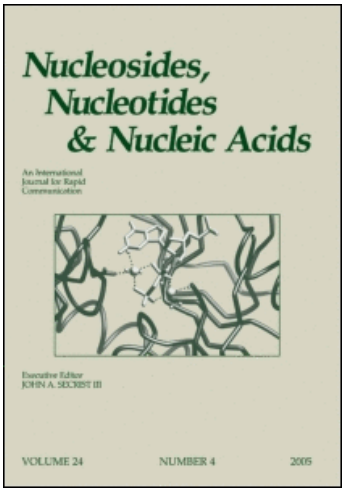

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SYNTHESIS, HYDROLYSIS AND ANTI-EBV ACTIVITY OF A SERIES OF 3′-MODIFIED *cyclo*Sal-BVDUMP PRONUCLEOTIDES

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ABSTRACT

A series of *cyclo*Sal-BVDUMP phosphate triesters has been prepared. The prototype compound was 3-methyl-*cyclo*Sal-BVDUMP **2**. Furthermore, a series of 3′-*O*-acyl-modified derivatives having carboxylic acids with different lipophilicity or a L-configurated α-amino acid (phenylalanine) was prepared. The hydrolysis properties in phosphate buffer PBS as well as in PBS containing pig liver esterase (PLE) will be described. Finally, the biological activity against EBV has been determined.

INTRODUCTION

Nucleosides are extensively used as antiviral or antitumor agents (1,2). The currently used nucleoside analogues against HIV in the clinic are 3′-azidothymidine (AZT), 2′,3′-dideoxy-2′,3′-didehydrothymidine (d4T), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyinosine (ddI) as well as 3′-thiacytidine (3TC) and the recently approved abacavir (1592U89). The general motif of these nucleoside analogues is a modified glycon residue as compared to the natural nucleosides. Beside the use against HIV, nucleosides are also used against herpes virus infections. Examples are the acyclic guanine bearing nucleoside analogue acyclovir (ACV) which is

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used against herpes-simplex virus as well as (E)-5-(2- bromovinyl)-2′-deoxyuridine (BVDU) against varicella-zoster virus which is the causative agent of chickenpox and shingles.

The major problem in the use of nucleoside analogues as antiviral agents is the prerequisite of being activated into their nucleoside triphosphates by three successive phos-phorylation steps. These phosphorylations are achieved by cellular or viral enzymes. Very often the exact procedure of bioactivation is not known and therefore one should hope for a newly developed nucleoside analogue that an efficient metabolism will take place ("black-box" bioactivation). However, for a few nucleoside analogues the metabolism has been studied in detail and it has been recognized that often the first conversion of the nucleoside analogue into its monophosphate is the rate limiting step. This has been shown for d4T as well as for ddA/ddI. Nevertheless, these nucleoside analogues exert their antiviral activity although the potential is not used efficiently. In an extreme case the nucleoside analogue can not be converted into its monophosphate and consequently no di- and triphosphate formation takes place and hence the compound is biologically absolutely inactive (e.g. ddU). In order to circumvent this limitation we and other have developed the so-called pronucleotide-concept (3). The basic idea is to develop a lipophilic precursor of the highly polar nucleoside monophosphate (nucleotide) that is able to penetrate the cell membrane and release the nucleotide efficiently under specific conditions (enzymatically or chemically) (Fig 1). In this context we designed the *cyclo*Sal-pronucleotides approach (4,5).

This concept has been introduced to deliver the nucleotide by means of chemical hydrolysis (5). The concept has been applied successfully to the intracellular delivery of d4TMP which lead to the thymidine-kinase(TK)-bypass in TK-deficient lymphocytes (6,7). Additionally, this concept has been used to achieve the adenosine-deaminase(ADA)-bypass for the nucleoside analogues ddA (8), d4A (8,9) and 2′-F-*ara*-ddA (10). Interestingly, the *cyclo*Sal-masking technique is able to convert an inactive nucleoside analogue into an active compound. This has been demonstrated by using 2′-F-*ribo*-ddA (10,11). On the other side, the *cyclo*Sal-concept was unable to restore the antiviral activity of AZT in TK-deficient CEM cells due to the delivery of AZTMP although we have clearly shown that again

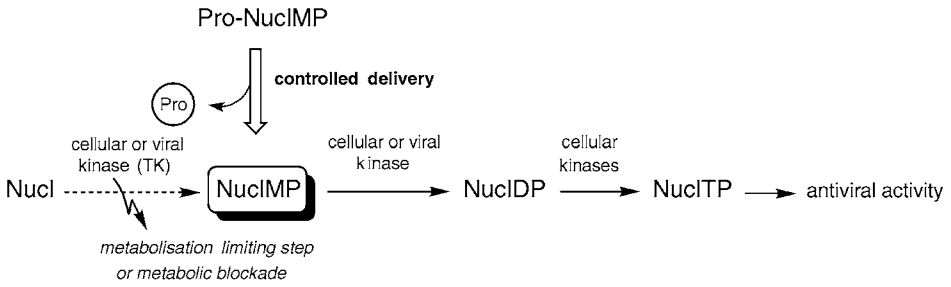

Figure 1. Metabolism of nucleosides and the effect of the corresponding pronucleotide.

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AZTMP was liberated from the lipophilic precursor triester in chemical hydrolysis studies (12). The reason for this failure has been found in a special metabolism of AZTMP (13). The first non-HIV directed example was the use of ACV in this concept (14). ACV is not activated by a cellular kinase but by the viral herpes-simplex thymidine-kinase. By preparing *cyclo*Sal-ACVMP compounds the antiviral activity of ACV could be regained in thymidine-kinase deficient virus lines (14,15). Here we report on the application of the *cyclo*Sal-methodology to the nucleoside analogue (E)-5-(2-bromovinyl)-2′-deoxyuridine **1** (BVDU or Brivudin).

BVDU **1** (16) is a potent and highly selective inhibitor of the replication of several herpes viruses (e.g. HSV-1 and VZV but not HSV-2) (17). BVDU **1** has been reported to inhibit also EBV infections (18) but these results could not be reproduced by our laboratories (19). The selectivity as inhibitor primarily depends upon a specific activation by the virus encoded thymidine-kinase (TK) to the mono- and diphosphate and finally by cellular enzymes to the triphosphate. In its triphosphate BVDU can act either as an inhibitor of the cellular DNA polymerase or alternate substrate which would render the DNA more prone to degradation when incorporated in DNA (20). Some limitations for the use of BVDU as antiviral agent are known. There is a lack of activity during virus latency because of missing viral TK. Moreover, drug resistant virus strains are known. Additionally, BVDU will be degraded from the bloodstream within 2–3 hours by pyrimidine nucleoside phosphorylase to the free pyrimidine nucleobase E-5-(2-bromovinyl)uracil (BVU) (20). Our aim in this study was to use the *cyclo*Sal-masking technology in order to broaden the application of BVDU **1** into the field of EBV associated lymphoproliferative disorders in immunosuppressed patients (21). It should be added that to our knowledge only two attempts have been published to develop prodrugs of BVDU **1**. Both were not successful (22,23).

RESULTS AND DISCUSSION

Here we report on the preparation of a series of 3′-unmodified (**2**) as well as 3′-*O*-modified *cyclo*Sal-BVDUMP derivatives (**3**–**6**). As 3′-modification carboxylic acids with different lipophilicity (**3**,**4**) as well as an α-amino acid (L-phenylalanine, **5**) have been used. Furthermore, the 3′-*O*-methyl ether derivative (**6**) of the prototype triester **2** has been prepared. In all cases we prepared the 3-methyl-substituted *cyclo*Sal-BVDUMP triester because this substituent gave the best antiviral results so far (6,8–12,14,15). The target phosphate triesters **2**–**6** are shown in Figure 2.

The preparation of 3-methyl-*cyclo*Sal-BVDUMP **2** has been carried out by direct phosphitylation of BVDU **1** using cyclic 3-methyl-*cyclo*Sal-chlorophosphite in a DMF/THF (2:1) solvent mixture at -20°C in the presence of diisopropylethylamine (DIPEA) as base. The intermediate phosphite triester was oxidized in the one-pot reaction procedure using t-butylhydroperoxide (TBHP) as published before (6,9,11). Evaporation of the solvent and purification by silica gel chromatography using the chromatotron technique gave the phosphate triester **2** in

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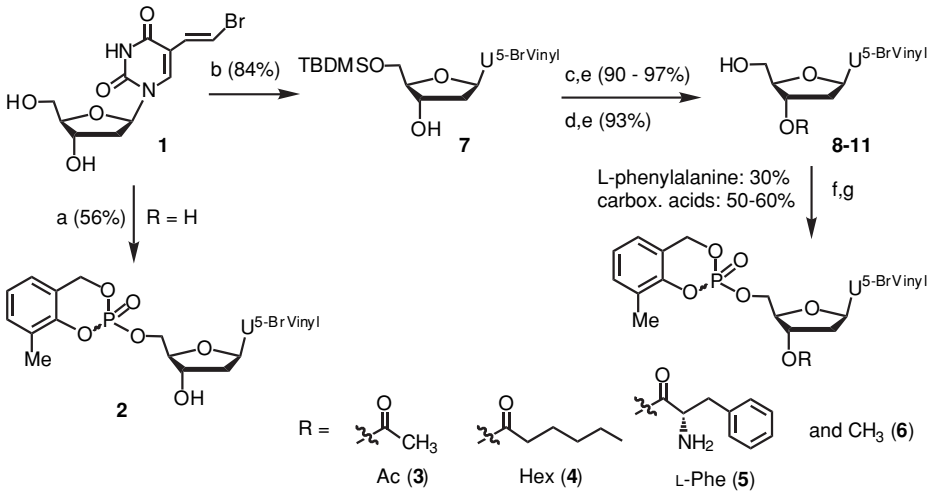

a) chlorophosphite, DIPEA, DMF/THF 2:1, 4h, -20°C then TBHP, 30 min, rt; b) TBDMSCl, pyridine, 12h, rt; c) carboxylic acid (*N*-Boc protected L-phenylalanine), DCC, DMAP, THF, 3h, rt; d) NaH, CH_3I, THF, 5h, rt; e) 2% TBAF in THF, 3h, rt; f) chlorophosphite, DIPEA, 2h, rt; then TBHP, 15 min, CH_3CN, rt; g) 5% TFA, CH_2Cl_2/MeOH 7:3, 30 min, rt

Figure 2. Synthesis of the target 3-methyl-*cyclo*Sal-BVDUMP triesters **2**–**6**.

56% yield. However, this reaction procedure was not suitable for the preparation of the 3′-*O*-modified compounds **3**–**6**.

Therefore, BVDU **1** was first protected selectively at the 5′-*O*-position by silylation using *t*-butyldimethylsilylchloride (TBDMS) in pyridine to give **7** in 84% yield. Next, compound **7** was treated with acetic acid, hexanoic acid and *N*-Boc L-phenylalanine in the presence of DCC and DMAP in THF at room temperature for 3 hours. Additionally, **7** was deprotonated using sodium hydride in THF and subsequently methylated by addition of methyl iodide to give 5′-OTBDMS-3′-*O*-methyl-BVDU in 80% yield (24). All derivatives were subsequently desilylated by treatment with a 2% solution of tetra-*n*-butylammonium fluoride (TBAF) in THF. By this reaction sequence the 3′-*O*-modified BVDU derivatives **8**–**11** were obtained in 90–97% yield. Compounds **8**–**11** were converted into the target phosphate triesters by 5′-*O*-phosphitylation using again 3-methyl-*cyclo*Sal-chlorophosphite and subsequent oxidation with TBHP to yield **3**, **4** and the *N*-Boc protected precursor of triester **5**. Here, the reactions were carried out in acetonitrile because the starting nucleoside analogues **8**–**11** were soluable in this solvent in contrast to BVDU **1**. In the case of the L-phenylalanine modified 3-methyl-*cyclo*Sal-BVDUMP triester the *N*-Boc protecting group was cleaved by treatment with 5% TFA in CH_2Cl_2/MeOH (7:3) to give **5**. The target phosphate triester **5** was obtained in 30% yield after purification. Characterization of the triesters **2**–**6** was done by all means of NMR spectroscopy as well as MS spectrometry. The purity was checked by HPLC analysis. The synthetic procedures are summarized in Figure 2.

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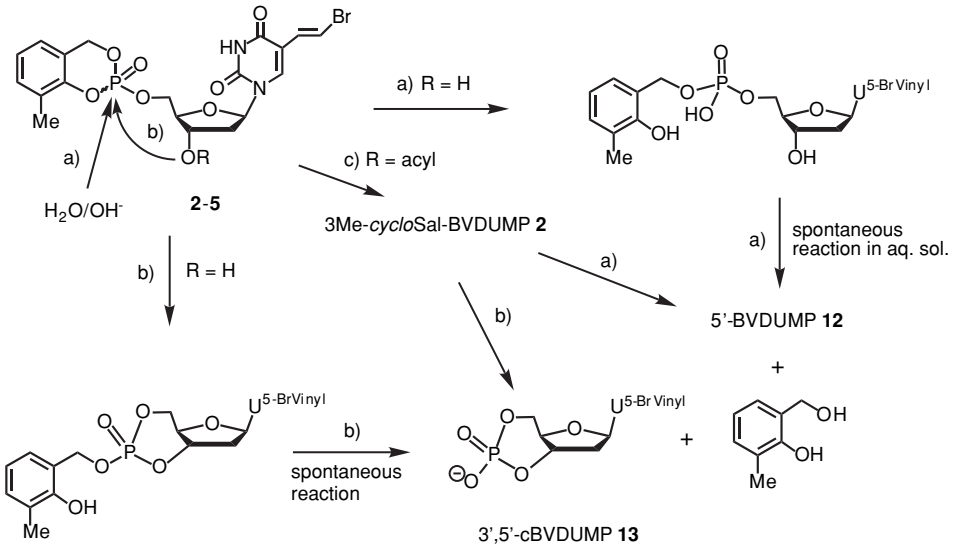

Figure 3. Hydrolysis pathways possible for the title phosphate triesters **2**–**5**.

Next, we conducted a series of hydrolysis studies in order to prove the selective delivery of BVDUMP **12** from the title phosphate triesters (pathway a, Fig 3). In contrast to our work published before on the corresponding triesters of d4T, ddA/d4A and 2′-F-*ara*/*ribo*-ddA (6, 9, 11), one should take into account that at least in the case of the 3′-*O*-unmodified 3-methyl-*cyclo*Sal-BVDUMP **2** a nucleophilic attack of the 3′-hydroxyl group at the phosphorus center may occur (pathway b, Fig 3). This would lead to the formation of undesired 3′,5′-cyclic BVDUMP **13** (cBVDUMP). We observed such a formation of an cyclic phosphate diester before in the case of the acyclic anti-herpetic agent penciclovir (25). This was also the reason why we decided to acylate the 3′-hydroxyl group in BVDU **1**. However, in the case of a rapid deesterification by cellular (carboxy)esterases in the cells this would lead back to triester **2** (pathway c, Fig 3), such a cyclisation reaction to cBVDUMP should again be possible in addition to the BVDUMP formation. Only triester **6** bearing the 3′-*O*-methyl ether should hydrolize only to yield the corresponding 3′-OMe-BVDUMP. The hydrolysis pathway leading to BVDUMP **12** as well as the mentioned possible concurrence reaction are depicted in Figure 3.

First, we hydrolyzed triesters **2**–**6** in a 25 mM phosphate buffer, pH 7.3. The reactions were followed by means of HPLC analysis. The hydrolysis reactions in these studies followed clearly pseudo-first-order-kinetics. The common and most important result of all reactions was that in all cases only BVDUMP **12** or the corresponding 3′-*O*-esterified BVDUMPs could be detected in the HPLC-chromatograms. There was definately no formation of the mentioned cBVDUMP **13**. This has been proven by separate preparation of **13** and subsequent coelution with the hydrolysis mixture of 3-methyl-*cyclo*Sal-BVDUMP **2** as well as by mass

spectrometry using an ESI-MS installation possessing an ion-trap, which allows tandem-MS analysis.

The prototype compound 3-methyl-*cyclo*Sal-BVDUMP **2** displayed a half-life of 6.7 h whereas the 3′-esterified triesters bearing a carboxylic acid showed half-lives in the range of 11 to 27 h. There seems to be a clear recognizable trend: the more lipophilic the carboxylic acid the more stable the phosphate triester. This may be due to the interaction of the attacking hydroxide nucleophile with the highly lipophilic 3′-*O*-acyl residue which result in a kind of protection of the phosphorus atom to nucleophilic attack. In contrast, the α-amino acid bearing *cyclo*Sal-BVDUMP triester **5** displayed a clearly lower $t_{1/2}$ value reflecting a lower hydrolytic stability in the slightly basic phosphate buffer. This may be due to the unprotected amino group present in the L-phenylalanine residue that may create a more polar surrounding in the vicinity of the phosphate group which makes a nucleophilic attack more probable. The half-lives are summarized in Table 1.

In order to study possible enzymatic contributions caused by intracellularly present (carboxy)esterases the title compounds **2**–**6** were treated in phosphate buffer, pH 7.3 with 50 units of pig liver esterase (PLE) as a reasonable model for contributing enzymes. Again, the hydrolyses were followed by means of HPLC analysis. The observed half-lives are also given in Table 1. Interestingly, the half-lives were found to be considerably lower as compared to the studies in phosphate buffer without PLE. More importantly, the hydrolysis product was in all cases the 3′-*O*-deesterified prototype triester **2** which points to an efficient enzymatically driven process. In the case of 3′-OAc-triester **3** we observed the formation of BVDUMP **12** from the initial product 3-methyl-*cyclo*Sal-BVDUMP **2**. In all other hydrolyses we were unable to detected the co-formation of the corresponding 3′-*O*-esterified BVDUMP.

Table 1. Log*P* Values, Hydrolysis Data and Antiviral Activity of the Title Triesters

Compound	Log*P* Value	Hydrolysis Half-Life: In Phosphate Buffer pH 7.3 [h]	Hydrolysis Half-Life: With 50 U PLE [h]	EC_{50} [μg/ml] DNA Synthesis	CC_{50} [μg/ml]
2	0.67	6.7[a]	n.d.[d]	2.1	63.0
3	1.16	11.3[a]	3.6[b,c]	>50	61.0
4	>2.11	27.6[a]	2.1[b]	>100	>200
5	1.66	1.2[a]	2.1[b]	31.5	16.0
6	1.43	8.0[a]	n.d.[d]	>50	>100
ACV		n.a.[e]	n.a.[e]	1.5	88.3
BVDU **1**	0.33	n.a.[e]	n.a.[e]	>100	75.0

EC_{50} : concentration required to reduce EBV DNA synthesis or VCA expression by 50%; CC_{50}: concentration required to reduce the growth of exponentially growing P3HR-1 cells by 50%; a) corresponding monophosphate; b) 3-Me-*cyclo*Sal-BVDUMP **2**; c) BVDUMP **12**; d) not determined; e) not available.

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It is worth mentioning that the 3′-*O*-α-amino acid containing triester **5** was cleaved to the same extent as compared to the 3′-*O*-carboxylic acid bearing derivatives. It seems that PLE has no preference to cleave α-amino acid esters as compared to esters formed from "normal" carboxylic acids. As a conclusion from these studies it should be possible to cleave all 3′-*O*-ester groups readily by enzymatic deesterification as a main degradation pathway to yield the prototype 3′-*O*-unmodified triester **2**.

In addition to the hydrolytic properties we determined the partition coefficients ($\log P$) of the triesters in a *n*-octanol/water mixture (Table 1). As can be seen all compounds **2–6** displayed considerably higher $\log P$ values as compared to the parent nucleoside **1**. Particularly, the $\log P$ of the 3′-OHex modified derivative **4** was extremely high ($\log P > 2.11$) so that one can not exclude micelle formation or lipid film formation in aqueous solution. Such formation will influence the hydrolytic behavior of the triester markedly.

The described phosphate triesters **2–6** were tested for their antiviral activity against EBV in human lymphoblastoid P3HR-1 cells. The EC_{50} values as well as the CC_{50} values are listed in Table 1. For comparison, the values of BVDU **1** and ACV are given. From the data it can be seen that only the prototype compound 3-methyl-*cyclo*Sal-BVDUMP **2** showed considerable antiviral activity against EBV. When compared with the parent nucleoside BVDU **1**, compound **2** was about a 100-fold more active (it should be added that **1** devoid of any antiviral activity below 100 mg/ml). The antiviral potency of triester **2** was in the same order of magnitude of the reference compound acyclovir (ACV). There was a striking loss in activity observed for the 3′-*O*-L-phenylalanine derivative **5** (15-fold). The other 3′-*O*-esterified compounds were completely inactive in the test assay. This observation was somewhat surprising because during the above described hydrolysis studies no general complication in the degradation has been observed. Therefore, the reasons for the pronounced differences in antiviral activity remain an secret. Moreover, these results clearly show that in this presented study the use of chemical hydrolysis and PLE catalyzed deesterification did not provide sufficient data in order to explain the behavior of the compounds in cellular media. As a consequence, hydrolysis studies in P3HR-1 cell extracts should be carried out. The data found in such experiments may give a more realistic "inside" view of the cell and therefore the degradation of the *cyclo*sal-BVDUMP described herein.

Nevertheless, the encouraging of the presented study is that the antiviral data of the prototype derivative **2** clearly demonstrate that by using the *cyclo*Sal-masking technology an antivirally inactive nucleoside analogue like BVDU **1** can be converted into a bioactive derivative against EBV. This will initiate further studies in our laboratories with respect to new α-amino acid bearing *cyclo*Sal-BVDUMP triesters as well as further substitution pattern in the *cyclo*Sal-masking group. Finally, it should be mentioned that to the best of our knowledge the work presented here represents the first example of a successful application of a pronucleotide strategy to a nucleoside analogue that contains a free 3′-hydroxyl group.

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