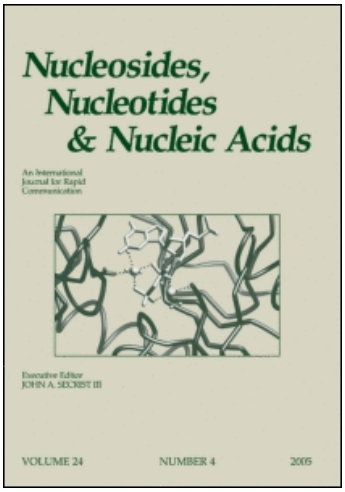

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SYNTHESIS AND BIOLOGICAL EVALUATION OF A CARBOCYCLIC AZANORARISTEROMYCIN SIDEROPHORE CONJUGATE

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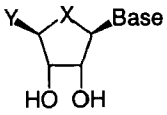
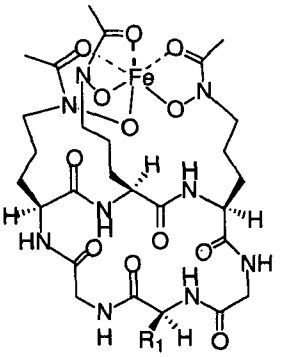
Abstract: Synthesis and biological evaluation of a carbocyclic azanoraristeromycin siderophore conjugate **22** is reported. Coupling of previously prepared L-alanyl-4'-azanoraristeromycin **19** with protected tripeptide trihydroxamate **20**, followed by hydrogenolytic removal of all protecting groups, provided the first carbocylic azanoraristeromycin siderophore conjugate (**22**, **8** with iron). Compounds **19** and **22** showed inhibitory activity against tumor cells, and conjugate **22**, in particular, displayed significant activity against those viruses (i.e. reo, parainfluenza, vaccinia, cytomegalo) that are known to be inhibited by *S*-adenosylhomocysteine hydrolase inhibitors.

Carbocyclic analogs (**1**) of nucleosides (**2**) are of considerable interest as antiviral, antitumor and antifungal agents. Isosteric replacement of the furanose oxygen of nucleosides by a methylene group results in enzymatic and hydrolytic resistance and often a decrease in mammalian toxicity.[1] Recent studies also suggest that other structural variations of nucleosides can enhance pharmacological properties. For example, replacement of the normal 5'-hydroxymethyl group of aristeromycin (**3**) with an hydroxyl group produced noraristeromycin (**4**) which, relative to aristeromycin, displays improved antiviral activity while losing the cytotoxicity associated with aristeromycin.[2] Other attempts to increase efficacy and diminish mammalian toxicity of drugs include derivatization that may allow them to localize at the biological target, whether it be a cancer cell, microbial pathogen or other source of disease. Recently, we have demonstrated that conjugation of siderophores [microbial iron chelators, such as ferrichrome (**6**)][3] or their iron-binding components (eg **7**) to antibiotics promotes microbially selective iron transport-mediated drug delivery.[4] Herein we report the synthesis and selected biological studies of a siderophore conjugate (**8**, shown with iron, **22**, without iron) of azanoraristeromycin (**5**).

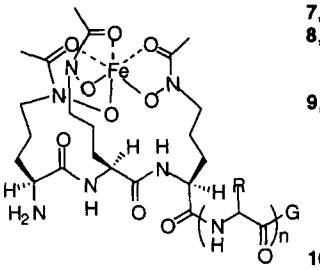


Base = A, T, G, U or analogs/derivatives

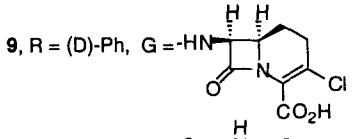
1, X = CH_2, Y = CH_2OH
2, X = O, Y = CH_2OH
3, X = O, Y = CH_2OH, Base = A
4, X = O, Y = OH, Base = A
5, X = CH_2, Y = NH_2



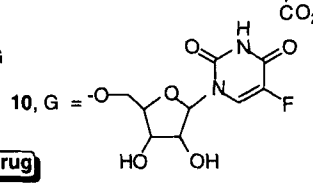
6, ferrichrome



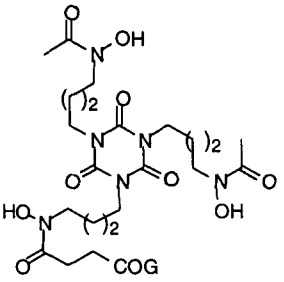
7, G = OH
8, R = CH_3, G = **5** (Y = NH, Base = A)



9, R = (D)-Ph, G = -HN-

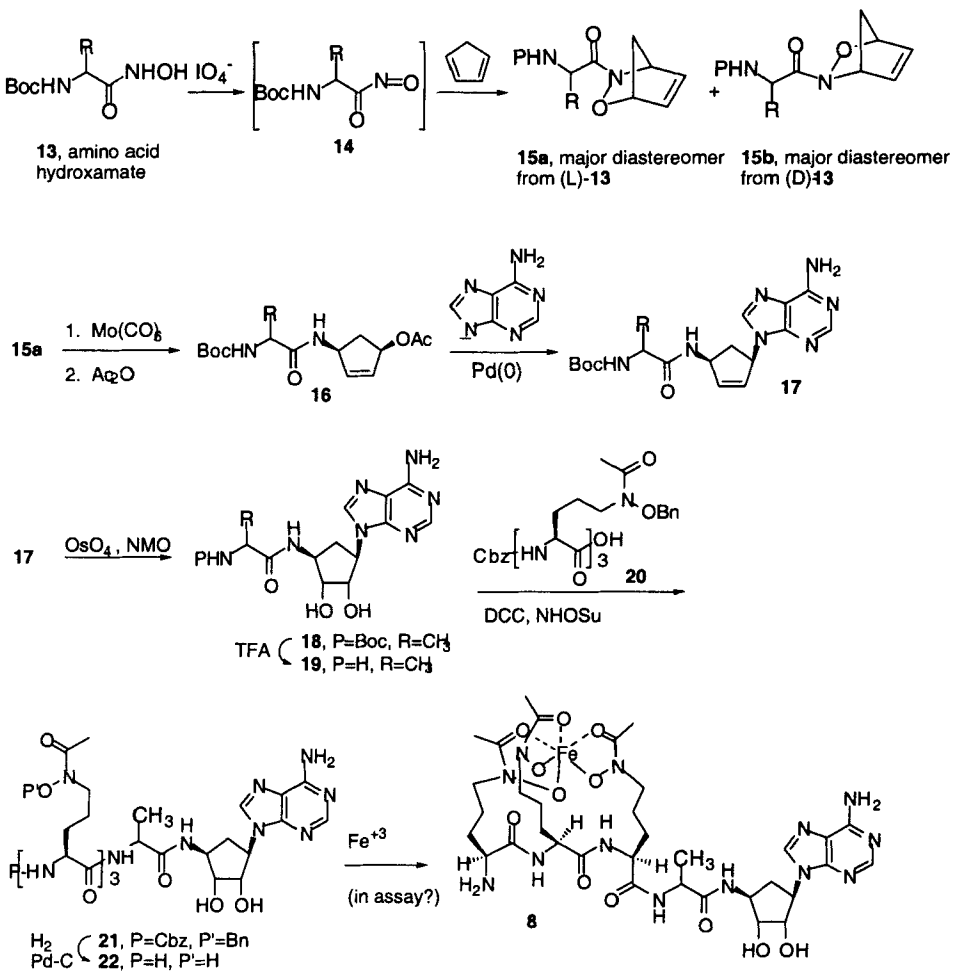


10, G =



11, G = OH
12, G = HNCHRCO-5-FU (as in **10**)

While most of the siderophore-drug conjugates synthesized and studied have incorporated classical antibiotics (eg. β-lactams as in Lorabid® conjugate **9**)[4] as the drug component, broader structure-activity studies are of interest. Our first siderophore-nucleoside conjugates (**10**[4f] and **12**[5]) consisted of either peptides (**7**) containing tri-N^{δ}-hydroxy-N^{δ}-acetyl-L-ornithine, the iron-binding tripeptide component of the natural microbial siderophore, ferrichrome, or the synthetic isocyanurate-based trihydroxamate (**11**) attached to 5-fluorouridine (5-FU) through amino acid spacers. As described earlier,[4f,5] these conjugates proved active against a variety of fungi, including *Candida albicans*, especially in low iron media or media that did not contain exogenous siderophores. The conjugates also were moderately active against several strains of Gram-positive bacteria, with notable activity (0.25-1.0 μg/mL) against strains of methicillin-resistant *Staphylococcus* (MRSA). While the 5' hydroxyl group was a convenient site for attachment of the spacer amino acids and siderophore component, we realized that it may be hydrolytically labile, and anticipated that hydrolysis might either beneficially serve as a drug release process *in vivo* or simple chemical hydrolysis might induce premature release of the drug before microbial assimilation of the conjugate. In fact, the rate of release of 5-FU from **10** in biological test media was readily determined by fluorine NMR and, as expected, was found to depend on stereoelectronic factors of the amino acid spacer (R=Ph; $t_{1/2}$=10h; R=H; $t_{1/2}$=13h; R=*i*Pr $t_{1/2}$=241h).[4f,5]



Scheme 1

Replacement of the "normal" 4'-hydroxymethyl group of nucleosides with an amine was anticipated to allow direct conjugation to siderophore components using amide linkages for comparison to the ester links described above. However, until recently, no 4'-azacarbocyclic nucleosides had been reported. Relevant aspects of our development of an asymmetric synthesis of 4'-azacarbocyclic nucleoside analogs[6] are briefly summarized below followed by extension to the synthesis of **22**, the deferri form of **8** (Scheme 1). The chemistry utilized asymmetric amino acid-based nitroso Diels-Alder reactions to assemble the carbocyclic nucleoside framework. Thus, oxidation of N^{α}-protected L-amino acid hydroxamates (**13**) to the corresponding nonisolable acylnitroso compounds (**14**) in the presence of cyclopentadiene produced cycloadducts

15. The diastereoselectivity depended on the amino acid used (phe, 60%; ala, 75%; val, 80%; *t*-leu, 85%: the major diastereomer is shown). The diastereomers were easily separated and demonstrated to be optically pure.[6,7] $Mo(CO)_6$-Mediated N-O bond reduction followed by acetylation produced amino acetates **16** cleanly. At this point, the amino acid chiral auxiliary can be removed by use of Edman degradation to eventually allow preparation of the parent 4'-azacarbocyclic nucleosides.[8] However, since incorporation of amino acid linkers onto previously described 5-FU derivatives (G of **10**) expanded the biological activity profile,[5] the amino acid was retained to serve as a linker between the siderophore and the new carbocyclic nucleoside. Direct Pd(0)-mediated reaction with the sodium salt of adenine gave 4'-azacarbocyclic adenosine precursor **17**, with traces of separable *N*-3 and/or *N*-7 alkylated products, as determined by detailed gated decoupling NMR studies.[6] The incorporation of the adenine base into the carbocycle proceeded with net retention as expected from the Pd(0) reaction.[9] Dihydroxylation of **17** with OsO_4/NMO produced the desired *anti* diol **18** as the major product (with contamination by the separable *syn* diol[10]). Removal of the Boc protecting group from the amino acid constituent (alanine, in this first case) gave free amine **19** which was coupled to protected trihydroxamate tripeptide **20**,[4] to give **21**. Subsequent deprotection provided conjugate **22**, the deferri form of **8** (R=Me). The carbocyclic adenosine conjugate was chosen as the first target because of the product's similarity to sinefungin,[11] an antifungal agent of considerable interest, as well as aristeromycin and noraristeromycin, two potent carbocyclic antiviral agents described previously.

Preliminary antifungal studies of Boc-protected alanylcarbocyclic azanoraristeromycin (**18**), the corresponding free amine (**19**), and conjugate **22** with *C. albicans* at Notre Dame indicated no anticandidal activity. Meanwhile, De Clercq's laboratory, had screened the Boc derivative, free amine and conjugate for antiviral and anticancer activity. While no selective activity was found against herpes simplex virus (HSV), human immunodeficiency virus (HIV) and a number of other viruses, as shown below, notable inhibitory effects were found for these novel carbocyclic nucleosides against some viruses and tumor cells (Tables 1-4). In particular, compound **22** proved active at a concentration ranging from 0.2 to 20 μg/mL against those viruses (i.e. reo, parainfluenza, vaccinia, cytomegalo) that are known to be inhibited by inhibitors of the *S*-adenosylhomocysteine hydrolase.[12] Whether the activity of **22** is attributable to the intact iron binding conjugate or release of the novel azanoraristeromycin after targeting by the conjugate remains to be determined.

The results described here, indicate that we will not only be able to prepare conjugates to siderophores by direct peptide coupling of the amino group of the

TABLE 1. Antiviral Activity* and Cytotoxicity** of Compounds **18**, **19**, and **22**.

VIRUS (STRAIN)	CELL	18	19	22	BVDU[a]	RIBAV[b]	ACV[c]	GC[d]
HSV-1 (KOS)	E_6SM	>400	>200	>200	0.007	20	0.07	0.002
HSV-2 (G)	E_6SM	>400	>200	>200	100	70	0.04	0.002
Vaccinia	E_6SM	>400	40	20	0.1	20	>400	>100
Vesicular stomatitis	E_6SM	>400	150	150	>400	70	>400	>100
HSV-1 (TK^-B2006)	E_6SM	300	150	40	40	20	20	0.7
HSV-1(TK^-VMW1837)	E_6SM	>400	>200	>200	10	40	2	0.1
Cytotoxicity	E_6SM	>400	>200	>200	>400	>400	>400	>100
							(*S*)-DHPA[e]	C-c[3] Ado[f]
Parainfluenza-3	Vero	>400	10	7	>400	150	40	4
Reovirus-1	Vero	>400	1	0.2	>400	7	4	0.4
Sindbis	Vero	>400	>200	>200	>400	>400	>400	>200
Coxsackie B4	Vero	>400	>200	>200	>400	>400	>400	>400
Semliki forest	Vero	>400	>200	>200	>400	40	>400	>400
Cytotoxicity	Vero	>400	>200	>200	>400	>400	>400	>400
Vesicular stomatitis	HeLa	>200	>200	>200	>200	20	>400	10
Coxsackie B4	HeLa	>200	>200	>200	>200	20	>400	>200
Polio virus-1	HeLa	>200	>200	>200	>200	20	>400	>200
Cytotoxicity	HeLa	≥400	>200	>200	≥400	>400	>400	>200

*Minimum inhibitory concentration (μg/mL) required to reduce virus-induced cytopathogenicity by 50%.
**Minimum cytotoxic concentration (μg/mL) required to cause a microscopically detectable alteration of normal cell morphology.
[a]Brivudin. [b]Ribavirin. [c]Acyclovir. [d]Ganciclovir. [e](*S*)-9-(2,3-Dihydroxypropyl)adenine. [f]Carbocyclic 3-deazaadenosine.

TABLE 2. Antiviral activity (EC_{50})* and Cytotoxicity (CC_{50})** of Compounds **18**, **19** and **22** Against Several Strains of Varicella-Zoster Virus (VZV) and Cytomegalovirus (CMV) in Human Embryonic Lung (HEL) Cells.

VIRUS (STRAIN)	18	19	22	BVDU[a]	
VZV[d]					
TK^+VZV (OKA strain)	>50	7	>50	0.0005	
TK^+VZV (YS strain)	>50	30	>50	0.0009	
TK^-VZV (07/1 strain)	>50	34	>50	50	
TK^-VZV (YS/R strain)	>50	9	>50	>50	
Cytotoxicity	200	50	>200	N.D.	
CMV				(*S*)-HPMPC[b]	GCV[c]
AD-169 strain (Assay-1)	>50	>50	20	0.04	0.2
Davis strain (Assay-1)	>50	>50	20	0.12	0.15
Cytotoxicity	200	50	>200	50	>200

*50% Inhibitory concentration, or concentration required to reduce virus plaque formation by 50%. Virus input was 20 plaque forming units PFU for VZM and 100 PFU for CMV. **50% Cytotoxic concentration or concentration required to reduce cell growth by 50%. [a]Brivudin. [b]Cidofovir. [c]Ganciclovir. [d]TK^+, thymidine kinase efficient, TK^-, thymidine kinase deficient.

TABLE 3. Anti-HIV-1 and HIV-2 Activity* of Compounds **18**, and **19** and **22** in Human T-lymphocyte (CEM/0) Cells.

VIRUS	EC_{50} or CC_{50}	18	19	22
HIV-1	EC_{50}	>40	>20	>20
HIV-2	EC_{50}	>200	>20	>20
Cytotoxicity	CC_{50}	>200	≥75	≥75

*50% Effective concentration, or concentration required to protect CEM cell against the cytopathogenicity of HIV by 50%.

TABLE 4. Inhibitory Effects [EC_{50} (μg/ml)] of Compounds **18**, **19**, and **22** on the Proliferation of Murine Leukemia Cells (L1210/0), Murine Mammary Carcinoma Cells (FM3A) and Human T-lymphocyte Cells (Molt4/C8), CEM/0). S.D. values provided.

CELL TYPE	18	19	22
L1210/0	>100	9.71 ± 2.15	21.5 ± 5.7
FM3A/0	>100	19.2 ± 16.2	16.5 ± 12.6
Molt4/C8	>100	43.5 ± 6.7	27.7 ± 24.9
CEM/0	>100	25.9 ± 12.1	77.3 ± 21.2

nucleoside analogs to the carboxy terminus of the siderophore, but the chemistry provides access to a whole new class of nucleoside analogs. Early test results indicate diverse biological potential, including anticancer and antiviral activity, for these novel carbocyclic nucleosides.

Experimental

L-Alanylcarbocyclic azanoraristeromycin (**19**) and its Boc-protected form (**18**) have been described earlier.[6]

N$^{\alpha}$-Cbz-tri-*N*$^{\delta}$-hydroxy-*N*$^{\delta}$-acetyl-L-ornithine-L-alanylcarbocyclic azanoraristeromycin (**21**). A solution of Boc-protected nucleoside **18** (68 mg, 0.16 mmol)[6] in anhydrous CH_2Cl_2/TFA (1:1, 3 mL) was stirred at 0 °C to 25 °C until complete consumption of the starting material was evident (TLC, 30 min). The volatile components were removed by rotary evaporation, and the residue was redissolved in 1 mL of anhydrous DMF under nitrogen. Triethylamine (68 μL, 0.48 mmol) was added to the solution, followed by tripeptide active ester obtained from overnight stirring of tripeptide acid **20**[4] (150 mg, 0.14 mmol), DCC (35 mg, 0.16 mmol), and *N*-hydroxysuccinimide (19 mg, 0.16 mmol) in dry THF at room temperature. The resulting reaction mixture was stirred at room temperature for 18 h, concentrated, and

the residue was rediluted with EtOAc. The organic layer was separated, washed with H_2O, brine, dried with anhydrous Na_2SO_4, filtered and concentrated. Flash silica gel column chromatography of the residue eluting with MeOH-$CHCl_3$ (1:20) provided fully protected conjugate **21** (120 mg, 69%) as a colorless gum: R_f = 0.35 (CH_3OH:$CHCl_3$; 3:17); HPLC RT = 14.02 min (silica gel, CH_2Cl_2:*i*PrOH; 80:20, flow rate = 0.5 mL/min); IR (KBr) 3340, 2980, 1690, 1640, 1368, 1165 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ (ppm): 8.30 (s, 1H), 8.20 (s, 1H), 7.38-7.22 (m, 20H), 5.06 (d, *J* = 13.0 Hz, 1H), 5.02 (d, *J* = 13.0 Hz, 1H), 4.81 (m, 6H), 4.61 (dd, *J* = 8.5, 5.0 Hz, 1H), 4.32 (m, 3H), 4.17 (m, 1H), 4.13 (m, 1H), 4.00 (m, 1H), 3.74-3.54 (m, 6H), 2.80 (m, 1H), 2.05 (m, 1H), 2.03 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.80-1.58 (m, 12H), 1.36 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 175.1, 174.3, 174.1, 173.7, 158.6, 157.4, 153.5, 150.2, 142.7, 138.1, 135.9, 130.7, 130.6, 130.1, 129.7, 129.5, 129.0, 128.9, 128.8, 121.0, 77.1, 76.8, 76.6, 67.7, 61.3, 56.3, 54.8, 54.7, 54.6, 50.9, 45.3, 33.3, 30.1, 29.9, 29.8, 24.6, 24.3, 20.5, 18.0; MS (FAB, *m/e*, rel. int.) 1264 [$(M+Na)^+$, 22], 1243 [$(M+H)^+$, 100], 1094 (10); HRMS (FAB, *m/e*) calcd. for $C_{63}H_{80}N_{13}O_{14}$ $[M+H]^+$ 1242.5948; found: 1242.5950.

Tri-N^{δ}-Hydroxy-N^{δ}-acetyl-L-ornithine-L-alanylcarbocyclic azanoraristeromycin (**22**). To a solution of protected conjugate **21** (20 mg, 0.016 mmol) in MeOH (3 mL, spectra grade) was added AcOH (5 μL, 0.08 mmol), followed by 10% Pd-C (5 mg, 25% w/w). The resulting suspension was stirred under hydrogen at atmospheric pressure for 5 h. The catalyst was removed by filtration through a short pad of celite, and the filtrate was concentrated to afford crude conjugate **22**. Purification by flash column chromatography (C_{18}-reverse phase silica gel, CH_3OH: H_2O; 45:55) gave 12 mg (89% yield) of the desired product (**22**) which was homogeneous by HPLC RT = 9.55 min (C_{18}-reverse phase silica gel, CH_3OH: H_2O; 45:55, 0.5 mL/min); TLC R_f = 0.10 ($CHCl_3$:CH_3OH: H_2O; 65:25:1); IR (KBr) 3345, 3165, 2960, 2900, 1680, 1640, 1620, 1340, 1120 cm^{-1}; ^1H NMR (600 MHz, CD_3OD) δ (ppm): 8.31 (s, 1H), 8.18 (s, 1H), 4.82 (m, 2H), 4.61 (dd, *J* = 8.4, 4.8 Hz, 1H), 4.37–4.27 (m, 3H), 4.19 (dt, *J* = 7.2, 3.0 Hz, 1H), 4.00 (m, 1H), 3.69–3.46 (m, 6H), 2.88 (ddd, *J* = 14.4, 10.2, 8.4 Hz, 1H), 2.12 (m, 1H), 2.09 (s, 3H), 2.08 (s, 6H), 2.06 (s, 3H), 1.80–1.60 (m, 12H), 1.41 (d, *J* = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ (ppm): 176.0, 174.4, 173.9, 174.2, 173.8, 173.7, 173.0, 157.5, 153.6, 150.1, 143.0, 121.1, 76.9, 76.7, 61.6, 61.5, 55.0, 54.8, 54.6, 54.5, 51.0, 48.3, 48.2, 33.1, 32.4, 30.0, 24.3, 24.1, 23.8, 23.7, 20.3, 20.2, 18.1; MS (FAB, *m/e*, rel. int.) 860 [$(M+Na)^+$, 1], 838 [$(M+H)^+$, 3], 307 (13), 154 (100); HRMS (FAB, *m/e*) calcd. for $C_{34}H_{56}N_{13}O_{12}$ [$(M+H)^+$]: 838.4171; found: 838.4182.

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