Antiviral Activity of Fusion Peptide Analogue Having Novel N-Protecting Groups

Takuo CHIBA, Naohito TAKAHASI* and Yuya TAKASUGI**

(Received November 25, 1997)

A series of oligopeptides have been synthesized with amino acid sequences that resemble those of the N-terminal regions of the F_1 polypeptides, N-termini generated by proteolytic cleavage which activates infectively. We synthesized the F_1 peptide analogue via the acetoacetyl amino acid intermediates. Amino acids reacted with diketene to afford quantitatively acetoacetyl amino acids which were useful synthones for the preparation of peptides. Acetoacetyl tripeptides were prepared by usual manner. Acetoacetyl group has two important properties, namely, one shows favorable release for changing the N-protect, and another one shows simple transformation into heterocycles. We applied the later for inducing heterocycles. Acetoacetyl group was transformed into oxazolyl, pyrazolyl and thiazolyl groups via brominated product. Unfortunately, antiviral active N-protected tripeptides were not found in this research.

Two glycoproteins which form spike-like projections from the surface of the virion are associated with the membrane of paramyxoviruses. The larger glycoprotein, designated HN, possesses the receptor-binding activity of the virus and except in the morbillivirus subgroup, this protein also exhibits neuraminidase activity[1]. The other glycoprotein, designated F, is involved in virus penetration, through fusion of viral and cell fusion and hemolysis, activities which are activated by proteolytic cleavage of the protein by a host protease to yield two disulfide-linked polypeptides, F_1 and F_2[2].

The proteolytic cleavage that activates these biological properties of the virus generates a new N-terminus on the F_1 polypeptides, which is the polypeptide whose C-terminus is associated with the lipid bilayer of the viral membrane. The ollowing lines of evidence suggested that the structure of new N-terminus is important for the expression of the biological activities of the F protein, each of which involves membrane fusion. As indicated above, the expression of the activities is dependent on the cleavage. The new N-terminal region is extremely hydrophobic, raising the possibility that it could be involved in interactions with the target cell membrane. The amino acid sequence in this region is highly conserved among different paramyxoviruses. Sheid et. al noted a similarity between the N-terminal amino acid sequence (Phe-Phe-Gly) of F_1 and that of an oligopeptide inhibitor of cell fusion by paramyxoviruises[3]. This oligopeptide, benzyloxycarbonyl-D-Phe-L-Phe-Gly, was found in 1968 to inhibit plaque formation by measles virus[4]. Because of the indications that the N-terminal region of F_1 was involoved in the biological activities of the protein, and with the hypothesis that it might be possible to inhibit these activities specifically, we synthesized a number of oligopeptides which resembled the region of the F_1 polypeptide, and investigated their ability to inhibit the replication of several different paramyxoviruses[5].

Oligopeptides, having amino acid sequences similar to those of the N-terminal regions of paramyxovirus F_1 peptide or the myxovirus HA_2 peptide, are highly active, specfic inhibitors of the infectivity of each virus, and of cell fusion and

* graduated from ANCT (Advanced)
**graduated from ANCT

hemolysis induced by paramyxoviruses[6]. Amino acid sequences of F_1 peptides of viruses (RSV and HIV) were shown on Table I.

Table I. Amino acid sequences of each viruses

Viruses	F_1 Peptide
RSV	**FLG**FLLGVGSAIASGVAVSK
HIV	**AVG**IGALFLGFLGAAGSTGA

We synthesized the hydrophobic N-terminal tripeptide (Ala-Val-Gly) having nobel N-protecting groups for the purpose of requiring the antiviral activity against HIV.

Materials and Methods

Cells

MT-4, a T4 lymphocyte line carring human T-cell lymphotropic type I (HTLV-1), and HUT-78, a T4 lymphocyte line not carring HTLV-1, were used for the anti-HIV assays. The cells were mycoplasma-negative. The cell lines used for the cytostatic assays, i.e. L1210, FM3A, Raji, Molt/4F, and CEM, have been described elsewhere[7].

Viruses

HIV type 1 (HIV-1) was obtained from the culture supernatant of HUT-78 cell line peristently infected with HTLV-III_B (HUT-78/HTLV-III_B) HIV type 2 (HIV-2) was obtained from the culture supernatant of CEM cells peristently infected with LAV-2 (CEM/LAV-2). Titers of the HIV-1 and HIV-2 stocks were $2x10^5$ and $5x10^4$ $CCID_{50}$ (50% cell culture infective dose) per ml, respectively. The method for preparing [5-^{3}H] uridine-labelled HIV-1 particles (30 Ci/mmol, Amersham, UK) has been reported[8].

Anti-HIV assays

Activity of the compounds against HIV-1 and HIV-2 replication was based on the inhibition of virus-induced cytopathogenicity, determined by trypan blue exclusion. MT-4 cells were suspended at $3x10^5$ cells/ml and infected with HIV at 1000 $CCID_{50}$/ml. Immediately after infection, 100 μl of the cell suspention was brought into each well of a flat bottomed microtiter tray containing various concentrations of the test compounds. After 5 days incubation at 37°C, the number of viable cells was determined microscopically in an hemacytometer by trypan blue exclusion.

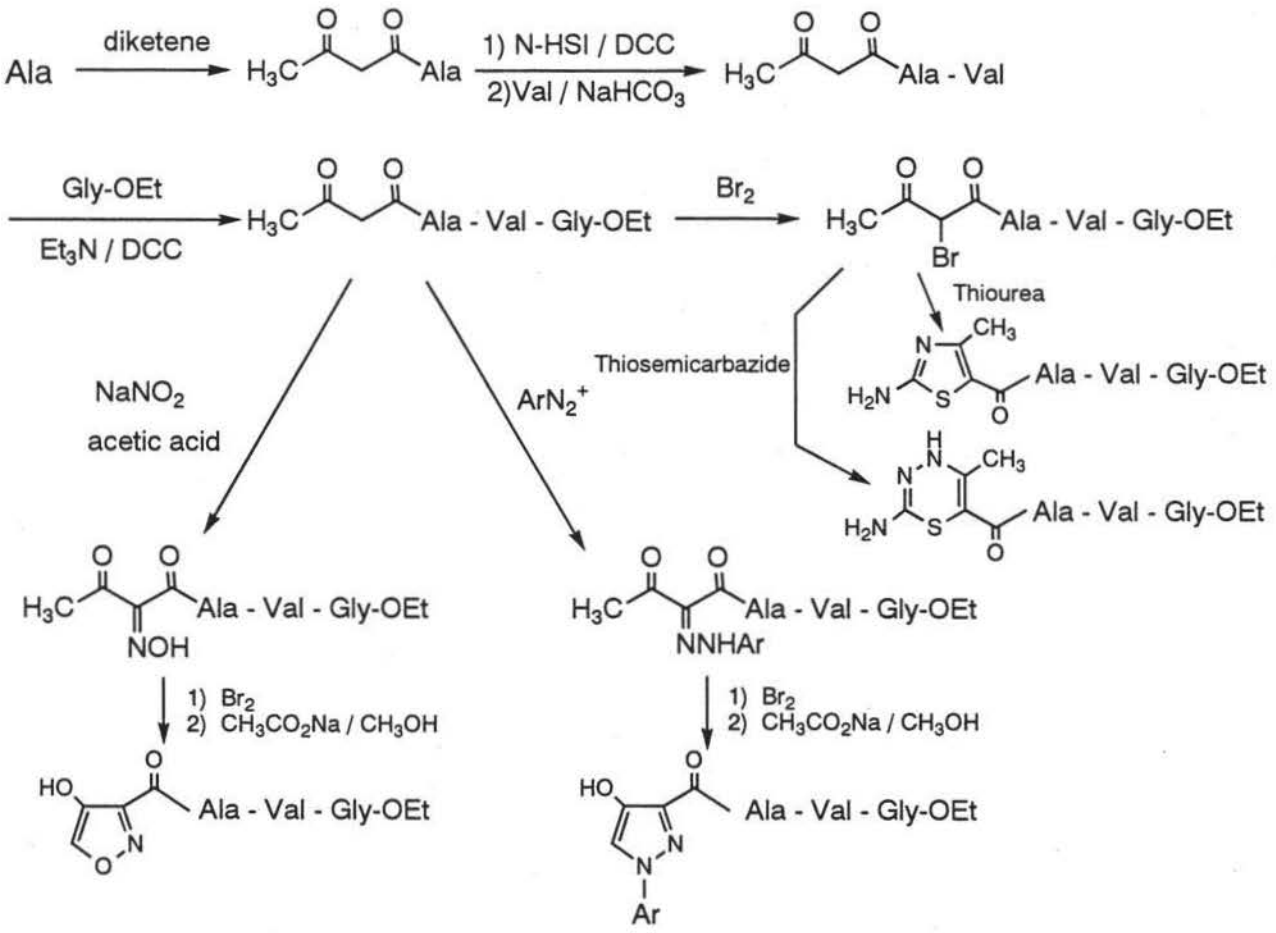
Anti-HIV activity was also determined by monitoring viral antigen expression in HUT-78 cells at day 12 after HIV-1 infection. Indirect immunofluorescence, using a polyclonal antibody as probe, and laser flow cytofluorographic analysis, were used for the determination of antigen-positive cells. HUT-78 cells were infected with HIV-1 at a multiplicity of infection of 0.4, and three quarters of the culture medium containing appropriate concentrations of the compounds were replenished every 4th day.

Cytotoxicity

Cytotoxicity of the compounds was determined by measuring viability of mock-infected MT-4 cells at day 5 and HUT-78 cells at day 12. The cytostatic effect of the compounds was evaluated in several cell lines, as described by Balzarini *et al.*[7].

Compounds

Acetoacetyl (AcAc)-Ala was easily prepared from the reaction of an equimolar amount of diketene with alanine in $NaHCO_3$ aqueous solution. After the reaction finished, the reaction mixture was acidified with conc. HCl, and then extracted with ethyl acetate to afford the product in 69% yield (mp 72-74°C ; $[\alpha]_D$-31.9). AcAc-Ala-Val was synthesized via an active ester intermediate of AcAc-Ala. Namely, equimolar amount of AcAc-Ala was disolved in a dioxane solution of N-hydroxysuccinimide. Dicyclohexylcarbodiimide (DCC) was added to this solution, and then mixture was stirred for 2h at room tempera-



Scheme 1

ture. After the cyclohexylurea was precipitated, a $NaHCO_3$ aqueous solution of Val was added. This reaction mixture was stirred at room temperature overnight, and was acidified with conc. HCl. The isolation of AcAc-Ala-Val was used usual manner. Yield of AcAc-Ala-Val was 95% (viscous oil). AcAc-Ala-Val-Gly-OEt was prepared by the reaction of AcAc-Ala-Val and Gly-OEt in the presence of DCC and triethylamine. AcAc-Ala-Val-Gly-OEt was obtained in 63% yield (mp 171–174°C ; $[\alpha]_D$-24.7).

Nitrosation of AcAc-Ala-Val-Gly-OEt by $NaNO_2$ in a 50% acetic acid solution gave 2-hydroxyimino-AcAc-Ala-Val-Gly-OEt in 92% yield (mp 156–157°C ; $[\alpha]_D$ 22.8).

Diazonium salts, obtaied from the reaction of $NaNO_2$-CH_3CO_2H with aniline derivatives, reacted with AcAc-Ala-Val-Gly-OEt to afford 2-arylamino-imino-AcAc-Ala-Val-Gly-OEt in 41–85% yields (arylamino = anilino, m-chloroanilino, p-chloroanilino, 2,4-dichloroanilino, p-anisidino).

Bromination of 2-hydroxyimino-AcAc-Ala-Val-Gly-OEt in a dioxane solution gave 2-hydroxyimino-4-bromo-AcAc-Ala-Val-Gly-OEt in 35% yield (mp 135–137°C).

Bromination of 2-arylaminoimino-AcAc-Ala-Val-Gly-OEt in a dioxane solution gave 2-arylaminoimino-4-bromo-AcAc-Ala-Val-Gly-OEt in 42–55% yields.

2-Hydroxyimino-4-bromo-AcAc-Ala-Val-Gly-OEt was treated with CH_3CO_2Na in methanol solution to give 4-hydroxy-isoxazole-3-carboxyl-Ala-Val-Gly-OEt in 35% yield.

2-Arylaminoimino-4-bromo-AcAc-Ala-Val-Gly-OEt was also treated with CH_3CO_2 Na in methanol solution to give 4-hydroxy-1-arylpyrazole-3-carboxyl-Ala-Val-Gly-OEt in 28–32% yield.

2-Bromo-AcAc-Ala-Val-Gly-OEt which was

the brominated product of AcAc-Ala-Val-Gly-OEt reacted with thiourea and thiosemicarbazide to give 2-amino-4-methylthiazole-5-carboxyl-Ala-Val-Gly-OEt and 2-amino-5-methyl-4H-1,3,4-thiadiazine-6-carboxyl-Ala-Val-Gly-OEt in 52.5% (mp 159.5–162.5°C ; $[\alpha]_D$-16.2) and 28% (mp 199–202°C ; $[\alpha]_D$-7.8) yields, respectively.

All compounds were identified by spectral measurements and elemental analyses.

Results

Based on the 20 hydrophobic amino acid sequences of HIV, tripeptide were synthesized to resemble the N-terminal region of F_1 peptide of HIV introducing addition of novel groups to the N-terminus. Acetoactyl group was used for the purpose of the synthesis of tripeptide. All compounds were tested for antiviral activity against HIV. Inhibition against replication of HIV was not found in all compounds. Some compounds were cytotoxic for host cells as CC_{50} 12.4–46.6 μg / ml (AcAc-Ala-Val-Gly-OEt ; 2-hydroxyimino-AcAc-Ala-Val-Gly-OEt ; hydroxy-1-arylpyrazole-3-carboxyl-Ala-Val-Gly-OEt ; 2-amino-4-methylthiazole-5-carboxyl-Ala-Val-Gly-OEt).

References

1) A. Sheid and P.W. Chopin, *J. Viol.*, **11**, 263–271 (1973).
2) A. Sheid and P.W. Chopin, *Virology*, **57**, 475–490 (1970).
3) A. Sheid, M.C. Graves, S.M. Silver, and P.W. Chopin, *Negative Strand Viruses and the Host Cell*, pp 181–193 (1978), Academic Press, London.
4) E. Nicolaides, H. DeWald, R. Westland, M. Lipnik, and J. Posler, *J. Med. Chem.*, **11**, 74–79 (1968).
5) T. Chiba, unpublished data.
6) C.D. Richardson, A. Sheid, and P.W. Chopin, *Virology*, **105**, 205–222 (1980).
7) J. Balzarini, E. De Clercq, P.F. Torrence, M. P. Mertes, J.S. Park, C.L. Schmidt, D. Shugar, P.J. Barr, A.S. Jones, G. Verherst, and R.T. Walker, *Biochem. Pharmacol.*, **31**, 1089–1095.
8) J. Balzarini, H. Mituya, E. De Clercq, and S. Broder, *Biochem. Biophys. Res. Commun.*, **136**, 64–71 (1986).