Analogues of the Cytostatic and Antimitogenic Agents Chlamydocin and HC-Toxin: Synthesis and Biological Activity of Chloromethyl Ketone and Diazomethyl Ketone Functionalized Cyclic Tetrapeptides†

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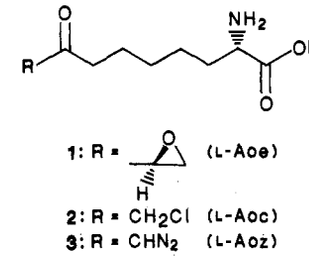
The synthesis and biological activity of four novel analogues of the cytostatic and antimitogenic agents chlamydocin and HC-toxin are reported in which the natural products' reactive epoxy ketone side-chain moiety is replaced by a chloromethyl or a diazomethyl ketone functionality, but the respective 12-membered cyclic tetrapeptide ring systems are retained. Syntheses of the linear tetrapeptide sequences were, in each case, achieved by conventional methodology and designed such that cyclization would be onto proline. The use of suitably protected L-2-aminosuberic acid (Asu) enabled the ready assimilation of the desired chloromethyl and diazomethyl ketone functionalities after cyclization. Cyclization was accomplished by using bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl). Yields of cyclic product were comparable to or, in the case of the HC-toxin ring system, better than those previously reported. Liberation of the Asu-side-chain acid and manipulation to the required functionalities via mixed anhydride to the diazomethyl ketone and quenching with HCl to yield the chloromethyl ketone was achieved in excellent yield for the HC-toxin analogues but in only moderate yield for the chlamydocin analogue. The antimitogenic activities of HC-toxin chloromethyl ketone (IC_{50} = 30–40 ng/mL) and chlamydocin chloromethyl ketone (IC_{50} = 3–10 ng/mL) were found to be 3–4-fold lower than those of the natural products themselves. The diazomethyl ketone analogue of HC-toxin was found to be inactive (IC_{50} > 2000 ng/mL). A modification of the HC-toxin peptide ring system, [L-Phe]^3-HC-toxin chloromethyl ketone was found not to be a more active analogue (IC_{50} = 40–100 ng/mL). The nature of the putative target molecule, the binding interactions of the various analogues and the contribution of rate of inhibition toward activity are briefly discussed. The chloromethyl ketones herein reported constitute the most potent synthetic antimitogenic cyclic tetrapeptide analogues yet designed.

Chlamydocin (*cyclo*[L-Aoe-Aib-L-Phe-D-Pro]) and HC-toxin (*cyclo*[L-Aoe-D-Pro-L-Ala-D-Ala]) are two representatives of a family of fungally derived natural products (Figure 1), all of whose members exhibit notable biological activity.[1-6] Perhaps of greater interest is the fact that the compounds demonstrate activity in widely differing biological systems. Chlamydocin, isolated from *Diheterospora chlamydosporia*,[7] and WF-3161 (*cyclo*[L-Leu-L-Pip-L-Aoe-D-Phe]), from *Petriella guttulata*,[8] have been shown to be potent in vitro cytostatic agents; indeed chlamydocin, with an ED_{50} value of 0.36 ng/mL for inhibition of cell growth in mouse P-815 mastocytoma cells, has activity 10–100 times greater than the clinical agents actinomycin D, vinblastine, vincristine, amethopterin, and colchicine in the same assay.[1] However, HC-toxin, isolated from *Helminthosporium carbonum*,[9] and Cyl-2 (*cyclo*[L-Aoe-D-Tyr(OMe)-L-Ile-L-Pip]), from *Cylindrocladium scoparium*,[5] are plant toxins. Cyl-2 is an agent with toxicity against a variety of plant species, whereas *Helm. carbonum* and its toxin are pathogenic only on susceptible varieties of maize.[2,3] Walton has demonstrated that chlamydocin is also toxic to maize and has the same host specificity as HC-toxin.[10] Similarly, we have independently shown[11,12] that HC-toxin possesses potent cytostatic and antimitogenic activities on murine lymphocytes, thus confirming complementary biological activity. Unfortunately, in vivo studies have shown chlamydocin to be only weakly active as a result, most probably, of enzymatic inactivation.[1] Nevertheless, these compounds remain potentially significant as antitumor agents and therefore a strong basis for further research into structural modifications to attempt to increase their in vivo potency.

Structurally, this family of natural products (Figure 2) is characterized by two important features; firstly, a 12-membered cyclic tetrapeptide ring system containing an imino acid, proline, or pipecolic acid and at least one D-configuration amino acid; secondly and probably most crucially with respect to biological activity, all the compounds contain the unusual amino acid L-2-amino-8-oxo-9(*S*),10-epoxydecanoic acid (L-Aoe) (1).[13] Furthermore,



studies have shown that both features together are essential for full activity.[11] Thus it seems likely that the peptide ring provides a very specific capability for the

† Abbreviations used in the text follow IUPAC–IUB tentative rules as described in *J. Biol. Chem.* **1972**, *247*, 977. Additional abbreviations used: L-AA = L-amino acid; Aib = α-aminoisobutyric acid; Aoe = L-2-amino-8-oxo-9,10-epoxydecanoic acid; Aoc = L-2-amino-8-oxo-9-chlorononanoic acid; Aoz = L-2-amino-8-oxo-9-diazononanoic acid; Asu = L-2-aminosuberic acid; BOP-Cl = bis(2-oxo-3-oxazolidinyl)phosphinic chloride; Con A = concanavalin A; DCC = dicyclohexylcarbodiimide; DIEA = diisopropylethylamine; EEDQ = *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; HOAc = acetic acid; HOBt = 1-hydroxybenzotriazole; NMM = *N*-methylmorpholine; ONp = *p*-nitrophenyl ester; Pip = pipecolic acid; TFA = trifluoroacetic acid.

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COMPOUND	SEQUENCE
Chlamydocin	cyclo[-L-Aoe^1-Aib^2-L-Phe^3-D-Pro^4-]
HC-Toxin	cyclo[-L-Aoe^1-D-Pro^2-L-Ala^3-D-Ala^4-]
WF-3161	cyclo[-L-Aoe^1-D-Phe^2-L-Leu^3-L-Pip^4-]
Cyl-2	cyclo[-L-Aoe^1-D-Tyr(OMe)^2-L-Ile^3-L-Pip^4-]

Figure 1. L-Aoe-containing cyclic tetrapeptide natural products.

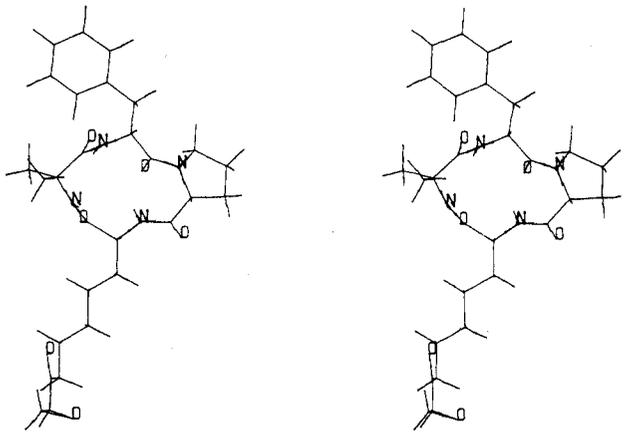
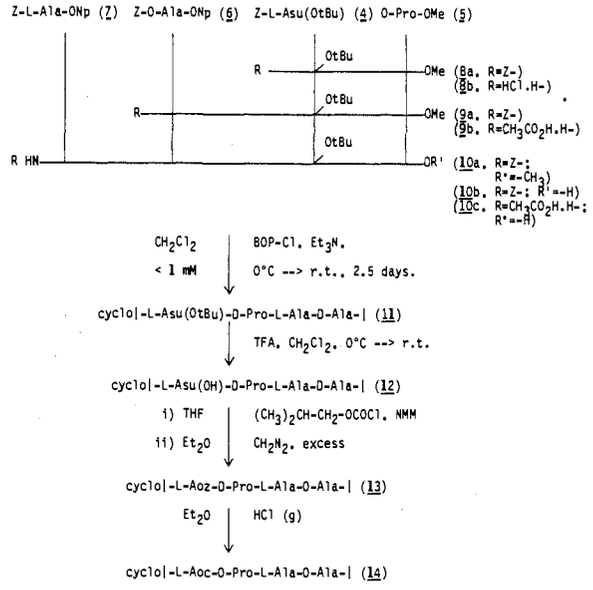


Figure 2. Stereo representation of chlamydocin in the all-transoid ring system conformation.[15]

molecules to bind at the putative receptor and that the highly electrophilic epoxy ketone moiety interacts with a crucial functional group at a distance from the peptide binding site described by the L-Aoe side chain. In order to understand these facets of structure–activity more fully, studies on both have been forthcoming.[1,7,14-21] Predominant, however, has been the study of the peptide ring system in attempts to identify the bioactive conformation.[14-20] Structural modifications of the unusual amino acid but with retention of the known biologically active ring systems have been less investigated.[1,7,21] This study describes chlamydocin and HC-toxin analogues in which the epoxy ketone group is replaced by a comparable biologically isosteric functionality.

The few studies to date on Aoe-modified cyclic tetrapeptides have indicated that an 8-oxo functionality on the amino acid is a prerequisite for activity. Reduction of the Aoe-side-chain carbonyl of chlamydocin to yield the epoxy alcohol dihydrochlamydocin[7,14] causes a 1000-fold reduction in activity.[1] Similar results were obtained in this laboratory when intermediates in the total syntheses of chlamydocin[22] and HC-toxin[23] with reduced or absent carbonyl functionality were tested.[12] If, as seems likely at present, the epoxy ketone is acting as a highly specific alkylating agent, an alternative alkylating ketone derivative would seem to be the most fruitful replacement. Such a functionality is exemplified by chloromethyl ketones and diazomethyl ketones, both classes of alkylating agent with

Scheme I



members possessing precedented biological activity, for example, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK),[24,25] a potent enzyme inhibitor, and 6-diazo-5-oxo-L-norleucine (DON),[26] an antitumor antibiotic. The target molecules therefore became chlamydocin and HC-toxin chloromethyl and diazomethyl ketones containing the novel amino acids L-2-amino-8-oxo-9-chlorononanoic acid (L-Aoc) (**2**) and L-2-amino-8-oxo-9-diazononanoic acid (L-Aoz) (**3**). It is the synthesis and biological activity of these compounds that are described in this work.

Chemistry

The synthesis of these agents was greatly assisted by the discovery that a suitably protected precursor to the desired amino acids, L-Aoc and L-Aoz was commercially available.[27] L-2-Aminosuberic acid differentially protected at the α amine with benzyloxycarbonyl and with side chain tertiary butyl ester protection (**4**) thus became the "lynchpin" of the synthetic strategy.

1. Synthesis of HC-Toxin Diazomethyl and Chloromethyl Ketones (13, 14). Synthesis of the linear HC-toxin tetrapeptide sequence was achieved (Scheme I), starting from methyl D-prolinate *p*-toluenesulfonate salt (**5**) by sequential coupling to Z-L-Asu(OtBu) via DCC/HOBt and thereafter to Z-D-Ala and Z-L-Ala by HOBt-catalyzed *p*-nitrophenyl active ester couplings[28] to yield the fully protected tetrapeptide Z-L-Ala-D-Ala-L-Asu(OtBu)-D-Pro-OMe (**10a**) in 70% overall yield.

In initial experiments on the hydrogenolysis of the dipeptide Z-L-Asu(OtBu)-D-Pro-OMe (**8a**) in methanol and in the presence of acetic acid, diketopiperazine formation was the predominant pathway. Such a side reaction is well-precedented, particularly in solid-phase peptide synthesis and most particularly in L,D-dipeptides where one

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of the amino acids is proline or another imino acid.[29-31] Furthermore, the reaction is catalyzed by carboxylic acids (e.g., formic and acetic acids). In this case, the problem was solved by using 1 equiv of HCl as the acidic component, hydrogenating in methanol at low temperature, and using excess palladium on charcoal catalyst to accelerate the deprotection, thus reducing the chances of side chain tertiary butyl ester removal. This procedure, in combination with the very rapid HOBt-catalyzed active ester coupling procedure of König and Geiger[28] and the utilization of the hindered tertiary base diisopropylethylamine[32] (DIEA) as the acid scavenger, almost totally eliminated this bothersome side reaction. Deprotection and coupling at the tripeptide stage was accomplished (**9a–10a**) by conventional methods. Final saponification and N-deprotection yielded the desired linear tetrapeptide acetate salt (**10c**) in 72% yield.

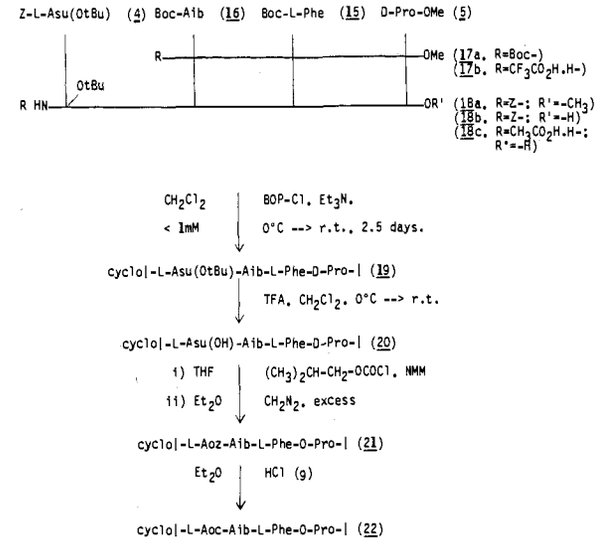
Perhaps the most crucial reaction in the synthesis of any cyclic peptide remains the cyclization procedure itself. In the field of cyclic tetrapeptides, as with other cyclic peptides, the reaction is highly sequence and procedure specific.[33] Previous work from this laboratory[22,23,33] suggested that in this system cyclization onto proline was likely to be the most productive, hence the choice of linear sequence above. More difficult to predict, however, was the choice of cyclization procedure. The high-yielding cyclization methodology of Schmidt et al.[34] was found to be incompatible with the side chain *tert*-butyl ester protection. Furthermore, diphenylphosphoryl azide (DPPA)[35] requires very careful control of reaction conditions, while other active ester and carboxyl activation procedures are unpredictable and frequently give high yields of byproduct dimers (cyclic octapeptide) and higher polymers. Currently in this group, the phosphorus-based coupling reagent bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl)[36] has been found to be an efficient coupling reagent,[37] and in separate experiments it was also found to be an effective and convenient cyclization reagent.[38,39] For these reasons BOP-Cl was utilized as the cyclization mediator in this study.

Cyclization of L-Ala-D-Ala-L-Asu(OtBu)-D-Pro acetate salt (**10c**) with BOP-Cl under high dilution yielded the cyclic tetrapeptide HC-toxin analogue (**11**) in 35% yield, compared with 21% using DPPA.[23] NMR (^{1}H and ^{13}C) spectral data of the product in deuteriochloroform was, apart from the Aoe side chain, identical with that of natural HC-toxin,[18] thus characterizing and confirming the chiral integrity of the product. Furthermore, this proved that the product possessed the all-transoid, bis-γ-turn conformation for the peptide ring as previously assigned to HC-toxin in this solvent.[18] Deprotection of the side-chain ester was accomplished quantitatively with trifluoroacetic acid. Treatment of the resultant acid (**12**) with isobutyl chloroformate, reaction of the mixed anhydride with excess diazomethane, and finally quenching of the diazo ketone (**13**) with HCl was achieved in a one-pot series to yield, after chromatography, HC-toxin chloromethyl ketone (**14**) in 94% yield. The presence of the new functionality was proved by NMR, by comparison with the known chloromethyl ketone TPCK,[25] and by high-resolution mass spectrometry.

It was found that the above one-pot series of reactions could be terminated prior to the HCl quench and the poorly stable intermediate diazo ketone (**13**) isolated in 63% yield by direct preparative layer chromatography on silica. This compound was characterized by the characteristic diazo stretch at ca. 2100 cm^{-1} as well as by ^{1}H NMR.

2. Synthesis of Chlamydocin Chloromethyl Ketone (22). In this case, as with the HC-toxin analogue, earlier work suggested that cyclization onto proline was likely to be the most efficient.[22,23] This entailed the incorporation of the Z-protected L-Aoc precursor (**4**) as the fourth and last amino acid and allowed the use of previous methodology involving *N*-Boc protection for the synthesis of the known tripeptide Boc-Aib-L-Phe-D-Pro-OMe (**17a**).[22,40] Deprotection using trifluoroacetic acid and coupling to Z-L-Asu(OtBu) using EEDQ[41] yielded the fully protected tetrapeptide Z-L-Asu(OtBu)-Aib-L-Phe-D-Pro-OMe (**18a**) in 47% overall yield (Shceme II). Amino and carboxyl deprotection was accomplished by conventional methods, and the resultant tetrapeptide acetate salt (**18c**) was cyclized by using BOP-Cl under high dilution to yield the chlamydocin analogue (**19**) in 24% yield. Once again, this result compared favorably with the previous active ester mediated cyclization (stereoselective 24% yield)[22] although not with the Schmidt procedure.[34] Comparison of the ^{1}H and ^{13}C NMR spectra in deuteriochloroform with chlamydocin again confirmed the integrity of the product and

Scheme II



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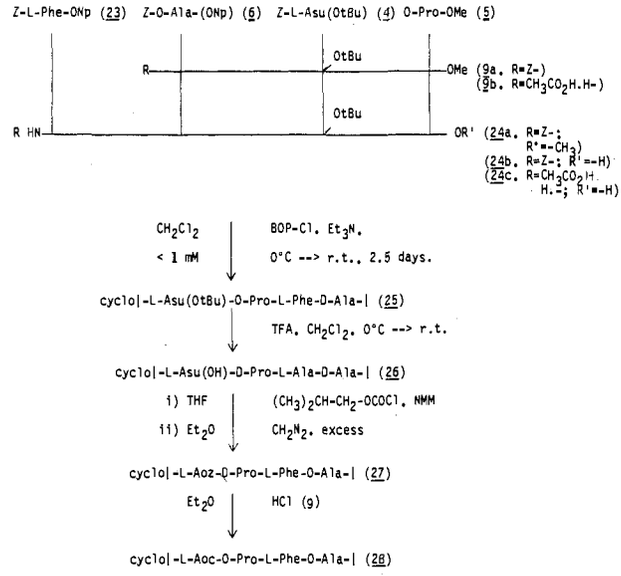
Scheme III



also the presence, in non-hydroxy bonding solvents, of the all-transoid, bis-γ-turn conformation for the peptide ring.[17] Deprotection of the side-chain ester and assimilation of the chloromethyl ketone functionality via the one-pot sequence (**19–22**) was accomplished in 48% yield. It should be noted that longer reaction times for the mixed anhydride, diazo ketone, and chloromethyl ketone formations were found to be necessary in this case if the product were not to be contaminated with the side-chain methyl ester analogue. The reason for this reduced reactivity has not been ascertained at present. As with the HC-toxin analogue, chlamydocin chloromethyl ketone (**22**) was characterized by ^{1}H and ^{13}C NMR and by high-resolution mass spectrometry.

3. Synthesis of [L-Phe]^3-HC-Toxin Chloromethyl Ketone (28). HC-toxin is approximately 5–10-fold less active than chlamydocin as a cytostatic agent against murine lymphocytes.[10-12] Examination of molecular models suggested that this could be due to the absence, in HC-toxin, of an aromatic amino acid residue, reducing binding affinity at an active-site lipophilic pocket. A closer appraisal indicated that replacement of the L-Ala residue at position 3 of HC-toxin with L-Phe would introduce the possibility for this binding interaction without adversely interfering with the peptide backbone geometry (Figure 1). Hence a further synthetic target became the doubly modified HC-toxin analogue [L-Phe]^3-HC-toxin chloromethyl ketone (**28**).

The synthesis of analogues at position 3 of HC-toxin is particularly convenient because this residue constitutes the fourth and last amino acid in the linear tetrapeptide sequence if cyclization onto proline is preferred. Thus, from a common, suitably protected tripeptide, in this case, Z-D-Ala-L-Asu(OtBu)-D-Pro-OMe (**9a**), a variety of position 3 analogues can be synthesized. Consequently, deprotection of the aforementioned tripeptide and coupling to Z-L-Phe-ONp (**23**) yielded the desired fully protected linear tetrapeptide (**24a**) in 72% overall yield (Scheme III). Amino and carboxyl deprotection and cyclization with BOP-Cl yielded the required cyclic tetrapeptide (**25**), albeit in only 11% yield. This poor yield provides further evidence for the fickle nature of these cyclization reactions; in this instance, however, optimization of the cyclization was not the primary goal of the study and hence was not further investigated. The chloromethyl ketone synthesis proceeded as before to yield the HC-toxin analogue (**28**) in 73% yield. Characterization by ^{1}H and ^{13}C NMR and by high-resolution mass spectrometry again confirmed the integrity of the product. A more detailed examination of the NMR data and close comparison with HC-toxin[18] suggested that this compound existed, in non-hydrogen bonding solvents, in the all-transoid, bis-γ-turn conformation like HC-toxin itself. It should be emphasized, however, that a complete conformational analysis of the compound by NMR has not been undertaken at present so the proposed conformation is yet to be absolutely proven.

Table I. Inhibition of Con A Stimulated Thymocyte Mitogenesis by Chlamydocin, HC-Toxin, and Synthetic Analogues

compound	IC_{50},[a] ng/mL
chlamydocin	1–3
HC-toxin	10–15
HC-toxin chloromethyl ketone (14)	30–40
HC-toxin diazomethyl ketone (13)	>2000
chlamydocin chloromethyl ketone (**22**)	3–10
[L-Phe]^3-HC-toxin chloromethyl ketone (**28**)	40–100

[a] Values represent range of results from at least three experiments.

Biology

The inhibition of concanavalin A (Con A) stimulated lymphocyte mitogenesis[42] was used to evaluate the relative biological activities of the analogues. Inhibition was measured as reduction of tritiated thymidine incorporation into Con A stimulated murine thymocytes.[43] Previous work in our laboratory has shown that chlamydocin and HC-toxin possess potent antimitogenic activities in this assay system with IC_{50} values for antimitogenesis[12] comparable with IC_{50} values for in vitro cytostatic activity against mouse P-815 mastocytoma cells.[10] The sensitivity and ease of quantifying the thymocyte assay has allowed us to compare synthetic analogues with widely different activities. The results of the antimitogenesis activities of chlamydocin, HC-toxin, and the synthetic analogues reported are shown in Table I.

Discussion

Chlamydocin and HC-toxin possess potent cytostatic and antimitogenic activity in vitro. Their activity in vivo is, however, considerably reduced by an inactivation process in whole serum that is presumably enzymatically mediated.[1] Two possible sites for this inactivation can be rationalized. The first is the peptide ring system and the second is the reactive epoxy ketone moiety on the side chain of the unusual amino acid L-Aoe.

The very nature of biological systems means that any peptide-derived therapeutic agent is highly prone to enzymatic degradation. To a degree, this can be circumvented by the use of "unnatural" amino acids, particularly D-amino acids, and by incorporation of cyclic structures.[44] This latter strategy has the double advantage, not only of reducing the likelihood of attack by proteinases and peptidases but also of restricting the compound conformationally. The potent cytostatic activity of the cyclic tetrapeptides vis a vis the linear precursors demonstrates the power of conformational mobility restriction in increasing biological activity. Similar observations of increased specificity, potency, and biological stability has arisen from

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studies on cyclic derivatives of the hormones somatostatin[45] and the enkephalins[46] as well as the potent cyclic undecapeptide immunosuppressive cyclosporin A.[47] It seems likely, therefore, that the peptide ring systems of chlamydocin and HC-toxin instill enzymatic stability due to the presence of unnatural amino acids and a cyclic structure while providing the specific binding capability essential for potent and specific biological activity. Thus the biological instability of these agents would appear to lie elsewhere in the molecules than the peptide portion, although this has yet to be categorically established.

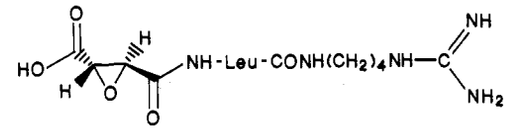
A more likely candidate for the site of biological inactivation is the reactive Aoe-side-chain epoxy ketone functionality. Indeed it is well-established that in vitro reduction of the ketone, ring opening, or reduction of the epoxide, all potential in vivo metabolic processes, causes a drastic reduction in cytostatic properties.[7] A modification of the epoxy ketone group that retains in vitro biological activity could, therefore, have potential as an antitumor agent, if the modification were shown to be metabolically stable.

To this end, we have shown that replacement of the epoxy ketone by a chloromethyl ketone functionality but with retention of the natural cyclic tetrapeptide ring systems does lead to compounds with potent in vitro antimitogenic properties. A 3–4-fold drop in IC_{50} values for the corresponding chloromethyl ketone analogues compared to the natural materials does, however, suggest a slightly less than perfect interaction with the putative target molecule. This difference is further emphasized by the complete inactivity of the analogous diazomethyl ketone compound derived from HC-toxin. Although both lack one carbon compared to the natural products, an implicitly more fundamental difference between the epoxy ketone/chloromethyl ketone active and the diazomethyl ketone inactive functionalities must exist. Such a difference may lie in the electronic nature of the methylene or methine carbon at C-9 of the unusual amino acids Aoc, Aoz, and Aoe.

The epoxy ketone methine of Aoe and the chloromethyl ketone methylene of Aoc are electron deficient and sp^3 hybridized whereas the corresponding carbon of Aoz is nominally sp^2 hybridized and possess a slight negative charge due to the resonance of the diazomethyl group. The diazomethyl ketone functionality is therefore a planar, dipolar, conjugated system that is itself inherently nucleophilic and only becomes a potential alkylating agent if it is protonated by an acidic active site grouping. Such differences suggest the absence of a suitable proton donor at the receptor that could activate the diazo group.

More difficult to rationalize is the basic difference in activity between chlamydocin and HC-toxin, a difference that is mirrored in their chloromethyl ketone analogues. This must be a function of the peptide ring system. As has been stated, examination of molecular models implied a difference in the lipophilic nature of the two agents, hence the rationale for the synthesis of the [L-Phe]^3-HC-toxin analogue (**28**) described above. The fact that this compound is not more active than HC-toxin chloromethyl ketone (**14**) despite the closer structural analogy to chlamydocin suggests that this lipophilic interaction is not sufficient for increased potency. Such an inference could be tested by the synthesis of the "HC-toxin hybrid" of chlamydocin, [L-Ala]^3-chlamydocin.

One consideration that has not previously been proposed to account for the activity differences in these compounds is that of rate of inhibition. It is possible that the higher activity of chlamydocin is a manifestation of a faster inhibition and, contrarily, the lower activity of HC-toxin is due to slower inhibition. The biological testing system yielding IC_{50} values is a fixed time-point assay (24 h) that gives no indication of the rate constants for the inhibitory process. For analogues of the epoxide-containing cysteine proteinase inhibitior E-64 (**29**), it has been observed that compounds with similar IC_{50} values can show very different second-order rate constants when tested on the isolated enzymes.[48] In other words, if an irreversible inhibitor is left in contact with its target for long enough, complete inhibition will ultimately result no matter how weak the initial binding.



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At present in this field, the precise target enzyme or enzyme system for cyclic tetrapeptides is not known nor is the stage in the cell cycle at which inhibition occurs, so IC_{50} studies are the only viable means of testing biological activity. Hopefully, after the target molecule for these agents has been identified, quantitative data on the rate of inhibition will be forthcoming that will discriminate more effectively among analogues by providing dissociation constants for the prealkylation binding process. It is at this step that differences in cyclic tetrapeptide sequence and conformation are likely to be most evident.

In this study we have shown that, in the cytostatic and antimitogenic cyclic tetrapeptides chlamydocin and HC-toxin, it is possible to replace the natural products' reactive epoxy ketone by a chloromethyl ketone functionality and yet retain potent antimitogenic activity. A modification of the HC-toxin peptide ring system, intended to assist receptor binding and increase activity, was found not to be a more active analogue. The consequences of these observations with respect to the nature of the putative receptor and to the interaction of these compounds at that receptor will be expanded in future publications.

Experimental Section

General Methods. All reagents were used as supplied unless otherwise specified. "pH 9.5 buffer" refers to a solution of sodium bicarbonate (28 g) and potassium carbonate (23 g) in water (1 L). The following reagents were purified as follows: hexane, distilled to boiling point 68–70 °C; tetrahydrofuran, distilled from sodium benzophenone ketyl; dimethylformamide, distilled under reduced pressure and stored over 4A molecular sieves; tertiary amines, distilled from phthalic anhydride followed by calcium hydride and then stored under nitrogen; *p*-nitrophenol, recrystallized from toluene. N-Protected amino acids and their *p*-nitrophenyl esters were used as commercially available.

Thin-layer and preparative layer chromatography was performed on commercially available prepared glass plates, silica gel thickness 0.25 mm and 0.5 or 2.0 mm, respectively. Silica gel column chromatography was performed on MN-Kieselgel 60 (60–270 mesh) under gravity. Melting points were determined

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with a Fisher-Johns apparatus and are uncorrected.

Proton NMR spectra were recorded in deuteriochloroform solutions with either a Varian EM-390 (90 MHz), Bruker WP-200 (200 MHz), or Bruker WH-270 (270 MHz) spectrometer. Chemical shifts are reported in ppm downfield from an internal tetramethylsilane (Me_4Si) standard. Carbon-13 NMR spectra were recorded on deuteriochloroform solutions with a JEOL FX-90Q (22.5 MHz) instrument and chemical shifts are also reported in ppm downfield from internal Me_4Si. Low-resolution mass spectra were determined on a Finnigan Model 1015 mass spectrometer and high-resolution mass spectra on an AEI MS 902 C instrument. Where linear peptides exhibited multiple conformations in the ^1H NMR, only the resonances for the major conformer are listed.

Optical rotations were determined on a Perkin-Elmer Model 251 automatic polarimeter (1.0 dm).

General Procedure A. Coupling Procedure Using Dicyclohexylcarbodiimide (DCC). The amine hydrochloride (3 mmol) was dissolved in dichloromethane (20 mL), the solution was cooled (−5 to 0 °C), and *N*-methylmorpholine (NMM) (3.3 mmol) was added. The acid component (3.3 mmol) dissolved in dichloromethane (15 mL) was added followed by solid HOBt (3.3 mmol). The resultant mixture was stirred at low temperature until the majority of the HOBt had dissolved (ca. 15 min). A solution of DCC (3.3 mmol) in dichloromethane (10 mL) was added, and the reaction mixture was stirred at low temperature for 2 h and then overnight at room temperature. The reaction mixture was then cooled to <−50 °C and filtered through Celite. The precipitated urea was washed with dichloromethane, and the combined filtrate and washings were evaporated in vacuo. The residue was dissolved in ethyl acetate (50 mL), washed successively with water (×2), 1 N HCl (×2), saturated $NaHCO_3$ (×2), and water (×2), dried ($MgSO_4$), and evaporated in vacuo. The crude peptide was purified by either crystallization or column chromatography.

General Procedure B. Removal of the Benzyloxycarbonyl Group. The protected peptide (1 mmol) was taken up in methanol (40 mL) containing acetic acid (66 mg, 1.1 mmol). After flushing with nitrogen, 10% palladium on charcoal (15–20% by weight of peptide) was added. The reaction mixture was then covered with a blanket of hydrogen and stirred under hydrogen for 3 h or until TLC indicated complete deprotection. The catalyst was filtered off through a pad of Celite and washed with methanol, and the combined filtrate and washings were evaporated. The residue was taken up in ether and reevaporated, and final traces of solvent were removed by storage under vacuum over P_2O_5. The amine salt was used without further purification.

General Procedure C. Coupling Reaction Using *p*-Nitrophenyl Active Esters.[28] The amine salt (1 mmol) was dissolved in DMF (5 mL) containing DIEA (1.5 mmol). To this solution were immediately added, with swirling, solid N-protected amino acid *p*-nitrophenyl ester (1.25 mmol) and HOBt (1.25 mmol). The resultant yellow solution was stored at room temperature for 2 h with occasional swirling. The reaction was then quenched by addition of *N,N*-diethylethylenediamine (1.5 mmol). After a further 10–15 min at room temperature, the reaction was diluted with ethyl acetate (40 mL), washed with copious amounts of water, 1 N HCl (×2), pH 9.5 buffer (6–8 times), and water (×3), dried ($MgSO_4$), and evaporated. The crude peptide was purified by crystallization or column chromatography.

General Procedure D. Saponification of Methyl Esters. The fully protected peptide (0.33 mmol) was dissolved in methanol (3 mL) and 1.0 N NaOH solution (1 mL, 3 equiv) was added. The reaction was stored at room temperature with occasional swirling for 3 h or until TLC indicated complete deprotection. The reaction mixture was then diluted with water (20 mL) and extracted carefully with ethyl acetate (10 mL). The aqueous phase was acidified with saturated potassium bisulfate solution and extracted with chloroform (3 × 10 mL). The combined organic extracts were washed with water, dried ($MgSO_4$), and evaporated. Final traces of solvent were removed by storage under vacuum over P_2O_5. The resultant acid was used without further purification.

General Procedure E. Cyclization Procedure.[39] To a solution of fully deprotected tetrapeptide acetate salt (1 mmol) in dichloromethane (1 L, i.e., 1 mM solution) at −5 to −10 °C was added, with stirring, a solution of triethylamine (3.3 mmol) in dichloromethane (10 mL). The mixture was covered with a blanket of nitrogen before solid BOP-Cl (1.1 mmol)[36] was added. The reaction mixture was stirred under nitrogen at low temperature for 4 h and then allowed to warm very slowly to room temperature with stirring for $2^1/_2$ days with rigorous exclusion of moisture. The reaction was evaporated to dryness, and the residue was partitioned between ethyl acetate (40 mL) and saturated brine (20 mL). The organic layer was separated and washed with saturated potassium bisulfate solution, saturated $NaHCO_3$, and brine, dried, and evaporated. The residue was chromatographed on silica gel to yield pure cyclic tetrapeptide.

General Procedure F. Removal of *tert*-Butyl Esters. To a solution of cyclic tetrapeptide *tert*-butyl ester (0.03 mmol) in dichloromethane (0.3 mL) was added ice-cold trifluoroacetic acid (5 mL). The solution was allowed to warm and stored at room temperature for 1 h and then evaporated at a temperature not greater than 35 °C. The residue was taken up in dichloromethane and reevaporated and the procedure repeated twice more. Final traces of acid and solvent were removed by storage under vacuum over KOH overnight.

General Procedure G. Chloromethyl Ketone Synthesis. Cyclic tetrapeptide acid (0.03 mmol) was dissolved in anhydrous THF (5 mL) and the solution cooled to 0 °C under nitrogen. *N*-Methylmorpholine (0.075 mmol) was added and the mixture stirred at 0 °C for 5–10 min. Isobutyl chloroformate (0.105 mmol) was added, and the reaction mixture was stirred under nitrogen for 30 min at a temperature not greater than 5 °C. The reaction mixture was then allowed to warm to room temperature and stirred for a further 15–30 min at the end of which time the mixed anhydride was quenched by addition of excess, ethereal, alcohol-free diazomethane. The resultant yellow mixture was stirred at room temperature under nitrogen for 2–3 h.

The diazo ketone was further quenched by addition of an excess of a saturated solution of HCl(g) in anhydrous ether The now-colorless reaction mixture was stirred at room temperature for 30–60 min and then evaporated to dryness in vacuo. The residue was suspended in dichloromethane and reevaporated, the procedure was repeated, and the product was purified directly by either silica gel preparative layer or column chromatography.

Methyl D-Prolinate *p*-Toluenesulfonate Salt (5).[49] A mixture of D-proline (2.3 g, 20 mmol), *p*-toluenesulfonic acid monohydrate (4.20 g, 22 mmol), and dimethyl sulfite (10 mL) was heated at 90 °C for 7 h and then poured into anhydrous ether (200 mL). Storage at −15 °C, collection, and recrystallization from chloroform/ether yielded the produced (5.70 g, 95%): mp 94.5–95 °C; $[\alpha]^{25}_D$ +18.2 (c 1.9, methanol) [lit.[49] L-prolinate, $[\alpha]^{25}_D$ −17.8 (c 6.7, methanol)]. Anal. ($C_{13}H_{19}NO_5S$) C, H, N.

***N*-(Benzyloxycarbonyl)-L-2-amino-7-(*tert*-butyloxycarbonyl)heptanoyl-D-proline Methyl Ester (8a).** The title compound was prepared from Z-L-Asu(OtBu) (liberated by careful acidification and extraction with ethyl acetate, from its dicyclohexylammonium salt, 2.0 g, 3.6 mmol) and methyl D-prolinate tosylate salt (1.0 g, 3.3 mmol) by general procedure A and purified by column chromatography (ethyl acetate/hexane, 3:2) to give a colorless oil quantitatively: R_f (ethyl acetate/hexane, 2:1) 0.57, (ethyl acetate/toluene, 20:1) 0.79; NMR δ 1.10–1.76 (m, 8 H), 1.43 (s, 9 H), 1.76–2.26 (m, 6 H), 3.50–3.74 (m, 2 H), 3.70 (s, 3 H), 4.35–4.63 (m, 2 H), 5.11 (s, 2 H), 5.60 (br, d, J = 9 Hz, 1 H), 7.37 (s, 5 H). [NB: The spectrum is complicated by cis and trans amide rotamers; data corresponding to only one rotamer is presented.] Anal. ($C_{26}H_{38}N_2O_7$) C, H, N.

***N*-(Benzyloxycarbonyl)-D-alanine *p*-Nitrophenyl Ester (6).** To a solution of Z-D-Ala (2.23 g, 10 mmol) and *p*-nitrophenol (1.67 g, 12 mmol) in ethyl acetate (20 mL) at −5 °C was added a solution of DCC (2.06 g, 10 mmol) in ethyl acetate (5 mL). The reaction mixture was stirred at low temperature for 30 min and at room temperature for 2 h. The precipitated urea was filtered off through Celite and washed with ethyl acetate. The combined filtrate and washings were evaporated, and the residue was taken up in ether, filtered again through Celite if necessary, washed with pH 9.5 buffer (repeatedly) and water (×3), dried ($MgSO_4$), and evaporated. The residue was dissolved in warm chloroform (5–10 mL), diluted with ether (10–20 mL), and crystallized by addition of hexane and storage at −15 °C to yield the product (2.50 g, 73%)

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as very pale yellow crystals: mp 77.5–78 °C; $[\alpha]^{25}_D$ +36.1 (c 2.1, ethyl acetate).

***N*-(Benzyloxycarbonyl)-D-alanyl-L-2-amino-7-(*tert*-butyloxycarbonyl)heptanoyl-D-proline Methyl Ester (9a).** Z-L-Asu(OtBu)-D-Pro-OMe (1.62 g, 3.3 mmol) was dissolved in methanol (60 ml) and, after flushing with nitrogen, 10% palladium on charcoal catalyst (0.5 g, 25–30% by weight of peptide) was added. The resultant mixture was cooled to −5 °C with stirring and concentrated hydrochloric acid (0.32 mL, 3.3 mmol) was added. The mixture was flushed with hydrogen and stirred under a blanket of hydrogen at low temperature for $1^1/_2$ h or until TLC indicated consumption of all starting material. Workup according to general procedure B yielded the dipeptide ester hydrochloride (**8b**), which was coupled without further purification to Z-D-Ala-ONp (**6**) (1.72 g, 5 mmol) by general procedure C. The crude product was purified by column chromatography (ethyl acetate/hexane, 5:1) and recrystallization from ether/hexane to yield the title product (1.53 g, 82%) as white crystals: mp 88–89 °C; $[\alpha]^{25}_D$ +24.9 (*c* 2.2, methanol); R_f (ethyl acetate/hexane, 5:1) 0.50, (ethyl acetate/toluene, 20:1) 0.66; NMR δ 1.18–1.79 (m, 11 H), 1.41 (s, 9 H), 1.79–2.27 (m, 6 H), 3.43–3.76 (m, 2 H), 3.63 (s, 3 H), 4.30–4.56 (m, 2 H), 4.68–4.89 (m, 1 H), 5.10 (s, 2 H), 5.72–5.81 (br d, *J* = 8 Hz, 1 H), 7.01–7.17 (br d, *J* = 9 Hz, 1 H), 7.33 (s, 5 H). [NB: major rotamer assignments.] Anal. ($C_{29}H_{43}N_3O_8$) C, H, N.

***N*-(Benzyloxycarbonyl)-L-alanyl-D-alanyl-L-2-amino-7-(*tert*-butyloxycarbonyl)heptanoyl-D-proline Methyl Ester (10a).** Tripeptide Z-D-Ala-L-Asu(OtBu)-D-Pro-OMe (**9a**) (0.28 g, 0.5 mmol) was deprotected according to general procedure B to give the acetate salt (**9b**) as a glass. The salt was coupled to Z-L-Ala-ONp (**7**) (0.25 g, 0.73 mmol) by general procedure C and the residue purified by column chromatography (ethyl acetate/methanol, 25:1) and precipitation from dichloromethane solution with hexane to yield the title product (0.27 g, 86%) as an amorphous solid: mp 76–78 °C; $[\alpha]^{25}_D$ +36.6 (c 1.1, chloroform); R_f (ethyl acetate/methanol, 20:1) 0.45; NMR δ 1.23–1.59 (m, 14 H), 1.43 (s, 9 H), 1.65–2.08 (m, 4 H), 2.13–2.21 (m, 2 H), 3.42–3.54 (m, 2 H), 3.66 (s, 3 H), 4.27 (m, 1 H), 4.41 (m, 1 H), 4.57 (m, 1 H), 4.76 (m, 1 H), 5.11 (AB q, *J* = 13 Hz, 2 H), 5.90 (d, *J* = 7.4 Hz, 1 H), 6.97 (d, *J* = 7.7 Hz, 1 H), 7.10 (d, *J* = 9.1 Hz, 1 H), 7.34 (s, 5 H). [NB: major rotamer assignments.] Anal. ($C_{32}H_{48}N_4O_9$) C, H, N.

***N*-(Benzyloxycarbonyl)-L-2-amino-7-(*tert*-butyloxycarbonyl)heptanoyl-α-aminoisobutyryl-L-phenylalanyl-D-proline Methyl Ester (18a).** Boc-Aib-L-Phe-D-Pro-OMe (**17a**)[22,40] (0.23 g, 0.5 mmol) was dissolved in ice-cold trifluoroacetic acid and stored at room temperature for 30–40 min. The solvent was removed in vacuo at a temperature not greater than 35 °C. The residue was taken up in chloroform and reevaporated. The procedure was repeated, and final traces of solvent were removed by storage over KOH overnight under vacuum. Without further purification, the glassy tripeptide ester trifluoroacetate salt (**17b**) was dissolved in ethyl acetate (1 mL) containing triethylamine (75 μL, 0.54 mmol). The solution was cooled to 0–5 °C and a solution of Z-L-Asu(OtBu) (**4**) (liberated by careful acidification and extraction with ethyl acetate, from its dicyclohexylammonium salt, 0.29 g, 0.52 mmol) in ethyl acetate (4 mL) was added. To the resultant solution at low temperature was added solid 97% EEDQ[41] (0.15 g, 0.59 mmol). The reaction was stirred at low temperature for 3 h and at room temperature for a further 24 h. The reaction mixture was washed with water (×2), 1 N HCl (×2), saturated $NaHCO_3$ (×2), and water (×2), dried ($MgSO_4$), and evaporated. Purification by column chromatography (ethyl acetate/methanol, 25:1) yielded the title compound (0.23 g, 64%) as a colorless oil: R_f (ethyl acetate/methanol, 20:1) 0.60, (chloroform/methanol, 25:1) 0.38; NMR δ 1.20–1.70 (m, 23 H including 1.43, s, 9 H; 1.49, s, 3 H; 1.57, s, 3 H), 1.71–1.99 (m, 4 H), 2.118 2.25 (m, 2 H), 2.72 (m, 1 H), 2.86–3.12 (m, 2 H), 3.46–3.62 (m, 1 H), 3.67 (s, 3 H), 4.07 (m, 1 H), 4.29 (m, 1 H), 4.90 (m, 1 H), 5.12 (s, 2 H), 5.43 (d, *J* = 7.6 Hz, 1 H), 6.77 (s, 1 H), 6.94 (br d, *J* = 8 Hz, 1 H), 7.22 (m, 5 H), 7.33 (s, 5 H). [NB: major rotamer assignments.] Anal. ($C_{39}H_{54}N_{O9}$) C, H, N.

***N*-(Benzyloxycarbonyl)-L-phenylalanyl-D-alanyl-L-2-amino-7-(*tert*-butyloxycarbonyl)heptanoyl-D-proline Methyl Ester (24a).** Tripeptide Z-D-Ala-L-Asu(OtBu)-D-Pro-OMe (**9a**) (0.28 g, 0.5 mmol) was deprotected according to general procedure B to give the acetate salt (**9b**) as a glass. The salt was coupled to Z-L-Phe-ONp (**23**) (0.3 g, 0.71 mmol) by general procedure C and the residue purified by column chromatography (ethyl acetate/methanol, 25:1) to yield the title product (0.31 g, 88%) as a glass: $[\alpha]^{25}_D$ +26.3 (*c* 0.9, chloroform); R_f (ethyl acetate/methanol, 20:1) 0.68; NMR δ 1.05–1.85 (m, 20 H including 1.20, d, *J* = 6 Hz, 3 H; 1.42, s, 9 H), 1.85–2.30 (m, 6 H), 3.08 (d, *J* = 7 Hz, 2 H), 3.30–3.80 (m, 5 H including 3.61, s, 3 H), 4.25–4.95 (m, 4 H), 5.08 (s, 2 H), 5.60–5.90 (br, 1 H), 6.38–6.62 (br, 1 H), 6.90–7.18 (br, 1 H), 7.28 (s, 5 H), 7.32 (s, 5 H). [NB: major rotamer assignments.] Anal. ($C_{38}H_{52}N_4O_9 \cdot {}^1/_2H_2O$) C, H, N.

***N*-(Benzyloxycarbonyl)-L-alanyl-D-alanyl-L-2-amino-7-(*tert*-butyloxycarbonyl)heptanoyl-D-proline (10b).** The title compound (0.19 g, 78%), a white foam, was prepared from fully protected tetrapeptide **10a** (0.25 g, 0.4 mmol) according to general procedure D: R_f (butan-1-ol/acetic acid/water, 4:1:1) 0.70.

***N*-(Benzyloxycarbonyl)-L-2-amino-7-(*tert*-butyloxycarbonyl)heptanoyl-α-aminoisobutyryl-L-phenylalanyl-D-proline (18b).** The title compound (0.85 g, 96%), an amorphous powder, was prepared from fully protected tetrapeptide **18a** (0.9 g, 1.24 mmol) according to general procedure D: R_f (butan-1-ol/acetic acid/water, 4:1:1) 0.81.

***N*-(Benzyloxycarbonyl)-L-phenylalanyl-D-alanyl-L-2-amino-7-(*tert*-butyloxycarbonyl)heptanoyl-D-proline (24b).** The title compound (0.18 g, 66%), a white foam, was prepared from fully protected tetrapeptide **24a** (0.28 g, 0.4 mmol) according to general procedure D: R_f (butan-1-ol/acetic acid/water, 4:1:1) 0.76.

L-Alanyl-D-alanyl-L-2-amino-7-(*tert*-butyloxycarbonyl)-heptanoyl-D-proline Acetate Salt (10c). The title compound (154 mg, 92%), an amorphous glass, was prepared from the N-protected tetrapeptide acid **10b** (0.19 g, 0.31 mmol) according to general procedure B: R_f (butan-1-ol/acetic acid/water, 4:1:1) 0.44.

L-2-Amino-7-(*tert*-butyloxycarbonyl)heptanoyl-α-amino-isobutyryl-L-phenylalanyl-D-proline Acetate Salt (18c). The title compound (0.21 g, 94%), an amorphous glass, was prepared from the N-protected tetrapeptide acid **18b** (0.25 g, 0.35 mmol) according to general procedure B: R_f (butan-1-ol/acetic acid/water, 4:1:1) 0.46.

L-Phenylalanyl-D-alanyl-L-2-amino-7-(*tert*-butyloxycarbonyl)heptanoyl-D-proline Acetate Salt (24c). The title compound (154 mg, 83%), an amorphous glass, was prepared from the N-protected tetrapeptide acid **24b** (0.18 g, 0.26 mmol) according to general procedure B: R_f (butan-1-ol/acetic acid/water, 4:1:1) 0.23.

***cyclo*[L-2-Amino-7-(*tert*-butyloxycarbonyl)heptanoyl-D-prolyl-L-alanyl-D-alanyl] (11).** The title compound (39 mg, 35%) was prepared from L-Ala-D-Ala-L-Asu(OtBu)-D-Pro acetate salt (**10c**) (130 mg, 0.24 mmol) according to general procedure E but using a modified workup procedure because of the water solubility of the product. The cyclization reaction mixture was evaporated almost to dryness and the residue was triturated with ethyl acetate. The solids were filtered off and washed with ethyl acetate and the combined filtrate and washings reevaporated; the procedure was repeated and the residue was purified directly by column chromatography (ethyl acetate/methanol, 25:1) to yield the pure cyclic tetrapeptide as an oil: R_f (ethyl acetate/methanol, 20:1) 0.66; ^{1}H NMR δ 1.29 (d, *J* = 7.7 Hz, 3 H), 1.32 (d, *J* = 6.8 Hz, 3 H), 1.44 (s, 9 H), 1.50–2.00 (m, 8 H), 2.02–2.47 (m, 4 H), 2.21 (m, 2 H), 3.51 (m, 1 H), 3.99 (m, 1 H), 4.47 (m, 1 H), 4.57 (m, 1 H), 4.71 (dd, *J* = 6.8 and 1.8 Hz, 1 H), 4.79 (m, 1 H), 6.29 (d, *J* = 9.3 Hz), 6.33 (d, *J* = 11.6 Hz, 1 H), 7.12 (d, *J* = 10.7 Hz, 1 H); ^{13}C NMR δ 173.68–171.46 (carbonyls), 80.02 (OtBu quaternary), 57.86 (Pro-α), 52.01 (Asu-α), 48.11 and 47.46 (Ala-2 × α), 47.08 (Pro-δ), 35.44 (Asu-ω), 29.31–25.52 (Asu side chain), 28.17 (OtBu Me), 25.14 (Pro-β), 24.87 (Pro-γ), 14.74 and 14.09 (Ala-2 × β); mass spectrum, M^+, 466; M^+ − isobutene (56), 410.

***cyclo*[L-2-Amino-7-(*tert*-butyloxycarbonyl)heptanoyl-α-aminoisobutyryl-L-phenylalanyl-D-prolyl] (19).** The title compound (44 mg, 24%), an oil, was prepared from L-Asu-(OtBu)-Aib-L-Phe-D-Pro acetate salt (**18c**) according to general procedure E and purified by column chromatography (ethyl acetate/hexane, 20:1): R_f (ethyl acetate) 0.74; ^{1}H NMR δ 1.34 (s, 3 H), 1.46 (s, 9 H), 1.25–1.90 (m, 8 H), 1.79 (s, 3 H), 2.05–2.39 (m, 4 H), 2.18 (t, *J* = 6.8 Hz, 2 H), 2.96 (dd, *J* = 13.2 and 6.3 Hz,

1 H), 3.13–3.25 (m, 1 H), 3.24 (dd, J = 13.2 and 10.5 Hz, 1 H), 3.87 (m, 1 H), 4.19 (m, 1 H), 4.66 (dd, J = 7.4 and 1.6 Hz, 1 H), 5.17 (dt, J = 10.5 and 6.3 Hz, 1 H), 5.95 (s, 1 H), 7.10 (d, J = 11.0 Hz, 1 H), 7.26 (m, 5 H), 7.52 (d, J = 10.5 Hz, 1 H); ^{13}C NMR δ 175.80 (Aib carbonyl), 174.50 (Pro carbonyl), 173.14 (ester carbonyl), 172.93 (Phe carbonyl), 171.95 (Asu carbonyl), 137.23, 129.21, 128.72, 126.82 (Phe aromatics), 80.13 (OtBu quaternary), 58.97 (Aib-α), 57.97 (Pro-α), 54.56 (Asu-α), 53.63 (Phe-α), 47.13 (Pro-δ), 35.98 (Phe-β), 35.60 (Asu-ω), 28.99, 28.88, 25.41, 25.14 (Asu side chain), 28.28 (OtBu Me), 26.66 (Aib-β), 25.03 (Pro-β), 24.87 (Pro-γ), 23.68 (Aib-β).

***cyclo*[L-2-Amino-7-(*tert*-butyloxycarbonyl)heptanoyl-D-prolyl-L-phenylalanyl-D-alanyl] (25).** The title compound (15 mg, 11%), an oil, was prepared from L-Phe-D-Ala-L-Asu-(OtBu)-D-Pro acetate salt (**24c**) (154 mg, 0.25 mmol according to general procedure E and purified by column chromatography (ethyl acetate/hexane 20:1): R_f (ethyl acetate/methanol, 25:1) 0.86, (ethyl acetate/hexane, 20:1) 0.67; ^{1}NMR δ 1.23 (d, J = 6 Hz, 3 H), 1.26–2.02 (m, 8 H), 1.42 (s, 9 H), 2.20 (t, J = 7 Hz, 2 H), 2.24–2.44 (m, 4 H), 2.93 (m, 1 H), 3.22 (m, 1 H), 3.51 (m, 1 H), 4.00 (m, 1 H), 4.44–4.67 (m, 2 H), 4.71 (dd, J = 7.4 and 1.1 Hz, 1 H), 4.79 (m, 1 H), 6.08 (d, J = 9.1 Hz, 1 H), 6.40 (d, J = 10.4 Hz, 1 H), 7.24 (m, 6 H); mass spectrum, M^+, 542; M^+ – isobutene (56), 486.

***cyclo*[L-2-Amino-8-oxo-9-chlorononanoyl-D-prolyl-L-alanyl-D-alanyl] (14).** The cyclic tetrapeptide *cyclo*[L-Asu-(OtBu)-D-Pro-L-Ala-D-Ala] (**11**) (19 mg, 0.04 mmol) was deprotected according to general procedure F to yield the cyclic tetrapeptide acid **12** as an oil: R_f (toluene/acetone/acetic acid, 10:7:1) 0.50, which was used without further purification. The title compound (17 mg, 94%), a pale yellow oil, was prepared from the aforementioned cyclic tetrapeptide acid (0.04 mmol) according to general procedure G and purified by column chromatography (ethyl acetate/methanol, 25:1): R_f (ethyl acetate/methanol, 20:1) 0.61 (ethyl acetate/methanol, 25:1) 0.51; ^{1}H NMR δ 1.27 (d, J = 6.5 Hz, 3 H), 1.31 (d, J = 6.5 Hz, 3 H), 1.52–2.08 (m, 8 H), 2.18–2.47 (m, 4 H), 2.59 (t, J = 8.1 Hz, 2 H), 3.51 (m, 1 H), 3.96 (m, 1 H), 4.07 (s, 2 H), 4.48 (m, 1 H), 4.58 (m, 1 H), 4.71 (dd, J = 6.1 and 1.5 Hz, 1 H), 6.19 (d, J = 10.1 Hz, 1 H), 6.31 (d, J = 10.6 Hz, 1 H), 7.12 (d, J δ 10.6 Hz, 1 H); ^{13}C NMR δ 202.50 (ketone carbonyl), 173.74, 173.30, 173.25, and 171.46 (amide carbonyls), 57.91 (Pro-α), 51.95 ([L-AA]^1-α), 48.11 (Ala-α and chloromethyl ketone CH_2), 47.46 (Ala-α), 47.13 (Pro-δ), 39.44 ([L-AA]^1-ω), 29.20, 28.71, 25.52, and 23.30 ([L-AA]^1 side chain), 25.14 (Pro-δ), 24.97 (Pro-γ), 14.74 and 14.14 (Ala 2 × β); high-resolution mass spectrum for $C_{20}H_{31}ClN_4O_5$ requires M^+ 442.1983, Found M^+, 442.1971. NB: In a separate experiment *cyclo*[L-Asu(OtBu-D-Pro-L-Ala-D-Ala] (**11**) (24 mg, 0.051 mmol) was deprotected according to general procedure F and the resultant cyclic tetrapeptide acid as above was processed according to general procedure G except that the intermediate diazo ketone **13** was not quenched by ethereal HCl. The intermediate was isolated as such by the passing of a stream of nitrogen through the reaction mixture to remove excess diazomethane, evaporation to dryness, redissolution in ethyl acetate, reevaporation, and purification by preparative layer chromatography (0.5-mm thickness, silica gel 20 × 20 plates; developing solvent ethyl acetate/methanol, 15:1) to yield the diazo ketone intermediate (14 mg, 63%) as a poorly stable pale yellow oil: R_f (ethyl acetate/methanol, 20:1) 0.29, (ethyl acetate) 0.17; IR ν_{max} 2110 cm^{-1}; NMR δ 1.28 (d, J = 7 Hz, 3 H), 1.33 (d, J = 7 Hz, 3 H), 1.62 (m, 2 H), 1.80 (m, 2 H), 1.86 (m, 2 H), 1.93 (m, 2 H), 2.23–2.36 (m, 4 H), 2.39 (m, 2 H), 3.51 (m, 1 H), 3.98 (m, 1 H), 4.45 (m, 1 H), 4.57 (m, 1 H), 4.69 (dd, J = 7.7 and 1.9 Hz, 1 H), 4.78 (m, 1 H), 5.23 (s, 1 H), 6.11 (d, J = 9.9 Hz, 1 H), 6.27 (d, J = 10.9 Hz, 1 H), 7.10 (d, J = 10.4 Hz, 1 H).

***cyclo*[L-2-Amino-8-oxo-9-chlorononanoyl-α-aminoisobutyryl-L-phenylalanyl-D-prolyl] (22).** The cyclic tetrapeptide *cyclo*[L-Asu(OtBu)-Aib-L-Phe-D-Pro] (**19**) (26 mg, 0.047 mmol) was deprotected according to general procedure F to yield the cyclic tetrapeptide acid **20** as an oil: R_f (butan-1-ol/acetic acid/water, 4:1:1) 0.37, which was used without further purification. The title compound (12 mg, 48%), an oil, was prepared from the aforementioned cyclic tetrapeptide acid (0.047 mmol) by general procedure G, using the long reaction times stated in the method, and purified by preparative layer chromatography (2-mm thickness, silica gel 20 × 20 plates; developing solvent ethyl acetate/hexane, 20:1): R_f (ethyl acetate/hexane, 20:1) 0.62; ^{1}H NMR δ 1.36 (s, 3 H), 1.49–1.73 (m, 8 H), 1.80 (s, 3 H), 2.01–2.44 (m, 4 H), 2.60 (t, J = 7.1 Hz, 2H), 2.96 (dd, J = 13.6 and 6.1 Hz, 1 H), 3.12–3.26 (m, 1 H), 3.27 (dd, J = 13.6 and 10.1 Hz, 1 H), 3.88 (m, 1 H), 4.07 (s, 2 H), 4.19 (m, 1 H), 4.66 (dd, J = 7.1 and 1.5 Hz, 1 H), 5.18 (dt, J = 10.1 and 6.1 Hz, 1 H), 5.93 (s, 1 H), 7.10 (d, J = 10.1 Hz), 7.25 (m, 5 H), 7.50 (d, J = 11.0 Hz, 1 H); ^{13}C NMR δ 202.50 (ketone), 175.58, 174.28, 172.82, and 171.79 (amide carbonyls), 137.06, 129.04, 128.61, and 126.71 (Phe aromatics), 58.83 (Aib-α), 57.80 (Pro-α), 54.34 ([L-AA]^1-α), 53.47 (Phe-α), 48.05 (chloromethyl ketone CH_2), 47.02 (Pro-δ), 39.44 ([L-AA]^1-ω), 35.86 (Phe-β), 28.77, 28.71, 25.25, and 23.30 ([L-AA]^1 side chain), 26.55 and 23.57 (Aib-β × 2), 25.03 (Pro-β), 24.46 (Pro-γ); high-resolution mass spectrum for $C_{27}H_{37}ClN_4O_5$ requires M^+, 532.2452, found M^+, 532.2458.

***cyclo*[L-2-Amino-8-oxo-9-chlorononanoyl-D-prolyl-L-phenylalanyl-D-alanyl] (28).** The cyclic tetrapeptide *cyclo*[L-Asu(OtBu)-D-Pro-L-Phe-D-Ala] (**25**) (15 mg, 0.028 mmol) was deprotected according to general procedure F to yield the cyclic tetrapeptide acid **26** as an oil: R_f (butan-1-ol/acetic acid/water, 4:1:1) 0.74, which was used without further purification. The title compound (10 mg, 73%), an oil, was prepared from the aforementioned cyclic tetrapeptide acid (0.028 mmol) according to general procedure G and purified by column chromatography (ethyl acetate/hexane, 10:1): R_f (ethyl acetate/hexane, 20:1) 0.60; ^{1}H NMR δ 1.24 (d, J = 7.7 Hz, 3 H), 1.49–2.05 (m, 8 H), 2.12–2.47 (m, 4 H), 2.58 (t, J = 6.9 Hz, 2 H), 2.90 (dd, J = 14.3 and 6.9 Hz, 1 H), 3.22 (dd, J = 14.3 and 9.0 Hz, 1 H), 3.50 (m, 1 H), 3.98 (m, 1 H), 4.07 (s, 2 H), 4.42–4.66 (m, 2 H), 4.70 (dd, J = 6.4 and 1.3 Hz, 1 H), 4.78 (m, 1 H), 6.08 (d, J δ 9.8 Hz, 1 H), 6.40 (d, J = 10.6 Hz, 1 H), 7.24 (m, 6 H); ^{13}C NMR δ 202.45 (ketone), 173.47, 173.30, 172.92, 171.62 (amide carbonyls), 136.74, 129.10, 128.56, and 126.71 (Phe aromatics), 57.80 (Pro-α), 53.90 (Phe-α), 51.84 ([L-AA]^1-α), 48.05 (chloromethyl ketone CH_2), 47.46 (Ala-α), 47.13 (Pro-δ), 39.38 ([L-AA]^1-ω), 35.27 (Phe-β), 29.20, 28.66, 25.46, and 23.24 ([L-AA]^1 side chain), 25.08 (Pro-β), 24.92 (Pro-γ), 13.98 (Ala-β); high-resolution mass spectrum for $C_{26}H_{35}ClN_4O_5$ requires M^+, 518.2296, found M^+, 518.2275.

Biological Methods. Female, 4–5-week-old Balb/c mice were obtained from Harlan Sprague–Dawley, Indianapolis, IN, and were maintained in our laboratories until used. Murine single-cell thymocyte suspensions were prepared as described previously.[42] Briefly, excised thymus tissue was minced, pressed through wire mesh with a syringe barrel, and centrifuged at 200G for 3 min, and the cells were washed once with media and distributed into 96-well microtiter plates (0.8 × 10^6 cells/well) in a final volume of 250 μL. Con A was added to the cell suspension at a final concentration of 1 μg/mL. Inhibitors were added in 10 μL of 10% aqueous ethanol (controls received 10 μL of 10% aqueous ethanol alone). In these experiments, Dulbecco's MEM—Hams F12 (1:1, v/v) medium containing 5 ng/L insulin, 5 ng/L transferrin, and 6 pg/L selenium was used in place of Eagle's high amino acid medium with mouse serum. The level of mitogenic activation was determined at 24 and 48 h by pulsing with [^{3}H]thymidine for 12 h, followed by harvesting and measurement of cellular [^{3}H]TdR incorporation by scintillation counting as described previously.[42]

Registry No. **1**, 103366-18-3; **4**, 49645-27-4; **5**, 104848-85-3; **6**, 30960-00-0; **7**, 1168-87-2; **8a**, 104848-86-4; **8b**, 104848-87-5; **9a**, 104848-88-6; **9b**, 104848-90-0; **10a**, 104848-91-1; **10b**, 104848-92-2; **10c**, 104848-94-4; **11**, 104848-95-5; **12**, 104848-96-6; **13**, 104848-97-7; **14**, 104848-98-8; **17a**, 104848-99-9; **17b**, 104849-00-5; **18a**, 104874-88-6; **18b**, 104849-01-6; **18c**, 104849-03-8; **19**, 104849-04-9; **20**, 104849-05-0; **21**, 104849-06-1; **22**, 104849-07-2; **23**, 2578-84-9; **24a**, 104849-08-3; **24b**, 104849-09-4; **24c**, 104849-11-8; **25**, 104849-12-9; **26**, 104994-59-4; **27**, 104849-13-0; **28**, 104849-14-1; D-Pro, 344-25-2; Z-D-Ala, 26607-51-2.