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# INHIBITORS OF THE ADENOVIRUS TYPE 2 PROTEINASE BASED ON SUBSTRATE-LIKE TETRAPEPTIDE NITRILES

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**Abstract:** Tetrapeptides containing the substrate derived $P_4$-$P_1$ residues Leu-Ala-Gly-Gly and C-terminal ester, acid, amide, nitrile and dimethylacetal functionalities were synthesised and tested as inhibitors of the adenovirus type 2 proteinase. The acids and amides did not act as inhibitors and the dimethyl acetals and the nitriles served as moderate and good reversible inhibitors respectively.

Adenoviruses were first isolated from adenoidal tissue in children by Rowe in 1953.[1] The type 2 (Ad2) causes sporadic[2,3] respiratory disease and adenovirus type 3 and 7 cause epidemic acute respiratory disease which can develop into pneumonia.[4] Adenoviruses are also associated with acute follicular conjunctivitis,[5] intussusception of the bowel in infants[6] and have been responsible for the mortality of immunocompromised patients through pneumonia and hepatic necrosis.[7] The proteinase is an important target for inhibition as it is responsible for processing six proteins in the virus, namely the preterminal protein, pVI, pVII, pVIII, IIIa and an 11 kDa protein.[8,9,10] These proteolytic cleavages are vital for virus maturation and, indeed, a mutant virus lacking the active proteinase has been shown to produce virus particles that are non infectious.[11]

The 23 kDa proteinases from 12 different adenovirus serotypes possess highly conserved amino acid sequences but share no recognisable motifs with other proteinases.[12] The pure proteinase is inactive and requires activation with a dimeric oxidised Cys containing 11-residue peptide cofactor derived from the viral pVI protein.[13] The enzyme is inhibited by thiol attacking reagents although, until now, specific inhibitors have not been described. Very recent studies have suggested that Cys-104 serves a catalytic role and have shown that its replacement by a serine residue gives an active proteinase which is sensitive to typical serine proteinase inhibitors but not to thiol specific agents.[14]

The substrate specificity of the proteinase[15] is defined as (M,L,I)XGG-X or (M,L,I)XGX-G. Based on the sequence upstream of the scissile bond a series of potential inhibitors of the type R-LAG-NH-$CH_2$-R' (Figure 1) were prepared using solution-phase coupling procedures as outlined below and summarised in Scheme 1.

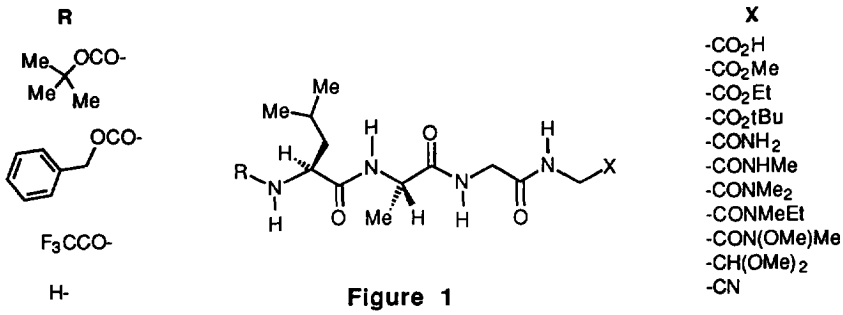
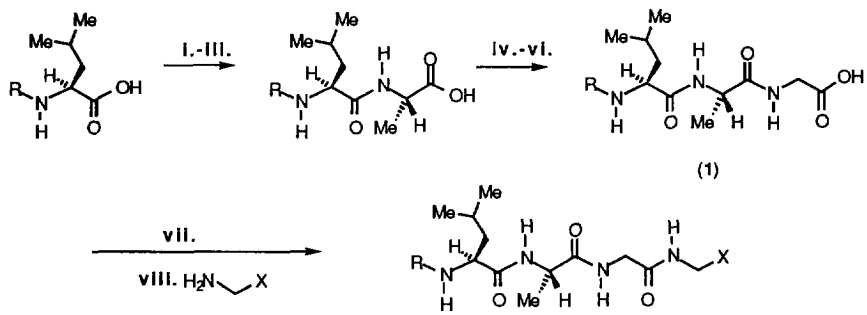



**Figure 1**

To prepare the parent N-protected tripeptides (**1**, R= $PhCH_2OCO$-, $Me_3COCO$- and $CF_3CO$-), the appropriate N-protected (2S)-leucine was coupled with either the methyl or benzyl ester of (2S)-alanine using the mixed anhydride procedure.[16] Saponification of the methyl ester or removal of the benzyl ester in each case gave the dipeptide which was further extended through reaction of the activated mixed anhydride with t-butylglycinate or benzylglycinate. Removal of the t-butyl or benzyl ester protection gave the required N-protected tripeptides. In the case of the benzyl esters $Pd(OAc)_2$, $Me_2EtSiH$ and $Et_3N$[17] in acetone, and then KF, was used to effect cleavage. Catalytic hydrogenolysis in methanol was accompanied by unwanted transesterification to the methyl ester.




**Scheme 1**

**Reagents.**
**Steps i-iii.**
For R = Cbz and t-Boc; **i**. N-methyl morpholine (NMM), i-Bu chloroformate in THF. **ii.** Ala methyl ester. HCl, NMM in DMF. **iii.** NaOH in MeOH; then HCl to pH 2. For R = $COCF_3$; **i**. NMM, i-Bu chloroformate in THF. **ii.** Ala benzyl ester tosylate, NMM in DMF. **iii.** $Pd(OAc)_2$, $Me_2EtSiH$, $Et_3N$ acetone; then KF.

**Steps iv-vi.**
For R = Cbz; **iv**. NMM, i-Bu chloroformate in THF. **v**. Gly t-Bu ester acetate, NMM in DMF. **vi.** 95% formic acid. For R = t-Boc and $COCF_3$; **iv**. NMM, i-Bu chloroformate in THF. **v**. Gly benzyl ester tosylate, 1 equiv. NMM in THF. **vi.** $Pd(OAc)_2$, $Me_2EtSiH$, $Et_3N$, DCM; then KF.

**Steps vii-viii.**
**vii.** TBTU in THF. **viii.** $H_2NCH_2$-X HCl; X = **a**) $-CO_2Me$ → (**2**); **b**) $-CO_2Et$ → (**3**); **c**) $-CO_2tBu$ → (**4**); **d**) $-CONH_2$ → (**6**); **e**) -CONHMe → (**7**); **f**) $-CONMe_2$ → (**8**); **g**) -CONMeEt → (**9**); **h**) -CON(OMe)Me → (**10**); **i**) -CN → (**13**-**15**).

The tripeptides (**1**, R= $PhCH_2OCO$-, $Me_3COCO$- and $CF_3CO$-) were further elaborated, after activation of the carboxylic acid, through reaction with the appropriate derivative of glycine ($H_2N$-$CH_2$-X, X = -$CO_2Me$, -$CO_2Et$, -$CO_2$-tBu, -$CONH_2$, CONHMe, $CONMe_2$, CONMeEt, CON(OMe)Me, or -CN). Due to the low solubility of the reactants in cold THF at -10 °C the mixed anhydride procedure failed to work in several instances and, therefore, O-benzotriazole-1-yl-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU)[18] in THF at 20 °C was used to activate the carboxylic acid for coupling. Compounds (**11**) and (**12**) were prepared from the appropriate dipeptide and glycyl-amidoacetaldehyde dimethyl acetal which was prepared through reaction with carboxybenzyl-glycine and aminoacetaldehyde dimethyl acetal. Compound (**5**) was prepared through base catalysed hydrolysis of ester (**2**) and was isolated as the sodium salt. Compounds (**16**), (**17**) and (**18**) were prepared from (**6**), (**7**) and (**3**) respectively. All compounds and intermediates gave the expected spectral and analytical data. Full details will be reported elsewhere.

**Table 1.** Inhibition of the Ad2 Proteinase by Tetrapeptide Substrate Analogues (**2**-**21**).

| | Compound | % Inhibition[a] | $IC_{50}$ | $K_i$ and/ or comments |
|---|---|---|---|---|
| 2 | CBZ-LAGG-OMe | 72 | - | Substrate |
| 3 | CBZ-LAGG-OEt | 67 | - | Substrate |
| 4 | BOC-LAGG-t-Bu | 40 | - | Substrate[b] |
| 5 | CBZ-LAGG-OH | 0 | - | Product |
| 6 | CBZ-LAGG-$NH_2$ | 0[c] | - | - |
| 7 | CBZ-LAGG-NHMe | 0[c] | - | - |
| 8 | CBZ-LAGG-$NMe_2$ | 0 | - | - |
| 9 | CBZ-LAGG-NMeEt | 0 | - | Not a substrate |
| 10 | CBZ-LAGG-N(OMe)Me | 0 | - | - |
| 11 | BOC-LAG-$NHCH_2CH(OCH_3)_2$ | 59 | 1800 μM | Non-linear, concave up |
| 12 | CBZ-LAG-$NHCH_2CH(OCH_3)_2$ | 59 | 400 μM | 380 μM Noncompetitive[d] |
| 13 | CBZ-LAG-$NHCH_2CN$ | 95 | 10 μM | 16 μM Noncompetitive[e] |
| 14 | BOC-LAG-$NHCH_2CN$ | 54 | - | - |
| 15 | TFA-LAG-$NHCH_2CN$ | 50 | 6900 μM | Linear |
| 16 | LAGG-$NH_2$ | 0 | - | - |
| 17 | LAGG-NHMe | 0 | - | Not a substrate |
| 18 | LAGG-OEt | - | - | Substrate |
| 19 | $NH_2CH_2CN$. HCl | 0 | - | - |
| 20 | TFA-G-$NHCH_2CN$ | 0 | - | - |
| 21 | TFA-AG-$NHCH_2CN$ | 5 | - | - |

[a]Rate determinations performed using 680 μM substrate, 200 nM proteinase and 1.35 mM of the tested compound in 0.6% DMSO in 2 mM phosphate buffer containing 100 mM NaCl at pH 7.8 and at 37 °C against controls containing no inhibitor. [b]Compound (**4**) was a substrate at two sites. [c]Not very soluble and inactive at maximum soluble concentration, see entries for compounds (**16**) and (**17**). [d]Compound (**12**) displayed non-linear inhibition. [e]Compound (**13**) displayed linear inhibition.

In the first instance, all of the compounds (**2** to **17**) [0.68-1.93 mM] were tested as inhibitors of the Ad2 proteinase in standard enzyme activity assays as previously described[19] or by using fluorescamine[20] to quantify the appearance of a new amino terminus, following the cleavage of the substrate, Ac-LRGA-GRSR (0.68 mM [$K_M$ = 3.4 mM]), Table 1. The acid (**5**) and the amides (**6-10** and **16** and **17**) showed no inhibitory activity whatsoever. The esters (**2-4**), the dimethylacetals (**11** and **12**) and the nitriles (**13-15**), however, showed significant inhibitory activity and their modes of inhibition were investigated further.

Plots of reciprocal initial rate versus inhibitor concentration were non-linear, concave-up for both of the dimethylacetals indicating that at high concentration of the inhibitor, more than one molecule can bind to the enzyme. At low concentrations it was possible to estimate the $IC_{50}$ values for both compounds from the near linear assymptotes, Table 1. Double reciprocal plots of initial rate versus substrate concentration at different low concentrations of inhibitor (**12**) were linear and intersected on the abscissa (*i.e.* the apparent value of $K_M$ was not affected) indicating that acetal (**12**) acts as a noncompetitive inhibitor.

The nitriles (**13-15**) all showed significant inhibitory activity. The Cbz- and trifluoroacetyl- protected nitriles both displayed linear plots of reciprocal initial rate versus inhibitor concentration and compound (**15**) acted as a noncompetive inhibitor. The removal of the $P_4$ binding Leu residue, to give compound (**21**), substantially reduced the activity of the inhibitor and removal of the $P_3$ binding Ala residue also, to give compound (**20**), abolished activity completely, Table 1.

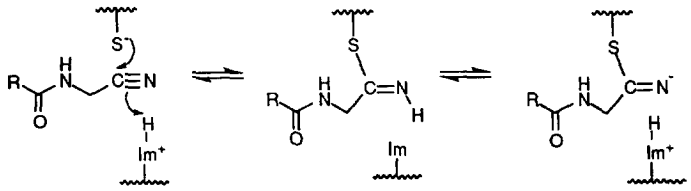
The finding that two of the best inhibitors for the proteinase are noncompetitve inhibitors is informative given that the compounds contain the $P_4$-$P_1$ binding potential of the substrate and might be expected to serve as competitive inhibitors. Nevertheless, the compounds strongly resemble the structure of the N-terminal cleavage product and evidently, are able to bind to the free enzyme (the form that the substrate binds to) and also to a form of the enzyme other than the free enzyme such that the apparent $K_M$ for the substrate is unaltered.

Endopeptidases such as the Ad2 proteinase produce two peptide products of which either can desorb from the active site first, depending on the mechanism and kinetics of the system. If the C-terminal product desorbs first, the N-terminal product would remain bound to the enzyme and block the entry of inhibitors containing similar $P_4$-$P_1$ peptide sequences. However, if the N-terminal product was released first, an inhibitor behaving as a $P_4$-$P_1$ mimick could enter and trap the C-terminal product on the enzyme. In combination with an ability to bind to the same region of the free enzyme (in the absence of the C-terminal product), the inhibitor would act in a noncompetitive mode and would best be considered as a product analogue. Thus, this analysis indicates that the N-terminal product is released first.

This result is of particular interest in the light of the proposal that the Ad2 proteinase is a thiol proteinase[14,19] since the well studied examples, including papain, release the C-terminal product upon the formation of the acyl enzyme.[21]

It is also interesting to consider the chemical mode of inhibition. In terms of size, all of the inhibitors are expected to be able to bind to the enzyme including compounds (**5-10, 16, 17**). The finding that compounds (**5-10, 16, 17**) did not inhibit coupled to the fact that the esters, including the large t-butyl ester (**4**), and the dimethylacetals (**11** and **12**) bind well indicates that a secondary chemical process must occur to cause inhibition. Covalent bond formation between the enzyme [or the amino group of the C-terminal product] and the inhibitor would seem to account for the properties of the compounds and, indeed, several other observations are in accord with these ideas. The nitrile Ac-Phe-$NHCH_2CN$ reversibly inhibits papain ($K_i$ = 380 μM) and forms a thioimidate adduct that does not hydrolyse while the corresponding acid and amide are very poor inhibitors ($K_i$

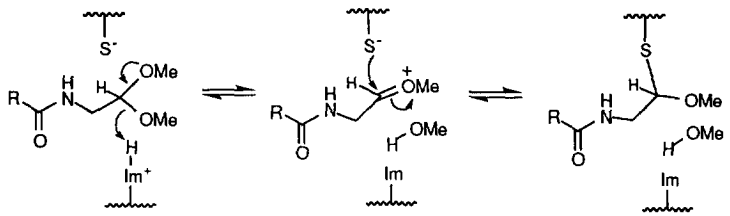
values > 10 mM).[22] The nitrile (**13**) reversibly inhibits the Ad2 proteinase (Table 1) but was not hydrolysed upon prolonged exposure to the enzyme as judged by NMR spectroscopy and thus could form a similar adduct, Scheme 2.



**Scheme 2**

All of the esters tested served as substrates. The t-butyl ester (**4**) served as a substrate for the Ad2 proteinase at pH 8.0 at two sites, as judged by NMR spectroscopy. Initially the t-butyl ester group was hydrolysed, and then, much more slowly, the N-terminal t-butyl ureathane group was hydrolysed. The non-inhibitory amides (**9**) and (**17**) did not serve as substrates under identical conditions.

The behaviour of the dimethyl acetal (**12**) is of particular interest since it was not hydrolytically processed by the enzyme, as judged by NMR spectroscopy, and yet served as a good inhibitor. The compound could function as a stable or transition state mimick for the catalytic process if the putative thiolate anion could nucleophilically displace one of the methoxy groups. As $S_N2$ displacement of methoxide seems most unlikely, it would appear that such a process, Scheme 3, would require an acidic group at the active site, possibly one analogous to the protonated His-159 of papain. Interestingly, specific alteration of the only conserved His residue in the adenovirus proteinase (His-54 in type 2) gives an inactive enzyme.[14]



**Scheme 3**

In summary, all of the results of this study are consistent with the notion that the adenovirus proteinases are thiol proteinases and form covalent adducts with substrates and with suitably functionalised inhibitors. The Ad2 proteinase appears to differ from papain in the order in which products are released from the active site and this property may provide alternative strategies to designing useful inhibitors.

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