or stored frozen until further use.

**Qualitative Analysis by Thin-Layer Chromatography.** Plasma was processed for TLC by placing a 50-μL aliquot into a 175-μL plastic tube designed for use in an airflo-type ultracentrifuge (Beckman Airfuge) and carefully layering 50 μL of PBS on top of the plasma. Centrifugation for 2 h at 165000*g*, followed by removal of the top 50 μL from the tube by means of a micropipet, yielded a solution which was nearly protein free. The same procedure was followed in order to obtain deproteinized tumor extracts. TLC was performed on cellulose sheets (Eastman 13254) by spotting 20-μL aliquots, developing with 0.1 M potassium phosphate buffer (pH 7.2), and visualizing the spots in an ultraviolet viewing chamber at 254 nm. The $R_f$ values for authentic reference samples of compound 12 and MTX in this solvent system were 0.60–0.65 and 0.80–0.85, respectively; deliberately prepared mixtures of the two compounds were readily separable. Only minute traces of MTX were detected in the sample of 12 used in this experiment. The 0.5-h plasma samples from mice treated with MTX or compound 12 contained enough drug to allow qualitative analysis by TLC, whereas the levels in tumor and brain extracts were too low for this method to be used. The only UV-absorbing spot in the 0.5-h plasma samples from MTX-treated mice according to TLC analysis was unchanged MTX. Similarly, there was no evidence of significant bioconversion to MTX in the 0.5-h plasma sample from the animal treated with compound 12. Thus, it appears that drug levels measured by radioimmunoassay (see below), at least in plasma at 0.5 h, represent mainly compound 12. However, because of the limited sensitivity of the TLC system at the very low concentrations involved in this experiment, it is possible that up to 10% hydrolysis to MTX could fail to be detected in dilute tissue samples.

**Quantitative Radioimmunoassay Measurements.** Levels of compound 12 or MTX were determined by means of an assay based on the competitive binding of [$^3$H]MTX to an anti-MTX antibody; the assay was adapted with some changes from a published procedure.[13] Goat anti-MTX antibody was obtained from Cappel Laboratories, Cochranville, PA, and was diluted 4000-fold prior to use. The $ID_{50}$ for competitive binding of [$^3$H]MTX to the antibody was 7.6 ± 0.91 nM for MTX and 10.0 ± 0.1 nM for compound 12, i.e., the antibody was almost completely cross-reactive toward these drugs. In a typical experiment, 100 μL of 0.1 M potassium phosphate buffer (pH 6.2) containing 0.1% BSA and approximately $1 \times 10^4$ counts/min of [$^3$H]MTX was added to each of eight wells of a microtiter plate. To each well was then added 25 μL of a 6 μM solution of compound 12 or MTX (or of an appropriately diluted unknown sample of plasma or tissue extract), and serial 5-fold dilutions were made as described above for the enzyme-binding assay. The eighth well (control) contained no competitive ligand. Buffer-diluted antibody (50 μL) was added to each well, and after 1 h at 37 °C the plate was cooled in ice for 5 min and 50 μL of Norite mixture was added to remove nonbound ligand. The charcoal was sedimented by centrifugation (7 min, 640*g*, 0 °C), and 100-μL aliquots of supernatant were removed and counted in 5.0 mL of Biofluor. Results were recorded on a logit-log scale, and the dilution of compound 12 or MTX causing a 50% decrease in binding was determined. Appropriate corrections were made for dilutions during workup of tissue samples in order to arrive at the concentration of drug in the original sample. The dose–response curve was linear over a 4-log range of concentration. Three mice were used for each time point in the MTX experiment, in order to compare interanimal differences with the experimental error in replicate measurements on a single sample. Interanimal variation was found to be equal to, or at most 3-fold greater than, this error.

**Acknowledgment.** This work was supported in part by Grant CH-23 from the American Cancer Society and by Project Grant CA 25394 and Cancer Center Support Grant CA 06516 from the National Cancer Institute, National Institutes of Health. The technical assistance of Linda Anderson (antitumor assays), Carol Ginty and Chitra Squires (enzyme inhibition and ligand-binding studies), and Selma Oppenheim and Gerda Swedowsky (cytotoxicity measurements) is gratefully acknowledged.

# Analogues of the Cytostatic Cyclic Tetrapeptide Chlamydocin. Synthesis of $N^{\beta}$-(*N*-Maleoylglycyl) and $N^{\beta}$-(*tert*-Butyloxycarbonyl) Derivatives of *cyclo*(Gly-L-Phe-D-Pro-L-Dap)[1]

Daniel H. Rich,* Ronald D. Jasensky,

*School of Pharmacy*

Gerald C. Mueller, and Kristin E. Anderson

*McArdle Research Laboratories, University of Wisconsin—Madison, Madison, Wisconsin 53706.* *Received October 20, 1980*

The synthesis of analogues of the cytostatic cyclic tetrapeptide chlamydocin is described. *cyclo*(Gly-L-Phe-D-Pro-$N^{\beta}$-Boc-L-Dap) (4) was prepared from $N^{\beta}$-(*tert*-butyloxycarbonyl)-L-diaminopropionic acid methyl ester (Dap) and Cbz-Gly-L-Phe-D-Pro using DCC/HOBt as the coupling reagent. The methyl ester was saponified to the acid, which was converted to the 2,4,5-trichlorophenyl (Tcp) ester by reaction with trichlorophenol and DCC. The *N*-(benzyloxycarbonyl) group was removed by hydrogenolysis and the amine active ester cyclized at 95 °C in pyridine. The Boc-protected cyclic tetrapeptide 4 was isolated in 14% yield. Cyclic tetrapeptide 4 was converted to *cyclo*-[Gly-L-Phe-D-Pro-$N^{\beta}$-(*N*-maleoylglycyl)-L-Dap] (5) to test for a possible sulfhydryl group at the chlamydocin receptor. Removal of the *tert*-butyloxycarbonyl group, followed by reaction with *N*-maleoylglycine and DCC/HOBt in methylene chloride, gave cyclic tetrapeptide 5 in 68% yield. The maleoyl cyclic tetrapeptide 5 did not inhibit [$^3$H]thymidine incorporation into calf thymus lymphocytes at concentrations 1000-fold higher than the $IC_{50}$ for chlamydocin (6 nM).

Chlamydocin, *cyclo*[α-aminoisobutyryl-L-phenylalanyl-D-prolyl-L-2-amino-8-oxo-9,10-epoxydecanoyl] (1), was isolated and characterized by Closse and Huguenin[2] as a result of their efforts to identify a cytostatic agent detected

0022-2623/81/1824-0567$01.25/0 

cyclo (A$^i$bu-L-Phe-D-Pro-L-Aoe) (1)

where Aoe = –NH–CH(CO–)(CH$_2$)$_5$–C(=O)–CH(O)–CH$_2$

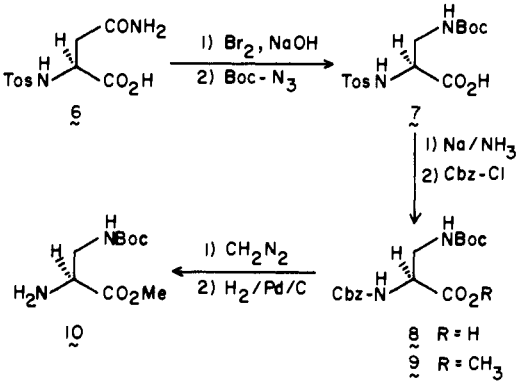
in culture filtrates of *Diheterospora chlamydosporia*. Stähelin and Trippmacher later reported that chlamydocin inhibited proliferation of P-815 mouse mastocytoma and C6 rat glial tumor cells at very low concentrations ($ED_{50}$ of $3 \times 10^{-10}$ g/mL),[3] concentrations about 10-fold lower than found for actinomycin D, vinblastine, vincristine, amethopterin, colchicine, or mechlorethamine in this assay system. In vivo chlamydocin was found to be less cytostatic. This appears to be due to rapid enzymatic inactivation and not sequestration or elimination, because plasma which had been heated to 65 °C for 30 min before addition of chlamydocin did not inactivate the compound.[3] The mode of activation is unknown, but the very low cytostatic activity of the reduced carbonyl derivative, dihydrochlamydocin (**2**), suggested that loss of activity could be due to modification of the epoxy ketone group.

While investigating the solution conformations of cyclic tetrapeptides related to chlamydocin,[4-6] we discovered that *cyclo*(Gly-L-Phe-D-Pro-L-Ala) (**3**) adopts a conformation in chloroform that is closely related to the conformation of chlamydocin observed in the crystalline state[7] and in solution.[4] This result established that the α-aminoisobutyryl residue is not essential for the novel conformation of the cyclic tetrapeptide ring system that had been observed for the first time in chlamydocin[4,7] and suggested that derivatives of **3** might become cytostatic when alanine is replaced by the epoxy ketone amino acid Aoe or by other amino acids with side chains capable of alkylating biological nucleophiles. Toward this objective we have developed a synthesis of *cyclo*(Gly-L-Phe-D-Pro-$N^{\beta}$-Boc-L-Dap) (**4**), a precursor for alkylating analogues of cyclic tetrapeptides **1** and **3**. The synthesis and biological properties of an *N*-maleoylglycyl derivative (**5**) are also described.

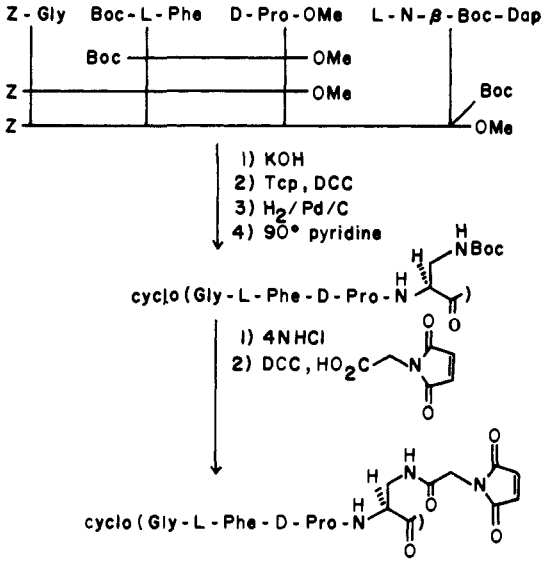
## Results

**Synthesis of $N^{\beta}$-(*tert*-Butyloxycarbonyl)-L-α,β-diaminopropionic Acid Methyl Ester (β-Boc-L-Dap-OMe) (9).** $N^{\alpha}$-Tos-L-Dap was prepared from Tos-L-Asn (**6**) in 51% yield (Scheme I) by using a modification of the reported procedure.[8] Protection of the β-amino group by reaction with *tert*-butyloxycarbonyl azide, removal of the tosyl group using sodium in liquid ammonia, and addition of the carbobenzoxy group gave $N^{\alpha}$-Cbz-$N^{\beta}$-Boc-L-Dap (**8**). The replacement of the α-tosyl group by the Cbz group was carried out to facilitate isolation of $N^{\beta}$-Boc-L-Dap from the reaction mixture obtained by metal reduction of

Scheme I




Scheme II




$N^{\alpha}$-Tos-$N^{\beta}$-Boc-L-Dap (**7**). Methylation of the carboxyl group gave ester **9**, which after careful hydrogenolysis under neutral conditions gave the desired $N^{\beta}$-Boc-L-Dap-OMe (**10**) in 22% overall yield from Tos-L-Asn (**6**).

**Synthesis of *cyclo*(Gly-L-Phe-D-Pro-$N^{\beta}$-Boc-L-Dap) (4) and *cyclo*[Gly-L-Phe-D-Pro-$N^{\beta}$-(*N*-Mal-Gly)-L-Dap] (5).** The Boc-protected cyclic peptide **4** was synthesized as shown in Scheme II. The linear tripeptide **12**, synthesized via the stepwise addition or protected amino acids to D-Pro-OMe in solution, was coupled to $N^{\beta}$-Boc-L-Dap-OMe **10** using DCC/HOBt to give tetrapeptide **13**. The usual deprotection–cyclization sequence[5] was modified to permit removal of the Cbz protecting group in the presence of a Boc protecting group. Saponification of the linear tetrapeptide **13** was followed by active ester formation, careful removal of the Cbz group by hydrogenation, and cyclization in pyridine to give Boc-protected cyclic peptide **4** (14%) as a highly crystalline, stable solid. The chiralities of the L-Phe and D-Pro residues were established by the L-amino acid oxidase procedure.[5,9] Because L-Dap is only weakly susceptible to L-amino acid oxidase, the chirality of this residue was established by comparing cyclic tetrapeptide **4** with cyclic tetrapeptide **3**, which contains an L-alanine in position 4. The $^{13}C$ and $^{1}H$ NMR data for **4** were consistent with a cyclic tetrapeptide containing an L-amino acid in position 4, and the circular dichroism spectra of **3** and **4** were nearly identical.

(1) Abstracted in part from: Jasensky, R. D. Ph.D. Thesis, University of Wisconsin, Madison, WI 1979. Abbreviations used follow IUPAC-IUB tentative rules as described in *J. Biol. Chem.* **1972**, *247*, 977. Additional abbreviations used are: DCC, dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; Boc, *tert*-butyloxycarbonyl; A$^i$bu, α-aminoisobutyric acid; Dap, α,β-diaminopropionic acid; Aoe, 2-amino-8-oxo-9,10-epoxydecanoic acid; *N*-Mal-Gly, *N*-maleoylglycine.

(2) Closse, A.; Huguenin, R. *Helv. Chim. Acta* **1974**, *57*, 533–545.
(3) Stähelin, H.; Trippmach, A. *Eur. J. Cancer* **1974**, *10*, 801–808.
(4) Rich, D. H.; Jasensky, R. D. "Peptides: Structure and Biological Function"; Gross, E.; Meienhofer, J. Eds.; Pierce Chemical Co.: Rockford, IL, 1979; pp 487–490.
(5) Rich, D. H.; Jasensky, R. D. *J. Am. Chem. Soc.* **1979**, *101*, 5412.
(6) Rich, D. H.; Jasensky, R. D. *J. Am. Chem. Soc.* **1980**, *102*, 1112.
(7) Flippen, J.; Karle, I. *Biopolymers* **1976**, *15*, 1081–1092.
(8) Rudinger, J.; Poduska, K.; Zaoral, M. *Collect. Czech. Chem. Commun.* **1960**, *25*, 2022.
(9) Sieber, P.; Riniker, B.; Brugger, M.; Kamber, B.; Rittel, W. *Helv. Chim. Acta* **1970**, *53*, 2135.

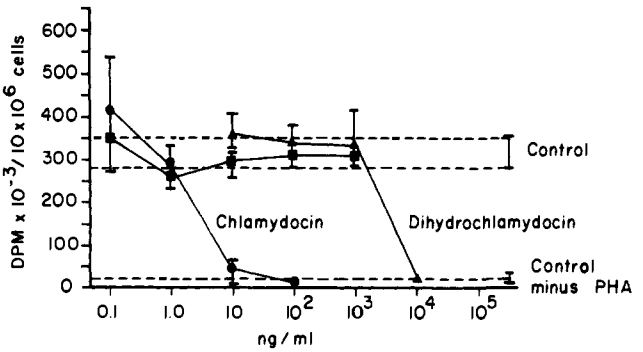



**Figure 1.** Inhibition of tritiated thymidine incorporation into calf thymus lymphocytes 48 h after stimulation with phytohemaglutinin (PHA): chlkamydocin (●); dihydrochlamydocin (△); *N*-maleoylglycyl cyclic tetrapeptide **5** (■). Control with and without added PHA is indicated by the horizontal dashed lines. The data are an average of 3 separate determinations.

These data establish that the chirality of the Dap residue in **4** must be L. Formation of the trichlorophenyl ester derivative racemized the C-terminal Dap residue as shown by a doubling of resonances in the $^1$H NMR and by the detection of two diastereomeric tetrapeptide active esters by thin-layer chromatography. The cyclization reaction leading to the Boc-protected cyclic peptide **4** via a racemic trichlorophenyl ester intermediate is stereoselective, since the diastereomer containing D-Dap was not isolated nor detected in the crude product mixture. The stereoselective cyclization of Gly-L-Phe-D-Pro-D-Ala-OTcp to the L-Ala cyclic tetrapeptide **3** has been described.[17]

The Boc group was removed from cyclic peptide **4** by reaction with hydrochloric acid in dioxane, and the amino group of Dap was derivatized with *N*-maleoylglycine (**14**; *N*-Mal-Gly)[10] to give the maleoyl cyclic peptide **5** in 68% yield (Scheme II). This peptide tended to decompose by reaction of the maleimide with the solvent and was difficult to purify. $^1$H NMR data indicated that small amounts of maleamic acid esters were formed during preparative layer chromatography purification using alcoholic solvents. Although the parent ion was not detected by mass spectrometry, maleoyl peptide **5** did give a satisfactory amino acid analysis. $^1$H NMR data established that the maleimide group was present in the purified sample.

**Biological Results.** The biological activities of maleoyl peptide **5**, chlamydocin (**1**), and dihydrochlamydocin (**2**) were measured in an in vitro assay, which measured the percent incorporation of [$^3$H]thymidine into calf thymus lymphocytes 48 h after stimulation with phytohemaglutinin (PHA).[12] This assay is sensitive to any agent which interfered with the rapid normal uptake of DNA precursors by the rapidly growing cells. As shown in Figure 1, chlamydocin is an extremely potent inhibitor in this system ($IC_{50}$ = 6 nM) and causes a return to prestimulatory levels of [$^3$H]thymidine incorporation at 10 ng/mL (20 nM). This strong inhibition parallels chlamydocin's behavior in the in vitro mouse mastocytoma assay ($ED_{50}$ = 0.3 ng/mL).[3] Dihydrochlamydocin (**2**) inhibits [$^3$H]thymidine uptake only at 1000-fold greater concentrations. Because this weak inhibition could be due to traces of chlamydocin formed from dihydrochlamydocin by oxidation in the assay, the dihydrochlamydocin was purified by

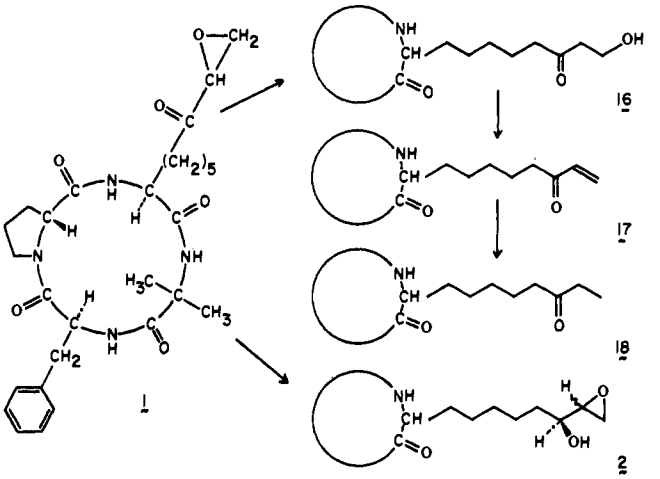



**Figure 2.** Synthetic, modified side-chain analogues of chlamydocin (**1**) reported by Closse and Huguenin.[2]

preparative layer chromatography and analyzed by TLC, NMR, or mass spectrometry. The dihydrochlamydocin contained less than 0.1% chlamydocin. A determination of the exact amount of chlamydocin in these samples has been deferred until labeled compound is available. The maleoyl peptide **5** does not inhibit [$^3$H]thymidine incorporation into calf thymus lymphocytes at concentrations of 1 μg/mL (2 μM) (Figure 1). In two separate experiments (data not shown), 20–40% inhibition was detected at very high concentrations of **5** (0.2 mM).

## Discussion

Chlamydocin possesses unusual cytostatic properties in in vitro assay systems, but the activity of chlamydocin in vivo has been limited by its rapid inactivation in whole serum by a process which appears to be enzymatically catalyzed. The mechanism by which chlamydocin is inactivated is not known, but one probable site of inactivation would be the epoxy ketone group found in the amino acid Aoe. Reduction of the carbonyl group, as in dihydrochlamydocin (**2**), or opening of the epoxide ring, as in derivatives **16**–**18** (Figure 2), has been shown to produce derivatives with drastically reduced cytostatic activities.[2,3] Each of these derivatives could be formed by known in vivo metabolic processes.

As part of a program to develop chlamydocin analogues which would be more resistant to degradation, we developed a route to cyclic tetrapeptide **4**. This derivative is formed from chlamydocin by replacing the α-aminoisobutyryl residue in position 1 with a glycyl group and the Aoe residue in position 4 with a diaminopropionic acid (Dap) unit. The 1st position substitution was chosen because conformational studies have shown that the ring conformation of the Gly-containing cyclic tetrapeptide **3** in chloroform is very similar to the ring conformation of chlamydocin,[4,6] and therefore this amino acid replacement is less likely to alter the biological properties of the cyclic tetrapeptide ring system. However, replacement of the Aoe unit in the 4th position with derivatives of L-Dap is clearly a more disruptive substitution, as two methylene groups are replaced by a larger and more conformationally constrained carboxamide group. We recognized that this replacement might preclude any biological activity; nevertheless, cyclic tetrapeptide **4** was chosen as the starting point in our search for more stable analogues because of its accessibility and because of the ease with which a number of potential alkylating ligands might be attached to the peptide ring system.

The synthesis of cyclic tetrapeptide **4** was carried out as shown in Scheme II. Removal of the benzyloxycarbonyl

(10) Rich, D. H.; Gesellchen, P. D.; Tong, A.; Cheung, A.; Buckner, C. K. *J. Med. Chem.* **1975**, *18*, 1004.
(11) Rittel, W.; Schwyzer, R. *Helv. Chim. Acta* **1961**, *44*, 159.
(12) Wertz, P. W.; Kensler, T. W.; Mueller, G. C.; Verma, A.; Boutwell, R. K.; *Nature (London)* **1979**, *277*, 227 and references therein.

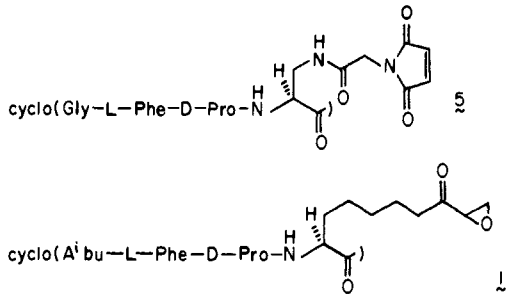



**Figure 3.** A comparison of the maleimide analogue **5** with chlamydocin (1). The site of alkylation in maleimide **5** would be 7 or 8 atoms removed from the cyclic peptide backbone. The same distance separates the epoxide group in 1 from the peptide backbone.

group from **13** required careful control of the hydrogenolysis conditions to avoid removing the β-(*tert*-butyloxycarbonyl) group from diaminopropionic acid, and the method of Schwyzer was utilized.[11] Conversion of **13** to the cyclic tetrapeptide **14** was achieved using standard conditions developed for synthesizing other cyclic tetrapeptides,[5] and no attempt was made to optimize conditions for the cyclization of this peptide. Interestingly, it was found that this cyclization reaction, like that of H-Gly-L-Phe-D-Pro-L-Ala-OTcp,[4,17] is stereospecific. No D-Dap derivative is formed even though preparation of the trichlorophenyl ester led to almost complete racemization of the C-terminal amino acid. A detailed study of this cyclization reaction will be reported separately.

The successful synthesis of the *N*-maleoylglycyl cyclic tetrapeptide **5** established that deprotection and subsequent acylation of the β-amino group in **4** can be carried out using standard methods for synthesizing side-chain functionalized peptides. Thus, the nonplanar amide bonds (ω-twist angle = ±15–25°) that have been observed in this cyclic tetrapeptide ring system[4,7] are not unusually reactive toward either the free β-amino group in deprotected **4** or the deprotection, neutralization, and coupling reagents used here.

Cyclic tetrapeptide **4** was converted to the *N*-maleoylglycyl derivative **5**, to test for possible sulfhydryl group involvement in the action of chlamydocin. Some antitumor agents are thought to alkylate thiol groups,[13] and retention of cytostatic activity would suggest the presence of a sulfhydryl group at or near the binding site of chlamydocin, since maleimides selectively alkylate sulfhydryl groups.[10] The *N*-maleoylglycyl group was chosen as the labeling group because the site of alkylation is the same number of atoms away from the cyclic tetrapeptide ring system in maleimide **5** as the epoxide group is in chlamydocin (Figure 3).

It is clear from the data presented in Figure 1 that the maleoyl derivative **5** does not inhibit proliferation of bovine lymphocytes as effectively as chlamydocin. Some inhibition was detected at very high concentrations (0.2 mM) but this appears to be relatively nonspecific and of the type expected for simple maleimides. Complete inhibition, even at millimolar concentrations of **5**, was not observed. These results do not clarify the nature of the functional groups at the chlamydocin site of action in bovine lymphocytes because cyclic tetrapeptide **5** differs from chlamydocin by three structural modifications. The extraordinarily high potency of chlamydocin in the in vitro assays suggests that chlamydocin exerts its action by binding to a receptor in a very specific fashion. Any of the structural modifications incorporated into **5** may have affected noncovalent binding or could have prevented covalent binding. Additional analogues which retain the Aoe unit at residue 4 or which have an epoxy ketone group attached to the β-amino group in **4** must be tested to distinguish these possibilities. This work is in progress using the synthetic methods reported here.

## Experimental Section

Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were determined on a Perkin-Elmer Model 241 automatic polarimeter (1.000-dm cell). Proton nuclear magnetic resonance spectra were recorded on Varian Model EM-390, Bruker HX-90E-pulse Fourier transform NMR interfaced with a Nicolet 1080 computer and disk unit, and Bruker WH270 spectrometers. Chemical shifts were reported as δ units (parts per million) relative to tetramethylsilane or 3-(trimethylsilyl-$d_4$)propionic acid, sodium salt, as internal standards. Low-resolution mass spectra were determined on a Finnigan Model 1015 mass spectrometer. High-resolution mass spectra were determined on AEI MS 902 C. TLC solvent systems used were methanol in chloroform: A, 6%; B, 15%; C, 4%; D, 10%.

**General Procedure A. Coupling Reaction Using Dicyclohexylcarbodiimide (DCC).** The amine hydrochloride (1 mmol) was dissolved in methylene chloride (5 mL), cooled (<0 °C), and triethylamine (1 mmol) was added. The acid component (1 mmol), HOBt (1 mmol),[13] and DCC (1 mmol, dissolved in methylene chloride) were added to the chilled solution, and the mixture was stirred overnight at 4 °C. The white suspension was filtered, concentrated in vacuo, suspended in ethyl acetate, chilled, and filtered. The filtrate was washed consecutively with cold 1 N HCl (3 × 20 mL), cold 0.1 N NaOH (3 × 30 mL), water (1 × 20 mL), and brine (1 × 20 mL). The solution was dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude peptide was usually purified by crystallization or column chromatography.

**General Procedure B. Cyclization Sequence to Prepare Cyclic Tetrapeptides.** The methyl ester was treated with 3 equiv of 1 N KOH in 50% aqueous ethanol. The progress of the reaction was monitored by TLC and was generally finished in 10 min. The solution was cooled, acidified to pH 2 (cold 1 N HCl), and extracted with ethyl acetate. The organic phase was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The resultant acid was used without further purification.

The 2,4,5-trichlorophenol (Tcp) ester was prepared in the following manner. The tetrapeptide acid (1 mmol) and Tcp (1.2 mmol) were dissolved in pyridine (2 mL), and the solution was cooled (0 °C) and treated with a preformed solution of DCC (1.2 mmol) in pyridine (3 mL). The solution was stirred under a drying tube at 4 °C for 20 h and then at 25 °C for 1 h. Workup consisted of chilling the mixture, filtration, concentration in vacuo (<30 °C), dissolving the residue in methylene chloride, chilling, filtration, and concentration in vacuo. The crude active ester, after thorough drying to remove pyridine, was deprotected as in general procedure C.

Cyclization of the crude ester hydrochloride was performed in the following manner. The crude salt was dissolved in 10 mL of purified DMF, filtered to remove insoluble materials, and added via syringe pump to 700 mL of pyridine at 90 °C under $N_2$. The pyridine solution was stirred vigorously and the rate of addition adjusted to conclude after 6 h. The solution was stirred an additional 2 h at 90 °C. The pyridine solution was concentrated in vacuo and the residue reevaporated twice from methanol. The brown residue was dried thoroughly in vacuo and chromatographed on a silica gel column.

**General Procedure C. Removal of the *tert*-Butyloxycarbonyl Group.** Using a modification of the reported procedure,[15] an excess of 4 N HCl in dioxane (10 mL) was added to the protected amino acid or peptide (5 mmol), and the mixture was stirred under a drying tube for 30 min at 25 °C. The solution was concentrated in vacuo, and the residue was dissolved and reevaporated twice from methylene chloride. After extensive

(13) Fujita, E.; Nagao, Y. *Bioorg. Chem.* **1977**, *6*, 287.
(14) König, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788.
(15) Carpino, L. A. *J. Am. Chem. Soc.* **1957**, *79*, 98.

drying, the amino acid or peptide hydrochlorides were used without further purification.

**$N^{\alpha}$-Tosyl-$N^{\beta}$-(*tert*-butoxycarbonyl)-L-$\alpha,\beta$-diaminopropanoic Acid (7).** Following the reported procedure,[8] bromine (8.63 mL, 167 mmol) in cold NaOH solution was allowed to react with Tos-L-Asn (6; 39.4 g, 138 mmol) to yield $\alpha$-Tos-L-diaminopropionic acid after crystallization (18.4 g, 51.7%): mp 219–220 °C; $[\alpha]^{21}_{D}$ +18.7° (*c* 2.4, 5 N HCl) [lit.[16] mp 218–219 °C (corrected); $[\alpha]^{23}_{D}$ +17.1° (*c* 2.4, 5 N HCl)]. The Tos-L-Dap (12.4 g, 47.9 mmol) was dissolved in a mixture of dioxane and 1 N NaOH and treated with *tert*-butoxycarbonyl azide (16.6 mL, 116 mmol) for 48 h at 45 °C to give after crystallization the title compound 7 as a white powder (15.2 g, 88.4%): mp 128–129 °C; $[\alpha]^{21}_{D}$ −65.8° (*c* 4, 1 N NaOH) [lit.[16] mp 128–129 °C (corrected; $[\alpha]^{22}_{D}$ −71.1° (*c* 4, 1 N NaOH)].

**$N^{\alpha}$-(Carbobenzoxy)-N-(*tert*-butoxycarbonyl)-L-$\alpha,\beta$-diaminopropanoic Acid (8).** Using a modification of the reported procedure,[15] $N^{\alpha}$-Tos-$N^{\beta}$-Boc-L-Dap (7; 12.5 g, 34.8 mmol) was dissolved in 345 mL of liquid $NH_3$. The flask was cooled to −50 °C and small pieces of sodium metal were added with vigorous stirring until the color passed from clear to yellow to green and remained blue for 10 min. The blue solution was quenched with $NH_4HCO_3$ (1 g) and allowed to evaporate overnight through a drying tube.

The resultant white solid was dissolved in ice–water (400 mL) and lyophilized. The powder was dissolved in ice–water (120 mL), cooled to 5 °C, and treated with benzyloxycarbonyl chloride (18 mL, 126 mmol) and 4 N NaOH such that the solution was pH 10–10.5. After stirring for 2 h at 25 °C, the basic solution was washed with ether (3 × 100 mL), cooled (5 °C), acidified (cold 1 N HCl) to pH 2, and extracted exhaustively with ethyl acetate. The ethyl acetate extract was washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo, and the oil crystallized (EtOAc, hexane) to yield a white powder (7.2 g, 61%): mp 135–139 °C (lit.[16] mp 145–146 °C); $R_f$ (A) 0.25, $R_f$ (B) 0.20; NMR ($CDCl_3$) $\delta$ 1.40 (s, 9 H), 3.52 (m, 2 H), 4.45 (m, 1 H), 5.1 (s, 2 H), 5.2 (br s, 1 H), 6.1 (s, 1 H), 7.34 (s, 5 H), 10.26 (s, 1 H); mass spectrum, *m/e* (relative intensity) 338 ($M^+$, 0.02).

**Methyl $N^{\beta}$-(*tert*-Butoxycarbonyl)-L-$\alpha,\beta$-diaminopropanoate (10).** Treatment of propanoic acid derivative 8 (3.92 g, 11.6 mmol) with ethereal diazomethane, followed by purification by column chromatography (1% MeOH, $CHCl_3$) and crystallization (EtOAc, hexane), gave the methyl ester 9 (3.25 g, 79.5%): mp 88–90 °C; $R_f$ (A) 0.57; NMR ($CDCl_3$) $\delta$ 1.32 (s, 9 H), 3.47 (m, 2 H), 3.64 (s, 3 H), 4.38 (m, 1 H), 5.05 (s, 2 H), 5.5 (t, 1 H, *J* = 6 Hz), 6.2 (d, 1 H, *J* = 7.8 Hz), 7.26 (s, 5 H). Boc methyl ester 9 (2.76 g, 7.84 mmol) was dissolved in 100 mL of MeOH containing 10% Pd on carbon (1.38 g). $H_2$ was passed over the stirred solution for 24 h, the mixture was filtered (Celite), and the filtrate was concentrated in vacuo (<20 °C) to give 10 as an oil (1.71 g, 100%). The product showed traces of higher $R_f$ impurities on TLC but was not further purified: $R_f$ (C) 0.24; NMR ($CDCl_3$) $\delta$ 1.42 (s, 9 H), 1.9 (br s, 2 H), 3.32 (m, 2 H), 3.57 (m, 1 H), 3.72 (s, 3 H), 5.37 (t, 1 H, *J* = 6.6 Hz); mass spectrum *m/e* (relative intensity) 219 (M + 1, 0.5), 187 (0.3), 173 (0.5), 162 (7), 145 (13), 130 (7), 117 (6), 115 (5), 103 (30), 89 (100), 74 (14), 57 (31), 41 (4), 30 (2).

**Methyl N-(*tert*-Butyloxycarbonyl)-L-phenylalanyl-D-prolinate (11).** The title compound was prepared from Boc-L-Phe (4.1 g, 15.5 mmol) and D-Pro-OMe·HCl (2.56 g, 15.5 mmol) by general procedure A and purified by column chromatography (1% MeOH, $CHCl_3$) to give a clear white oil (4.21 g, 72;): $R_f$ (C) 0.49; NMR ($CDCl_3$) $\delta$ 1.39 (s, 9 H), 1.84 (m, 4 H), 2.7 (m, 1 H), 2.95 (m, 2 H), 3.50 (m, 1 H), 3.67 (s, 3 H), 4.28 (m, 1 H), 4.58 (m, 1 H), 5.39 (d, 1 H, *J* = 8 Hz), 7.18 (s, 5 H); mass spectrum, *m/e* (relative intensity) 376 ($M^+$, 0.8).

**Methyl N-(Benzyloxycarbonyl)glycyl-L-phenylalanyl-D-prolinate (12).** The title compound was prepared from Z-Gly (1.58 g, 7.54 mmol) and L-Phe-D-Pro-OMe·HCl (2.35 g, 7.54 mmol) by general procedure A and purified by column chromatography (1% EtOH, EtOAc) to give a clear oil (3.1 g, 88%): $R_f$ (C) 0.16; NMR ($CDCl_3$) $\delta$ 1.72 (m, 4 H), 2.68 (m, 1 H), 2.97 (m, 2 H), 3.52 (m, 1 H), 3.67 (s, 3 H) 3.80 (d, 2 H, *J* = 6 Hz), 4.26 (m, 1 H), 5.0 (m, 1 H), 5.09 (s, 2 H), 6.1 (t, 1 H, *J* = 6 Hz), 7.14 (s, 5 H), 7.23 (s, 5 H), 7.73 (d, 1 H, *J* = 6.3 Hz); mass spectrum, *m/e* (relative intensity) 476 ($M^+$, 0.1). Anal. Calcd for $C_{25}H_{29}O_6N_3$: C, 64.23; H, 6.25; N, 9.39. Found: C, 63.76; H, 6.24; N, 8.79.

**Methyl N-(Benzyloxycarbonyl)glycyl-L-phenylalanyl-D-prolyl-$N^{\beta}$-(*tert*-butoxycarbonyl)-L-$\alpha,\beta$-diaminopropanoate (13).** The title compound was prepared from Z-Gly-L-Phe-D-Pro-OH (1.5 g, 3.31 mmol) and methyl $N^{\beta}$-Boc-L-Dap (10; 722 mg, 3.31 mmol) by general procedure A (except no base was added) and purified by column chromatography (2% EtOH, EtOAc) to give a clear white oil (1.55 g, 72%): $R_f$ (A) 0.32; NMR ($CDCl_3$) $\delta$ 1.4 (s, 9 H), 1.52–2.23 (m, 4 H), 2.57 (m, 1 H), 3.0 (m, 2 H), 3.50 (m, 3 H), 3.67 (s, 3 H), 3.9 (d, 2 H, *J* = 6 Hz), 4.49 (m, 3 H), 5.09 (s, 2 H), 6.12 (m, 2 H), 7.19 (s, 5 H), 7.28 (s, 5 H, 8.72 (m, 2 H); mass spectrum, exact *m/e* 553.2559 (calcd for $C_{28}H_{35}O_7N_5$, 553.2537).

***cyclo*(Glycyl-L-phenylalanyl-D-prolyl-$N^{\beta}$-(*tert*-butoxycarbonyl)-L-$\alpha,\beta$-diaminopropanoyl) (4).** Successful cyclization of the linear precursor of the title compound was strongly dependent on the purity of the tetrapeptide 13 and the careful removal of the Cbz group. Pure tetrapeptide 13 (1.56 g, 2.4 mmol) was saponified and the Tcp active ester prepared as described in general procedure B to give 2.2 g of yellow foam. TLC of the crude active ester showed two main ninhydrin, tolidine, and UV positive spots consistent with the presence of a racemized alanine residue.

To remove the Cbz group, the active ester (1.0 g, 1.22 mmol) was dissolved in methanol (50 mL), cooled to 0 °C, and treated with acetic acid (74 μL, 1.28 mmol) and Pd on carbon (10%, 1 g). The mixture was hydrogenated at atmospheric pressure at 0 °C and monitored via TLC. After 1–3 h the reaction was filtered through Celite, and the filtrate was concentrated in vacuo (<20 °C) and dried in vacuo (4 °C) for 16 h to give a clear white glass (765 mg, 84%). The glass was dissolved in 10 mL of purified DMF and cyclized as described in general procedure B. The crude reaction mixture was purified by column chromatography (1% EtOH, EtOAc) to yield the pure title compound as an amorphous white solid (86 mg, 14.5%). An analytical sample was crystallized (ether, EtOAc) to give a white powder: mp 128–130 °C; $[\alpha]^{23}_{D}$ −18.3° (*c* 0.282, TFE); $R_f$ (D) 0.48; UV $\lambda_{max}$ (MeOH) 260 nm sh ($\epsilon$ 365), 235 sh (3491); $^1$H NMR ($CDCl_3$) $\delta$ 1.44 (s, 9 H), 1.6–2.4 (m, 4 H), 2.91 (dd, 1 H, *J* = 5.6 and 13 Hz), 3.05–3.24 (m, 3 H), 3.46 (m, 2 H), 3.81 (m 1 H), 4.36–4.65 (m, 3 H), 5.02 (m, 2 H), 6.36 (d, 1 H, *J* = 10 Hz), 6.74 (br d, 1 H, *J* = 7.5 Hz), 7.03–7.28 (m, 6 H); $^{13}$C NMR ($CDCl_3$) 173.42–171.75 (carbonyls), 155.95 (Boc carbonyl), 136.63–126.87 (aromatic), 79.83 (Boc quaternary carbon), 57.93 ($\alpha$ carbon), 53.46 (Phe $\alpha$), 52.05 ($\alpha$ carbon), 46.98 ($\alpha$ carbon), 44.29 (Pro $\delta$), 39.76 (Dap $\beta$), 35.76 (Phe $\beta$), 28.37 (Boc Me), 24.93 (Pro $\beta$ and $\gamma$); mass spectrum, exact *m/e* 487.2432 (calcd for $C_{24}H_{33}O_6N_5$, 487.2430). Amino Acid Analysis (control, L-amino acid oxidase treated) Found: Gly (1.0, 1.08), L-Phe (1.04, 0.04), D-Pro (1.0, 1.0), L-Dap (1.04, 0.70). Anal. Calcd for $C_{24}H_{33}O_6N_5$: C, 59.12; H, 6.82; N, 14.36. Found: C, 58.91; H, 7.00; N, 14.16.

***cyclo*(Glycyl-L-phenylalanyl-D-propyl-$N^{\beta}$-(*N*-maleoylglycyl)-L-$\alpha,\beta$-diaminopropanoyl) (5).** Cyclic tetrapeptide 4 (76 mg, 145 μmol) was deprotected according to general procedure C (5 mL of 4 N HCl in dioxane) to give, after thorough drying in vacuo the HCl salt as a white crust. The salt was dissolved in DMF (3 mL), chilled to 0 °C, and treated with *N*-maleoylglycine[10] (49.4 mg, 319 μmol), HOBt (24.4 mg, 159 μmol), and TEA (16.1 mg, 159 μmol). DCC (32.8 mg, 159 μmol) dissolved in DMF (1 mL) was added, and the solution was stirred for 10 h at 6 °C and then for 2 h at 25 °C. The white suspension was filtered and the DCU cake was washed (3 × 1 mL) with cold DMF. The filtrate was concentrated in vacuo (<1 mmHg, 2 h, 35 °C) and the residue was purified by TLC (15% MeOH, $CHCl_3$). The product was washed from the silica gel using 25% MeOH/$CHCl_3$ and the solution was concentrated in vacuo to give an amorphous white powder (55 mg, 67%) (a minor low $R_f$ impurity formed on exposure to alcoholic solvents): UV $\lambda_{max}$ (MeOH) 295 nm br ($\epsilon$ 346), 264 sh (812), 229 sh (7367); $R_f$ (D) 0.25; $^1$H NMR ($Me_2SO$-$d_6$/$CDCl_3$, 5:95) $\delta$ 1.67–2.5 (m, 4 H), 2.99 (dd, 1 H, *J* = 14 and 7.2 Hz), 3.24

(16) Broadbent, W.; Morley, J. S.; Stone, B. E. *J. Chem. Soc. C* **1967**, 2632.
(17) Rich, D. H.; Jasensky, R. D.; Singh, J. In "Proceedings of the Symposium on Neurohypophyseal Hormones and Other Biologically Active Peptides"; Schlesinger, D., Ed., Elsevier North Holland: New York, in press.

(m, 3 H), 3.54-4.1 (m, 2 H), 3.89 (m, 1 H), 4.28 (pq, 2 H, $J$ = 19.6 Hz) 4.5 (dd, 1 H, $J$ = 12.8 and 8.8 Hz), 4.72 (m, 1 H), 4.78 (br d, 1 H, $J$ = 7.6 Hz), 5.17 (m, 1 H), 6.83 (d, 1 H, $J$ = 10.4 Hz), 7.27-7.63 (m, 7 H), 7.87 (dd, 1 H, $J$ = 3.1 and 9.6 Hz); mass spectrum, $m/e$ (relative intensity) 524 ($M^+$, not observed), 153 (4), 149 (2), 143 (2), 129 (1.5), 125 (2), 119 (2), 115 (2), 101 (8), 83 (9), 73 (42), 59 (45), 56 (31), 45 (83), 43 (100), 31 (87), 28 (85).

Amino Acid Analysis (control, L-amino acid oxidase treated) Found: Gly (1.97, 2.05), L-Phe (1.07, 0.03), D-Pro (1.0, 1.0), L-Dap (1.07, 0.59).

**Acknowledgment.** This work was supported in part by Biomedical Research Support Grant SO7RR-05456 and by CA 27985.

# Structure-Sweetness Relationship of L-Aspartyl Dipeptide Analogues. A Receptor Site Topology

Hajime Iwamura

*Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan.*
*Received September 2, 1980*

The relationship between structure and the sweet potency of L-aspartyl dipeptide analogues was investigated by physicochemical parameters and regression analysis. The dipeptide analogues reported were divided into the following four classes: L-aspartic acid amides, L-aspartylaminoethyl esters, L-aspartylaminopropionates, and L-aspartylaminoacetates. The analysis carried out for each class indicated that the electron-withdrawing effect of the substituents directed to the peptide bond and the steric dimensions of the molecules are important in eliciting the sweet taste. The values of coefficients of the electronic $\sigma^*$ terms in the correlations for L-aspartic acid amides, L-aspartylaminoethyl esters, and L-aspartylaminopropionates were ~0.7, indicating a common basic site on the receptor surface. The value for L-aspartylaminoacetates was ~1.5, and this value suggests, together with the factor of the participation of steric parameters, a closer or geometrically more proper fit to the receptor, explaining the generally higher potency of this class compared to the other three. The receptor model drawn based on these quantitative analyses appears to be consistent with other classes of sweeteners of apparently unrelated structures.

A wide variety of structurally unrelated compounds are known to elicit a common sweet taste. On the relationship between the structure and taste of these compounds, Shallenberger's A-H/B model[1] and Kier's dispersion site[2] are most commonly quoted, probably because of their diverse applicability to various kinds of sweet compounds. The limitations of these hypotheses are, however, obvious. Many nonsweet compounds which contain heteroatoms or double bonds possess an acidic proton and a basic site corresponding to the A-H and B sites, respectively, along with a dispersion site which is generally a methylene or methine group. In some cases, for example, in perillartines and 5-nitro- and 5-cyanoanilines, it is difficult to find the A-H site in a practical sense and in some cases, such as dihydrochalcones and phyllodulsins, multiple A-H/B units are a possibility.[3] Clearly, these oversimplified theories lack predictive value. One should, however, appreciate the attempts made by Shallenberger[1] and Kier[2] to seek common peculiarities among different classes of sweeteners. Efforts made since then to correlate sweetness or taste with chemical structure have been summarized in recent literature,[4] outlining the possibility of further developing structure-activity studies in this field. Although some workers have presented evidence in support of the existence of multiple sweet receptors and of differences in the sweet and bitter sites,[3] it is more useful in terms of predictive power to try to explain the possibly diverse classes of sweeteners in terms of a single, common receptor site.

Recently, the relationship between structure and taste of perillartine and nitro- and cyanoaniline derivatives has been quantitatively analyzed by physicochemical parameters and regression analysis,[5] indicating that the mode of interactions of these two classes of sweeteners is very similar. Furthermore, although only semiquatitatively, it has been shown that the sweet and bitter taste of the perillartine derivatives can be explained as a function of the steric dimensions of the molecules.[5] These ressults are suggestive of the usefulness or the predictive value of this approach. In this study, an attempt was made to correlate the relationship between the structure and the sweet potency of L-aspartyl dipeptide analogues in which activity had been estimated quantitatively and to compare the results with those of a previous paper.[5]

## Methods

Activity data used for analyses were taken from literature reported by Mazur et al.,[6,7] Ariyoshi et al.,[8,9] Brussel et al.,[10] Fujino et al.,[11] and Miyoshi et al.[12]

The steric parameters used were calculated by the STERIMOL program developed by Verloop et al.[13] The $L$ parameter expresses the length of a substituent along the bond axis which connects the substituent to the rest of the molecule. The $W_r$, $W_l$, $W_u$, and $W_d$ parameters are the molecular width in directions perpendicular to the $L$ axis and rectangular to each other. The $W_r$ parameter is defined as the width in the direction in which the longest chain of the substituent extends in the fully extended (staggered) conformation. $W_l$ is the width in the direction opposite to $W_r$. The $W_u$ and $W_d$ parameters are the widths upward and downward, respectively, of the substituent when one views it from the con-

(1) R. S. Shallenberger and T. E. Acree, *Nature (London)*, **216**, 480 (1967).
(2) L. B. Kier, *J. Pharm. Sci.*, **61**, 1394 (1972).
(3) G. A. Crosby, G. E. DuBois, and R. E. Wingard, Jr., *Drug Des.*, 8, 215 (1979).
(4) J. C. Boudreau, Ed., *ACS Symp. Ser.*, No. 115, 93, 149 (1979).
(5) H. Iwamura, *J. Med. Chem.*, **23**, 308 (1980).
(6) R. H. Mazur, A. H. Goldkamp, P. A. James, and J. M. Schlatter, *J. Med. Chem.*, **13**, 1217 (1970).
(7) R. H. Mazur, J. A. Reuter, K. A. Swiatek, and J. M. Schlater, *J. Med. Chem.*, **16**, 1284 (1973).
(8) Y. Ariyoshi, N. Yasuda, and T. Yamatani, *Bull. Chem. Soc. Jpn.*, **47**, 326 (1974).
(9) Y. Ariyoshi, *Agric. Biol. Chem.*, **40**, 983 (1976).
(10) L. B. P. Brussel, H. G. Peer, and A. van der Heijden, *Z. Lebensm. Unter-Forsch.*, **159**, 337 (1975).
(11) M. Fujino, M. Wakimasa, K. Tanaka, H. Aoki, and N. Nakajima, *Chem. Pharm. Bull.*, **24**, 2112 (1976).
(12) M. Miyoshi, K. Nunami, H. Sugano, and T. Fujii, *Bull. Chem. Soc. Jpn.*, **51**, 1433 (1978).
(13) A. Verloop, W. Hoogenstraaten, and J. Tipker, *Drug Des.*, **7**, 165 (1976).

0022-2623/81/1824-0572$01.25/0 