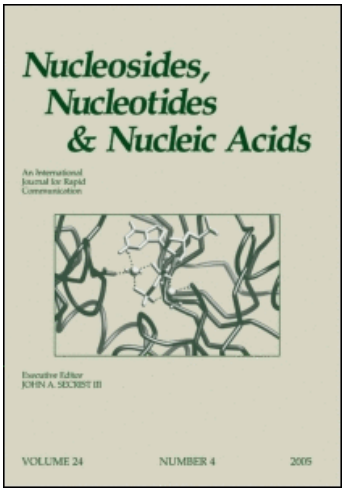

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Synthesis and Biological Evaluation of a New N^4-(N-Formyl Peptide)- 2',3'-Dideoxy-3'-Thiacytidine as Anti-Hiv Prodrug

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SYNTHESIS AND BIOLOGICAL EVALUATION OF A NEW N^4-(N-FORMYL PEPTIDE)- 2',3'-DIDEOXY-3'-THIACYTIDINE AS ANTI-HIV PRODRUG.

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Abstract:

The synthesis of a new analogue of 2',3'-dideoxy-3'-thiacytidine 9 covalently linked to an N-formyl methionyl leucyl phenylalanine peptide is described. This new prodrug analogue has been tested on the one hand as activator of human polymorphonuclear leukocytes (an EC_{50} value of 1.8 10^{-5} M was determined from dose-response curve for superoxide production) and on the other hand as inhibitor of the syncitium formation caused by HIV-1 in MT4-cells (IC_{50} = 8.0 $\pm$ 0.8 μM). In so far as this new prodrug possesses these two biological properties, it represents a useful "chemical-head" capable of targeting specific receptors located on leukocytes membranes.

An important aspect of the infection by the human immunodeficiency virus type 1 (HIV-1) is its clinical latency, suggesting that the virus itself or the provirus remains latent for extended periods of time after primary infection. Certain heterologous viral proteins (1-5), certain cytokines (6-8) or chemical and physical agents (9-13) are able to reactivate latent virus. It has been observed that a property on capacity shared by these agents is the ability

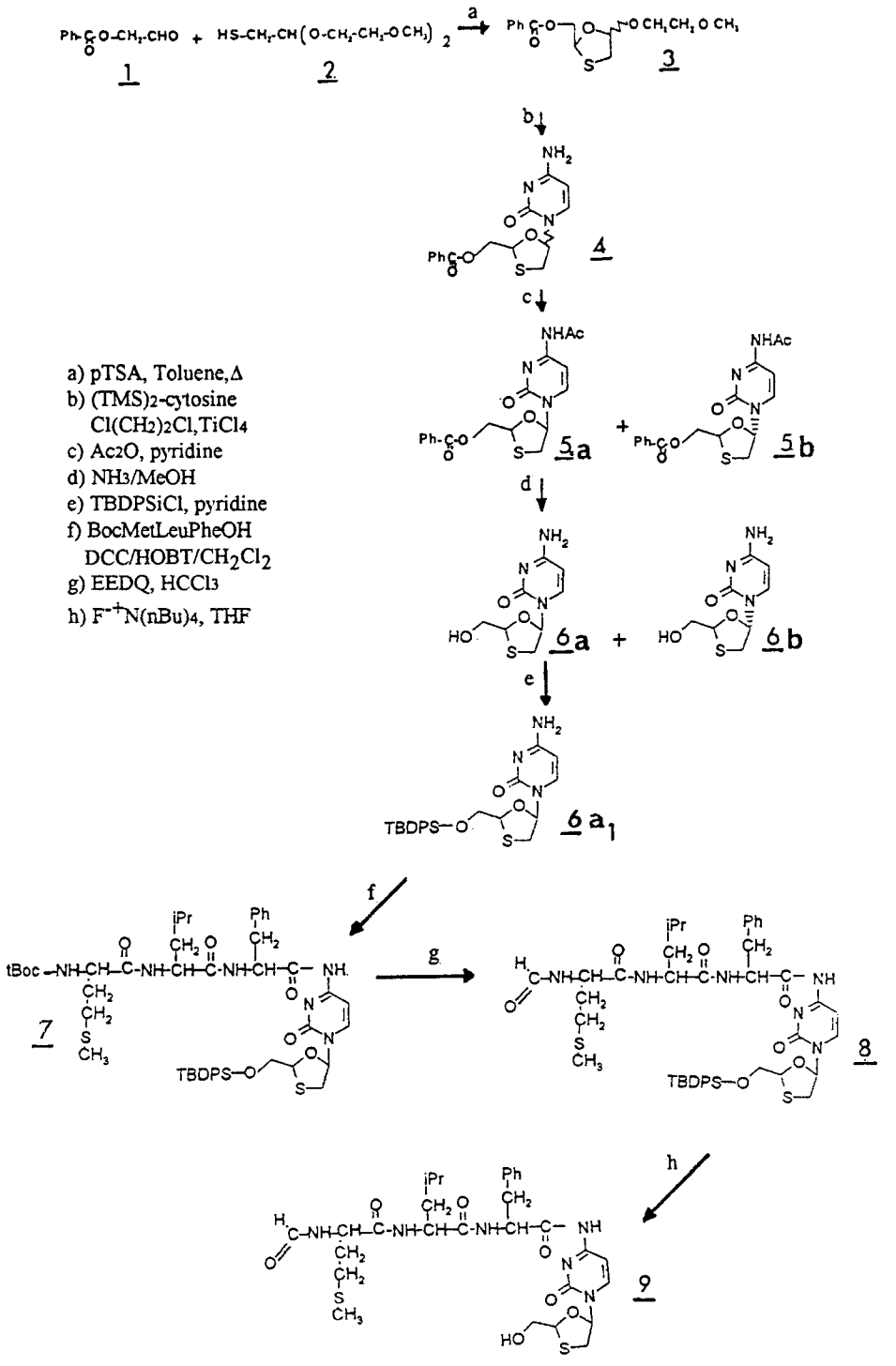
to cause stress response in cells (14), and among them, the oxidative stress mediated by hydrogen peroxide on HIV-1 latently infected cells appears to be very efficient (13). Following these studies the authors suggested that during inflammatory reactions, blood cells containing latent virus can be exposed to activated oxygen species released by stimulated leukocytes and such a stress could convert a latent into a productive infection, leading to the development of the acquired immune deficiency syndrome. However, more recently it was demonstrated (15) that when PMN (polymorphonuclear leukocytes) are stimulated, MPO (myeloperoxidase) released by degranulation reacts with H_2O_2 formed by the respiratory burst to oxidize chloride to a product (presumably hypochlorous acid) that is toxic to HIV-1. These findings prompted us to design new antiretroviral molecules which could induce an oxidative stress. Indeed, taking into account that N-formyl-peptides are known to elicite superoxide anion production by human leukocytes (16-18), it was tempting to utilize these peptides as "chemical heads" capable of targeting specific receptors located on PMN membranes. Due to its high affinity for these receptors this "N-formyl-peptide head" coupled to antiretroviral nucleoside moiety could mediate internalization processes (19) and therefore by combined effects through the release of the MPO/H_2O_2/Chloride system enhance the antiretroviral activity. Based on this concept, we report the total synthesis of a new N^4-(N-formyl-methionyl-leucyl-phenylalanyl)-2',3'-dideoxy-3'-thiacytidine prototype 9 and the studies of its biological activities : superoxide production and antiviral activity. The nucleoside 2',3'-dideoxy-3'-thiacytidine was selected because it is now clear that this derivative, which is in the present under clinical trials, appears to be very promising (20-22) since it is virtually non toxic to normal cells (23).

RESULTS AND DISCUSSION

CHEMISTRY

The synthetic strategy used for the preparation of this new conjugated nucleoside prototype is summarized in scheme 1.

2-benzoyloxymethyl-5-[(2-methoxy)ethoxy]-1,3-oxathiolane 3 was obtained through the condensation of benzoyloxyacetaldehyde 1 with 2-mercaptoacetaldehyde di-[2-methoxyethyl] acetal 2 according to a procedure described under reference 23. Reaction of the anomeric mixture 3 with silylated cytosine in the presence of $TiCl_4$ in 1,2-dichloroethane at room temperature resulted in the formation of a mixture of N-glycosylated anomers 4 (β:α = 6:1). As already reported by Choi et al. (25) the level of selectivity of β:α anomer ratio is under the control of the Lewis acid catalyst, the solvent and the structure of the sugar ring. N-acetylation of the mixture 4 using Ac_2O in pyridine, followed by flash column chromatography separation led to compounds 5a and 5b, which



Scheme 1

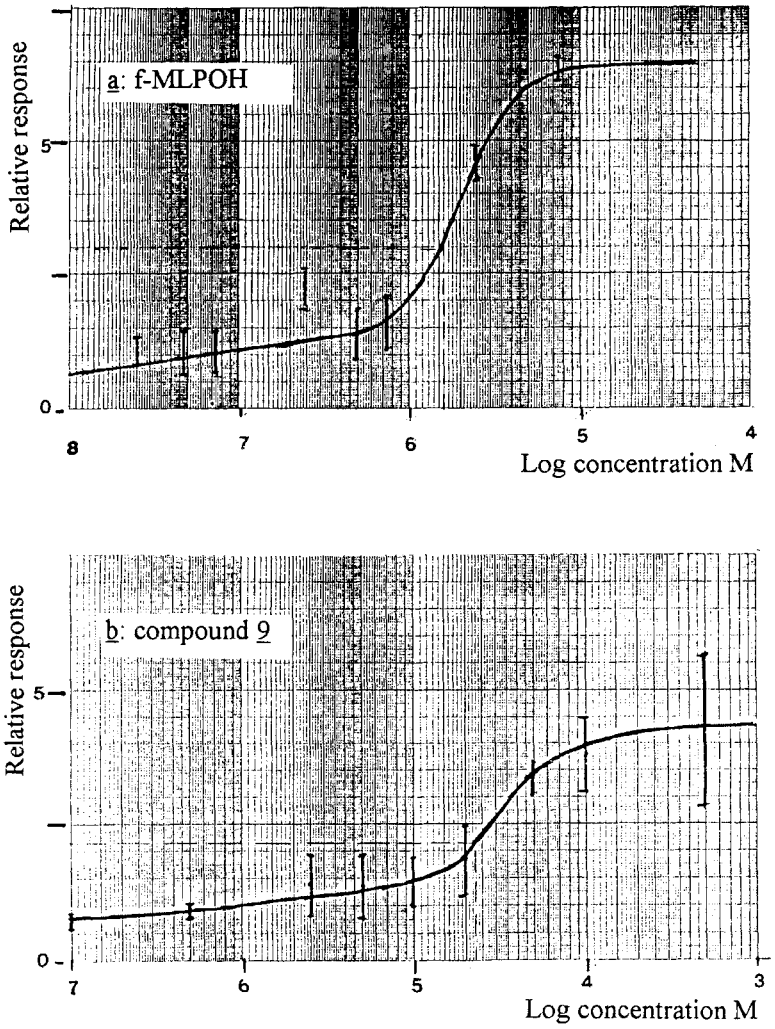


FIG. 1: typical dose-response curve for superoxide anion production elicited by the standart peptide f-MLPOH and the corresponding N^4-(N-formyl MLP)-2',3'-dideoxy-3'-thiacytidine 9.

The results of each experiment carried out with a different blood sample were normalized by taking the response in the analogue concentration range 1 to 5.10^{-6} M as equal to 1. f-MLPOH curve a (8 independent experiments) and f-MLP-nucleoside 9 curve b (4 independent experiments). The response was taken to be the maximum rate of superoxide production at each analogue concentration.

TABLE 1 : EC_{50} values for superoxide anion production.

Compounds	EC_{50} (M)
9	$3.2 .10^{-5}$
fMetLeuPheOH	$1.6 .10^{-6}$
2',3'-dideoxy-3'-thiacytidine 6a	no activation

after methanolysis in presence of ammonia gave the two corresponding isomers 6a and 6b. Since only the β-isomers exhibit useful antiviral activity as a consequence of their low toxicity, isomer 6a alone was selected as candidate for the next steps of the synthesis. After silylation of 6a using ter-butyldiphenylsilyl chloride as reagent, the resulting intermediate 6a1 was submitted to the coupling reaction with the tripeptide BocMetLeuPheOH. Thus compound 6a1 was added to BocMetLeuPheOH in the presence of 1.1 equivalent of DCC/HOBT (N,N'-dicyclohexylcarbodiimide/1-hydroxybenzotriazole hydrate) in dichloromethane, the key intermediate 7 was isolated in 38 % yield. The N-Boc-Methionine protecting group was removed in formic acid, and the resulting nucleoside-peptide was formylated using EEDQ (2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline) in chloroform as formylating reagent (25). When the corresponding N^4-(N-formyl-methionyl-leucyl-phenylalanyl) nucleoside derivative 8 (recovered in 75 % yield) was treated with one equivalent of a solution of tetra-n-butyl-ammonium fluoride in THF (27) the final compound 9 was isolated pure at 99.8 % by HPLC analysis, and gave satisfactory analytical and spectral data as reported in the experimental part.

BIOLOGICAL EVALUATION

Superoxide ion production : As described in the experimental part, the production of superoxide was measured as the superoxide dismutase-inhibitable reduction of cytochrome *c* in a continuous spectrophometric assay (28). FIG. 1 illustrates the classical dose-response curves for superoxide anion production obtained with the peptido-nucleoside 9 and with the standard peptide fMetLeuPheOH. EC_{50} values for superoxide production were determined from the curves shown on FIG. 1 and reported in TABLE 1.

The values presented are the average ± SD of EC_{50} values determined for each individual dose-response curve (4 to 8 experiments for each).

ANTIVIRAL ACTIVITY

Antiviral activity of the peptido-nucleoside 9 against HIV-1 was determined *in vitro,* and the anti-HIV activity was compared with that of 2',3'-dideoxy-3'-thiacytidine 6a. Virus

TABLE 2 : Antiviral activity on the replication of HIV-1 BRU

Compounds	Inhibition of syncitium formation IC_{50} (μM)	Ref
9	8.0 ± 0.8	-
2',3'-dideoxy-3'-thiacytidine 6a	0.1 ± 0.2	30
AZT	0.1 ± 0.09	30
ddI	8.5 ± 0.5	30

replication was measured by syncitium formation in MT4 cells infected with HIV-1 strain BRU. Cells were infected at a multiplicity of infection of 0.001 infectious units of HIV per cell and virus was adsorbed for 1 h at room temperature. The cells were washed free of unadsorbed virus, and cells aliquots were placed in 24-well plates containing serial dilutions of tests compounds. After incubation at 37°C for 5 to 6 days in 5 % CO_2, the cells were examined for HIV induced syncitia, and these were counted. By reference to untreated infected controls, the IC_{50} values were calculated and reported in TABLE 2.

The primary objective of this project was to design and synthesize the new N^4-(N-formyl-methionyl-leucyl-phenylalanyl)-2',3'-dideoxy-3'-thiacytidine analogue which can interfere with superoxide anion production by PMN and induce inhibition of HIV-1 cytopathogenicity in MT4 cells. On the one hand the results reported on TABLE 1 illustrate that compound 9 stimulated superoxide production (with an EC_{50} value which was one order of magnitude higher than the value found for the single peptide fMetLeuPheOH); on the other hand compound 9 induced inhibition of HIV-1 cytopathogenicity in MT4 cells, at a concentration non toxic to the host cells with 50 % antiviral effective concentration of 8.0 μM. Although this latter activity could be partially due to the enzymatic hydrolysis of compound 9 during cell culture experiments, this novel peptido-nucleoside appears to be less active than the parent nucleoside 2',3'-dideoxy-3'-thiacytidine 6a or the standard anti-HIV drug (AZT) but equivalent with ddI. Moreover in preliminary experiments we did not observe any synergistic effects when both compounds 6a and fMLPOH were simultaneously added to the culture medium. In so far as 2',3'-dideoxy-3'-thiacytidine 6a has no activity on superoxide anion production, compound 9 must bind to the fMetLeuPheOH receptor and thus induce the oxidative stress. The specific binding of 9 to human leukocytes could be of interest since it could mediate the cellular entry of the nucleoside moiety. This rationally designed antiviral agent that is targeted to specific cellular components could help the understanding of viral infections. In this respect such a prodrug modification may be extended with other antiviral agents.

EXPERIMENTAL SECTION

CHEMISTRY

Analytical Methods. Proton magnetic spectra were recorded on a Varian XL 200 spectrometer. Chemical shifts are reported as values in parts per million downfield from internal tetramethylsilane. Analytical thin layer chromatography was carried out on aluminium based sheets precoated with Kieselgel 60 F_{254} 0.2 mm thick (Merck Co, Darmstadt). Column and flash chromatography were performed with Merck silica gel (230-400 mesh). Mass spectra were recorded at Laboratoire de chimie organique des substances naturelles, Strasbourg, France.

Reagents. All solvents were used from sealed bottles purchased from Aldrich Company. All amino acids used as starting materials , were of the L-configuration and used as supplied from the manufacturer (Aldrich) for the following peptides : Methionine, Leucine, Phenylalanine. EEDQ was recrystallized from ether. HOBT, DCC, EEDQ and ter-butylchlorodiphenylsilane were purchased from Aldrich company.

Compounds. 2-benzoyloxyacetaldehyde 1 has been prepared according to known procedures (31). 2-mercaptoacetaldehyde di-[2-methoxyethyl] acetal 2 was prepared according to the method of Hesse and Jorder (32) starting from 2-bromoacetaldehyde di-[2-methoxyethyl] acetal.

2-benzoyloxymethyl-5-[2 -methoxy-ethyloxy] - 1,3 oxathiolane 3.

2-benzoyloxyacetaldehyde 1 (0.980 g; 6 mM) and 2-mercaptoacetaldehyde di-[2-methoxyethyl] acetal 2 (1.25 g ; 6 mM) were dissolved in toluene (200 ml) in the presence of a catalytic amount of p-toluenesulfonic acid. The resulting mixture was refluxed for 3 h. The solvent was removed under *vacuum* and the oily residue was chromatographed on a silica gel column using EtOAc/Toluene (1:9) as eluent ; yield 65 % mixture cis and trans.
^{1}H NMR ($CDCl_3$); δ= 3.15 (d,2H,C_5-O-CH_2-); 3.35 (s,3H,OCH_3); 3.45 (m,2H,CH_3-O-CH_2); 3.65 (m,1H,C_4-H_2); 3.80 (m,1H,C_4-H_2); 4.35 (dd,1H,,C_2-CH_2-O-); 4.55 (m,1H,,C_2-CH_2-O-); 5.5 (t,1H,C_5-H); 5.60 (dd,1H,C_2-H) ; 7.4 - 8.0 (m,5H,aromatic).

2-benzoyloxymethyl-5-[cytosin-1-yl]-1,3-oxathiolane mixture 4.

To a solution of silylated cytosine (1.1 eq, 2.1 mM) in 1,2-dichloroethane (10 ml) under argon was added the protected 1,3-oxathiolane 3 (1 eq, 2 mM). The reaction mixture was stirred one night at room temperature. The organic solution was washed with a saturated $NaHCO_3$ solution, extracted with CH_2Cl_2 and dried over $MgSO_4$. After solvent

evaporation the residue was purified by flash column chromatography over silica gel using 10 % MeOH in EtOAc as eluent yielding the mixture of 4 (56 %).

2-hydroxymethyl-5-[cytosin-1-yl]-1,3-oxathiolane 6a.

To the purified mixture 4 (1 eq, 1.1 mM) dissolved in dry pyridine (10 ml) was added acetic anhydride (1.4 ml) and a catalytic amount of DMAP. After extraction with CH_2Cl_2 (3 x 8 ml), washing with water, drying over $MgSO_4$ and evaporation, the oily residue was purified by flash column chromatography over silica gel using 1 % MeOH in EtOAc as eluent. The corresponding N^4-acetylated 5a and 5b were isolated and characterized. 5a (1 eq, 0.6 mM) dissolved in 20 ml of methanolic ammonia was stirred at room temperature for 12 h. After solvent evaporation, the oily residue was crystallized in a mixture of EtOH/Et_2O.

yield: 85 %; 6a mp = 172°C; ^1H NMR (DMSO-d_6): δ= 2.9 (dd,1H, C_4-H_2); 3.4 (dd,1H,C_4-H_2); 3.7 (m,2H,C_2-CH_2-O); 5.12 (t,1H,-OH); 5.30 (t,1H,C_5-H); 5.7 (d,1H,C_5'-H); 6.2 (t,1H,C_2-H); 7.2 (broad s,2H,NH_2); 7.8 (d,1H,C_6'-H)

Cis isomer of 2-terbutyldiphenylsilyloxymethyl-5-(cytosin-1-yl)-1,3-oxathiolane 6a1.

To a solution of 6a (104 mg, 0.45 mmole) in dry pyridine (6 ml) was added under nitrogen diphenylterbutylsilyl chloride (135 µl, 0.52 mmole, 1.15 eq). After stirring for one day at room temperature the resulting mixture was evaporated under reduced pressure. H_2O (30 ml) was added to the residue and extraction with ethyl acetate (3x20 ml) gave a white solid after drying over Na_2SO_4 and solvent evaporation. Quantitative yield. Rf (EtOAc/MeOH 2:1) = 0.62; ^1H NMR ($CDCl_3$): δ=1.1 (s,9H,tBu); 3.1-3.6 (m,2H,C_2-CH_2-O-); 3.7-4.2 (m,2H,-C_4-H_2-); 5.25 (t,1H,C_5-H); 5.5 (d,1H,C_5'-H); 6.35 (q,1H,C_2-H); 7.4-7.8 (m,10H,2Ph); 8.0 (d,1H,C_6'-H).

Cis isomer of 2-terbutyldiphenylsilyloxymethyl-5-[N^4-(terbutoxycarbonyl-methionyl-leucyl-phenylalanyl)-cytosin-1-yl]-1,3-oxathiolane 7.

To a solution of BocMetLeuPheOH (114 mg, 0.23 mM, 1.1 eq) in dry CH_2Cl_2 was added under nitrogen at 0°C, HOBT (31 mg, 0.23 mM, 1.1 eq) and DCC (47 mg, 0.23 mM, 1.1 eq). The reaction mixture was stirred for 2 hours at room temperature and 6a1 (100 mg, 0.21 mM, 1 eq) was added. After 24 hrs, precipitated DCU was filtrated and the filtrate washed successively with 5 % aqueous $NaHCO_3$ and 5 % aqueous citric acid. Extraction with dichloromethane, drying over Na_2SO_4 and solvent evaporation gave a residue which was purified by flash column chromatography over silica gel. Yield (38 %); Rf (Toluene/MeOH 9:1) = 0.46; ^1H NMR ($CDCl_3$); δ= 0.8 (m,6H,2CH_3δLeu); 1.1 (s,9H,tBuSi-); 1.4 (s,9H,BOC), 2.1 (s,3H,S-CH_3); 2.6 (m,2H,CH_2βMet); 3.1-3.6

(m,2H,C_2-CH_2-O); 4.0 (m,1H,CH_αLeu); 4.2 (m,1H,CH_αMet); 5.25 (s,1H,NHCO); 6.3 (m,1H,NHCO); 7.4 (m,10H,2Ph); 8.2 (m,1H,C6'-H).

Cis isomer of 2-terbutyldiphenylsilyloxymethyl-5-[N^4-(N-formyl-methionyl-leucyl-phenylalanyl)-cytosin-1-yl]-1,3-oxathiolane 8.

To 7 (75 mg, 0.08 mM) was added formic acid (1.5 ml). After 5 hrs stirring at room temperature EEDQ (39 mg, 0.16 mM, 2 eq) in chloroform (4 ml) was added. The reaction controlled by TLC was stopped after 15 hrs. Evaporation of the mixture gave a residue which was purified by flash column chromatography over silica gel. Yield (36 %) ; Rf (EtOAc/MeOH 9:1) = 0.57; ^{1}H NMR ($CDCl_3$): δ= 0.8 (m,6H,2CH_3δLeu); 1.1 (s,9H,tBuSi-); 2.0 (s,3H,S-CH_3); 2.45 (t,2H,CH_2βMet); 3.0-3.1 (m,2H,CH_2βPhe); 3.2-3.6 (m,2H,C_2-CH_2-O-); 3.9-4.3 (m,2H,C_4-H_2); 4.74 (m,1H,CH Phe); 5.0 (m,1H,NHCO), 5.25 (t,1H,C5-H); 5.5 (m,1H,C5'-H); 6.35 (t,1H,C2-H); 7.0-7.2 (m,7H, H Phe aromatic and NHCO); 7.3-7.7 (m,10H,2Ph); 8.0 (d,1H,C6'-H); 10.5-10.8 (m,1H,-C(O)H).

Cis isomer of 2-hydroxymethyl-5-[N^4-(N-formyl-methionyl-leucyl-phenylalanyl)-cytosin-1-yl]-1,3-oxathiolane 9.

To a solution of 8 (26 mg, 0.03 mmole) in dry THF (2 ml) was added under nitrogen tetrabutylammonium fluoride (81 µl, 0.09 mmole, 3 eq). After 5 hrs the reaction mixture was concentrated under reduced pressure and the residue was purified by flash column chromatography over silica gel. Yield (66 %), 99.8 % pure by HPLC, Rf (EtOAc/MeOH 9:1) = 0.41; ^{1}H NMR ($CDCl_3$) : δ= 0.8 (m,6H,2CH_3δLeu); 2.0 (s,3H,S-CH_3); 2.45 (t,2H,CH_2βMet); 3.0-3.2 (m,2H,CH_2βPhe); 3.2-3.6 (m,2H,C_2-CH_2-O-); 3.9-4.3 (m,2H,C_4-H_2); 5.35 (m,1H,C5-H); 6.2 (m,1H,C2-H); 7.2 (m,5H,Ph); 8.3 (d,1H,C6'-H); 10.9 (m,1H,-C(O)H).

MS : MH^+ (FAB) m/z 649,2. Anal. $C_{29}H_{40}O_7N_6S_2$ C,H,N

BIOLOGICAL EVALUATION

1. SUPEROXIDE PRODUCTION.

Materials. The chemoattractants were dissolved in dimethylsulfoxide. The organic solvent concentration in the assays as well as in the controls was 1 %.

Isolation of human neutrophils. PMN were prepared from peripheral venous blood of human volunteers according to the technique of Böyum (29) with a few modifications. After a 1 h dextran sedimentation, the leukocyte-rich plasma was layered over Ficoll-Hypaque and centrifuged for 30 min at 400 g. Residual red blood cells were removed from the resultant PMN pellet by a 5 min hypotonic lysis. The preparation usually contained 97

% PMN and 3 % lymphocytes. Cell viability was tested with the trypan blue exclusion test. PMN were resuspended in calcium and magnesium free Hank's balanced salt solution (HBSS) (pH = 7.5) at 10^8 cells/ml for superoxide assays.

Assay of superoxide ion production. The formation of superoxide was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome *c* in a continuous spectrophotometric assay (28). Duplicate reaction mixtures containing PMN(10^6 cells/ml), cytochrome *c* (200 µM), cytochalasin B (5 µg/ml) in Ca^{2+} and Mg^{2+} containing HBSS were allowed to equilibrate at 37°C for 2 min in spectrophotometer cuvettes. The reference, in addition, contained superoxide dismutase (10 µg/ml). The reaction was initiated simultaneously in the two cuvettes by addition of the tested compound. The reaction was continuously monitored at 550 nm for 2 min with a Uvikon 930 spectrophotometer. The maximal initial rate of cytochrome *c* reduction was calculated using the following expression $E_{550}^{red\text{-}ox} = 21\ mM^{-1}.cm^{-1}$

2. ANTIVIRAL ACTIVITY.

The antiviral activity was tested by the syncitium formation assay in duplicate and in two independent experiments. The assessment of antiviral potency was based on the cytopathic effect of HIV-1 (HIV-1 BRU prototype) on MT4 cells, 4 to 7 days after HIV-1 infection. Syncitium formation was evaluated under an inverted optical microscope.

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