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ADA-Bypass by lipophilic *Cyclo*Sal-ddAMP Pro-Nucleotides A second Example of the Efficiency of the *cyclo*Sal-Concept

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Abstract: The synthesis of lipophilic pro-nucleotides of ddAMP **2** based on *cyclo*Sal-ddAMP **3a-c** is described. Phosphotriesters **3** released ddAMP **2** selectively by a controlled, chemically induced tandem reaction. *Cyclo*Sal-phosphotriesters **3** exhibited antiviral activity against HIV-1/ HIV-2 in CEM cells that where by a factor up to hundred higher as compared to ddA **1**.
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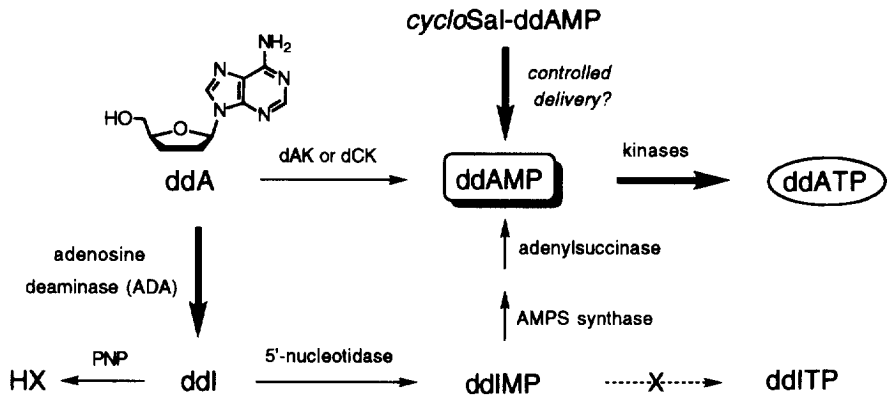
Nucleoside analogues, e.g. 3'-azido-2',3'-dideoxythymidine (AZT) or 2',3'-dideoxy-2',3'-didehydro-thymidine (d4T), are used as antiviral agents in the treatment for AIDS and the AIDS related complex (ARC). The only purine nucleoside analogue approved so far is 2',3'-dideoxyinosine (ddI; Didanosine, Videx®), the deamination product of 2',3'-dideoxyadenosine (ddA **1**)[1,2]. After penetration through cell membranes, all these compounds have to be converted intracellularly into their 5'-triphosphate for the expression of the biological activity. The major drawback in the use of ddA **1** is the high enzymatic instability against deamination into ddI by the enzyme adenosine deaminase (ADA). The major limitation of the metabolization of ddI into ddATP is the conversion of ddIMP into ddAMP by two enzymatically catalyzed steps (AMPS synthase and adenylsuccinase) and the high susceptibility of ddI to degradation by purine nucleoside phosphorylase (PNP; scheme 1)[3].

Scheme 1: Metabolization and ADA-bypass of 2',3'-dideoxyadenosine (ddA **1**)



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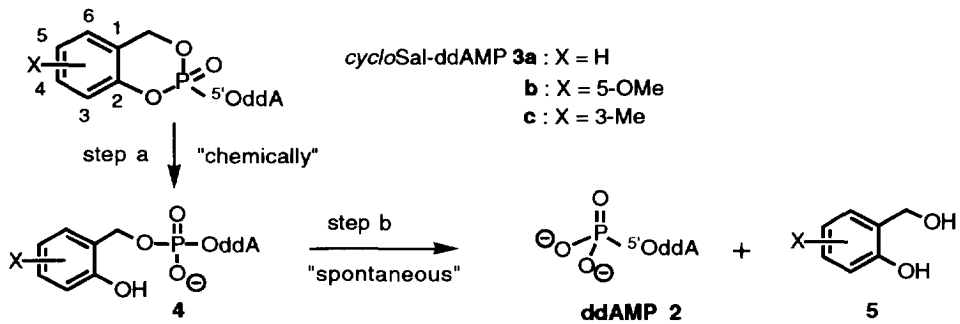
Consequently, the direct introduction of ddAMP **2** should bypass all these limiting steps and should have advantages for the biological activity (scheme 1). Unfortunately, the highly polar monophosphate **2** is not able to penetrate membranes due to low lipophilicity. In principle, *pro-nucleotides* should be able to circumvent this limitation[4]. For these reasons, we decided to apply our *cyclo*Sal-pro-nucleotide concept[5] directly to ddA as a new delivery system of ddAMP **2** inside cells. This concept was introduced successfully for the nucleoside analogue d4T as thymidine-kinase bypass[6]. In contrast to the situation of d4T, the pro-nucleotide-approach described here should result in a bypass of the degradative action of adenosine deaminase (***ADA-bypass***; scheme 1).

Here we present the synthesis, the hydrolytic behaviour, the anti-HIV activity and the properties against ADA degradation of the corresponding ***cyclo*saligenyl ddA-monophosphates 3a-c** (*cyclo*Sal-ddAMP) as neutral pro-nucleotides of ddAMP **2**[7].

Our *cyclo*Sal-pro-nucleotide concept was designed to release the nucleotide **2 selectively** by *controlled, chemically induced hydrolysis* following a tandem-mechanism[5,6]. The main characteristic of this pro-nucleotide system is the *coupled* cleavage of the phenyl- and the benzylester bond of the phosphotriester **3**, which leads exclusively to ddAMP **2** without any second activation (scheme 2).

Scheme 2: The hydrolysis pathways of *cyclo*Sal-ddAMP phosphotriesters **3**

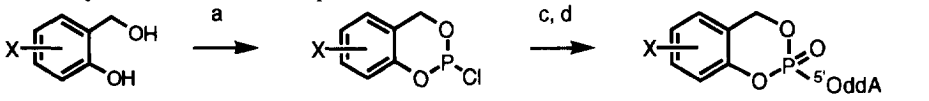


This tandem reaction has been experimentally verified by a NMR hydrolysis experiment in "wet" DMSO-d_6 using 5-nitro-*cyclo*Sal-d4TMP and has been published before[6a]. The major advantage of this tripartate prodrug approach is the avoidance of the formation of a *stable* intermediate phosphodiester which requires a highly selective activation in order to deliver exclusively the nucleotide[8]. An additional advantage of the approach applied here is that in all studied cases so far we never observed any evidence for the pseudorotation process normally observed in chemically driven hydrolyses of phosphodiesters[9,10]. A pseudorotation process would lead additionally in the liberation of the nucleoside analogue. As a consequence of this observation the second hydrolysis step could not proceed via a nucleophilic attack at the phosphorus atom but should proceed via a nucleophilic attack at the C_{benzyl}-atom or via a heterolytic cleavage of the O-C_{benzyl}-bond. Moreover, it was shown that we are able to control the hydrolysis rates of the *cyclo*Sal-derivatives by substituents in the aromatic ring. Here we introduced the most promising candidates (X = H, 5-OMe and 3-Me) with regard to the hydrolytic stability and antiviral activity to the *cyclo*Sal-ddAMP derivatives **3a-c**. Before hydrolysis takes place, **3a-c** should serve as lipophilic, neutral prodrugs.

The syntheses of the title compounds were carried out as published before[5,6]: The appropriate salicyl-alcohols **5** were reacted with phosphorus trichloride under anhydrous conditions to yield the cyclic saligenyl-

chlorophosphanes **6** (50-85% yield), which were treated with ddA **1**[11]. In contrast to the sooner published reaction sequence, we used mixtures of N,N-dimethylformamide (DMF) and tetrahydrofuran (THF) as solvent at low temperature. Using these conditions we obtained the target triesters **3a-c** in 60-70% yield. The first reason why we changed the solvent was due to the low solubility of ddA in acetonitrile. The second reason is related to the use of *N-unprotected* ddA which was reacted with cyclic chlorophosphanes **6**. Consequently, a regioselective reaction at the 5'-oxygen atom of ddA was required. The regioselectivity was best in a 2:1 mixture of DMF/THF at -40°C. We isolated a 4:1 ratio of the desired 5'-O phosphorylated triesters **3a-c** vs. the 5'-O,N-diphosphorylated side products. It should be mentioned that in all reactions carried out, we never isolated the N-monophosphorylated derivatives. The reaction is outlined in scheme 3. After purification on a chromatotron the *cyclo*Sal-ddAMPs **3** were obtained as 1:1 diastereomeric mixtures with respect to the configuration at the phosphorus atom. The two diastereomers could be distinguished by ^{1}H-, ^{13}C- and ^{31}P NMR spectroscopy[12] but could not be separated by common chromatographic methods. Furthermore, **3** were characterized by means of UV spectroscopy and electrospray (ESI; positive mode) mass spectrometry. The purity was checked by means of analytical HPLC analysis.

Scheme 3: Synthesis of the title compounds **3a-c**



5a-c
X = H, 5-OMe, 3-Me

6a-c

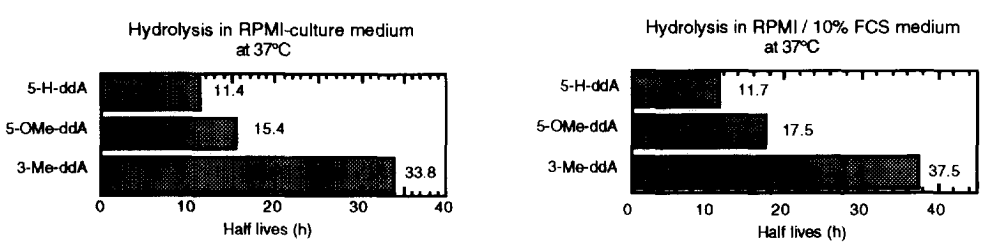
*cyclo*Sal-ddAMP **3a-c**
+ 5'-O, N-bis*cyclo*Sal-ddAMP **7a-c**
(minor amounts)

a) PCl_3, pyridine, Et_2O, -10°C, 2h; c) ddA **1**, di-*i*-propylethylamine, DMF / THF 2:1, -40°C, 30 min; d) *t*-butylhydroperoxide, DMF / THF 2:1, -40°C - rt, 1 h.

The partition coefficients (PC values) in 1-octanol / phosphate buffer (pH 6.8) are a qualitative estimation of the lipophilicity of *cyclo*Sal-ddAMPs **3**[13]. The calculated log(PC) values are listed in table 1 and show that all phosphotriesters **3** have partition coefficients that are by a factor of 7 to 15 higher as compared to ddA **1**. All log(PC) values are positive. Consequently, an improved cellular uptake should to be possible.

The hydrolytic stability of **3a-c** was studied in 50 mmol phosphate buffer, pH 7.3, in RPMI culture medium as well as in RPMI medium containing 10% heat inactivated fetal calf serum (FCS) at 37 °C and followed by means of HPL-chromatography at 260 nm. The half lives of the degradation are summarized in table 1 (phosphate buffer) and in figure 1 (RPMI and RPMI/FCS medium).

Figure 1: Hydrolysis of *cyclo*Sal-ddAMPs **3** in RPMI culture medium and RPMI /FCS medium



It should be mentioned that in the first two studies the sole hydrolysis products were ddAMP **2** and diols **5** (pseudo-first order kinetics) whereas in the latter study also ddA **1** was detected. This is due to dephosphorylation of **2** by enzymes included in FCS. As before[6], the intermediate phosphodiester could not be observed (scheme 2). Furthermore, the identification of **1**, **2** and **5** was accomplished directly from the hydrolysis mixtures by electrospray mass spectrometry (negative mode).

Again, in addition to the selective hydrolysis to ddAMP **2**, the expected effect of the substituents of the aromatic ring on the $t_{1/2}$ values was observed (table 1; figure 1). All these results are in fully agreement with the postulated hydrolysis pathway shown in scheme 1. Consequently, according to their hydrolytic stability ($t_{1/2}$ value) the reported derivatives of **3** should act as suitable pro-nucleotides in biological systems[14].

This expectation was proven by evaluation of the antiviral activity of *cyclo*Sal-ddAMPs **3** in HIV-1 and HIV-2 infected CEM/O cells[15]. The results are summarized in table 1. As can be seen, all *cyclo*Sal-ddAMPs **3** showed considerably higher antiviral potency as compared to the parent nucleoside ddA **1**. The best activity was observed in the case of the 3-methyl-substituted derivative **3c** which is more than a 100-fold more active than **1** against HIV-1 and HIV-2. Compounds **3a-c** exhibited identical antiviral activity against HIV-1 and HIV-2. Furthermore, a correlation of the biological activity with the hydrolysis half lives of the *cyclo*Sal-ddAMPs **3** was observed: the more stable the compound in the different media, the higher the antiviral activity. Although the cytotoxicity of the title compounds **3** increase (probably due to the higher amount of bioactive ddATP levels) the potency of our approach is additionally expressed in the calculated selectivity indices (SI value; table 1). Whereas the parent nucleoside **1** exhibited SI values of 37 and 54, respectively, *cyclo*Sal-ddAMPs **3** showed SI values of 200 to 460. This is an improvement of at least a factor of 4. The 3-Me-*cyclo*Sal-ddAMP **3a** which exhibited the best antiviral activity, showed a SI value of 300, hence a seven-fold enhancement of the SI value. It should be added that in parallel experiments the diols **5** showed neither an antiviral activity nor an cytotoxic effect up to concentrations of >250 µM. The results reported above clearly demonstrate the ability of *cyclo*Sal-ddAMPs to deliver ddAMP **2** *inside* the cells[14].

Table 1: Hydrolysis in phosphate buffers, biological activity and log(PC) values of *cyclo*Sal-ddAMPs **3**

3 (X) or ddA **1**	Hydrolysis ($t_{1/2}$) in phosphate buffer pH 7.3 [h]	Antiviral Activity EC_{50} [µM] CEM/O HIV-1	Antiviral Activity EC_{50} [µM] CEM/O HIV-2	Cytotoxicity CC_{50} [µM]	SI value	log(PC)[13]
3a (H)	8.2	0.12	0.11	21.9	200	0.54
3b (5-OMe)	14.0	0.065	0.09	30.4	460	0.61
3c (3-Me)	35.1	0.03	0.035	9.54	320	0.91
1	--	3.67	5.33	200	54	-0.28

Additionally to the evaluation of the biological activity we studied the stability of *cyclo*Sal-ddAMPs **3** against degradation by adenosine deaminase (ADA). The experiment was carried out using 0.15 U ADA in a 10 mmol citrate buffer, pH 6.3[16] at 37°C. The concentration of ddA **1** and the *cyclo*Sal-ddAMPs **3**, respectively, were 80 µM. The deamination reactions were followed in an UV spectrometer at 260 nm. Under these conditions, **1** was completely converted to ddI within 3.5 min whereas the 5'-phosphorylated ddA derivatives **3** were as expected[17] completely stable against ADA deamination for 12 hours. The integrity of *cyclo*Sal-

ddAMPs **3** has been proven after the reaction time (12 h) by their UV spectra and by analytical HPLC. From this study we can exclude the formation of the corresponding *cyclo*Sal-ddIMPs. As a consequence the measured biological effects described above are clearly related to ddAMP **2** delivered from *cyclo*Sal-ddAMPs **3** and not from ddIMP. ddIMP would be delivered from *cyclo*Sal-ddIMPs after deamination of *cyclo*Sal-ddAMPs **3**. Because ddA **1** would be fastly degraded by adenosine deaminase to ddI that is cleared by different detoxification mechanisms, the reason for the higher antiviral activity and toxicity of **3a-c** as compared to **1** (table 1) is a result of the considerably higher quantities of bioactive ddATP inside the cells.

In summary, after the successful thymidine kinase-bypass reported before, this is a second example that the *cyclo*Sal pro-nucleotide concept is suitable to deliver selectively nucleoside monophosphates by controlled, non-enzymatic cleavage at physiological pH according to the designed tandem-reaction. In the case reported here, we synthesized triester derivatives which served as successful ADA-bypass compounds with high biological activity. Further work is currently in progress in our laboratory in order to explore this pro-nucleotide concept.

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EXPERIMENTAL

General Procedure: To a solution of 0.6 mmol of dried ddA **1** in 10 ml dimethylformamide (DMF) 0.9 mmol di*iso*propylethylamine (DIPEA) was added at -40 °C. To this solution 1.5 equiv. (0.9 mmol in 1 ml tetrahydrofuran) of the cyclic chlorophosphanes **6a-c** were slowly added and stirring was continued for 30 min. The formed cyclophosphites were oxidized *in-situ* using 200 µl *t*-butylhydroperoxide (TBHP) within 1 h by warming the mixture from -40 °C to room temperature. After evaporation of the solvent the residue was purified on a Chromatotron using a gradient of ethyl acetate / methanol (0-30%) or dichloromethane / methanol (0-20%). To be sure to eliminate even contaminations of the parent nucleoside ddA **1** for the biological evaluation, small samples of **3** were additionally purified by semi-preparative HPLC (isocratic elution in water / acetonitrile) and isolated by lyophilisation.

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11. ddA **1** was synthesized by the following procedure: regioselective 5'-O benzoylation of deoxyadenosine with benzoic acid catalyzed by N,N'-bis-(2-oxo-oxazolidin-3-yl)phosphorodiamidic chloride (BOPDC; Liguori, A.; Perri, E.; Sindona, G.; Uccella, N.; *Tetrahedron* **1988**, *44*, 229-234) in pyridine (65% yield), regioselective 3'-O esterification with thiocarbonyldiimidazole and subsequent reaction with methanol (77% yield; note: esterification using phenoxythiocarbonylchloride was not sufficiently regioselective), subsequent Barton desoxygenation (88% yield) followed by a 12 hour treatment of the intermediate with ammonia in methanol to yield ddA (94% yield).
12. The characteristic spectroscopic data of *cyclo*Sal-ddAMP **3c** is given representatively: ^{1}H NMR: δ [ppm] (400 MHz, DMSO-d_6) 8.20 (2x s, 1H, H2; diastereomeric mixture); 8.10 (2x s, 1H, H8); 7.17-7.21 (m, 3H, $H4_{arom.}$, NH_2); 7.00-7.07 (m, 2H, H5 and $H6_{arom.}$) 6.19-6.24 (m, 1H, H1'); 5.26-5.46 (m, 2H, CH_2); 4.17-4.36 (m, 3H, H4', H5', H5"); 2.40-2.49 (m, 2H, H3', H3"); 2.04-2.14 (m, 1H, H2', H2"); 2.18 and 2.11 (2x s, 3H, CH_3); ^{13}C NMR: δ [ppm] (100.6 MHz, DMSO-d_6) 155.98 (C6), 152.46 (C2), 148.86 (C4), 147.82 ($C2_{arom.}$), 138.87 (C8), 130.75 ($C4_{arom.}$), 126.77 ($C3_{arom.}$), 123.81 ($C5_{arom.}$) , 123.40 ($C6_{arom.}$), 120.84 ($C1_{arom.}$), 119.05 (C5), 84.26 (C1'), 78.54 (C4'), 69.19 (C5'), 68.20 (CH_2), 30.67 (C3'), 25.77 (C2'), 14.77 (CH_3); ^{31}P NMR: δ [ppm] (162 MHz, DMSO-d_6) -8.24; -8.13; (1:1 ratio); MS (ESI+): calc.: 417.36; obs.: 418.10 (M+1); UV (water): λ_{max} = 261.76 nm; λ_{min} = 230.57 nm. In all cases the resonance signals of the two diastereomers of **3** were found to be well separated in the ^{31}P NMR spectra: **3a** (H; δ = -8.84 ppm/ -8.78 ppm); **3b** (5-OMe; δ = -8.64 ppm / 8.58 ppm); **3c** (3-Me; δ = -8.24 ppm / -8.13 ppm).
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