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ADDITIONAL PEPTIDYL DIAZOMETHYL KETONES, INCLUDING BIOTINYL DERIVATIVES, WHICH AFFINITY-LABEL CALPAIN AND RELATED CYSTEINYL PROTEINASES

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Calpain, the calcium-activated cysteinyl proteinase, can be irreversibly inactivated by peptidyl diazomethyl ketones in which the peptide portion contains a penultimate leucine residue. Some new derivatives of this type have been synthesized and examined for their rates of inactivation of chicken gizzard and human platelet calpain. Two derivatives containing a C-terminal biotin residue, Biot-Aca-Leu-TyrCHN$_2$ and Biot-Aca-Leu-Leu-TyrCHN$_2$, have also been prepared in the expectation that their application to the study of the function of calpain and related proteases will prove fruitful.

KEY WORDS: Calpain, cysteinyl proteases, inhibitors, peptidyl diazomethyl ketones.

ABBREVIATION: Aca, ε-aminocaproic acid.

INTRODUCTION

Cysteine proteinases belonging to the papain family are important components of cell metabolism, both lysosomal as in the case of cathepsins B, H, and L[1] and cytoplasmic, as in the case of calpain, the calcium-activated protease.[2] Irreversible inhibitors which penetrate cells and inactivate these proteases are of increasing value for the delineation of their role in cellular activities. In initial studies carried out *in vitro*, peptidyl diazomethyl ketones,[3–6] fluoromethyl ketones[7–11] and epoxides[12–16] have been shown to be effective inactivators of these proteases which favor binding of hydrophobic amino acid side chains in their S_1–S_3 subsites, using the nomenclature of Schechter and Berger.[17,18] In these small peptide derivatives, a Phe residue in P_2 promotes binding to cathepsins B and L, but not to calpain. On the other hand a Leu in P_2 promotes binding to both cathepsin L and calpain. The development of these and other affinity-labeling reagents for cysteinyl proteinases has been reviewed.[19]

Inhibitors of this type have the additional useful property of diffusing into cells and inactivating the same proteinases *in situ*.[20,21] With ^{125}I-Tyr derivatives, cathepsin B and L[22,23] and calpain[24] can be inactivated intracellularly and confirmation of the protein modifications can be obtained by autoradiography. This is an important criterion for establishing the selectivity of an inhibitor before applying it to deduce

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the state or function of an enzyme. It represents an advantage over reversible inhibitors whose intracellular actions are a matter of surmise.

Affinity-labelling inactivators of the calcium activated protease previously described[6] such as Cbz-Leu-Leu-Tyr-CHN_2 were based on the observations of Sasaki *et al.*[25] on peptide substrates that the specificity of the protease is directed towards the Leu-Leu sequence in the P_2 and P_3 subsites. Reversible inhibitors, Cbz-Leu-Pheal[26] and Cbz-Leu-Leu-Leual[27] have also been developed guided by the evidence on substrate preferences.[21]

It may be that this view of the specificity of calpain is incomplete. In studies with some protein substrates thought to be important in calpain action, for example erythrocyte Ca^{2+}-ATPase, four cleavage sites have been identified[28] which do not permit generalization and appear unrelated to the findings with peptides. Possibly in this case the conformation of the protein determines the available sites for proteolysis. On the other hand, in other substrates, brain spectrin[29] and tyrosine hydroxylase[30] the calpain cleavage site has a penultimate valine or leucine, respectively, thus resembling the situation with peptides. An endogenous inhibitor of calpain, known as calpastatin, exists in the cytoplasm[31–33] and is a polypeptide with four domains capable of inhibiting the protease and a sequence Thr-Ile-Pro-Pro-X-Tyr-Arg has been identified as the conserved inhibitory sequence[33] which, however, has not demonstrated inhibition unless present in considerably longer sequences.[33]

We have extended our studies on diazomethyl ketone derivatives of small peptides with hydrophobic side chains to explore the relationship of structure and selectivity of action among cysteinyl proteinases. Since the N-terminal region of the inhibitor is generally less vital to its function and will permit various modifications to be made without loss of activity, the incorporation of a biotinyl residue there was viewed as an alternative to the utilization of a radioiodinated tyrosine residue for following the action of peptidyl diazomethyl ketones (cf earlier applications[34,35]) and as a potential aid for the isolation of new proteases by affinity chromatography.

EXPERIMENTAL SECTION

Materials

Peptide intermediates were purchased from Bachem, CH-4416 Bubendorf, Switzerland. Biotin and other reagents were obtained from Fluka, CH-9470 Buchs, Switzerland.

Syntheses

Fmoc-Leu-Leu-Ala-CHN_2. The tripeptide was obtained via Fmoc-Leu-OSu coupled to H-Leu-Ala-OH according to standard methods. The diazomethylketone was formed via a mixed anhydride reacted with ethereal diazomethane as described by Green and Shaw.[4] The crude product was chromatographed on Kieselgel 60 with (1:20) MeOH-$CHCl_3$ as solvent. Purity was confirmed by elemental analysis as shown in Table I.

TABLE I
Analytical data for peptidyldiazo- and haloalkanes

Peptidyldiazomethane	M_v	Elementary analysis (%) C	H	N	I	m.p. (°C)	FAB-MS $(M + H-N_2)^+$
H-Leu-Leu-Ala-CHN_2							312
($C_{16}H_{29}N_5O_3$)	339.4						
Fmoc-Leu-Leu-Ala-CHN_2		66.29	7.00	12.47		160–161	
($C_{31}H_{39}N_5O_5$)	561.7	66.30	7.01	12.07			
Z-Leu-Leu-Met-CHN_2		58.26	7.48	13.09		140 dec	
($C_{26}H_{39}N_5O_5S$)	533.7	58.51	7.37	13.12			
Z-Leu-Leu-Pro-CHN_2		62.57	7.80				472
($C_{26}H_{37}N_5O_5$)	499.6	62.51	7.46				
Fmoc-Leu-Leu-Val-CHN_2		67.21	7.35	11.88		202–205	
($C_{33}H_{43}N_5O_5$)	589.7	67.23	7.32	12.00			
PAB-Leu-Leu-Val-CHN_2		61.71	7.87	17.27		142–146	
($C_{25}H_{38}N_6O_4$)	486.6	61.35	8.08	17.75			
Z-Tyr(I)-Leu-Leu-Ala-CHN_2		51.97	5.68	11.02	16.64		
($C_{33}H_{43}N_6O_7I$)	762.7	51.98	5.84	10.77	16.01		
Boc-His-Leu-Leu-Tyr-CHN_2							641
($C_{33}H_{48}N_8O_7$)	668.8						
Z-Tyr-Leu-Leu-Val-CHN_2		63.23	7.28	12.64		156–157	637
($C_{35}H_{51}N_7O_8$)	664.8	62.75	7.49	12.07			
Z-His-Val-Leu-Leu-Ala-CHN_2							682
($C_{35}H_{51}N_9O_7$)	709.9						
Z-Tyr-Pro-Leu-Leu-Ala-CHN_2		62.19	7.01	13.36		amorph.	706
($C_{38}H_{51}N_7O_8$)	733.9	61.41	6.98	13.09			
Biotinyl-Aca-Leu-Leu-Ala-CHN_2							651
($C_{32}H_{54}N_8O_6S$)	678.9						
Biotinyl-Aca-Leu-Tyr-CHN_2							628
($C_{32}H_{47}N_7O_6S$)	657.8						
Peptidyldiazoethane							
Z-Leu-Leu-Tyr-$C(CH_3)N_2$							552
($C_{31}H_{41}N_5O_6$)	579.7						

Fmoc-Leu-Leu-Val-CHN_2. Fmoc-Leu-Leu-OH as the mixed anhydride was coupled to Val-O-t-Bu and the ester converted to the acid by overnight treatment with TFA. The resultant acid was converted to the mixed anhydride for reaction with diazomethane to form the diazomethyl ketone. This was isolated by chromatography on silica in $CHCl_3$-MeOH (1:50), and recrystallized from ethyl acetate.

H-Leu-Leu-Ala-CHN_2. 0.38 g (0.7 mmol) of the Fmoc-derivative was deprotected and purified as described by Crawford *et al.*[6] and 0.16 g (56% yield) of pure product was obtained, supported by MS/FAB. H-Leu-Leu-Ala-CHN_2 served as an intermediate in the preparation of Z-Tyr(I)-Leu-Leu-Ala-CHN_2, Z-Tyr-Pro-Leu-Leu-Ala-CHN_2, Z-His(Z)-Val-Leu-Leu-Ala-CHN_2, and Biotinyl-Aca-Leu-Leu-Ala-CHN_2.

Z-Leu-Leu-Pro-CHN_2. Z-Leu-Leu-OH was coupled with H-Pro-OMe via the mixed anhydride. After hydrolysis with NaOH the resulting acid was converted to the diazomethyl ketone as described above.

Biotinyl-Aca-Leu-Leu-Ala-CHN_2. Biotinyl-Aca-OH (294 mg, 0.82 mmole) was dissolved in *N*-methyl pyrollidone (8 ml) with heat, treated with *N*-methylmorpholine (90 μl, 0.82 mmole), brought to $-20°C$ and isobutylchloroformate (107 μl, 0.82 mmole) added. To this was added after 15 min H-Leu-Leu-Ala-CHN_2 (220 mg, 0.65 mmole) in THF (10 ml) and the mixture stirred 2 h at 0°C then overnight at room temperature. After addition of water, the reaction was extracted with chloroform which was washed with aqueous $NaHCO_3$, dried and concentrated. The residual syrup contains *N*-methylpyrollidone which was removed by a batchwise precipitation of the product from 0.6 g of the residue in ethyl acetate (1 ml) on addition of hexane (6 ml). All precipitates were combined and chromatographed on silica-60 poured in methylene chloride. The sample was applied in MeOH-CH_2Cl_2 (1:12.5) and eluted with CH_2Cl_2 containing stepwise increases in methanol eventually to 10%. The effluent was monitored with tlc and hplc and the combined fractions yielded 113 mg (26%).

Biotinyl-Aca-Leu-Tyr-CHN_2. Fmoc-Aca-Leu-Tyr-OH was prepared from the dipeptide and Fmoc-Aca-OSu and converted to the diazomethyl ketone in the usual way. This was purified on silica with elution by CH_2Cl_2 and increasing methanol content and yielded 33% based on Leu-Tyr.

After deblocking with piperidine (cf. above) the product, H-Aca-Leu-Tyr-CHN_2 (0.25 g, 0.58 mmole) in THF (6 ml), was treated with biotinyl-OSu (198 mg, 0.58 mmole) in *N*-methyl pyrollidone (1.5 ml) followed by $NaHCO_3$ (49 mg, 0.58 mmole) in water (1 ml). The reaction was monitored by hplc and when the disappearance of starting diazomethyl ketone levelled off, a second portion of biotinyl-OSu (50 mg, 0.15 mmole) in *N*-methyl-pyrrolidone (0.5 ml) was added, and stirring continued overnight. The reaction was partitioned between water (50 ml) and 150 ml each of ethyl acetate and CH_2Cl_2. After drying and removal of solvents, the residual oil was heated at 45–50°C in high vacuum to remove the methylpyrollidone. When a constant weight was reached, the residue (0.26 g) was taken up in 3 ml of CH_2Cl_2 containing 10% methanol and applied to a silica column (1 cm diameter, 6.5 g of silica) which was eluted with methylene chloride and methanol (5% to 15%) and monitored by tlc in CH_2Cl_2 and methanol (20%) to yield 180 mg, 46% of product with the expected mass (Table I).

Other syntheses. Boc-His-Leu-Leu-Tyr-CHN_2 was prepared from H-Leu-Leu-Tyr-CHN_2; PAB-Leu-Leu-Val-CHN_2 and Z-Tyr-Leu-Leu-Val-CHN_2 from H-Leu-Leu-Val-CHN_2.

Enzymes

Some observations were made with chicken gizzard calpain purified as described previously.[11] Platelet calpain was obtained as described below. Cathepsin L was a rat liver preparation kindly provided by Dr. H. Kirschke, Halle University.

Purification of platelet Calpain

Various methods have been described which at various stages were either not reproducible or were otherwise unsatisfactory. A procedure was evolved by slight modification of published methods which provided pure calpain consistently. Two-day-old human platelet concentrates were purchased from the Swiss Red Cross Central Laboratory in Bern. A washed platelet suspension (5×10^9 platelets/ml) in 10 mM Tris-HCl, containing 154 mM NaCl, 5 mM EDTA, 1 mM DTT, pH 7.5 (homogenizing buffer) was freeze-thawed thrice and sonicated (Labsonic 2000, Braun; max output for 15 s × 7 cycles). The platelet lysate was ultracentrifuged at 235,000 × g for 1 h. Proteins were precipitated from the supernatant solution with solid $(NH_4)_2SO_4$ added to 70% saturation. The precipitate was dialyzed against 10 mM Tris-HCl, 0.25 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 0.01% NaN_3, pH 7.5 (buffer A), and the crude extract thus obtained was chromatographed on a DEAE-Sepharose CL-6B column[36] using a linear gradient of 0.07–0.7 M NaCl in buffer A. Active fractions of calpain I were pooled and further purified by successive chromatographic procedures previously described utilizing Cibacron 3GA-Agarose,[11] Phenyl-Sepharose CL-4b and FPLC mono Q 5/5 columns;[15] all but the last procedure were carried out at 4°C. The final preparation showed a single band on gel electrophoresis. Activity was monitored fluorimetrically using Cbz-Leu-Tyr aminomethylcoumaranylamide as substrate.[6,25]

Assay Procedures

The inhibitors were evaluated by either of two standard procedures. Timed aliquots were removed from an incubated mixture of inhibitor and calpain and residual activity determined or, in the second method, the cleavage of substrate was followed in a mixture of all three components with a recording fluorimeter.[6] The final concentration of calpain in the reaction mixture was 5 $\mu g/\mu l$.

RESULTS AND DISCUSSION

Our initial group of inhibitors for the affinity-labelling of calpain was based on the effectiveness of Leu-Leu in P_2 and P_3 as demonstrated in *in vitro* studies with a checken gizzard preparation.[6] Observations on the use of such inhibitors for the inactivation of calpain within cells began with platelets and employed chiefly radioiodinatable Z-Leu-Leu-Tyr-CHN_2 and the corresponding fluoromethyl ketone.[24] Meanwhile, studies on variation in inhibitor structures evaluated *in vitro*

eventually involved a transition to a platelet preparation to correlate more closely with the cellular studies. The nature of the P_1 residue has been varied to include Ala, Pro, Met, and Val and some variations have been made in the N-terminal region to improve water solubility (His, Cit) or, in the case of biotinyl derivatives, to provide another tool for the detection and handling of modified proteases.

The retention of Leu-Leu in P_2 and P_3, ensures the effectiveness of a number of alterations in P_1, however, the presence of proline caused a major loss of reactivity (Table II). The loss was comparable to that seen with the conversion of diazomethyl ketone to a diazoethyl ketone which was shown earlier to reduce effectiveness towards cathepsins B and L. The proline in the conserved region of calpastatin[33] may not be positioned as we have imagined it or, if so, considerable assistance in binding comes from other regions of the natural inhibitor while the proline provides a difficultly cleavable bond for preservation of the inhibitor.

N-terminal variations were found to be tolerated with considerable retention of inhibitory activity as demonstrated by many entries in Tables II and III. These values represent different kinetic methods since those in Table III were observed in the presence of substrate (cf., also Figures 1–3). Z-Leu-Leu-Tyr-CHN_2 is included in each Table for reference. An unexpected result was the superiority of Biot-Aca-Leu-Tyr-CHN_2 to Biot-Aca-Leu-Leu-Tyr-CHN_2 in the inactivation of calpain. Biotin and the spacer aminocaproic acid provide a large lipophilic addition

TABLE II
Inactivation of chicken gizzard calpain by peptidyl diazomethyl ketones

Inhibitor	[I] (μM)	k_2 ($M^{-1} s^{-1}$)
Z-Leu-Leu-Tyr-CHN_2	0.02	3.5×10^4
Boc-His-Leu-Leu-Tyr-CHN_2	0.2	3.4×10^3
Z-Tyr-Leu-Leu-Val-CHN_2	1	1.4×10^3
Biot-Aca-Leu-Leu-Ala-CHN_2	25	2.4×10^2
Z-Tyr(I)-Leu-Leu-Ala-CHN_2	10	2.1×10^2
PAB-Leu-Leu-Val-CHN_2	200	5.3
Z-Leu-Leu-Pro-CHN_2	80	3.8
Z-Leu-Leu-Tyr-$C(CH_3)N_2$	200	3.2

TABLE III
Inhibition of human platelet calpain by peptidyl diazomethyl ketones observed in the presence of substrate,[a] 37°C

Inhibitor	$10^{-3} \times k_2$ ($M^{-1} s^{-1}$)
Z-Leu-Leu-Tyr-CHN_2	190
Z-Leu-Leu-Met-CHN_2	23
Z-Tyr-Pro-Leu-Leu-Ala-CHN_2	6.5
Z-Cit-Leu-Leu-Ala-CHN_2	3
Biot-Aca-Leu-Tyr-CHN_2	2.9
Z-His-Val-Leu-Leu-Ala-CHN_2	1.4

[a]0.25 mM Succ-Leu-Tyr-AMC, in 50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$, 2.5 mM DTT, 0.1% Triton X-100, 2% DMSO at 37°C.

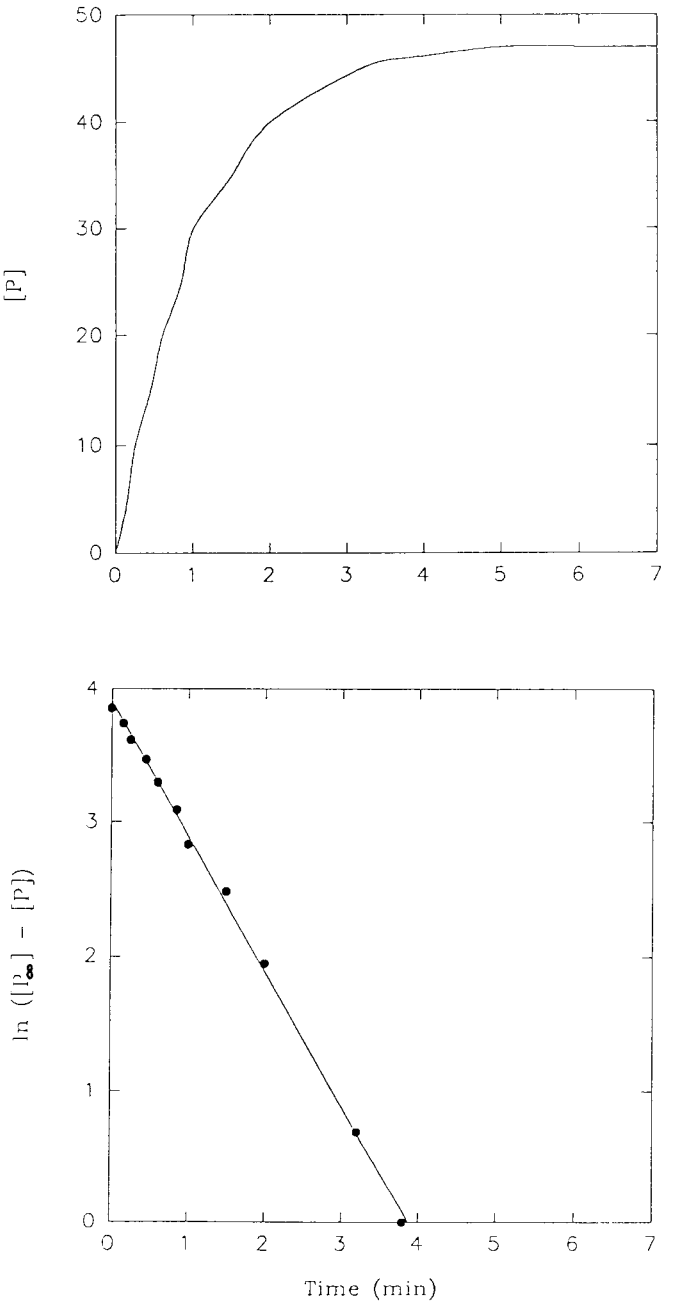

FIGURE 1 Inhibition of platelet calpain by Z-Tyr-Pro-Leu-Leu-Ala-CHN_2 (0.005 mM) observed in the presence of Succ-Leu-Tyr-NHMec (0.25 mM).

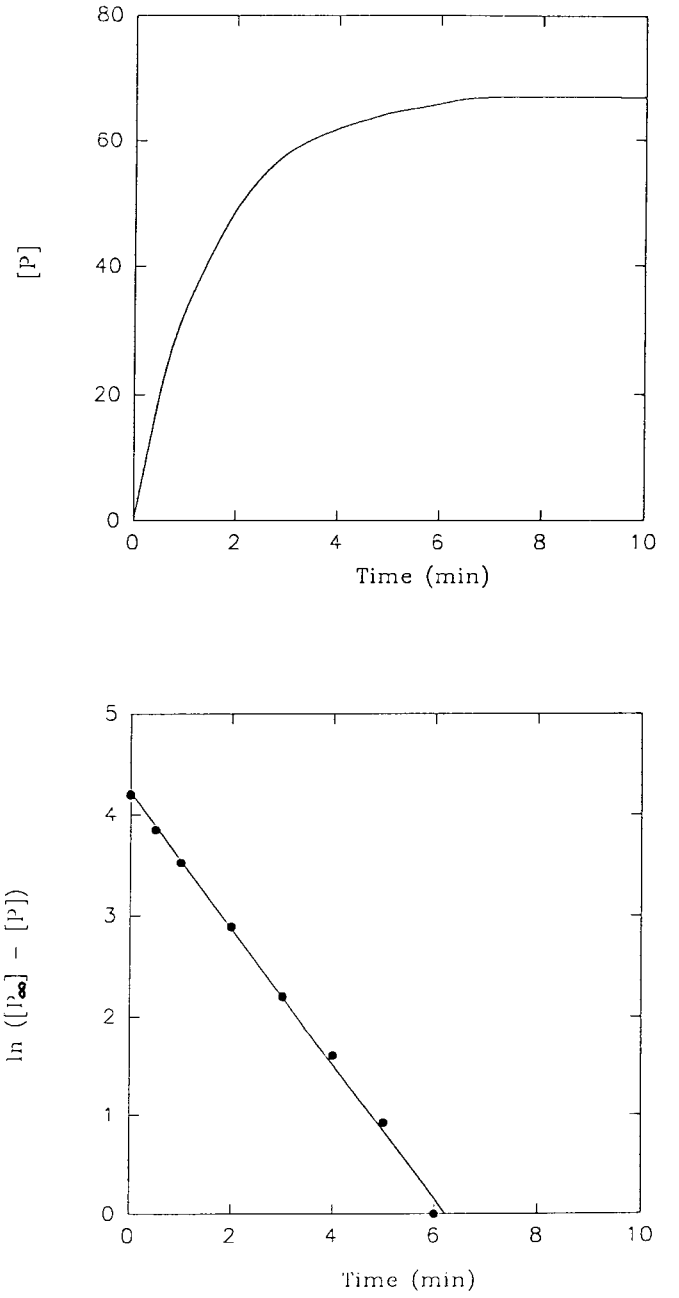

FIGURE 2 Inhibition of platelet calpain by Z-Leu-Leu-Met-CHN_2 (0.50 μM) observed in the presence of Succ-Leu-Tyr-NHMec.

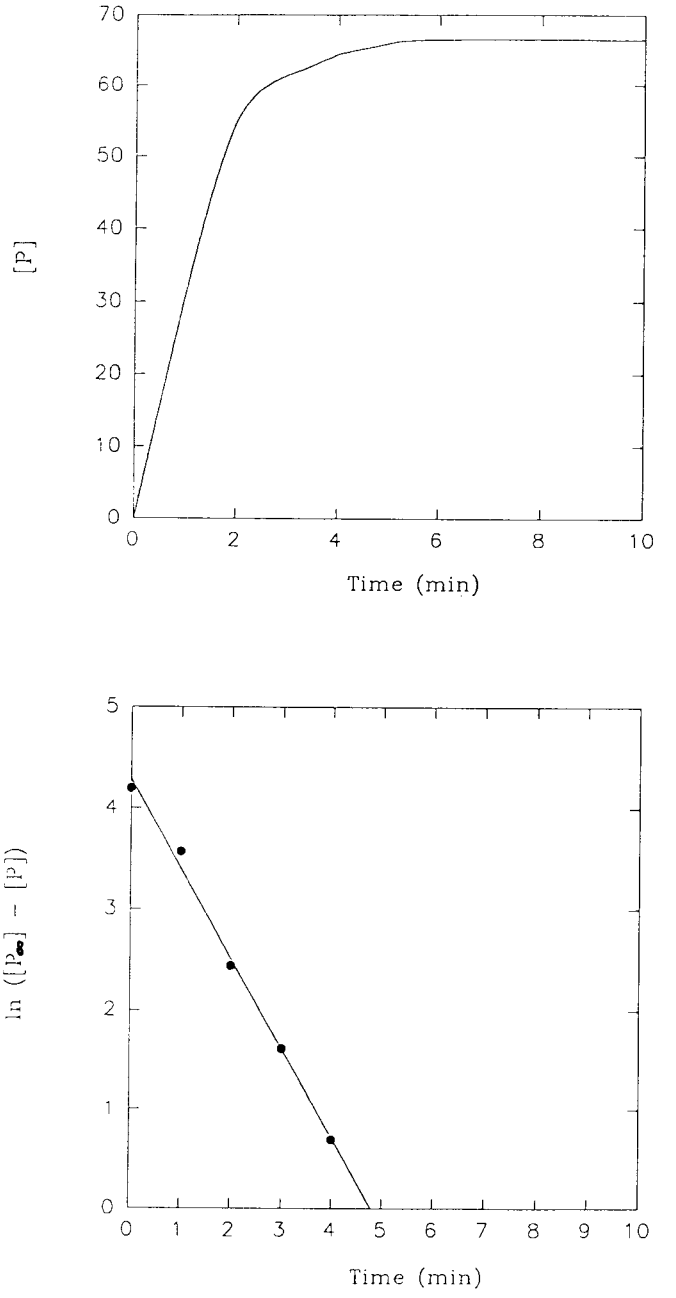

Figure 3 Inhibition of platelet calpain by Biot-Aca-Leu-Tyr-CHN_2 observed in the presence of Succ-Leu-Tyr-NHMec (0.25 mM).

which is apparently promoting binding in the shorter peptide overcoming the lack of the P_3 Leu. We have included the spacer because it occasionally improves access of avidin or related protein to the biotin.

It is customary in altering inhibitor structures to attempt to maximize activity in *in vitro* studies. However, we have found in concurrent studies with intact platelets that other important differences may become apparent. Thus with iodinated derivatives, it has been shown Cbz-Leu-Leu-TyrCHN_2 is more selective than the corresponding fluoromethyl ketone; although the latter appears to be more effective in intact cells, its action is accompanied by modification of additional proteins besides calpain (Anagli *et al.*, Biochem J., in press).

A recent development of interest in the area of cysteinyl proteases is the growing evidence[37] that these proteases are of importance in the life cycle of parasites that have a considerable impact on the well-being of man and animals. Many have been shown by studies at the DNA level or protein level to possess sequences homologous to the papain family. Inhibitors of cysteinyl proteinases have a lethal effect on malaria parasites, although the target proteinase(s) has not yet been characterized. Some of the materials we describe may be of value in the study of this growing family of proteinases.[38–42]

References

1. Bohley, P., Kirschke, H., Langner, J., Miehe, M., Riemann, S., Slama, Z., Schon, E., Wiederanders, B. and Ansorge, S. (1979) In *Biological Function of Proteinases* (S. Holzer and H. Tschesche, eds.). Berlin: Springer-Verlag, pp. 17.
2. Murachi, T., Tanaka, K., Hatanaka, M. and Murakami, T. (1981) *Adv. Enzyme Regul.*, **19**, 407.
3. Leary, R., Larsen, D., Watanabe, H. and Shaw, E. (1977) *Biochemistry*, **16**, 5857.
4. Green, G.D.J. and Shaw, E. (1981) *J. Biol. Chem.*, **256**, 1923.
5. Kirschke, H.. Wikstrom, P. and Shaw, E. (1988) *FEBS Lett.*, **228**, 128.
6. Crawford, C., Mason, R.W., Wikstrom, P. and Shaw, E. (1988) *Biochem. J.*, **253**, 751.
7. Rasnick, D. (1985) *Anal. Biochem.*, **149**, 461.
8. Rauber, P., Angliker, H., Walker, B. and Shaw, E. (1986) *Biochem. J.*, **239**, 633.
9. Angliker, H., Wikstrom, P., Rauber, P. and Shaw, E. (1987) *Biochem. J.*, **241**, 871.
10. Angliker, H., Wikstrom, P., Rauber, P., Stone, S. and Shaw, E. (1988) *Biochem. J.*, **256**, 481.
11. Angliker, A., Anagli, J. and Shaw, E. (1992) *J. Med. Chem.*, **35**, 217.
12. Hanada, H., Tamai, M., Yamagishi, M., Ohmura, S., Sawada, J. and Tanaka, I. (1978) *Agric. Biol. Chem.*, **42**, 523.
13. Barrett, A.J., Kembhavi, A.A., Brown, M.A., Kirschke, H., Knight, G., Tamai, M. and Hanada, K. (1982) *Biochem. J*, **201**, 189.
14. Suzuki, K. (1983) *J. Biochem.*, **93**, 1305.
15. Parkes, C., Kembavi, A.A. and Barrett, A.J. (1985) *Biochem. J.*, **230**, 509.
16. McGowan, E.B., Becker, E. and Detwiler, T.C. (1989) *Biochem. Biophys. Res. Commun.*, **158**, 432.
17. Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.*, **27**, 157.
18. Schechter, I. and Berger, A. (1968) *Biochem. Biophys. Res. Commun.*, **32**, 898.
19. Shaw, E. (1990) *Adv. Enzymol. Rel. Areas Mol. Biol.*, **63**, 271.
20. Shaw, E. and Dean, R. (1980) *Biochem. J.*, **186**, 385.
21. Salminen, A. and Gottesman, M.M. (1990) *Biochem. J.*, **272**, 39.
22. Mason, R.W., Wilcox, D., Wikstrom, P. and Shaw, E. (1989) *Biochem. J.*, **257**, 125.
23. Wilcox, D. and Mason, R.W. (1992) *Biochem. J.*, **285**, 495.
24. Anagli, J., Hagmann, J. and Shaw, E. (1991) *Biochem. J.*, **274**, 497.
25. Sasaki, T., Kikuchi, T., Yumoto, N., Yoshimura, N. and Murachi, T. (1984) *J. Biol. Chem.*, **259**, 12489.
26. Angelastro, M.R., Mehdi, S., Burkhart, J.P., Peet, N.P. and Bey, P. (1990) *J. Med. Chem.*, **33**, 13.
27. Hayashi, M., Inomata, M., Saito, Y., Ito, H. and Kawashima, S. (1991) *Biochim. Biophys. Acta*, **1094**, 249.
28. James, P., Vorherr, T., Krebs, J., Morelli, A., Castello, G., McCormick, D.J., Penniston, J.T., DeFlora, A. and Carafoli, E. (1989) *J. Biol. Chem.*, **264**, 8289.

29. Kiuchi, K., Kuichi, K., Titani, K., Suzuki, K. and Nagatsu, T. (1991) *Biochemistry*, **30**, 10416.
30. Harris, A.S., Croall, D.E. and Morrow, J.S. (1988) *J. Biol. Chem.*, **263**, 15754.
31. Suzuki, K., Imajoh, S., Emori, Y., Kawasaki, H., Minami, Y. and Ohno, S. (1987) *FEBS Lett.*, **220**, 271.
32. Kawasaki, H., Emori, Y., Imajoh-Ohmi, S., Minami, Y. and Suzuki, K. (1989) *J. Biochem.*, **106**, 274.
33. Uemori, T., Shimojo, T., Asada, K., Asano, T., Kimizuka, F., Kato, I., Maki, M., Hatanaka, M., Murachi, T., Hanzawa, H. and Arata, Y. (1990) *Biochem. Biophys. Res. Commun.*, **166**, 1485.
34. Walker, B., Cullen, B.M., Kay, G., Halliday, I.M., McGinty, A. and Nelson, J. (1992) *Biochem. J.*, **283**, 449.
35. Cullen, B.M., Halliday, I.M., Kay, G., Nelson, J. and Walker, B. (1992) *Biochem. J.*, **283**, 461.
36. Kambayashi, J.-I. and Sakon, M. (1989) *Meth. Enzymol.*, **169**, 445.
37. North, M.J., Mottram, J.C. and Coombs, G.H. (1990) *Parasitology Today*, **6**, 270.
38. Mbawa, Z.R., Gumm, I.D., Shaw, E. and Lonsdale-Eccles, J.D. (1992) *Eur. J. Biochem.*, **204**, 3771.
39. Cazzulo, J.J., Franke, M.C.C., Martinez, J. and de Cazzulo, B.M.F. (1990) *Biochim. Biophys. Acta*, **1037**, 186.
40. Luaces, A.L. and Barrett, A.J. (1988) *Biochem. J.*, **250**, 903.
41. Mottram, J.C., North, M.J., Barry, J.D. and Coombs, G.H. (1989) *FEBS Lett.*, **258**, 211.
42. Klinkert, M.-Q., Felleisen, R., Link, G., Ruppel, A. and Beck, E. (1989) *Mol. Biochem. Parasitol.*, **33**, 113.