetic peptides was determined *in vitro* by the frog skin method (Shizume *et al.*, 1954; Ramachandran, 1970). Measurement of the lipolytic activity was performed in isolated rabbit fat cells as previously described (Ramachandran *et al.*, 1972). The natural hormone was employed in each assay for comparison.

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References

Bewley, T. A., and Li, C. H. (1967), *Biochim. Biophys. Acta 140,* 201.

Bodanszky, M., and du Vigneaud, V. (1959), *J. Amer. Chem. Soc. 81,* 5688.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Geschwind, I. I., Li, C. H., and Barnafi, L. (1957), *J. Amer. Chem. Soc. 79,* 6394.

Gutte, B., and Merrifield, R. B. (1969), *J. Amer. Chem. Soc. 91,* 501.

IUPAC–IUB Commission on Biochemical Nomenclature (1972), *J. Biol. Chem. 247,* 277.

Lenard, J., and Robinson, A. B. (1967), *J. Amer. Chem. Soc. 89,* 181.

Li, C. H., Danho, W., Chung, D., and Rao, A. J. (1975), preceding paper.

Liu, T.-Y. and Chang, Y. H. (1971), *J. Biol. Chem. 246,* 2842.

Loffet, A. (1971), *Int. J. Peptide Protein Res. 3,* 297.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Mazur, R. H., and Plum, G. (1968), *Experientia 24,* 661.

Merrifield, R. B. (1964), *Biochemistry 3,* 1385.

Ohno, M., Tsukamoto, S., Makisumi, S., and Izumiya, N. (1972), *Bull. Chem. Soc. Jap. 45,* 2852.

Ohno, M., Tsukamoto, S., Sato, S., and Izumiya, N. (1973), *Bull. Chem. Soc. Jap. 46,* 3280.

Peterson, E. A., and Sober, H. A. (1956), *J. Amer. Chem. Soc. 78,* 751.

Ramachandran, J. (1970), *Biochem. Biophys. Res. Commun. 41,* 353.

Ramachandran, J., Lee, V., and Li, C. H. (1972), *Biochem. Biophys. Res. Commun. 48,* 274.

Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M., and Sugihara, H. (1967), *Bull. Chem. Soc. Jap. 40,* 2164.

Schwyzer, R., Iselin, B., Kappeler, H., Riniker, B., Rittel, W., and Zuber, H. (1963), *Helv. Chim. Acta 46,* 1975.

Scotchler, J., Lozier, R., and Robinson, A. B. (1970), *J. Org. Chem. 35,* 3151.

Sheehan, J. C., and Hess, G. P. (1955), *J. Amer. Chem. Soc. 77,* 1067.

Shizume, K., Lerner, A. B., and Fitzpatrick, T. B. (1954), *Endocrinology 54,* 553.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.

Wang, K. T., Blake, J., and Li, C. H. (1973), *Int. J. Peptide Protein Res. 5,* 33.

Yajima, H., Kawasaki, K., Minami, H., Kawatani, H., Mizokami, N., Kiso, Y., and Tamura, F. (1970), *Chem. Pharm. Bull. 18,* 1394.

Yajima, H., Okada, Y., Kimomura, Y., Mizohami, N., and Kawatani, H. (1969), *Chem. Pharm. Bull. 17,* 1237.

Yamashiro, D. (1964), *Nature (London) 201,* 76.

Yamashiro, D., Blake, J., and Li, C. H. (1972), *J. Amer. Chem. Soc. 94,* 2855.

Yamashiro, D., and Li, C. H. (1973a), *Biochem. Biophys. Res. Commun. 54,* 882.

Yamashiro, D., and Li, C. H. (1973b), *J. Amer. Chem. Soc. 95,* 1310.

Yamashiro, D., and Li, C. H. (1973c), *J. Org. Chem. 38,* 591.

Yamashiro, D., and Li, C. H. (1973d), *J. Org. Chem. 38,* 2594.