are found to be quite shallow (cf. Table I). Clearly, previous single-determinant calculations on planar singlet CR_4 have produced the lower of the two zwitterionic states.[3-6] The charge distributions given in Table I are indicative.

It is necessary to note that triplet planar methane derivatives were calculated as early as 1972 to be either the most stable electronic state or energetically competitive with the closed-shell singlet **1** (* = ··).[6] Our calculations yield a similar result (Table I). The importance of a biradical constitution for flattened carbon structures has been alluded to by the observation that electron correlation preferentially stabilizes $^1A_1$ singlets.[4,7] In spite of these reports, the closed-shell singlet embellished by a lone electron pair has been the standard for comparison and the electronic entity consistently described as the experimental goal.[1b,2b,3b,4,5c,7-9]

The biradical description for planar or near-planar CH_4 is not limited to the parent. Cyclopropane, suggested as a candidate for stereomutation via the planar transition state **5**,[2a] and cyclo-

5 **6** **7** **8** **9** **10** **11**

propene likewise prefer open-shell singlet states by ΔE(open − closed) = 17.3 and 53.3 kcal/mol, respectively (Table II). It should be noted that open-shell singlet and triplet energies are comparable. On the other hand, the five- and six-membered ring structures **7**–**11** are all predicted to exist as closed-shell molecules with a carbon lone electron pair as depicted by **1** (* = ··) (Table II). The relative ordering of singlet states for planar methane, **5**, and **6** on the one hand and cycles **7**–**11** on the other appears to arise from a combination of MO reorganization and electron repulsion effects. Specifically, the electron repulsion implied by the p-type orbital of **1** (* = ··), unless mediated by delocalization, is sufficient to uncouple the electron pair.[14]

As has been noted previously, the incorporation of a planar carbon moiety in a three-membered ring or in a cyclic unit with $(4N + 2)\pi$ electrons lowers the planarization barrier relative to methane.[1c,3,4a] The effect persists regardless of the pairing scheme favored by PRDDO. However, in the practical design of synthetic routes to or through tetracoordinate planar carbon, the most expeditious substrates would appear to be those employing a delocalized π system and molecular charge. In this spirit, the planar D_{4h} ammonium ion, NH_4^+, lies 166 kcal/mol above the tetrahedral geometry, a gain of 90 kcal/mol relative to CH_4,[15] and is predicted by PRDDO–GVB to be a closed-shell ground state. All-carbon structures such as fenestrane **2** and the paddlanes,[1b,5c,8,9] on the contrary, are suggested to evidence closed-shell qualities only by deviating sufficiently from the sought-after planarity.

Acknowledgment. We are grateful to the Danish Natural Science Research Council for generous provision of computer funds (J.nr. 511-15446) and to the University of Copenhagen for a scholar stipendium to D.C. We are likewise indebted to Professor Thomas Halgren (City College of the City University of New York) for patient instruction in the manipulation of PRDDO and for an appreciation of its utility for describing biradicals. Provocative discussions with Professor Paul von R. Schleyer (Universität Erlangen-Nürnberg) and the pioneering work of his group provided the impetus for our work.

Debbie C. Crans, James P. Snyder*
Department of General and Organic Chemistry
H. C. Ørsted Institute, The University of Copenhagen
DK-2100 Copenhagen Ø, Denmark

Received February 25, 1980

(14) For example, the HOMO–LUMO gap for planar methane at the PRDDO–RHF level is larger than that for **5**, **6**, and **7**–**10**. Calculated values of two-electron repulsion integrals intimate that the dominating factor stabilizing the open-shell singlets is intraorbital Coulomb repulsion.

(15) The GAUSSIAN 70 program series yields $\Delta E_{NH_4^+}$(tetrahedral − planar) = 165 (STO-3G) and 129 kcal/mol (4-31G), corresponding to reduced barriers to flattening of 75 and 39 kcal/mol, respectively, relative to $^1A_1$ methane.[5b] More recent calculations at the 6-31G* and MP2/6-31G* levels lead to a similar reduction in planarization energies for the ammonium ion.[4b,16]

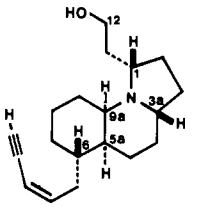
(16) We are grateful to Professor Schleyer for informing us of these results prior to publication.

(17) D. Döhnert and J. Koutecký, *J. Am. Chem. Soc.*, **102**, 1789 (1980).

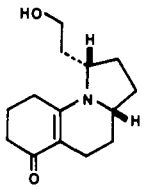
Total Synthesis of (±)-Gephyrotoxin

Sir:

Gephyrotoxin (**1**) is one of a variety of alkaloids isolated from the skin extracts of the Columbian frog, *Dendrobates histrionicus*. The structure of gephyrotoxin, including its absolute configuration,



1, gephyrotoxin



2

was elucidated by chemical[1,2] and X-ray[2] studies. Recent publication of a total synthesis of (±)-perhydrogephyrotoxin by Overman[3] and a model study for the ring construction of the alkaloid by Hart[4] prompted us to report the first, highly stereoselective total synthesis of (±)-gephyrotoxin. The unique aspect of this synthesis is that all five asymmetric centers were stereoselectively introduced through hydrogenation reactions. One of the key strategies of this synthesis involved the use of the hydroxyethyl side chain to direct the stereochemical course of the hydrogenation of the vinylogous amide **2**.

The synthesis of the vinylogous amide **2**[5a] (mp 153–154 °C; NMR ($CDCl_3$) δ 3.8–4.2 (2 H, m), 3.69 (2 H, t, J = 6.0 Hz); UV (MeOH) λ_{max} 319 nm (ϵ 30 900)) is summarized in Scheme I. Introduction of the first two asymmetric centers at C-1 and C-3a[6] was achieved by the hydrogenation of compound **4**, yielding a 12:1 ratio[7a] of the *cis*- and *trans*-pyrrolidines. The overall yield of **2** from **3** was about 20%.

Hydrogenation of the vinylogous amide **2** by using 10% palladium on charcoal in ethyl acetate under 60 psi hydrogen pressure at room temperature gave a single amino alcohol, **8**[5b] (51% yield; NMR ($CDCl_3$) δ 3.72 (2 H, dt, J = 3.0, 7.0 Hz)) along with the hydrogenolysis product **9**[5b] (19% yield). The stereochemistry of the amino alcohol **8** was determined by the X-ray analysis of its *p*-bromobenzenesulfonate salt[5a] (mp 160–162 °C).[8] The ste-

(1) Tokuyama, T.; Uenoyama, K.; Brown, G.; Daly, J. W.; Witkop, B. *Helv. Chim. Acta* **1974**, *57*, 2597.

(2) Daly, J. W.; Witkop, B.; Tokuyama, T.; Nishikawa, T.; Karle, I. L. *Helv. Chim. Acta* **1977**, *60*, 1128.

(3) Overman, L. E.; Fukaya, C. *J. Am. Chem. Soc.* **1980**, *102*, 1454.

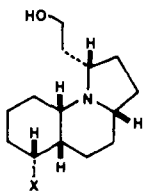
(4) Hart, D. J. *J. Am. Chem. Soc.* **1980**, *102*, 397.

(5) (a) Satisfactory elemental analysis and spectroscopic data (NMR, IR, UV, MS) have been obtained for this substance. (b) Satisfactory spectroscopic data (NMR, IR, UV, MS) have been obtained for this substance.

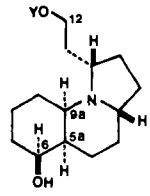
(6) Numbering in this paper corresponds to that of gephyrotoxin; see ref 2 and also structure **1** of this paper.

(7) This ratio was based on (a) the isolated yield of **5** and its stereoisomer; (b) the isolated yield of **10** and its stereoisomer and also of **11** and its stereoisomer; (c) the high-performance LC analysis. Base-line to base-line separation of the two diastereomers was observed for the compounds having X = CH_2CO_2Et, Y = COC_6H_5 in structures **15** and **16**.

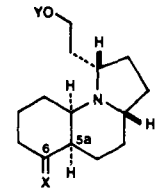
0002-7863/80/1502-7154$01.00/0



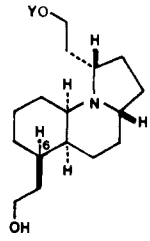
8, X = OH
9, X = H



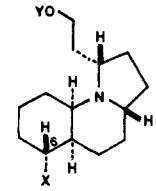
10, Y = H
11, Y = Ac



12, X = O, Y = Ac
13, X = $CHCO_2Et$, Y = H
14, X = $CHCO_2Et$, Y = Ac



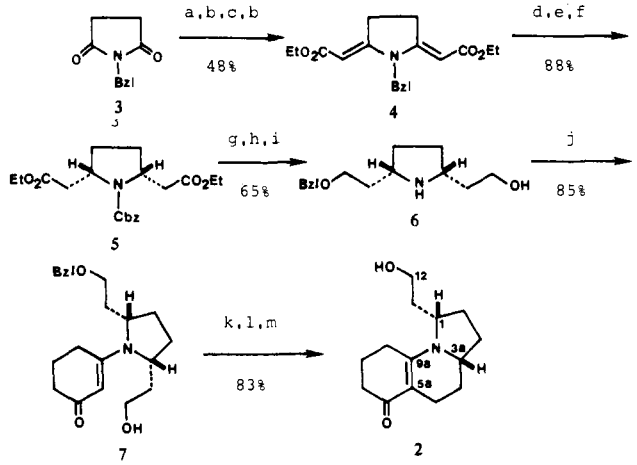
15, Y = $Si(C_6H_5)_2Bu$-*t*



16, X = CH_2CH_2OH; Y = $Si(C_6H_5)_2Bu$-*t*
17, X = $CH_2CH_2CH_2CH_2CH_3$; Y = H
18, X = CH_2CHO; Y = $Si(C_6H_5)_2Bu$-*t*
19, X = $CH_2CH{=}CHCHO$ (cis),
Y = $Si(C_6H_5)_2Bu$-*t*

reochemical outcome of the hydrogenation was found to be reversed by using catalysts on an alumina support. The best result, a 12:1 ratio[7b] of the amino alcohol **10**[5b] over **8**, was realized by using 5% platinum on alumina in anhydrous ethyl acetate at 60 psi hydrogen pressure at room temperature. In practice, the amino alcohol **10** was conveniently isolated as its monoacetate **11**[5b] (Ac_2O, room temperature; NMR ($CDCl_3$) δ 4.09 (2 H, t, J = 7.0 Hz), 3.71 (1 H, m), 2.02 (3 H, s)). The overal yield of **11** from **2** was 61%. The C-5a, C-6, and C-9a stereochemistry of **11** was tentatively assigned as indicated from the following considerations: First, the ketone prepared from **11** was different from the ketone obtained from the monoacetate of **8**. Assuming that the hydrogenation of **7** took place in the cis fashion on the C-5a and C-9a olefinic bond, this experiment concluded the C-5a and C-9a stereochemistry. Second, the reduction of the ketone **12** yielded exclusively **11** (vide infra). Assuming the stereochemistry assignment made for the C-5a and C-9a positions was correct, this experiment suggested the assignment of the C-6 stereochemistry—see a molecular model of **12**. It is interesting

Scheme I[a]



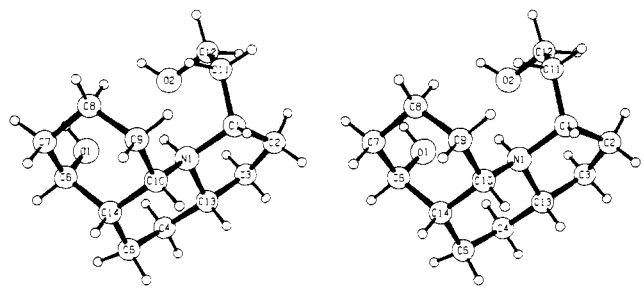
[a] Reagents: (a) EtOC≡CMgCl/THF/RT. (b) 5% HCl/0 °C. (c) EtOC≡CMgBr/THF/RT. (d) H_2 (60 psi)/10% Pd-C/$HClO_4$/MeOH/RT. (e) C_6H_5OCOCl/Py/CH_2Cl_2/RT. (f) Chromatographic separation. (g) $LiBH_4$/THF/RT. (h) (1) KH/THF/RT, (2) $C_6H_5CH_2Br$/DMF/RT. (i) $Ba(OH)_2/H_2O/\Delta$. (j) Cyclohexane-1,3-dione/py·TsOH/C_6H_6/Δ. (k) MsCl/Et_3N/CH_2Cl_2/RT. (l) LiBr/DMF/RT. (m) H_2/10% Pd-C/$HClO_4$/MeOH/RT. RT = room temperature.

to point out the fact that hydrogenation of the acetate, the silyl ether, or the C-12 deoxy derivative of **2** gave no reduction under the same conditions.[9]

Oxidation of **11**[5b] ($(COCl)_2$/Me_2SO, Et_3N/−65 °C → room temperature)[10] gave the ketone ($CDCl_3$) (NMR ($CDCl_3$) δ 4.10 (2 H, t, J = 7.0 Hz, 2.03 (3 H, s)) in 89% yield. Upon sodium borohydride or L-Selectride reduction, **12** gave back the alcohol **11** quantitatively, which confirmed that there was no epimerization at the C-5a position during the oxidation. Reaction of the ketone **12** with ethoxyacetylenemagnesium chloride in THF, followed by addition of methylmagnesium bromide to cleave the C-12 acetate group and then by acid workup, gave a 1:1 mixture of *E*- and *Z*-unsaturated esters **13**. Although hydrogenation (5% Rh on Al_2O_3/EtOH/60 psi H_2/room temperature) gave a 1:1 mixture of the C-6 stereoisomers of the saturated esters, we were able to obtain either isomer with high stereoselectivity in the following manner: The acetates **14**, prepared from **13** under standard conditions, were reduced by lithium in liquid ammonia to a saturated ester. Silylation (*t*-Bu$(C_6H_5)_2SiCl$/imidazole/DMF/room temperature) followed by lithium aluminum hydride reduction gave a 35:1 mixture[7c] of the diastereomeric alcohols **15**[5b] (NMR ($CDCl_3$) δ 3.64 (4 H, m), 0.96 (9 H, s)) and **16**[5b] (NMR ($CDCl_3$) δ 3.66 (4 H, m), 1.05 (9 H, s)). On the other hand, hydrogenation (5% Rh on Al_2O_3/1 atm H_2/hexane/−20 °C) of the *tert*-butyldiphenylsilyl ether of **13** (*t*-Bu$(C_6H_5)_2SiCl$/imidazole/DMF/room temperature) followed by lithium aluminum hydride reduction yielded a 10:1 mixture[7c] favoring **16** over **15**.

Since the introduction of the bulky silyl group had a dramatic effect upon the stereoselectivity of the last hydrogenation, we felt confident that the hydrogenation sequence gave the axial isomer **16**, while the dissolving metal reduction sequence gave the equatorial isomer **15**. These assignments were confirmed by successful conversion of the alcohol **16** to perhydrogephyrotoxin in four steps [(1) PCC/CH_2Cl_2/room temperature; (2) $CH_3CH_2CH{=}P(C_6H_5)_3$/THF/0 °C; (3) 5% Rh on Al_2O_3/EtOH/60 psi H_2/room temperature; (4) $Bu_4N^+F^-$/DMF/room temperature] in 84% overall yield. Synthetic (±)-perhydrogephyrotoxin (**17**) was found to be identical (NMR, IR, MS,

(8) Crystals of the *p*-bromobenzenesulfonate salt of **8** are orthorhombic, space group $Pbc2_1$, with a = 10.848 (1), b = 12.717 (1), c = 15.433 (1) Å, and d_{calcd} = 1.486 g cm^{-3} for Z = 4 ($C_{14}H_{25}NO_2{\cdot}C_6H_5BrO_3S$, M_r = 476.44). The intensity data were measured on a Hilger–Watts diffractometer (Ni filtered Cu Kα radiation, θ–2θ scans, pulse height discrimination). A crystal measuring approximately 0.08 × 0.12 × 0.4 mm was used for data collection; the data were corrected for absorption (μ = 40.4 cm^{-1}). A total of 1503 reflections were measured for $\theta > 57°$, of which 1385 were considered to be observed ($I > 2.5\sigma(I)$). The structure was solved by the heavy atom method and was refined by full-matrix least squares. In the final refinement anisotropic thermal parameters were used for the heavier atoms and isotropic temperature factors were used for the hydrogen atoms. The hydrogen atoms were included in the structure factor calculations but their parameters were not refined. The final discrepancy indices are R = 0.023 and wR = 0.026 for the 1385 observed reflections. The final difference map has no peaks greater than ±0.2 e Å^{-3}. The stereoscopic drawing of the cation is shown below. Note that the numbering of the drawing does not correspond to that of gephyrotoxin.



(9) For the directing effect of a hydroxy group in hydrogenation, for example, see: Eisenbraun, E. J.; George, T.; Riniker, B.; Djerassi, C. *J. Am. Chem. Soc.* **1960**, *82*, 3648. Thompson, H. W.; Naipawer, R. E. *J. Am. Chem. Soc.* **1973**, *95*, 6379.

(10) Omura, K.; Swern, D. *Tetrahedron* **1978**, *34*, 1651.

TLC) with the authentic sample[11] derived from natural gephyrotoxin.

Having confirmed the stereochemical assignment, all that remained was the introduction of the *cis*-enyne unit. The alcohol **16** was oxidized to the aldehyde **18**[5b] (PCC/CH_2Cl_2/room temperature; NMR ($CDCl_3$) δ 9.69 (1 H, br s), 3.62 (2 H br t, J = 6.0 Hz)). Wittig reaction of **18** ($EtOCH{=}CHP^+(C_6H_5)_3Br^-$/NaOEt/room temperature)[12] followed by acid hydrolysis (*p*-TSA/acetone/H_2O/0 °C) yielded the unstable cis-unsaturated aldehyde **19**[5b,13] (NMR ($CDCl_3$) δ 10.02 (1 H, d, J = 8.0 Hz), 6.56 (1 H, m), 6.02 (1 H, dd, J = 10.0, 8.0 Hz), 3.61 (2 H, t, J = 6.0 Hz)). The Corey method to convert *cis*-enals to *cis*-enynes [(1) $ClCH_2P^+(C_6H_5)_3Cl^-$/BuLi/THF; (2) MeLi/THF, Me_3SiCl; (3) $Bu_4N^+F^-$/DMF][14] was applied to **19** to give synthetic (±)-gephyrotoxin (**1**) in about 45% overall yield from **16**. The synthetic substance was found to be identical with natural gephyrotoxin[13,15] by comparison of ^1H NMR (C_6D_6) and mass spectra as well as TLC behavior (Merck Al_2O_3 (1:4 acetone–hexane); Merck silica gel (22:1:0.15 chloroform–2-propanol–aqueous ammonia)).

Acknowledgment. Financial assistance from the National Institutes of Health, NS 12108, is gratefully acknowledged.

Supplementary Material Available: Spectra (NMR, IR, MS) of new compounds described in this paper (33 pages). Ordering information is given on any current masthead page.

(11) We are indebted to Professor Overman, University of California, Irvine, for providing a sample of perhydrogephyrotoxin.
(12) Bestmann, H. J.; Roth, K.; Ettlinger, M. *Angew. Chem., Int. Ed. Engl.* **1979**, *18*, 687.
(13) Contamination by the corresponding trans isomer, if any, is less than 5% (^1H NMR in $CDCl_3$).
(14) Corey, E. J.; Ruden, R. A. *Tetrahedron Lett.* **1973**, 1495.
(15) We are indebted to Dr. Daly, National Institutes of Health, for providing a sample of natural gephyrotoxin.

Roger Fujimoto, Yoshito Kishi*
Department of Chemistry, Harvard University
Cambridge, Massachusetts 02138

John F. Blount
Research Division, Hoffmann-La Roche Inc.
Nutley, New Jersey 07110

Received July 3, 1980

Development of a Convenient Spectrophotometric Assay for Peptide Phosphorylation Catalyzed by Adenosine 3′,5′-Monophosphate Dependent Protein Kinase

Sir:

Bovine cardiac muscle adenosine 3′,5′-monophosphate dependent protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase) catalyzes the transfer of the terminal phosphoryl group from adenosine 5′-triphosphate to the serine hydroxyl of peptide or protein substrates.[1] Currently, protein kinase is assayed by using adenosine 5′-[γ-^{32}P]triphosphate and quantitating the transfer of the labeled phosphoryl residue to a peptide or protein substrate. This method is inconvenient since (1) it does not permit continuous assay and requires removal of unreacted nucleotide before the amount of radioactive material transferred is determined for each time point; (2) adenosine 5′-[γ-^{32}P]triphosphate is expensive and has a limited shelf life; (3) the use of radioactive materials requires special handling. If a reactive peptide substrate for protein kinase could be prepared which would undergo a significant spectral change upon phosphorylation, kinetic and mechanistic studies of the enzyme would be greatly facilitated. In the present report we wish to describe our finding that phosphorylation of the Ser residue in the reporter group labeled heptapeptide Leu-Arg-Arg-(*o*-NO_2)Tyr-Ser-Leu-Gly (**1**) catalyzed by the catalytic subunit of protein kinase at pH 7.5 causes a spectral change at 430 nm which permits us to monitor this reaction continuously.

The synthesis of peptide **1** was carried out by the solid-phase method employing polystyrene-bound *p*-nitrobenzophenone oxime as the support.[2] Starting with BocLeu resin (substitution level 0.5 mmol/g), the symmetric anhydrides of BocSer(Bzl), Boc(*o*-NO_2)Tyr(Bzl),[3] BocN^{g}TosArg, and BocLeu were coupled in threefold excess, resulting in BocLeu-(Tos)Arg-(Tos)Arg-(*o*-NO_2)Tyr(Bzl)-Ser(Bzl)-Leu-oxime polymer. The fully protected heptapeptide was obtained by using GlyOBzl·HOAc to displace hexapeptide from the oxime resin and was purified by chromatography on LH 20. Removal of all protecting groups by treatment with HF[4] followed by chromatography on Sephadex G-15 and CM Sephadex C-25 gave peptide **1** in 28% yield, based on the initial substitution level on the resin. The amino acid analysis (Arg (2.0), Gly (1.0), Leu (2.0), Ser (0.91), and (*o*-NO_2)Tyr (1.0)), the 270-MHz NMR spectrum, and the UV-visible spectrum of peptide **1** were consistent with the structure postulated. No impurities were detected by thin-layer chromatography.

The protein kinase catalyzed phosphorylation of peptide **1** was carried out at 30.0 °C in 50 mM Tris buffer, pH 7.5, containing 10 mM $MgCl_2$, 0.15 M KCl, 0.2 mM dithiothreitol, and 0.2 mg/mL bovine serum albumin. Typically, 6.54 nM catalytic subunit, 2.00 mM ATP, and between 25 and 200 μM peptide **1** were employed in the spectrophotometric assays which were performed by using a Cary 219 spectrophotometer. A decrease in ϵ_{430} of 210 M^{-1} was measured when peptide **1** was phosphorylated, and the entire time course of reaction was monitored in the spectrophotometric experiments. To check the validity of the spectrophotometric assays, rates for the transfer of the γ-phosphoryl group from adenosine 5′-[γ-^{32}P]triphosphate to peptide **1** were measured as described previously,[5] except that the entire course was observed. The kinetics of peptide phosphorylation catalyzed by bovine cardiac muscle catalytic subunit have been shown to be consistent with the sequential mechanism[6] illustrated in eq 1.[7] Since the phosphopeptide product is not inhibitory and

$$\begin{aligned} E + MgATP \rightleftharpoons E{\cdot}MgATP + peptide \rightleftharpoons \\ E{\cdot}MgATP{\cdot}peptide \rightarrow E{\cdot}MgADP + phosphopeptide \rightleftharpoons \\ E + MgADP \end{aligned} \quad (1)$$

$$v = \frac{k_{cat}[E]_0[peptide]}{[peptide] + K_{m,peptide}} \quad (2)$$

MgATP is present in large excess, the kinetics of the phosphorylation of peptide **1** measured under the conditions of our experiments can be analyzed by using eq 2. For those reactions where the entire time course was monitored, values for k_{cat} and $K_{m,peptide}$ were obtained by using an iterative curve fitting program (by B. Blumenstein of Emory University) for an IBM 370 com-

(1) Kemp, B. E.; Graves, D. J.; Benjamini, E.; Krebs, E. G. *J. Biol. Chem.* **1977**, *252*, 4888.
(2) DeGrado, W. F.; Kaiser, E. T. *J. Org. Chem.* **1980**, *45*, 1295.
(3) L-*o*-NO_2-Tyrosine (Aldrich) was benzylated by a modification of the procedure described by Erickson et al. [Erickson, B. W.; Merrifield, R. B. *J. Am. Chem. Soc.* **1973**, *95*, 3750] for the synthesis of L-*o*-(2,6-dichlorobenzyl)tyrosine. The product was treated with di-*tert*-butyl dicarbonate (Pierce) according to the general protocol described by: Moroder, L.; Hallet, A.; Wunsch, E.; Keller, O.; Wersin, G. *Hoppe-Seyler's Z. Physiol. Chem.* **1976**, *357*, 1651. Because the product did not crystallize, 1 equiv of dicyclohexylamine (DCHA) was added to yield DCHA N^{α}-Boc-*O*-benzyl-L-*o*-nitrotyrosine. After filtration the product was recrystallized at −20 °C from a minimum of methanol to which an equivalent amount of ether and a small amount of hexane had been added. The purified DCHA salt had mp 125 °C, $[\alpha]_D$ 20.9° (*c* 0.028, CH_3OH), and a satisfactory elemental analysis. Prior to coupling it was suspended in 5% citric acid and ethyl acetate (1:1, v/v). Boc-L-*O*-benzyl-nitrotyrosine was extracted into the organic layer which was dried over $MgSO_4$ and evaporated to give a viscous oil.
(4) Sakakibara, S.; Shimonishi, Y.; Kishida, Y.; Okada, M.; Sugihara, H. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 2164.
(5) Armstrong, R. N.; Kondo, H.; Granot, J.; Kaiser, E. T.; Mildvan, A. S. *Biochemistry* **1979**, *18*, 1230.
(6) Pomerantz, A. H.; Allfrey, G.; Merrifield, R. B.; Johnson, E. M. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 4261.
(7) Bolen, D. W.; Stingelin, J.; Bramson, H. N.; Kaiser, E. T. *Biochemistry* **1980**, *19*, 1176.

0002-7863/80/1502-7156$01.00/0