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Antiviral Activity of Cyclopentenyl Nucleosides Against Orthopox Viruses (Smallpox, Monkeypox and Cowpox)

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Abstract—An improved method for the synthesis of enantiomerically pure D-cyclopentenyl nucleosides has been accomplished and their antiviral activity against orthopox viruses have been evaluated. The key intermediate, L-cyclopent-2-enone **13** was prepared from D-ribose using a ring closing metathesis reaction in eight steps. Among the synthesized nucleosides, the adenine **2** (Neplanocin A), cytosine **14**, and 5-F-cytosine **15** analogues exhibited potent anti-orthopox virus activity, including smallpox virus.

Due to their interesting biological activity, carbocyclic nucleosides have received much attention in recent years. Various synthetic methodologies have been reported not only for naturally occurring carbocyclic nucleosides, such as (−)-aristeromycin **1**[1] and neplanocin A **2**[2] but also for synthetic analogues, including abacavir **3**[3] and carbovir **4**,[4] which demonstrated potent anti-HIV activity (Fig. 1). Neplanocin A **2**, an unsaturated analogue of (−)-aristeromycin **1**, has potent antiviral activity as well as cytotoxicity.[5] The antiviral effect may be due to the inhibition of *S*-adenosylhomocysteine (AdoHcy) hydrolase.[6] AdoHcy is a potential feedback inhibitor of cellular transmethylation using *S*-adenosyl-L-methionine as a methyl donor, which is essential for mRNA maturation.[7] Therefore, inhibition of AdoHcy hydrolase would provide broad-spectrum of antiviral activity for DNA as well as RNA viruses. Although neplanocin A **2** itself is not an effective antiviral agent due to its cytotoxicity to the host cell, it is a lead compound for the discovery of structurally related chemotherapeutic agents.[8] Therefore, a number of neplanocin A analogues have been synthesized and evaluated as potential antiviral and antitumor agents.[9]

Previously, we have developed a synthetic method for enantiomerically pure D- and L-cyclopentenyl and cyclopentyl nucleosides, including (+)-aristeromycin and neplanocin A, using L-cyclopent-2-enone **13** as the key intermediate.[10] Optically pure starting material, such as D-ribose, D-mannose and D-ribonolactone, have been used for the synthesis of L-cyclopent-2-enone **13**.[11] However, the synthesis of **13** from these carbohydrates has been problematic due to low and inconsistent yield. Some of the reactions are very sensitive to the reaction conditions, such as moisture and temperature, resulting in low yield. Therefore, an efficient synthetic method for the key intermediate, such as D-cyclopent-2-enone **13**, is highly desirable in order to carry out studies for structural–activity relationships of optically pure carbocyclic nucleosides. Recently, we have developed an efficient and practical methodology for the synthesis of D-cyclopent-2-enone using ring closure metathesis (RCM)

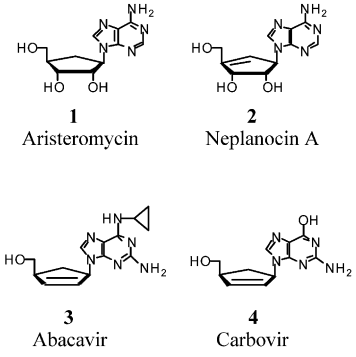

Figure 1. Biologically active natural and synthetic carbocyclic nucleosides cyclopentenyl nucleosides as reported in this article.

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reaction from D-ribose in excellent overall yield in preparative scale.[12] Therefore, our efforts were directed toward the synthesis of the L-cyclopent-2-enone **13** as the key intermediate for the synthesis of D-cyclopentenyl nucleosides as reported in this article.

Smallpox (Variola virus),[13] which has only human as the natural host, has been responsible for worldwide epidemics. Smallpox is highly contagious virus with a case fatality rate of 30% in unvaccinated individuals.[14] Under the massive vaccination program by World Health Organization (WHO), it has been more than 22 years since the last case of smallpox was confirmed,[15] and WHO finally declared in 1980 that the global eradication had been achieved. Smallpox is a particularly dangerous biological threat because of its clinical and epidemiological properties,[16] and no clinically proven antiviral treatment exists at this time. The recent increasing concern of biological terrorism affects all segments of the population and the growing concern of smallpox virus as a prime bioterrorism agent prompted us to search for antiviral agents to prevent and to treat the smallpox virus.

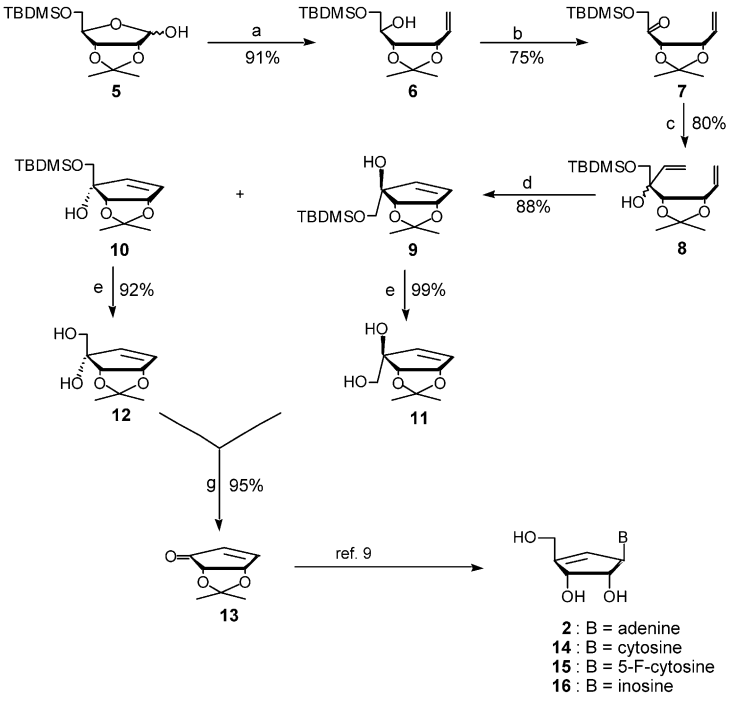
In this communication, we describe an improved synthesis of the key intermediate L-cyclopenten-2-one **13** for D-cyclopentenyl nucleosides and their potent anti-orthopox virus (smallpox, monkeypox, cowpox and vaccinia virus) activity.

Chemistry

The protected D-ribose **5** was readily available from D-ribose in two steps,[11] which underwent Wittig reaction using NaH, DMSO and methyltriphenylphosphonium bromide in THF to give olefin **6** in 91% yield (Scheme 1). The carbonyl compound **7** was obtained by oxidation of the secondary hydroxyl group. Several oxidation conditions were investigated to optimize the yield. Among them, Moffatt oxidation with dicyclohexyl carbodiimide, DMSO, pyridine and trifluoroacetic acid in toluene gave the best result to afford ketone **7** in 75% yield. To introduce another olefin moiety, Grignard reaction was carried out with vinylmagnesium bromide to provide inseparable mixture of diene **8** in 80% yield. This underwent the ring-closing metathesis (RCM) reaction using 5% Grubbs catalyst at refluxing temperature in anhydrous CH_2Cl_2 to provide α and β cyclopentanol **9** and **10** (**9**/**10** ratio = 10/1) in 88% yield. The α- and β-configurations were assigned by ^1H NMR and NOE experiments. The removal of primary hydroxyl protecting group using 1 M solution of tetrabutylammonium fluoride in THF to form a vicinal diol **11** and **12**, followed by an oxidative cleavage with sodium periodate to afford the key intermediate cylopentenone **13**[17] in 95% yield. The prepared cyclopentenone **13** was converted to adenine (Neplanocin A) **2**, cytosine **14**, 5-Fluoro-cytosine **15**, and inosine **16** analogues as we previously reported.[10a]

Antiviral Activity

The synthesized nucleosides were evaluated for their antiviral activity against smallpox, monkeypox, cowpox, and vaccinia viruses as well as their cytotoxicity in Vero and MK2 cells.[18] The results are summarized in Table 1. It was found that adenine **2** (Neplanocin A), cytosine **14**, and 5-fluoro-cytosine **15** analogues exhibited



Scheme 1. (a) NaH, DMSO, methyltriphenylphosphonium bromide, THF, 0 °C to reflux, 2 h; (b) dicyclohexyl carbodiimide, DMSO, pyridine, trifluoroacetic acid, toluene, rt, 4 h; (c) vinylmagnesium bromide, anhydrous THF, −78 °C to rt, 1 h; (d) Grubbs catalyst, anhydrous CH_2Cl_2, reflux, 24 h; (e) TBAF, THF, rt, 1 h; (g) $NaIO_4$, H_2O, rt, 0.5 h.

Table 1. Antiviral activity and cytotoxicity against orthopox viruses of D-cyclopentenyl nucleosides in MK2 and Vero cells

Compd	Cell	Virus	Activity EC_{50} (μg/mL)	Cytotoxicity IC_{50} (μg/mL)	TI[h]
Adenine **2**	MK2[a]	7124[c]	0.10	23	230
		BSH[d]	0.10	36	360
		CPX[e]	100	100	1.0
		MPX[f]	0.26	42	161
		VAC[g]	2.62	31	12
	Vero[b]	7124	0.03	50	1667
		BSH	0.14	10	71
		CPX	>100	20	<0.2
		MPX	0.21	57	271
		VAC	>100	>100	1.0
Cytosine **14**	MK2	7124	0.08	44	550
		BSH	0.03	3	100
		CPX	0.06	10	>166
		MPX	0.1	21	210
		VAC	0.12	36	300
	Vero	7124	<0.05	>100	>2000
		BSH	>100	30	0.3
		CPX	0.05	1	>20
		MPX	<0.05	4	>80
		VAC	0.04	40	1000
5-F-Cytosine **15**	MK2	7124	1.73	>100	>58
		BSH	0.51	>100	>196
		CPX	0.43	70	162
		MPX	0.61	>100	>164
		VAC	0.53	100	189
	Vero	7124	2.63	39	15
		BSH	1.17	100	86
		CPX	1.35	>100	>74
		MPX	2.05	100	49
		VAC	1.46	>100	>69
Inosine **16**	MK2	7124	>100	>100	—
		BSH	>100	>100	—
		CPX	>100	>100	—
		MPX	>100	>100	—
		VAC	>100	>100	—
	Vero	7124	>100	>100	—
		BSH	>100	>100	—
		CPX	>100	>100	—
		MPX	>100	>100	—
		VAC	>100	>100	—

[a]LLC-MK2 (ATTC CRL 7).
[b]Vero 76 (ATTC CRL 1587).
[c,d]Smallpox virus.
[e]Cowpox virus.
[f]Monkeypox virus.
[g]Vaccinia virus.
[h]$TI = IC_{50}/EC_{50}$.

potent anti-smallpox, -cowpox, -monkeypox, and -vaccinia virus activity.

These preliminary studies against orthopoxviruses showed that D-cyclopentenyl nucleoside analogues, particularly adenine, cytosine and 5-F-cytosine derivatives, possess potent anti-smallpox virus activities. Therefore we plan to further investigate the structure–activity relationships as well as biochemical studies of these carbocyclic nucleosides.

In summary, we have developed an improved synthesis of L-cyclopentenone **13** using the ring closure metathesis reaction which was applied to synthesize several unsaturated carbocyclic nucleosides, and it was found that adenine, cytosine and 5-F-cytosine derivatives possess potent antiviral activity against orthopox viruses, including smallpox virus.

Acknowledgements

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17. Data for **6**: $[\alpha]_D$ −9.97° (*c* 0.39, MeOH); ^{1}H NMR ($CDCl_3$) δ 6.07–5.98 (m, 1H), 5.41 (dd, *J* = 1.26 and 17.1 Hz, 1H), 5.28 (dd, *J* = 0.97 and 10.4 Hz, 1H), 4.68 (t, *J* = 6.0 Hz, 1H), 4.05 (t, *J* = 7.7 Hz, 1H), 3.70–3.64 (m, 3H), 2.53 (d, *J* = 4.7 Hz, 1H), 1.46 (s, 3H), 1.36 (s, 3H), 0.90 (s, 9H), 0.08 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 134.16, 117.55, 108.74, 78.80, 77.44, 69.55, 64.32, 27.82, 25.42, 18.31, −5.37, −5.45. Anal. calcd for $C_{15}H_{29}O_4Si$: C, 59.56; H, 10.00. Found: C, 60.17; H, 9.60.

Data for **7**: $[\alpha]_D$ −20.34° (C 0.70, MeOH); ^{1}H NMR ($CDCl_3$) δ 5.74–5.66 (m, 1H), 5.41 (d, *J* = 16.6 Hz, 1H), 5.24 (d, *J* = 10.5 Hz, 1H), 4.92–4.87 (m, 2H), 4.47 (d, *J* = 18.9 Hz, 1H), 4.22 (d, *J* = 18.9 Hz, 1H), 1.61 (s, 3H), 1.40 (s, 3H), 0.92 (s, 9H), 0.08 (s, 6H); ^{13}C NMR ($CDCl_3$) δ 132.70, 118.89, 81.90, 78.22, 68.62, 31.42, 26.99, 25.83, 24.86, 22.64, 13.71, −5.48. Anal. calcd for $C_{15}H_{28}O_4Si$: C, 59.96; H, 9.39. Found: C, 59.92; H, 9.17.

Data for **8**: ^{1}H NMR ($CDCl_3$) δ 6.14–5.88 (m, 2H), 5.43–5.14 (m, 4H), 4.66 (t, *J* = 7.1 Hz, 0.9H), 4.54 (t, *J* = 6.6 Hz, 0.1H), 4.38 (d, *J* = 6.4 Hz, 0.1H), 4.29 (d, *J* = 6.9 Hz, 0.9H), 2.77 (s, OH, D_2O exchangeable, 0.9H), 2.51 (s, OH, D_2O exchangeable, 0.1H), 1.53 (s, 0.3H), 1.51 (s, 2.7H), 1.38 (s, 0.3H), 1.36 (s, 2.7H), 0.89 (s, 8.1H), 0.87 (s, 0.9H), 0.05 (s, 5.4H), 0.03 (s, 0.6H); ^{13}C NMR ($CDCl_3$) δ 138.07, 138.01, 135.70, 135.29, 117.56, 117.05, 115.80, 115.70, 108.16, 107.92, 79.33, 78.57, 78.32, 75.08, 76.69, 75.08, 74.79, 68.15, 67.99, 27.67, 27.29, 25.82, 25.80, 25.43, 24.88, 18.31, 18.20, −5.40, −5.49, −5.54. Anal. calcd for $C_{17}H_{32}O_4Si$: C, 62.15; H, 9.82. Found: C, 62.05; H, 9.76.

Data for **9**: $[\alpha]_D$ +55.97° (*c* 0.37, MeOH); ^{1}H NMR ($CDCl_3$) δ 5.98 (d, *J* = 5.7 Hz, 1H), 5.74 (d, *J* = 5.7 Hz, 1H), 5.31 (d, *J* = 5.3 Hz, 1H), 4.47 (d, *J* = 5.4 Hz, 1H), 3.92 (d, *J* = 9.9 Hz, 1H), 3.62 (d, *J* = 9.9 Hz, 1H), 3.22 (s, OH, D_2O exchangeable, 1H), 1.38 (s, 3H), 1.32 (s, 3H), 0.92 (s, 9H), 0.10 (s, 3H), 0.09 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 135.20, 135.02, 112.06, 84.82, 84.66, 64.97, 27.49, 25.95, 25.88, 18.38, −5.38, −5.41. Anal. calcd for $C_{15}H_{29}O_4Si$: C, 59.96; H, 9.39. Found: C, 60.05; H, 9.48.

Data for **10**: $[\alpha]_D$ +72.04° (*c* 0.35, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.90 (d, *J* = 5.7 Hz, 1H), 5.66 (d, *J* = 5.7 Hz, 1H), 5.00 (d, *J* = 5.3 Hz, 1H), 4.47 (d, *J* = 5.2 Hz, 1H), 3.69 (ddd, *J* = 1.5, 9.7 and 38.7 Hz, 2H), 3.12 (s, OH, D_2O exchangeable, 1H), 1.43 (s, 3H), 1.39 (s, 3H), 0.85 (s, 9H), 0.03 (s, 3H), 0.01 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 136.90, 133.15, 112.41, 84.13, 82.47, 80.84, 67.04, 27.85, 26.75, 25.80, −5.49. Anal. calcd for $C_{15}H_{29}O_4Si$: C, 59.96; H, 9.39. Found: C, 60.10; H, 9.39.

Data for **11**: mp 103–104 °C; $[\alpha]_D$ +104.12° (*c* 0.28, MeOH); ^{1}H NMR ($CDCl_3$) δ 5.96 (dd, *J* = 1.6 and 5.8 Hz, 1H), 5.64 (d, *J* = 5.8 Hz, 1H), 5.25 (d, *J* = 5.8 Hz, 1H), 4.49 (d, *J* = 5.8 Hz, 1H), 3.84 (dd, *J* = 4.2 and 11.4 Hz, 1H), 3.56 (dd, *J* = 8.7 and 11.2 Hz, 1H), 2.89 (s, OH, D_2O exchangeable, 1H), 2.32 (dd, *J* = 4.7 and 8.6 Hz, OH, D_2O exchangeable, 1H), 1.38 (s, 3H), 1.29 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 135.31, 135.12, 112.94, 86.34, 84.42, 65.84, 27.08, 25.41. Anal. calcd for $C_9H_{14}O_4$: C, 58.05; H, 7.58. Found: C, 58.06; H, 7.61.

Data for **12**: $[\alpha]_D$ +88.18° (*c* 0.27, MeOH); ^{1}H NMR ($CDCl_3$) δ 5.95 (dd, *J* = 1.7 and 5.8 Hz, 1H), 5.72 (d, *J* = 5.8 Hz, 1H), 5.07 (d, *J* = 5.5 Hz, 1H), 4.61 (d, *J* = 5.6 Hz, 1H), 3.73 (d, *J* = 11.5 Hz, 1H), 3.31 (bs, OH, D_2O exchangeable, 1H), 3.26 (d, *J* = 11.5 Hz, 1H), 2.13 (bs, OH, D_2O exchangeable, 1H), 1.46 (s, 3H), 1.41 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 136.42, 133.60, 112.83, 83.43, 82.58, 79.12, 66.38, 27.67, 26.48. Anal. calcd for $C_9H_{14}O_4$: C, 58.05; H, 7.58. Found: C, 58.10; H, 7.63.

Data for **13**: mp 68.1–69.4 °C; $[\alpha]_D$ +69.1° (*c* 0.77, $CHCl_3$); [reported:[10c] mp 68.7–69.8 °C; $[\alpha]_D$ +69.1° (*c* 1.98, $CHCl_3$)]; ^{1}H NMR ($CDCl_3$) δ 7.57 (dd, *J* = 2.0 and 5.8 Hz, 1H), 6.17 (d, *J* = 5.9 Hz, 1H), 5.23 (dd, *J* = 2.3 and 5.4 Hz, 1H), 4.42 (d, *J* = 5.4 Hz, 1H), 1.37 (s, 3H), 1.36 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 203.17, 159.65, 134.28, 115.46, 78.56, 76.46, 27.37, 26.10.

18. Neutral red uptake assay. Stocks of antiviral compounds were made by dissolving each compound in DMSO to a concentration of 20 mg/mL. Drugs were then diluted to 400 μg/mL in RPMI-1640, serially diluted 3-fold in RPMI-1640, and 50 μL added to 96-well microtiter plates of confluent Vero 76 and LLC-MK2 cells already containing 100 μL of medium. At each drug concentration, three wells were infected with 10^5 pfu/well (MOI = 0.1) of orthopoxvirus in 50 μL of medium, while three were left uninfected for toxicity determination (50 μL of medium added to each well). Plates were examined daily, and were stained once virus-infected, untreated cells showed 4+ cytopathic effect (CPE). 50 μL neutral red (1.11 mg/mL) was added to the medium to give a final concentration of 0.22 mg/mL, and cells returned to the incubator for 90 min. The medium was removed, the wells were rinsed twice with buffered saline solution, and retained stain was solubilized by adding 100 μL of developing solution (50% ethanol, 5 mM ammonium phosphate ($NH_4H_2PO_4$), pH 3.5). Plates were rocked for 30 min at 150 RPM, and the optical density (OD) of the wells at a wavelength of 450 nm was measured on a plate reader. The data were graphed and analyzed by using the four parameter–log it curve fit option of a curve-fitting program (Molecular Devices, Menlo Park, CA, USA) to determine the 50% inhibitory and cytotoxic drug concentration.