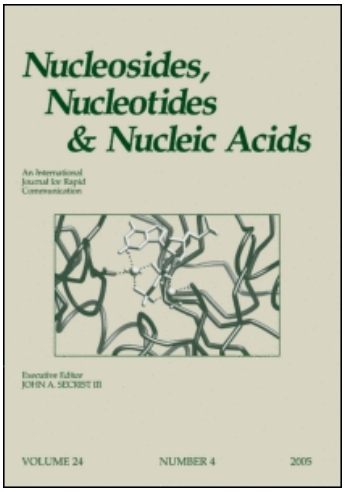

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A NEW FACILE SYNTHESIS AND ANTIVIRAL ACTIVITY OF OXAZOFURIN

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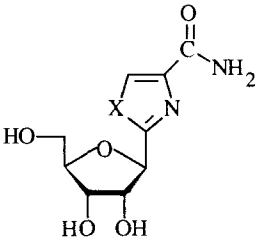
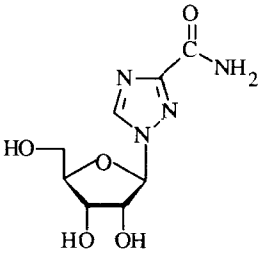
Abstract: A new, more facile synthesis of oxazofurin, a structural analogue of tiazofurin, selenazofurin and ribavirin, has been carried out by rhodium catalyzed reaction of ethyl α-formyl-diazoacetate with 2,3,5-tri-*O*-benzoyl-β-D-ribofu-ranosyl cyanide. When evaluated against DNA and RNA viruses, HIV-1 inclu-ded, oxazofurin was found inactive. It was also ineffective in potentiating the anti-HIV activity of 2',3'-dideoxyadenosine.

Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide, NSC-286193, **1b**) and selenazofurin (2-β-D-ribofuranosylselenazole-4-carboxamide, NSC-340847, **1c**) are effective antitumor agents both *in vitro* and *in vivo*.[1-3] Their antitumor activity has been related to inhibition of inosine monophosphate dehydrogenase (IMPD), to consequent IMP accumulation and depletion of the intracellular pools of GTP and dGTP.[4,5] Tiazo- and selenazofurin, which are structurally related to ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, NSC-163039, **1d**), are also endowed with broad spectrum antiviral activity.[1,6,7] More recently, both ribavirin and tiazofurin have been shown to potentiate *in vitro* the anti-HIV activity of several purine 2',3'-dideoxynucleosides,[8-10] a phenomenon also ascribed to IMPD inhibition by the two compounds. In this case, the increased IMP levels lead to increased levels of inosine and guanosine dideoxynucleoside 5'-triphosphates due to the fact that IMP is the phosphate donor in the 5'-nucleotidase catalyzed conversion of purine dideoxynucleosides into corresponding monophosphates.[10]



1a X = O, Oxazofurin
1b X = S, Tiazofurin
1c X = Se, Selenazofurin



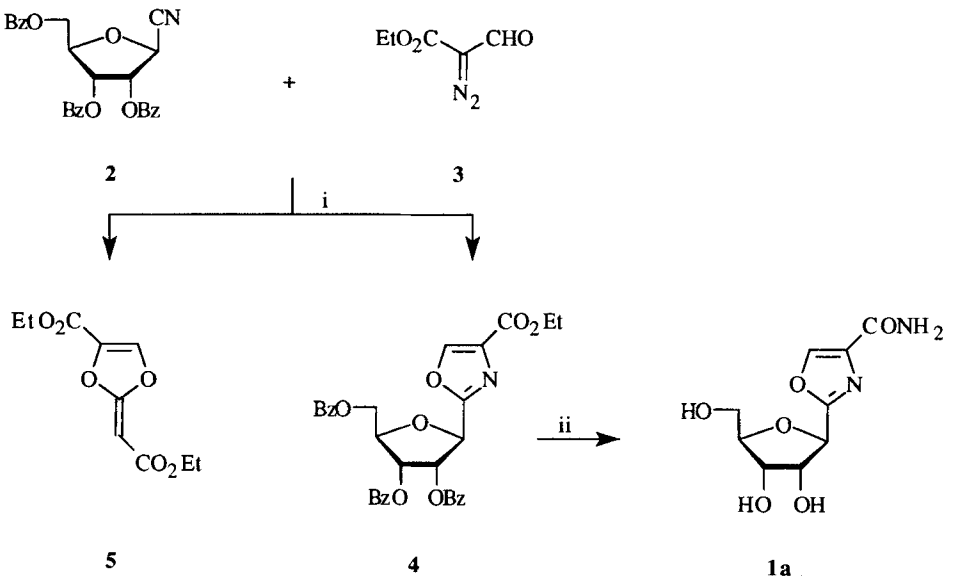
1d Ribavirin

Recently we have synthesized, in 7 steps starting from 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl cyanide (**2**), oxazofurin (2-β-D-ribofuranosyloxazole-4-carboxamide, **1a**),[11] a new structural analogue of tiazo- and selenazofurin possessing an oxygen atom in the place of sulfur or selenium atoms.

We now report on a new facile synthesis of oxazofurin that can be carried out in only two steps starting from nitrile **2**. Moreover, in order to extend structure-activity relationship studies of this type of nucleoside, we deemed it useful to evaluate the antiviral activity of oxazofurin against selected DNA and RNA viruses and its capability to potentiate the anti-HIV activity of 2',3'-dideoxyadenosine (ddAdo).

CHEMISTRY

Connell et al.[12] have recently reported that 2-substituted-4-carbethoxy oxazoles may be obtained in one simple step by the 3+2 cycloaddition of ethyl α-formyldiazoacetate (**3**) to nitriles, via an α-formylcarbene generated in situ in the presence of a catalytic amount of rhodium(II) acetate. This finding prompted us to apply a similar reaction to the synthesis of oxazofurin (Scheme 1). Thus the reaction of 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl cyanide[13] (**2**) with ethyl α-formyldiazoacetate[14] (**3**) in the presence of rhodium(II) acetate at 85 °C for 15 h gave ethyl 2-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)oxazole-4-carboxylate (**4**) and (*E*)-4-carbethoxy-2-(2-ethoxy-2-oxoethylidene)-1,3-dioxole (**5**) in 11.7% and 2.1% yields respectively. The structure of dioxole **5** was



Reagents: i) $Rh_2(OAc)_4$, 85 °C, 15 h; ii) NH_3/EtOH, r.t., 35 h.

SCHEME 1

confirmed by the ^1H-, ^{13}C-NMR and mass spectra. When the reaction was carried out in the presence of CH_2Cl_2 as solvent at 70°C for 19 h, compound **4** was produced in 4.7 % yield, whereas a much higher yield of dioxolane **5** (36%) was obtained.

Although the reaction yield was not very high in the synthesis of **4**, this method was found much more convenient than the previously reported multi-step procedure.[11] Treatment of ester **4** with saturated ethanolic ammonia at room temperature for 35 h, gave oxazofurin in 54 % yield.

BIOLOGICAL EVALUATION AND DISCUSSION

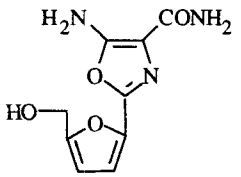
Antiviral activity. Oxazofurin (**1a**) and compounds **6** and **7**, two furan analogues of oxazofurin whose synthesis has been previously reported,[11] were tested in plaque reduction assays against selected DNA and RNA viruses.

TABLE 1. Comparative antiviral effects of oxazofurin (**1a**), oxazofurin analogues **6** and **7**, tiazofurin (**1b**) and ribavirin (**1d**) in Vero cells.

Compd	MCC[a] (μM)	ED_{50}[b]						
	Vero	HSV-1	HSV-2	VV	ASFV	Sb-1	Coxs	VSV
1a	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
6	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
7	> 500	> 500	> 500	> 500	> 500	> 500	> 500	> 500
1b	> 500	384	> 500	170	248	> 500	> 500	> 500
1d	> 500	390	320	82	ND	400	400	> 500

[a] Compound dose required to cause a microscopically detectable alteration of normal Vero cell morphology.
[b] Compound dose required to reduce by 50% the number of plaques. Plaque numbers in untreated cultures were 130 (HSV-1), 120 (HSV-2), 115 (VV), 145 (ASFV), 140 (Sb-1), 120 (Coxs.), 200 (VSV).
ND = Not determined.

Tiazofurin[15] (**1b**) and ribavirin[15] (**1d**) were used as reference compounds. The data in Table 1 show that in Vero cells neither oxazofurin nor its furan analogues were inhibitory to any of the viruses tested. Both tiazofurin and ribavirin were confirmed as broad spectrum antiviral agents although, as already reported,[6] their activity in Vero cells was neither potent nor very selective.



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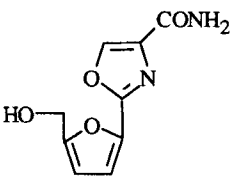

7

The activity of the test compounds against HIV-1 (HTLV-IIIB strain) multiplication in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 cells.

As shown in Table 2, oxazofurin and its furan analogues were non-toxic to MT-4 cells, confirming previous results on their poor antitumor cell cytotoxicity.[11]

TABLE 2. Comparative anti-HIV-1 effects of oxazofurin, oxazofurin analogues **6** and **7**, tiazofurin and ribavirin in MT-4 cells.

Compd	CC_{50}[a]	EC_{50}[b]
1 a	> 500	> 500
6	> 500	> 500
7	> 500	> 500
1 b	1.9	> 1.9
1 d	35	> 35
ddAdo	> 500	12

[a] Cytotoxic concentration of compound required to reduce the viability of mock-infected MT-4 cells by 50%.
[b] Effective concentration of compound required to achieve 50% protection of MT-4 cells against the cytopathic effect of HIV-1.

TABLE 3. Anti-HIV-1 effect of ddAdo, alone or in combination with ribavirin or oxazofurin in MT-4 cells.

ddAdo concentration (μM)	% protection against CPE of HIV-1		
	alone	+Ribavirin (5 μM)	+Oxazofurin (200 μM)
5	25	61	28
10	44	98	47

Cell cultures were exposed to ribavirin or oxazofurin one hour before the addition of ddAdo.

Tiazofurin was the most inhibitory compound to MT-4 cell growth (CC_{50} = 1.9 μM), followed by ribavirin (CC_{50} = 35 μM). When used singly, none of these analogues was effective against HIV-1, while ddAdo afforded a 50% protection against the HIV-induced cytopathogenicity at 12 μM. When the combined inhibitory effect of ddAdo with oxazofurin was examined (Table 3), oxazofurin proved totally ineffective in potentiating the anti-HIV activity of the dideoxynucleoside analogue even at 200 μM. On the contrary, ribavirin, which was preferred to tiazofurin as a control due to its lower cytotoxicity, confirmed its ability to potentiate the anti-HIV-1 activity of ddAdo.

Overall, these results suggest that the isosteric substitution of the sulfur and selenium atoms of tiazofurin and selenazofurin with oxygen is detrimental for the biological activity of this type of ribofuranosyl-carboxamide nucleosides.

The biological effects of tiazo- and selenazofurin appear related to a shutdown of guanine nucleotide synthesis produced by dinucleotide anabolites TAD and SAD which act as inhibitors of IMPD.[4,5] Crystallographic studies of tiazo- and selenazofurin have demonstrated close contacts between the thiazole and selenazole heteroatoms and the furanose oxygen. This close contact has been attributed to an electrostatic attraction between the positively charged sulfur or selenium and the lone-pair of electrons on the furanose oxygen,[16,17] a hypothesis confirmed by molecular orbital calculations. This interaction would constrain rotation about the C-glycosidic bond in TAD and SAD, enabling these dinucleotides to bind to IMPD. The inactivity of oxazofurin might be due to the weak attractive interaction between the oxazole and furanose oxygens which allows free rotation about the C-glycosidic bond; this would negatively influence the phosphorylation of oxazofurin or the binding of the putative anabolite OAD to the target enzyme.[18]

EXPERIMENTAL SECTION

Chemistry. Melting points were determined with a Buchi apparatus and are uncorrected. Elemental analyses were determined on a Carlo Erba Model 1106 analyzer. Thin layer chromatography (TLC) was run on silica gel 60 F-254 plates (Merck); silica gel 60 (Merck) for column chromatography was used. Nuclear magnetic resonance ^{1}H and ^{13}C spectra were determined, respectively, at 300 and 75 MHz with a Varian VXR-300 spectrometer. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard. All exchangeable protons were confirmed by addition of D_2O. Mass spectra were determined on a Hewlett-Packard mass spectrometer 59-71 A.

Ethyl 2-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)oxazole-4-carboxylate (4) and (E)-4-carbethoxy-2-(2-ethoxy-2-oxoethylidene)-1,3-dioxole (5). To 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl cyanide[13] (**2**) (5.4 g, 11.4 mmol) melted at 85°C, were added rhodium(II) acetate (75.4 mg, 0.17 mmol)

and ethyl α-formyldiazoacetate[14] (**3**) (9.18g, 60.0 mmol). The mixture was stirred at 85°C under nitrogen for 15 h, then purified by silica gel column chromatography eluting with a mixture of benzene-ethyl acetate (85:15). Evaporation of the first eluate gave 0.7 g (11.7 %) of compound **4**. T L C (benzene-ethyl acetate 90:10): Rf = 0.43. ^{1}H-NMR spectrum was identical to that reported in the literature.[11]

Compound **5** was separated in the second eluate and obtained as white solid (30 mg, 2.1 %). M.p. 126-127 °C; TLC (benzene-ethyl acetate 90:10): Rf = 0.37. U V (MeOH): λ_{max} 258 nm (ε 24100); ^{1}H-NMR (Me_2SO-d_6): δ 1.18-1.25 (2t, 6H, -CH_3); 4.08, 4.32 (2 dd, 4H, -CH_2); 4.95 (s, 1H, H-2'); 8.70 (s, 1H, H-4). ^{13}C-NMR (Me_2SO-d_6): δ 167.1 (C=0); 164.7 (C=O); 156.1 (C-2); 138.9 (C-5); 134.0 (C-4); 68.7 (C-2'); 61.8 (C-H_2); 58.7 (C-H_2); 14.4 (C-H_3); 13.9 (C- H_3). MS m/e 228, 200, 183, 172, 155, 128. Anal. Calcd. for $C_9H_{12}O_6$: C 50.0; H 5.59; O 44.4. Found: C 49.89; H 5.47; O 44.0.

2-β-D-Ribofuranosyloxazole-4-carboxamide (1a).[11] To compound **4** (0.24 g, 0.45 mmol) ethanolic ammonia (saturated at 0 °C) (10 mL) was added and the mixture was stirred at room temperature for 35 h. Evaporation to dryness afforded a product which was chromatographed on a silica gel column eluting with chloroform-methanol (85:15) to give 62 mg (54 %) of **1a**. $[\alpha]^{25}_D$ - 17.3° (c 1.04, H_20). ^{13}C-NMR (Me_2SO-d_6): δ 162.1 (C-2); 162.0 (C=O); 142.8 (C-5) 136.6 (C-4); 86.0 (C-1'); 77.0 (C-4'); 74.3 (C-2'); 71.5 (C-3') 62.1 (C-5'). MS, m/e 244, 155, 141, 138, 124.

Biological determination

Drugs. Test compounds were solubilized in DMSO at 100 mg/mL and then diluted in culture media.

Cells. MT-4 cells, used for anti-HIV assays, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 UI/mL penicillin G and 100 UI/mL streptomycin. Monolayers of Vero cells, used in assay with the other RNA and DNA viruses, were grown in Dulbecco's modified minimal essential medium (MEM) supplemented with 10% FCS, 100 UI/mL penicillin G and 100 µg/mL streptomycin. All cell lines were checked periodically for the absence of mycoplasma contamination.

Viruses. HIV-1 (HTLV/IIIB strain) from supernatants of H9/IIIB cells was used in the anti-HIV assay. HIV stocks were titrated in C8166 cells and stored at -80 °C until use. Virus stock of Herpes Simplex type 1 (HSV-1, ATCC VR 733),

Herpes Simplex type 2 (HSV-2, ATCC VR 734), Vaccinia (VV, ATCC VR 117), African Swine Fever (ASFV, Istituto Zooprofilattico di Sassari), Polio type 1 (Sabin strain, Sb-1), Coxsackie B1 (Coxs., ATCC VR 28) and Vescicular Stomatitis (VSV, ATCC VR 158) were obtained in Vero cell at 37 °C.

Antiviral assays. Antiviral assays against HSV-1, HSV-2, VV, ASFV, Sb-1, Coxs and VSV viruses were based on the plaque reduction test, carried out in Vero cells according to Collins and Bauer.[19] Activity of the compounds against HIV-1 replication was based on the inhibition of virus-induced cytopa-thogenicity in MT-4 cells. Briefly, 50 μL of growth medium containing $1x10^4$ MT-4 cells were added to each well of flat-bottomed microtiter trays containing 50 μL of culture medium, with or without various concentrations of the test compounds. A HIV-1 suspension (20 μL) was then added to obtain a multiplicity of infection (m.o.i.) of 0.01. After a 4-day incubation at 37 °C, the number of viable MT-4 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method.[20] The cytotoxicity of compounds for MT-4 cells was evaluated in parallel with their antiviral activity. It was based on the viability of mock-infected cells, as monitored by the MTT method.

Cell viability and plaque counts values obtained in the presence of the compounds were expressed as percentage of those obtained in untreated controls and plotted against the logarithm of drug concentrations. Dose-response lines were drawn by linear regression technique and 50% cytotoxic concentrations (CC_{50}) or 50% effective concentrations (EC50) were calculated.

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