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Synthesis of Chemoreversible Prodrugs of *ara*-C with Variable Time-Release Profiles. Biological Evaluation of Their Apoptotic Activity

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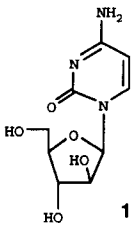
Abstract—N^4-Dipeptidyl slow-release forms of the anticancer drug *ara*-C were prepared by acylation of the lithiated nucleotide with 4,4-dialkyloxazolinones. An azapeptide prodrug of *ara*-C was obtained by condensation of an amino acid hydrazide with an activated nucleotide urea. The use of unnatural amino acid residues at N^4 prevented nonspecific proteolytic cleavage in biological medium. *Ara*-C prodrugs **10**, **15**, **17**, and **19** released active drug with half-lives from a few minutes to several days, respectively. Activation via intramolecular N^4-deacylation did not require enzymatic intervention but was strictly dependent on the structure of the peptide chain. The prodrugs **10**, **15**, and **17** produced similar growth inhibition as *ara*-C in cultured murine leukemia cells while the azapeptide prodrug **19** was less potent reflecting the slow release of active drug with this compound. All four prodrugs retained the ability to induce apoptosis in human HL-60 leukemia cells with kinetics dictated by the rate of intramolecular N^4-deacylation. This the first demonstration for the control of apoptotic cell death by the modulation of drug release from prodrugs. Copyright © 1996 Elsevier Science Ltd

Introduction

Ara-C (**1**, cytosine arabinose, cytarabine, 1-(β-D-arabino-furanosyl)cytosine) is a pyrimidine nucleotide analogue and one of the most effective anticancer drugs for the treatment of acute myelogenous leukemia.[1–3]



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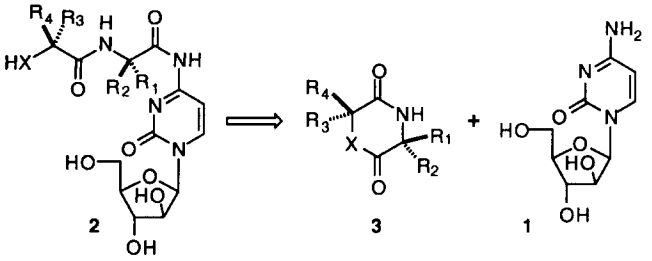
Ara-C is less effective, however, in the treatment of other leukemias and solid tumors. The lack of activity against these types of malignancies is thought to be a result of the pharmacokinetic and pharmacodynamic properties of this agent. Specifically, upon administration, *ara*-C enters cells through a facilitated diffusion process and is converted to the triphosphate derivative (and active metabolite) *ara*-CTP.[4] *Ara*-CTP is a competitive inhibitor of DNA polymerase α and is incorporated into replicating DNA as a function of both concentration and duration of exposure to *ara*-C.[5] The major impediments to a broad use of *ara*-C include the rapid metabolism of the drug in plasma to its inactive metabolite *ara*-U by the enzyme deoxycytidine deaminase and its cell cycle (S-phase) specificity.[6] Consequently, *ara*-C is administered by continuous iv infusion or frequent administration of high doses that are sometimes associated with significant untoward effects.[6] To generally improve the pharmacological profile of *ara*-C, derivatives and prodrugs of this nucleotide have been synthesized.[7] In this article, we report the preparation of dipeptidyl prodrugs of *ara*-C with a wide range of biological half-lives that do not require the intervention of external agents for activation. These chemoreversible[8,9] prodrugs induce apoptosis, a form of physiological cell death, in the human promyelocytic leukemia HL-60 cell line, with a time–activity profile that closely correlates to the release of *ara*-C. This is the first example for the control of apoptotic cell death by the modulation of drug release from prodrugs.

Synthesis of Peptidyl Prodrugs of *ara*-C

Peptides are readily available and relatively easy to modify. They are therefore ideal candidates for the preparation of prodrugs. However, fast degradation of peptides by a variety of peptidases restricts the use of peptidyl derivatives of active drugs. Peptides incorporating nonproteinogenic amino acids have significantly increased stabilities toward proteolytic enzymes. The rate of cleavage of the amide bond in peptides with α,α-disubstituted amino acids is significantly reduced.[10] Therefore, we considered the acylation of *ara*-C with short peptides containing α,α-dialkyl amino acids such as 2-aminoisobutyric acid (Aib) an attractive way for the evaluation of peptide–nucleoside conjugates such as **2** as reversible prodrugs (Scheme 1).[8]



Scheme 1.

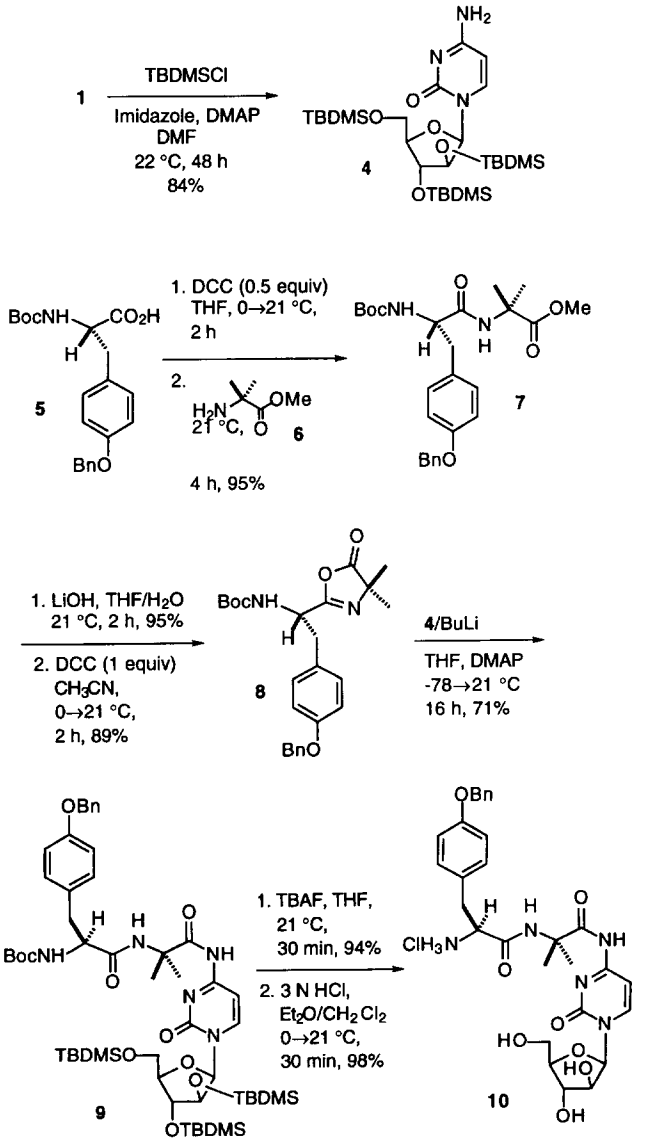
N^4-Acylation of *ara*-C circumvents the deamination of the drug by cytidine deaminase.[11] Also, as a consequence of the *gem*-dialkyl effect,[12] the presence of α,α-disubstituted amino acids significantly facilitates intramolecular cyclization reactions. This characteristic property can be used for the selective cleavage of the amide bond between peptide and nucleoside. Thus, N^4-dipeptidyl derivatives **2** were considered to be practical prodrugs with the potential of releasing the active drug by an intramolecular cyclization process to the six-membered heterocycles **3**.

Peptide bond formation at the N^4-position of *ara*-C is complicated by the intrinsically low nucleophilicity of this amino function and the presence of three arabinose hydroxyl groups that contribute to the low solubility of the compound in common organic solvents. Therefore, trisilylated nucleoside **4** was first prepared by treatment of *ara*-C with 4.5 equiv of TBDMSCl and imidazole in the presence of 50 mol% of DMAP (Scheme 2). Subsequent amide bond formation between Aib and **4** was accomplished by condensation of the lithium salt of **4** with the oxazolinone **8**, which was prepared by acylation of Aib-methyl ester **6** with the symmetrical anhydride of Boc-L-Tyr(OBn)-OH (**5**), hydrolysis and cyclodehydration with 1 equiv of DCC in acetonitrile.[8,13] After *O*-desilylation and acidic cleavage of the Boc-carbamate, the desired dipeptidyl nucleotide **10** was isolated in 65% overall yield from **8**. This route proved to be an efficient and general way to N^4-Aib derivatives of *ara*-C.

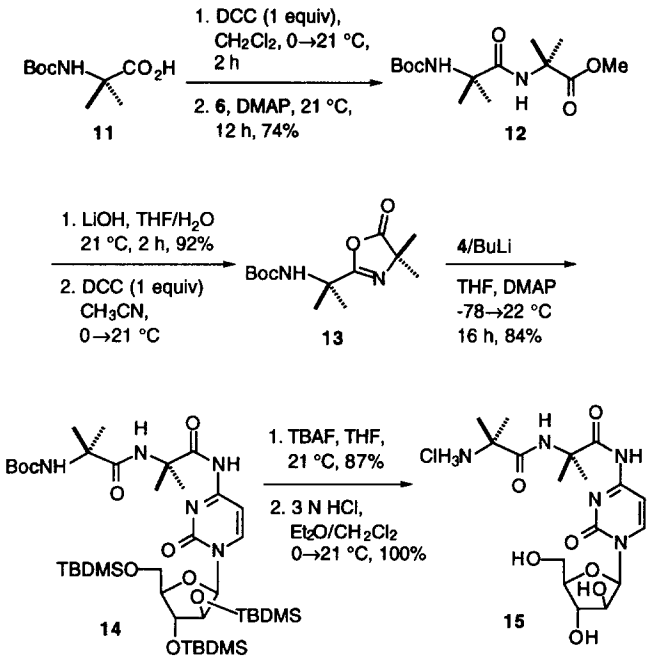
For the preparation of the *bis*-Aib prodrug of *ara*-C, dipeptide **12** was synthesized in 74% yield from *N*-Boc protected aminoisobutyric acid and ester **6** via the in situ prepared oxazolinone of **11** (Scheme 3). Ester cleavage and cyclodehydration led to oxazolinone **13**, which smoothly acylated the lithium salt of silylated *ara*-C in 84% yield. In an analogous fashion and in similarly high efficiency, the glycolate-Aib prodrug **17** was synthesized (Scheme 4). Protection of the glycolate hydroxyl function as a TBDMS-ether allowed the simultanous removal of all protective groups with TBAF after acylation of the nucleoside.[14]

Besides the α-alkylation of amino acid residues, modifications of the peptide bond and replacements of backbone carbon atoms with heteroatoms can also be used to increase hydrolytic stability in biological media.[15] Azapeptides, for example, are quite resistant to enzymatic or acidic cleavage, while they often retain the biological activity of the parent peptides.[16] Little is known about the tendency for cyclization of peptides containing α-aza amino acid residues, but, in general, these peptidomimetics appear to prefer extended conformations in solution that would be considered unfavorable for the formation of small- or medium-sized heterocycles.[16a] Therefore, we envisioned the azapeptide prodrug **19** to be a particulary stable and slow-releasing precursor of *ara*-C in a biological environment.

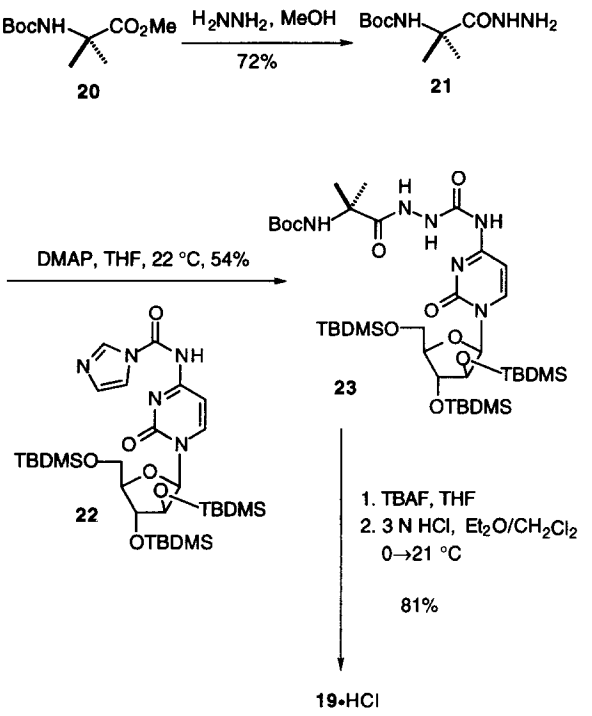
An initial approach to synthesize **19** by ring-opening of the aza-oxazolinone **18** failed due to the low electrophilicity of this heterocycle[17] and the insufficient nucleophilicity even of *N*-lithiated **4** (Scheme 5). In contrast, Boc-Aib-hydrazide **21** was smoothly acylated with imidazolide **22**, prepared by in situ condensation of lithiated **4** with *N,N'*-carbonyldiimidazole (CDI, Scheme 6). The desired hydrochloride of **19** was isolated in 44% overall yield after removal of protective groups from **23**.



Scheme 2.



Scheme 3.

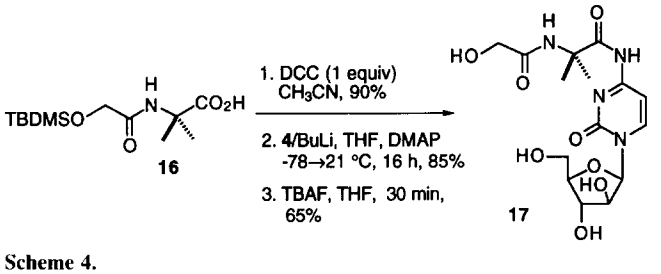


Scheme 6.

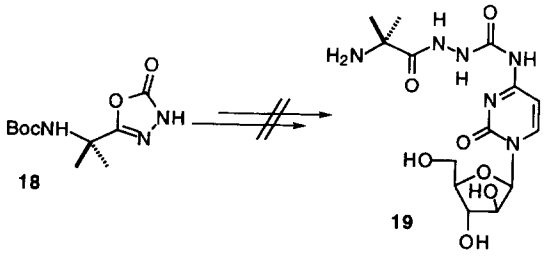
The strategies demonstrated here for the preparation of peptidyl prodrugs of the pyrimidine nucleotide *ara*-C can also be applied for the preparation of analogous derivatives of purine nucleotides.[18,19] They represent a general synthetic protocol for the reversible *N*-protection of nucleotide drugs.

NMR and HPLC Studies of Drug Release

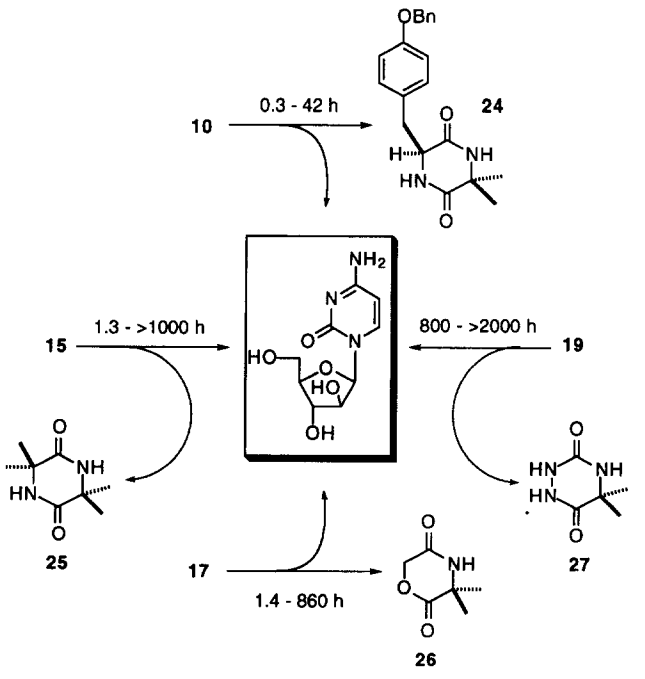
The stability of the *ara*-C derivatives **10**, **15**, **17**, and **19** in various reaction media was investigated by NMR and HPLC analyses. The rate of release of *ara*-C was dependent upon the peptide structure and the pH of the medium. In the NMR studies, the samples were dissolved in CD_3OD or D_2O and sodium acetate or acetic acid were added to the solution to control the pH. The disappearance of characteristic signals of the prodrugs was accompanied by the appearance of signals of *ara*-C and the corresponding heterocycles **24**–**27** (Scheme 7). Kinetic analysis showed that the drug was formed following a first-order rate law (Fig. 1–3). Relevant half-lives are summarized in Table 1.



Scheme 4.



Scheme 5.



Scheme 7.

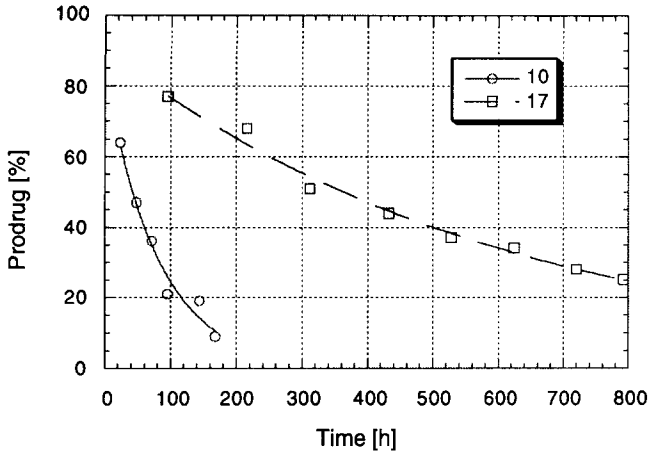


Figure 1. Release of *ara*-C from **10** and **17** in MeOH-d_4 at 22 °C monitored by ^{1}H NMR (exponential curve fit).

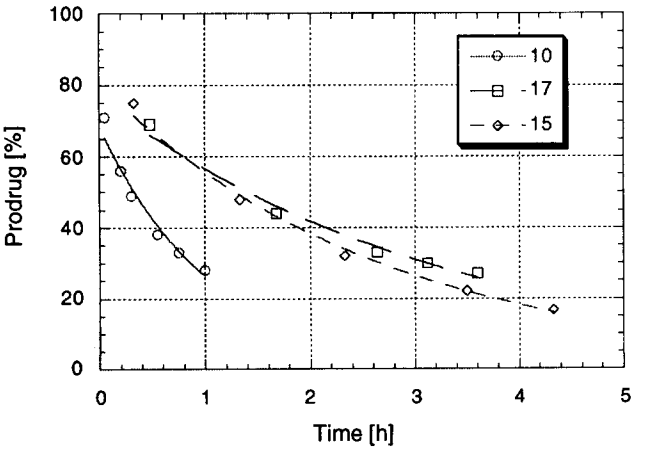


Figure 2. Release of *ara*-C from **10**, **15**, and **17** in the presence of NaOAc in MeOH-d_4 at 22 °C monitored by ^{1}H NMR (exponential curve fit).

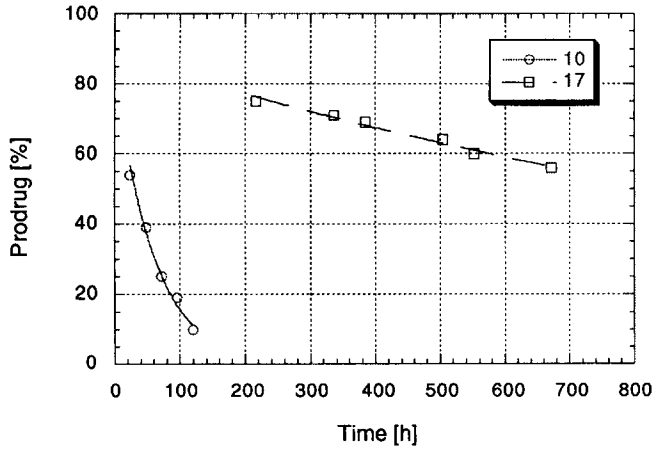
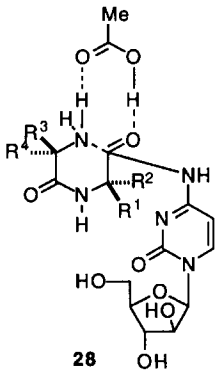


Figure 3. Release of *ara*-C from **10** and **17** in the presence of HOAc in MeOH-d_4 at 22 °C monitored by ^{1}H NMR (exponential curve fit).

Tyrosyl-Aib prodrug **10** was found to have shortest half-life in CD_3OD among all the derivatives, and the process of drug-release was additionally greatly enhanced in the presence of NaOAc (Table 1, entry 2). This could be due to the increase in the concentration of free amine in the reaction medium. Alternatively, acetate can also directly participate and assist in the ring closure of **28** (Scheme 8). In the presence of HOAc the intramolecular cyclization was only slighly accelerated, or, with glycolate **17**, actually decelerated. Clearly, the rate of cyclization of amines **10** and **15** is dependent on the concentration of free amine versus ammonium salt and should therefore be accelerated in neutral or basic environment. However, the presence of a bidentate proton acceptor/donor moiety appears to be especially advantageous for the intramolecular carbonyl addition. The addition of triethylamine to the reaction media, in contrast, did not lead to any significant rate increases for drug release. In addition, the half-life of **10** in CD_3OD in the presence of NaCl was found to be the same as in the absence of NaCl. This indicates that acetate, not Na^+ ions exerted an enhancement on prodrug cyclization. Upon change of the reaction medium from CD_3OD to D_2O, **10** was found to cyclize ca. 50% faster (entry 4).

The hydroxyl compound **17** cyclized slower than **10** due to the lower nucleophilicity of the hydroxyl versus an amino group. However, compound **17** cyclized significantly more slowly in acidic medium than in neutral one. Aib-Aib derivative **15** cyclized readily in the presence of NaOAc. However, its half-life was



Scheme 8.

Table 1. Half-lives (h) for *ara*-C release of prodrugs **10**, **15**, **17**, and **19** determined by NMR (entries 1–4) and HPLC (entries 5–6) analyses

		$t_{1/2}$			
Entry	Conditions	**10**	**15**	**17**	**19**
1	CD_3OD, 22 °C	42	>1000	360	>2000
2	CD_3OD, 22 °C, NaOAc	0.3	1.3	1.4	800
3	CD_3OD, 22 °C, HOAc	31	>1000	860	>2000
4	D_2O, 22 °C	21	ND	ND	ND
5	Bovine plasma, pH 7.4, 22 °C	0.4	0.5	0.4	>10
6	Human plasma, pH 7.4, 22 °C	0.3	0.4	0.3	>10

extremely long in the absence of NaOAc or with excess of HOAc; the cyclization process was still incomplete after 1 month. This could be the result of unfavorable ground state conformations or steric interactions of the hindered amine in the transition state of cyclization. Probably similarly due to conformational effects, the aza peptide derivative **19** did not undergo cyclization to release the drug in neutral or acid conditions, and cyclization was extremely slow even in the presence of NaOAc.

The stability of these compounds in bovine and human plasma was determined by HPLC analysis. Compounds **10**, **15**, and **17** were found to cyclize readily in bovine or human plasma (Table 1, entries 5 and 6). In contrast, compound **19** showed good stability in plasma. Only traces of *ara*-C were detected after 24 h.

Biological Studies of the Effect of *ara*-C Prodrugs

The overall antiproliferation activity of *ara*-C prodrugs in an L-1210 cell line growth inhibition assay correlated closely with our NMR studies. IC_{50} and IC_{90} values of **10**, **15**, and **17** were essentially identical to the *ara*-C control, whereas azapeptide **19** was ca. 500 times less toxic in this assay (Table 2).

Since conventional proliferation assays do not readily distinguish between cytostasis and cytotoxicity, we examined the ability of the prodrugs to kill human tumor cells. Apoptosis is the mode of cell death that occurs in drug-induced tumor regression and is now recognized to be the primary effect of most, if not all, chemotherapeutic agents including *ara*-C.[20,21] Altered nuclear morphology is the preeminent feature of apoptosis and includes extensive DNA condensation and margination.[20,22] Consequently, we evaluated the *ara*-C prodrugs **10**, **15**, **17**, and **19** for their ability to induce apoptosis in the human promyelocytic leukemia HL-60 cell line, as these cells are known to undergo apoptosis following exposure to *ara*-C.[23] In addition to morphology, the ability of these prodrugs to cause internucleosomal DNA fragmentation, a characteristic feature of apoptosis in HL-60 cells,[24] was investigated. The results are shown in Figures 4–9.

As indicated in Figure 4, a 24 h exposure to 10 mM *ara*-C resulted in the appearance of obvious apoptotic bodies in HL-60 cells (Panel B). Analogues **10**, **15**, and **17** produced similar results (panels C–E), but not analogue **19** (panel F). No significant difference in the

Table 2.[25] L-1210 cell growth inhibition (ng/mL) for **10**, **15**, **17**, **19**, and *ara*-C

Entry	Compd	IC_{50}	IC_{90}
1	**10**	1.3	5
2	**15**	1.1	3.4
3	**17**	1.0	3.4
4	**19**	600	1400
5	*ara*-C (**1**)	1.5	4.3

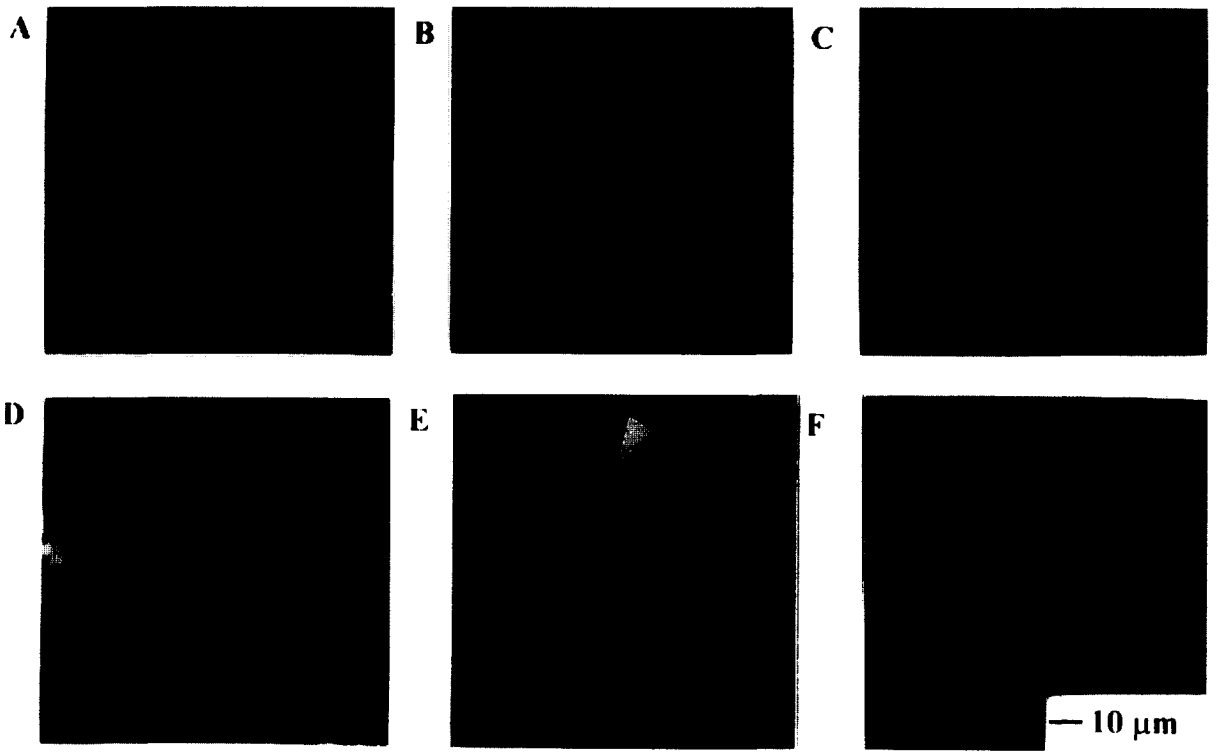


Figure 4. Induction of morphological apoptosis by *ara*-C analogues. Exponentially growing cells were treated with 10 μM of each of the *ara*-C analogues **10**, **15**, **17**, and **19** or with an equiv amount of DMSO (0.1%) at 0 h. After 24 h of continuous drug exposure, cells were harvested, stained with 20 μg/mL Hoechst 33342 in Puck's Saline A, and visualized using fluorescence microscopy. panel A: DMSO controls; panel B: *Ara*-C; panel C: **17**; panel D: **15**; panel E: **10**; panel F: **19**. These photographs are representative fields for four individual experiments. The arrow head indicates an apoptotic cell.

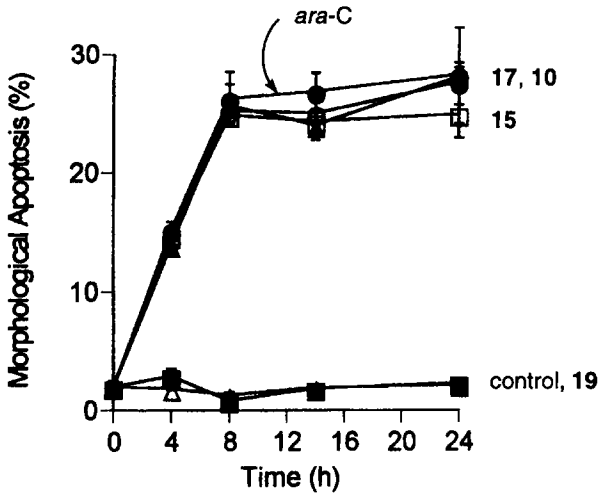


Figure 5. Time-dependent induction of apoptosis by *ara*-C analogues. Exponentially growing cells were treated with 10 μM of each of the *ara*-C analogues or with an equiv amount of DMSO (0.1%) at 0 h. At indicated times, aliquots of cells were stained with 20 μg/mL Hoechst 33342 in Puck's Saline A, and visualized using fluorescence microscopy. The percentage of cells exhibiting apoptotic morphology was determined by examination of 100–200 cells. The symbols ■, ●, ▲, □, ○, and △ represent DMSO controls, *ara*-C, **17**, **15**, **10**, and **19**, respectively. The symbols are the mean values of triplicate determinations from a representative experiment; error bars are SE.

efficacy of the active compounds was noted with a maximum of approximately 25% of cells treated with *ara*-C, **10**, **15**, and **17** exhibiting a frank apoptotic morphology (Fig. 5). The formation of apoptotic bodies occurred with a maximal half time of 4 h and there was no noticeable difference in the kinetics of apoptosis among *ara*-C, **10**, **15**, and **17**. The *ara*-C analogue **19** produced no obvious increase in apoptosis during the 24 h observation period.

One hallmark of apoptosis is ordered DNA fragmentation; HL-60 cells treated with 10 mM *ara*-C for 4 h displayed internucleosomal 180–200 base pair DNA fragments (Fig. 6). Similar DNA ladders were seen with *ara*-C analogues **17**, **15**, and **10**, but not **19**. Due to the relatively long in vitro half life of analogue **19**, we preincubated prodrug **19** in serum-free medium for 15 days. Cells were then incubated with the resulting medium and morphological apoptosis was assessed. Preincubation for 15 days at 37°C in serum-free medium did not affect the efficacy of *ara*-C at several concentrations tested (Fig. 7). As anticipated, analogue **19** had little activity when freshly administered, but preincubation for 15 days enhanced the apoptotic activity to levels that were slightly less than the parent compound (Fig. 8). Cells exposed for 4 h to 5–20 mM preincubated *ara*-C also displayed DNA ladders (Fig. 9). DNA fragmentation was also seen with 10 and 20 μM preincubated **19**.

Conclusions

The intramolecular cyclization of dipeptyl derivatives of *ara*-C to heterocycles and *ara*-C provides a versatile time-dependent delivery method for the modified nucleotide. No enzymatic interaction is required in the prodrug cleavage, and a considerable increase of the in vitro stability of the prodrugs can be achieved by appropriate structural modifications of the peptidyl moiety. Thus, an adoption to specific delivery requirements appears possible. Our findings indicate that the use of nonproteinogenic peptidyl derivatives of active but otherwise poorly bioavailable drugs offers an attractive alternative to standard prodrug protocols. This approach is principally useful not only for *ara*-C, but also for a large variety of antitumor and antiviral nucleosides containing amino functions. Azapeptide-based prodrugs deserve particular attention due to their extraordinary stability to intra- and intermolecular solvolysis.

Ara-C, like many other antineoplastic agents, can kill cells by either a necrotic or an apoptotic process depending at least partially on drug concentrations.[22] Lower concentrations are generally associated with apoptotic death and, thus, this form of cell death is probably the most important pharmacological action. In our HL-60-based assays, we found that prodrugs retained the concentration-dependent apoptotic activity

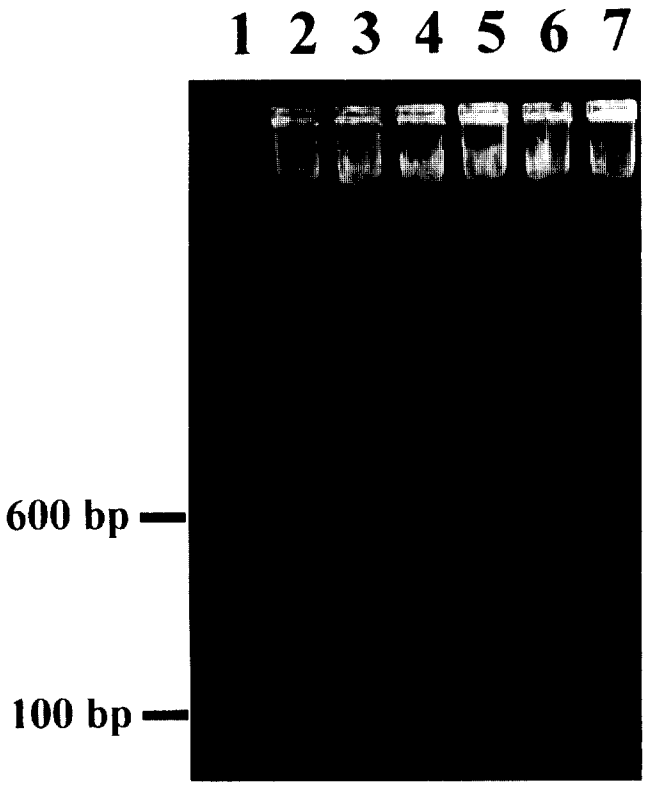


Figure 6. Effects of *ara*-C analogues on generation of internucleosomal DNA fragmentation. Exponentially growing cells were treated with 10 μM of each of the *ara*-C prodrugs or with an equivalent amount of DMSO (0.1%). After 4 h, cells were harvested and 1×10^6 cells were digested with proteinase K and RNase A, and the digests were electrophoresed in each lane of a 1.8% agarose gel using TPE buffer. The gel was visualized and photographed using an UV transilluminator after staining with ethidium bromide (1 μg/mL in water). Lane 1: 100 bp DNA ladder; lane 2: DMSO control; lane 3: *ara*-C; lane 4: **17**; lane 5: **15**; lane 6: **10**; lane 7: **19**. This figure is representative of five independent experiments.

of the parent drug. Therefore, these studies represent the first example of potent apoptotic activity by a prodrug. Interestingly, we found that the hydrazide peptide isostere **19** induces programmed cell death only after prolonged (ca. 15 days) incubation at 37 °C. This is in accord with the NMR and plasma study models and the resulting half-life characterization of these prodrugs (Table 1). The lack of cytotoxicity by analogue **19** that has not been preincubated is encouraging as it reinforces the importance of an unencumbered N^4 for biological activity. It seems likely, therefore, that N^4-dipeptidyl derivatives can not be transported into cells and used as inhibitors of DNA polymerase or incorporated in DNA without first undergoing requisite chemoreversible activation.

Considerable efforts have been directed at an enhancement of the therapeutic efficacy of *ara*-C, because it is one of the major drugs successfully used in newly diagnosed acute myelogenous leukemia. During the last decade there have been numerous clinical trials to test new treatment schedules and doses including attempts to use high dose *ara*-C; these studies have not markedly improved therapeutic outcome. Thus, there continues to be a great need for improved methods to deliver pharmacological active *ara*-C. Since *ara*-C is at least partially S-phase specific, increased duration of drug exposure may be a desirable characteristic to maximize biological activity. Studies to evaluate the in vivo biological potential of **19** and related slow-acting delivery forms of *ara*-C are planned for the near future.

Experimental

General methods

All glassware was dried in an oven at 150 °C prior to use. THF and dioxane were dried by distillation over Na/benzophenone under a nitrogen atmosphere. Dry CH_2Cl_2, DMF, and CH_3CN were obtained by distillation from CaH_2.

^{1}H NMR studies of prodrug half-lives in CD_3OD and H_2O

NMR studies of the cyclization process were performed on a 4 mg/mL solution of prodrug in CD_3OD or D_2O. Sodium acetate (2 mg) or acetic acid (2 μL) was added neat to the samples. The spectra were collected in appropriate time-intervals and the ratio of prodrug/drug was determined by comparing the integration of the H-C(6) on the nucleoside of prodrug and *ara*-C. The half-life was the time required

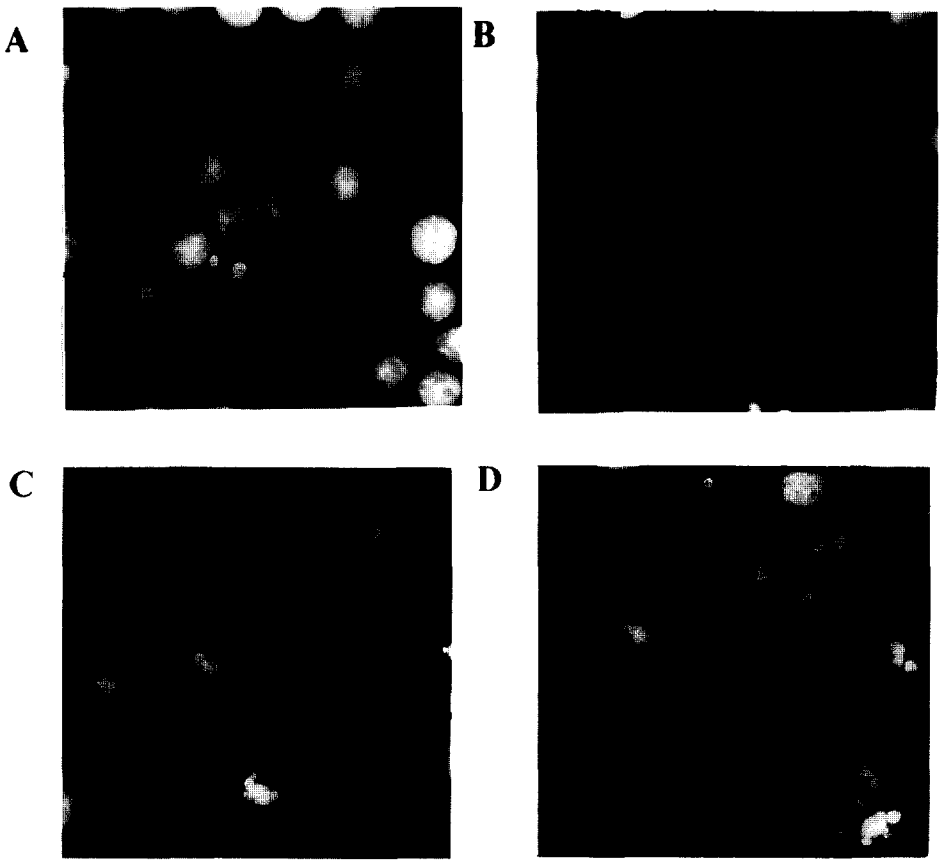


Figure 7. Induction of morphological apoptosis using 19 after a 15 day preincubation. DMSO (panel A) or freshly resuspended **19** (panel B) were added to exponentially growing cells. Alternatively, *ara*-C (panel C) or **19** (panel D) were resuspended in DMSO and the compounds were preincubated in serum-free medium for 15 days at 37 °C prior to addition to cells. In either case, cells were harvested, stained with 20 μg/mL Hoechst 33342 in Puck's Saline A, and visualized using fluorescence microscopy 24 h after drug (10 μM) addition. These photographs are representative fields that were consistent with duplicate experiments.

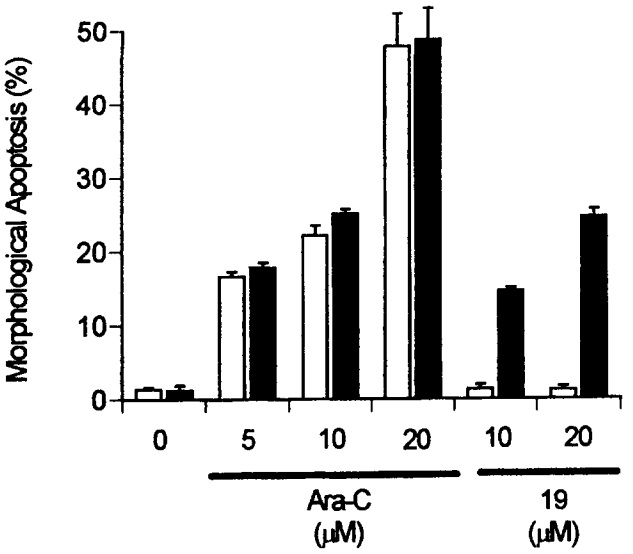


Figure 8. Concentration-dependent induction of apoptosis by preincubated *ara*-C and **19**. *Ara*-C or compound **19** were either freshly resuspended in DMSO and added to cells (open bars) or these compounds were preincubated in serum-free medium for 15 days at 37 °C prior to addition to cells (filled bars). In either case, cells were harvested, stained with 20 μg/mL Hoechst 33342 in Puck's Saline A, and visualized using fluorescence microscopy 24 h after drug addition. The percentage of cells exhibiting apoptotic morphology was determined by examination of 100–200 cells. The bars represent the mean value of triplicate determinations; error bars are SE.

for 50% conversion of prodrug to drug and was calculated via first-order kinetics using Cricket Graph software.

HPLC studies of prodrug half-lives in plasma

Studies of the cyclization process in plasma were performed on a 4 mg/mL solution of prodrug in plasma. At appropriate time-intervals, aliquots (25 μL) were removed and 0.20 mL of CH_3CN was added to precipitate the protein. After centrifugation, clear supernatent liquid (25 μL) was injected directly into the HPLC port. The HPLC analysis was performed with a C-18 reverse-phase column using a H_2O:CH_3CN (1:1) eluent and the UV detector was set at 250 nm. The relative concentration of *ara*-C was determined by the relative peak-areas. The half-life was the time required for 50% conversion of prodrug to drug.

Cell culture

Murine L1210 leukemia cells and human promyelocytic leukemia HL-60 cells were grown in suspension using Dulbecco's medium (Gibco/BRL, Iscoue's modification used for HL-60 cells) supplemented with 10% fetal bovine serum (FBS, HyClone), 2 mM L-glutamine, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin (Gibco/BRL). Cells were grown in a humidified incubator at 37 °C under an atmosphere of 95% air and 5% CO_2. Cells were routinely passaged at a 1:5 ratio and found to be free of mycoplasma contamination.

Drug treatments

Ara-C (Sigma) or the specified prodrugs were dissolved in DMSO and added to cells (ca. 3×10^5 cells/mL) at indicated concentrations in 0.1% DMSO (final concentration); controls received an equivalent amount of DMSO. In several experiments, the hydrazide derivative **19** and *ara*-C were incubated in medium containing all of the above supplements except for FBS for 15 days at 37 °C. After this preincubation period, the drug-containing medium was mixed with an equal volume of medium containing the above supplements, 20% FBS, and 6×10^5 cells/mL. Thus, the final concentrations of FBS and cells in all experiments herein were 10% and 3×10^5, respectively.

Cell growth assays[25]

The growth-inhibiting effects of *ara*-C and prodrugs were examined in L1210 cells using various concentrations and continuous exposure. Cell numbers were determined 72 h after initial drug exposure. The values in Table 2 represent the mean values of triplicate determinations.

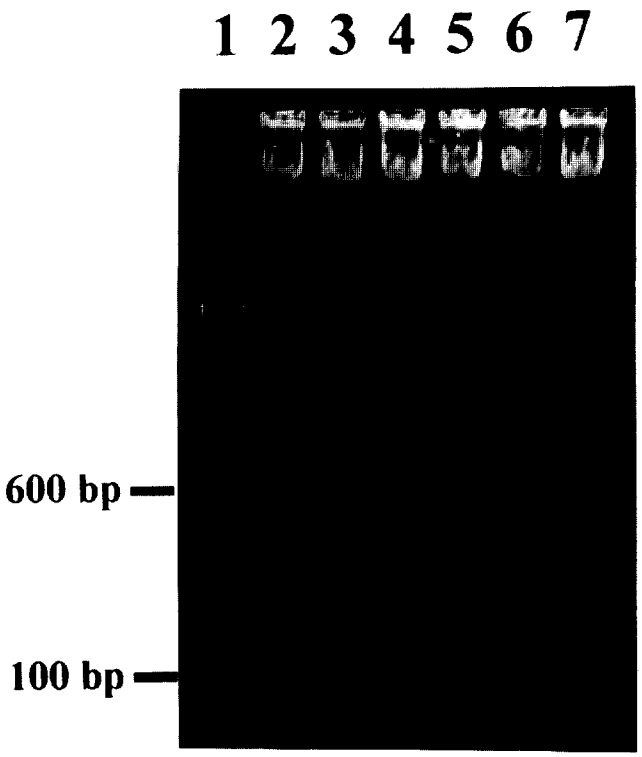


Figure 9. Effects of preincubation of *ara*-C or **19** on the generation of internucleosomal DNA fragmentation. *Ara*-C or compound **19** were preincubated in serum-free medium for 15 days at 37 °C prior to addition to exponentially growing cells. Four hours after drug addition, cells were harvested and 1×10^6 cells were digested with proteinase K and RNase A, and the digests were electrophoresed in each lane of a 1.8% agarose gel using TPE buffer. The gel was visualized and photographed using an UV transilluminator after staining with ethidium bromide (1 μg/mL in water). Lane 1: 100 bp DNA ladder; lane 2: DMSO control; lane 3: 5 μM *ara*-C; lane 4: 10 μM *ara*-C; lane 5: 20 μM *ara*-C; lane 6: 10 μM **19**; lane 7: 20 μM **19**. This figure is representative of duplicate experiments.

Morphological examination of cells

Aliquots of cells were centrifuged at $1000 \times g$ for 5 min and resuspended in Puck's Saline A (Gibco/BRL) containing 20 μg/mL Hoechst 33342 (Molecular Probes) for 15–30 min at room temperature. The nuclei of the stained cells were visualized and photographed using a Nikon Microphot-PX photomicroscope with an epifluorescence attachment. The DNA from normal cells exhibited a fluorescence pattern that was wispy and mottled in appearance. In contrast, the fluorescence pattern from apoptotic cells revealed highly condensed DNA that was divided into numerous individual bodies within a cell or localized as a crescentic cap along the nuclear membrane. The percentage of apoptotic cells was determined by examination of 100–200 cells.

Internucleosomal DNA fragmentation

The integrity of DNA was evaluated as previously described.[26] Briefly, following indicated drug treatments, aliquots of cells (1×10^6) were pelleted by centrifugation at $1000 \times g$ for 5 min, washed with PBS, solubilized with 20 μL of lysis buffer (10 mM EDTA, 0.5% sarkosyl, 1 mg/mL proteinase K, 50 mM Tris, pH 8), and incubated at 50 °C for 1 h. After incubation, RNase A (Boehringer Mannheim) was added to a final concentration of 0.33 mg/mL and incubated for an additional hour at 37 °C. The lysate was loaded into dry wells of a 1.8% agarose gel, the wells were sealed with low melting-point agarose, and the DNA was electrophoresed using Tris–phosphate–EDTA (TPE) running buffer. After electrophoresis, the DNA was stained by immersion of the gel in water containing 1 μg/mL ethidium bromide (Sigma). The DNA was visualized and photographed using an ultraviolet transilluminator.

2′,3′,5′-Tri-(*tert*-butyldimethylsilyl)-*ara*-C (4). To a solution of 2.50 g of *ara*-C (**1**, 10.29 mmol) in 55 mL of dry DMF were added 6.98 g of TBDMSCl (46.30 mmol), 3.15 g of imidazole (46.30 mmol), and 630 mg of DMAP (5.14 mmol). The clear solution was stirred at 22 °C and formation of the product and disappearance of the starting material were monitored by TLC (MeOH:$CHCl_3$, 1:9). After 2 days, the reaction mixture was poured into 200 mL of CH_2Cl_2 and extracted with water (3×50 mL). The organic layer was dried (Na_2SO_4), evaporated to dryness, and the residue was chromatographed on SiO_2 (MeOH:$CHCl_3$, 1:19) to give 3.61 g of **4** as a colorless solid and 2.16 g of *tetra*-silylated product (*N*-silylation). The later compound was dissolved in 40 mL of THF and treated with 40 mL of 10% aq NH_4OH soln. After 4 h of stirring at 22 °C, the organic solvent was evaporated and the residue was extracted with CH_2Cl_2 (3×25 mL). The combined organic layers were dried over Na_2SO_4, evaporated to dryness, and the residue was chromatographed on SiO_2 (MeOH:$CHCl_3$, 1:19) to give 1.45 g of **4** (overall yield: 84%): IR ($CHCl_3$) 3365, 3175, 2950, 1674, 1480, 1410, 1263, 1108, 1070, 915, 840, 780 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.55 (d, 1 H, $J = 7.4$ Hz), 6.23 (d, 1 H, $J = 2.8$ Hz), 5.64 (d, 1 H, $J = 7.4$ Hz), 4.20 (d, 1 H, $J = 2.7$ Hz), 4.14 (s, 1 H), 3.98–3.94 (m, 1 H), 3.83–3.72 (m, 2 H), 0.90, 0.89 (2s, 18 H), 0.79 (s, 9 H), 0.10–0.03 (m, 15 H), −0.15 (s, 3 H); ^{13}C NMR ($CDCl_3$): δ 165.2, 155.1, 142.9, 93.1, 87.7, 86.8, 78.4, 76.5, 62.9, 25.8, 25.7, 17.8, −4.7, −5.3, −5.5; MS (EI): *m/z* (relative intensity) 528 ([M-*t*-butyl]$^+$, 25), 396 (14), 168 (100), 147 (20), 115 (20), 89 (20), 73 (90); HRMS (EI): *m/z* calcd for $C_{23}H_{46}N_3O_5Si_3$ (M-*t*-butyl): 528.2745, found: 528.2745.

Boc-Tyr(OBn)-Aib-OMe (7). A solution of 1.50 g of Boc-Tyr(OBn)-OH (**5**, 4.04 mmol) in 10 mL of dry CH_2Cl_2 was treated at 0 °C with 417 mg of DCC (2.02 mmol). The mixture was stirred at 0 °C for 5 min and treated with the solution of 620 mg of Aib-OMe hydrochloride **6** (4.04 mmol) and 490 mg of NMM (1.20 equiv) in 2 mL of dry DMF. After stirring at 22 °C for 12 h, the reaction mixture was extracted with a satd aq soln of NH_4Cl. The organic layer was dried (Na_2SO_4) and evaporated to dryness. The residue was chromatographed on SiO_2 (EtOAc:hexanes, 3:7) to give 900 mg (95%) of **7** as a white solid: IR ($CHCl_3$): 2977, 2916, 1734, 1656, 1509, 1246, 1146, 1020, 690, 665 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.44–7.29 (m, 6 H), 7.15 (d, 2 H, $J = 8.4$ Hz), 6.90 (d, 2 H, $J = 8.5$ Hz), 6.22 (br s, 1 H), 5.05 (s, 2 H), 4.22 (m, 1 H), 3.71 (s, 3 H), 3.04, 2.92 (AB part of ABX, 2 H, $J = 13.7$, 6.1, 7.7 Hz), 1.44, 1.43 (2s, 15 H); ^{13}C NMR ($CDCl_3$): δ 174.3, 170.9, 157.4, 155.3, 136.8, 130.3, 128.9, 128.3, 127.7, 127.1, 114.6, 79.6, 69.6, 55.9, 55.5, 52.2, 37.4, 28.0, 24.5, 24.4; MS (CI): *m/z* (relative intensity) 471 ([M + 1]$^+$, 10), 415 (50), 397 (40), 371 (100), 353 (45), 339 (20), 197 (10), 91 (25), 69 (20).

Boc-Tyr(OBn)-Aib-2′,3′,5′-tri-(*tert*-butyldimethylsilyl)-*ara*-C (9). A solution of 260 mg of **7** (0.55 mmol) and 91.5 mg of LiOH monohydrate (2.1 mmol) in 7.5 mL of THF:H_2O (2:1) was stirred at 22 °C for 3 h. The organic solvent was evaporated and the aqueous solution was extracted with Et_2O (2×10 mL) before it was acidified to pH < 1 with 1 N HCl. The mixture was extracted again with Et_2O (3×10 mL) and the combined organic extracts were dried (Na_2SO_4) and evaporated to yield 240 mg (95%) of Boc-Tyr(OBn)-Aib-OH as a colorless solid, which was used for the next reaction without further purification.

A solution of 200 mg (0.44 mmol) of Boc-Tyr(OBn)-Aib-OH in 10 mL of dry CH_3CN was treated at 0 °C with 95 mg (0.46 mmol) of DCC and stirred for 2 h. After filtration through florisil, the solution was evaporated to dryness. The resulting crude **8** (175 mg, 89%) was dissolved in 2 mL of dry THF and added at −78 °C to a mixture of 130 mg (0.22 mmol) of **4** and 165 μM (0.25 mmol) of a 1.5 M solution of *n*-BuLi in hexanes in 4 mL of dry THF. After addition of 13 mg (0.10 mmol) of DMAP, the cold bath was removed and the reaction mixture was stirred at 22 °C for 2 h. The solvent was evaporated and 4 mL of H_2O was added. After extraction with CH_2Cl_2 (3×10 mL), the combined organic layers were dried (Na_2SO_4), filtered,

and evaporated to dryness. The residue was purified by column chromatography on SiO_2 (EtOAc:hexanes, 1:1) to give 285 mg (71%) of **9** as a viscous oil: IR (neat): 2928, 2855, 1653, 1507, 1312, 1252, 1103, 837, 777 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.87 (d, 1 H, $J=7.5$ Hz), 7.26–7.44 (m, 7 H), 7.04 (d, 2 H, $J=7.7$ Hz), 6.84 (m, 2 H), 6.20 (d, 1 H, $J=2.8$ Hz), 5.25 (br s, 1 H), 4.99 (s, 2 H), 4.35–4.25 (m, 2 H), 4.16 (s, 1 H), 4.00 (dd, 1 H, $J=6.2$, 8.4 Hz), 3.86–3.71 (m, 2 H), 2.95 (m, 2 H), 1.35–1.45 (m, 15 H), 0.90, 0.88 (2s, 18 H), 0.76 (s, 9 H), 0.10, 0.09, 0.08, 0.07 (4s, 12 H), 0.02 (s, 3 H), −0.33 (s, 3 H); ^{13}C NMR ($CDCl_3$): δ 174.3, 171.6, 162.5, 157.6, 155.5, 155.0, 146.2, 137.0, 130.4, 128.5, 127.8, 127.3, 114.9, 95.3, 88.6, 87.4, 80.0, 78.3, 76.3, 69.8, 62.9, 57.7, 55.7, 37.5, 28.2, 25.8, 25.7, 25.6, 25.0, 24.6, 23.8, 18.2, 17.8, 17.7, −4.7, −5.2, −5.4; FABMS: *m/z* (relative intensity) 1024 ($[M+1]^+$, 30).

Tyr(OBn)-Aib-*ara*-C hydrochloride (10). A solution of 250 mg (0.24 mmol) of **9** in 2 mL of THF was treated with 0.93 mL (0.93 mmol) of a 1 M solution of TBAF in THF stirred for 40 min at 22 °C, evaporated to dryness, and the residue was chromatographed on SiO_2 (MeOH:$CHCl_3$, 1:9) to yield 154 mg (94%) of Boc-Tyr(OBn)-Aib-*ara*-C: IR (neat): 3324 (br), 2980, 2932, 1647, 1489, 1316, 1244, 1171, 1123, 1055, 806 cm^{-1}; ^1H NMR (acetone-d_6, CD_3OD): δ 8.24 (d, 1 H, $J=7.5$ Hz), 7.42–7.27 (m, 6 H), 7.13 (d, 2 H, $J=8.5$ Hz), 6.88 (d, 2 H, $J=8.5$ Hz), 6.19 (d, 1 H, $J=3.8$ Hz), 5.05 (s, 2 H), 4.26–4.19 (m, 2 H), 4.09 (t, 1 H, $J=2.5$ Hz), 4.03–3.99 (m, 1 H), 3.81 (d, 2 H, $J=4.5$ Hz), 2.94, 2.78 (AB part of ABX, 2 H, $J=13.6$, 6.8, 8.1 Hz), 1.40–1.34 (m, 15 H); ^{13}C NMR (acetone-d_6): δ 175.2, 172.8, 163.6, 158.3, 156.5, 156.4, 147.3, 138.3, 131.2, 130.3, 129.1, 128.4, 128.2, 115.3, 96.0, 89.2, 87.2, 79.6, 77.9, 75.8, 70.2, 62.4, 58.3, 56.8, 37.4, 28.4, 25.0, 24.4; FABMS: *m/z* (relative intensity) 682 ($[M+1]^+$, 40).

A solution of 89 mg (0.13 mmol) of Boc-Tyr(OBn)-Aib-*ara*-C in 1.5 mL of CH_2Cl_2 was treated at 0 °C with 1.5 mL of 3 N solution of HCl (gas) in Et_2O. The reaction mixture was stirred at 22 °C for 30 min. The colorless precipitate was filtered to yield 81 mg of **10** (98%): IR (neat): 3343 (br), 2932, 1718, 1609, 1511, 1246, 1121, 1064, 814, 740, 700 cm^{-1}; ^1H NMR (CD_3OD): δ 8.73 (d, 1 H, $J=6.5$ Hz), 7.22–7.42 (m, 8 H), 6.97 (d, 2 H, $J=7.6$ Hz), 6.23 (s, 1 H), 5.07 (s, 2 H), 4.32 (s, 1 H), 4.30–4.20 (m, 1 H), 4.15 and 4.05 (m, 2 H), 3.85–3.80 (m, 2 H), 3.25–3.15 (m, 1 H), 3.10–3.00 (m, 1 H), 1.47 (s, 3 H), 1.35 (s, 3 H); ^{13}C NMR (CD_3OD): δ 176.8, 169.8, 159.6, 153.5, 146.9, 138.5, 131.8, 129.5, 128.8, 128.5, 127.4, 116.4, 95.2, 90.1, 87.9, 77.0, 76.5, 70.8, 62.1, 59.1, 55.7, 37.2, 25.4, 23.4; FABHRMS (MNBA/MeOH): *m/z* 582.269 ($[M+H]^+$, $C_{29}H_{36}N_5O_8$ requires 582.256).

Boc-Aib-Aib-OMe (12). To a solution of 400 mg of Boc-Aib-OH (**11**, 1.97 mmol) in 10 mL of dry CH_2Cl_2 was added 200 mg of Et_3N (1.98 mmol) and 100 mg of DMAP (0.82 mmol). The clear solution was cooled in an ice bath and treated with a solution of 408 mg of DCC (1.98 mmol) in 5 mL of dry CH_2Cl_2. The reaction mixture was stirred at 22 °C for 12 h and the resultant slurry was filtered through florosil. The filtrate was evaporated in vacuo and the residue was chromatographed on SiO_2 (AcOEt:hexanes, 2:3) to yield 440 mg (74%) of **12**: IR (neat): 3339, 2982, 2936, 1736, 1686, 1508, 1366, 1159, 1076, 770, 668 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.04 (br s, 1 H), 5.08 (s, 1 H), 3.67 (s, 3 H), 1.48 (s, 6 H), 1.41 (s, 6 H), 1.39 (s, 9 H); ^{13}C NMR (CD_3OD): δ 177.1, 176.6, 156.5, 80.6, 57.2, 52.8, 28.7, 25.5, 25.1; MS (EI): *m/z* (relative intensity) 229 ($[M-t\text{-BuO}]^+$, 2), 169 (8), 158 (20), 116 (20), 102 (40), 84 (15), 69 (15), 58 (100); HRMS (EI): *m/z* calcd for $C_{10}H_{17}N_2O_4$ ([M-*t*-BuO]): 229.1188, found: 229.1148.

Boc-Aib-Aib-2′,3′,5′-tri-(*tert*-butyldimethylsilyl)-*ara*-C (14). According to the procedure used for **9**, 300 mg (1.00 mmol) of **12** yielded 645 mg (75%) of **14**: IR (neat): 2930, 2856, 1684, 1558, 1254, 1103, 837, 779, 668 cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.86 (br s, 1 H), 8.02 (br s, 1 H), 7.84 (d, 1 H, $J=7.5$ Hz), 7.40 (d, 2 H, $J=7.5$ Hz), 6.18 (d, 1 H, $J=2.8$ Hz), 4.24 (d, 1 H, $J=2.6$ Hz), 4.12 (s, 1 H), 3.99 (m, 1 H), 3.84–3.78 (m, 1 H), 3.73–3.67 (m, 1 H), 1.50–1.44 (m, 12 H), 1.39 (s, 9 H), 0.87, 0.86 (2s, 18 H), 0.66 (s, 9 H), 0.08, 0.07, 0.05, 0.04 (4s, 12 H), −0.01 (s, 3 H), −0.23 (s, 3 H); ^{13}C NMR ($CDCl_3$): δ 175.2, 174.7, 162.6, 155.3, 154.9, 145.9, 95.1, 88.7, 87.6, 79.9, 78.3, 76.3, 62.8, 57.5, 56.5, 28.3, 25.8, 25.7, 25.6, 25.0, 24.4, 24.1, 18.2, 17.9, 17.7, −4.6, −5.1, −5.4; FABMS: *m/z* (relative intensity) 856 ($[M+1]^+$, 100).

Aib-Aib-*ara*-C hydrochloride (15). According to the procedure used for **10**, 250 mg (0.29 mmol) of **14** yielded 105 mg (87%) of **15**: IR (neat): 3371 (br), 2995, 2943, 1715, 1652, 1559, 1505, 1389, 1115, 1052, 785 cm^{-1}; ^1H NMR (CD_3OD): δ 8.53 (d, 1 H, $J=7.6$ Hz), 7.27 (br s, 1 H), 6.21 (d, 1 H, $J=4.0$ Hz), 4.28 (m, 1 H), 4.09 (m, 1 H), 4.04 (m, 1 H), 3.83 (m, 2 H), 1.63 (s, 6 H), 1.55 (s, 6 H); ^{13}C NMR (CD_3OD): δ 177.0, 173.4, 160.0, 153.3, 147.3, 95.1, 90.1, 87.9, 77.1, 76.6, 62.2, 59.5, 58.2, 24.1; FABHRMS (MNBA/MeOH): *m/z* 414.181 ($[M+H]^+$, $C_{17}H_{29}N_5O_7$ requires 414.199).

Glc-Aib-ara-C (17). According to the procedure used for **9**, 200 mg (0.73 mmol) of **16** yielded 468 mg (77%) of TBDMS-Glc-Aib-2′,3′,5′-tri-(*tert*-butyldimethylsilyl)-*ara*-C: IR (neat): 2932, 2365, 1686, 1655, 1105, 839, 779 cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.83 (br s, 1 H), 7.89 (d, 1 H, $J=7.5$ Hz), 7.38 (br d, 1 H, $J=6.2$ Hz), 7.04 (br s, 1 H), 6.22 (d, 1 H, $J=3.0$ Hz), 4.30 (d, 1 H, $J=2.8$ Hz), 4.16 (s, 1 H), 4.06 (s, 2 H), 4.01 (dd, 1H, $J=6.3$, 8.4 Hz), 3.86–3.70 (m, 2 H), 1.59 (s, 6 H), 0.98–0.89 (m, 27 H), 0.76 (s, 9 H), 0.13–0.07 (m, 18 H), 0.02 (s, 3 H), −0.19 (s, 3 H); ^{13}C NMR (CD_3OD): δ 175.8, 173.2, 164.5, 157.2, 147.4, 97.2, 90.3, 89.0, 79.5, 77.7, 64.1, 63.7, 58.5, 26.5, 26.4, 26.3, 26.2, 24.6 (2C), 19.1, 18.8, 16.7, −4.2, −4.3, −4.6, −5.1, −5.2, −5.4; FABMS: *m/z* (relative intensity) 843 ($[M+1]^+$, 70).

According to the procedure used for **10**, 330 mg (0.39 mmol) of TBDMS-Glc-Aib-2′,3′,5′-tri-(*tert*-butyldimethylsilyl)-*ara*-C yielded 98 mg (65%) of **17**: IR (neat): 3341 (br), 2930, 1651, 1491, 1316, 1127, 1074, 804 cm^{-1}; ^1H NMR (CD_3OD): δ 8.26 (d, 1 H, $J=7.6$ Hz),

7.47 (d, 1 H, $J=7.5$ Hz), 6.19 (d, 1 H, $J=3.8$ Hz), 4.24 (dd, 1 H, $J=2.3$, 3.4 Hz), 4.08 (t, 1 H, $J=2.4$ Hz), 4.03–3.98 (m, 3 H), 3.81 (d, 2 H, $J=4.6$ Hz), 1.54 (s, 6 H); ^{13}C NMR (CD_3OD): δ 176.1, 174.9, 164.5, 157.7, 148.0, 96.9, 89.5, 87.4, 79.5, 78.1, 76.5, 62.7, 58.4, 24.8; FABHRMS (MNBA/MeOH): *m/z* 387.140 ($[M+H]^+$, $C_{15}H_{23}N_4O_8$ requires 387.152).

2-Boc-1′-aminoisopropyl-1,3,4-oxadiazol-5-one (18). A solution of 160 mg (0.74 mmol) of **22** in 4 mL of THF was treated at 22 °C with 144 mg (0.88 mmol) of *N,N′*-carbonyldiimidazole (CDI). The reaction mixture was stirred for 3 h. The solvent was evaporated and the residue was chromatographed on SiO_2 (AcOEt:hexanes, 2:3) to yield 152 mg (85%) of **18**: IR (neat): 3318 (br), 2984, 1779, 1694, 1520, 1393, 1084, 907, 849, 750, 668 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 9.79 (s, 1 H), 5.06 (br s, 1 H), 1.59 (s, 6 H), 1.40 (s, 9 H); ^{13}C NMR ($CDCl_3$): δ 160.2, 155.4, 154.4, 80.8, 51.0, 28.3, 25.8; MS (EI): *m/z* (relative intensity) 187 ($[M-C_4H_8]^+$, 20), 172 (20), 128 (20), 119 (100), 57 (60); HRMS (EI): *m/z* calculated for $C_6H_9N_3O_4$ ($[M-t$-butyl$+H]$): 187.0593, found: 187.0597.

Boc-Aib-NHNH$_2$ (21). A solution of 570 mg (2.63 mmol) of Boc-Aib-OMe (**20**) in 1.5 mL of MeOH was treated at 22 °C with 622 mg (12.4 mmol) of H_2NNH_2 monohydrate. The solution was heated up and stirred at 55 °C for 24 h and then recooled to 22 °C. The solvent was evaporated in vacuo to yield 410 mg of crude **21** (72%) as a white solid, which was used for the next step without further purification.

Boc-Aib-2-azagly-2′,3′,5′-tri-(*tert*-butyldimethylsilyl)-*ara*-C (23). A solution of 130 mg (0.22 mmol) of **4** in 2 mL of dry THF was cooled to −78 °C and treated with 100 μL of a 2.5 M solution of *n*-BuLi in hexanes. After 5 min, a solution of 54 mg (0.33 mmol) of CDI in 2 mL of THF was added. The dry ice–acetone bath was removed after 10 min, and the reaction mixture was stirred for 1 h before 96 mg (0.44 mmol) of **21** and 13 mg of DMAP were added. After stirring for 20 h, the solvent was evaporated and the residue was chromatographed on SiO_2 (AcOEt:hexanes, 4:1) to yield 58 mg of **4** (45%) and 55 mg (30%, 54% based on recovered starting material) of **23** as a colorless solid: IR (neat): 2957, 2861, 1651, 1507, 1257, 1105, 910, 839, 781 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 11.12 (br s, 1 H), 7.78 (d, 1 H, $J=7.6$ Hz), 7.45 (br s, 1 H), 6.16 (d, 1 H, $J=2.9$ Hz), 5.18 (br s, 1 H), 4.22 (d, 1 H, $J=2.8$ Hz), 4.15 (s, 1 H), 3.99 (dd, 1 H, $J=6.3$, 8.1 Hz), 3.85–3.67 (m, 2 H), 1.56 (s, 6 H), 1.45 (s, 9 H), 0.90, 0.89 (2s, 18 H), 0.76 (s, 9 H), 0.11, 0.10, 0.07, 0.06 (4s, 12 H), 0.02 (s, 3 H), −0.20 (s, 3 H); ^{13}C NMR ($CDCl_3$): δ 173.8, 164.3, 155.8, 155.2, 154.0, 145.0, 96.2, 88.5, 87.5, 78.5, 76.2, 62.9, 56.4, 28.4, 25.9, 25.8, 25.7, 18.4, 18.0, 17.8, −4.5, −5.3; FABMS: *m/z* (relative intensity) 830 ($[M+1]^+$, 50).

Aib-2-azagly-*ara*-C hydrochloride (19). According to the procedure used for **10**, 90 mg (0.11 mmol) of **23** yielded 34 mg (81%) of **19**: IR (neat): 3208 (br), 1728, 1626, 1500, 1304, 1190, 1057, 808, 706 cm^{-1}; ^{1}H NMR (CD_3OD): δ 8.55 (d, 1 H, $J=7.5$ Hz), 6.58 (br d, 1 H, $J=6.4$ Hz), 6.20 (d, 1 H, $J=3.9$ Hz), 4.28 (m, 1 H), 4.11 (m, 1 H), 4.04 (dd, 1 H, $J=4.6$, 8.2 Hz), 3.82 (m, 2 H), 1.68 (s, 6 H); ^{13}C NMR (CD_3OD): δ 173.1, 160.8, 154.7, 151.2, 149.1, 94.6, 89.8, 87.8, 77.3, 76.6, 62.3, 57.8, 24.1; FABHRMS (MNBA/MeOH): *m/z* 387.167 ($[M+H]^+$, $C_{14}H_{23}N_6O_7$ requires 387.163).

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