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Activation of Antibacterial Prodrugs by Peptide Deformylase

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Abstract—5′-Dipeptidyl derivatives of 5-fluorodeoxyuridine (FdU) (**1a–d**) were synthesized. These compounds are biologically inactive but can be activated by peptide deformylase, which removes the N-terminal formyl group of the dipeptide, to release the active drug FdU via an intramolecular cyclization reaction. Because the deformylase is ubiquitous among bacteria but absent in mammalian cells, **1a–d** provide a novel class of potential antibacterial agents. © 2000 Elsevier Science Ltd. All rights reserved.

The emergence of bacterial pathogens that are resistant to multiple classes of existing antibiotics has created an urgent demand for new antibacterial agents with novel mechanisms of action.[1] Recent studies from several laboratories have suggested that peptide deformylase (PDF), an enzyme responsible for removing the N-terminal formyl group from newly synthesized polypeptides, may be a suitable target for antibacterial drug design.[2,3] While PDF is present in all bacteria and essential for bacterial survival,[2] it is apparently absent in mammalian cells.[3] Indeed, PDF inhibitors have recently been shown to act as broad-spectrum antibacterial agents.[4] Here we report a novel, alternative approach to antibacterial drug design, in which a pharmacologically inactive prodrug is selectively processed by PDF to release an active drug inside the bacterial pathogen.

To demonstrate the viability of this approach, the 5′-OH group of 5-fluorodeoxyuridine (FdU) was acylated with an *N*-formylated dipeptidyl unit to give compounds **1a–d** (Scheme 1) as potential prodrugs. In vivo, FdU is converted by thymidine kinase into FdU 5′-phosphate, which is cytotoxic by inhibiting thymidylate synthetase.[5] As acylation of the 5′-OH prevents 5′-phosphorylation of FdU, **1a–d** should have little or no inhibitory activity. However, when bacterial cells take up **1a–d**, their PDF would remove the *N*-formyl group, thereby triggering the onset of a cyclization reaction[6] to release FdU, which would be converted into the active thymidylate synthetase inhibitor in situ by thymidine kinase (Scheme 1). Because of the unique presence of PDF in bacterial cells, *active drugs should be produced only inside the bacterial cells*, thus minimizing the cytotoxicity of these prodrugs to the host cells.

Prodrugs **1a–d** were prepared from the *t*-BOC-protected amino acids **5a–d**, which were selectively coupled to the 5′-OH of FdU (**4**) via Mitsunobu condensation[7] to afford carboxylic esters **6a–d** (Scheme 2). Treatment of **6a–d** with trifluoroacetic acid (TFA) followed by condensation with *N*-formylmethionine (for **1c**) or its activated ester **7** led to prodrugs **1a–d** in 25–73% overall yields (from **5**).

Prodrug **1a**, which contains an L-proline as the penultimate residue, is somewhat unstable under aqueous conditions (20 mM sodium phosphate, pH 7.4); incubation for 18 h at 37 °C led to 16% degradation (Fig. 1, tracing a), due to hydrolysis of the ester linkage and background deformylation followed by cyclization. Prodrug **1a** was then tested for enzymatic degradation by adding 5% (final) fetal bovine serum to the phosphate buffer. Significant degradation (55%) into FdU and two other unidentified species was observed after 18 h of incubation (Fig. 1, tracing b). Judging from the elution pattern of the products, the cleavage occurred primarily at the ester linkage to produce FdU and formyl-Met-Pro.

To improve the prodrug stability against protease/esterase action, unnatural amino acids were used to construct prodrugs **1b–d** (Scheme 1). L-Methionine is still used as the N-terminal residue, because PDF strongly prefers an L-methionine at this position.[8–11] At the second position, D-proline (**1b**), *N*-methyl-L-leucine (**1c**), and α-aminoisobutyrate (**1d**) were substituted for

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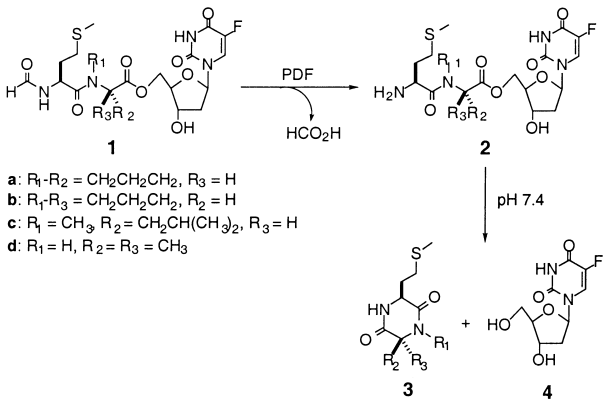
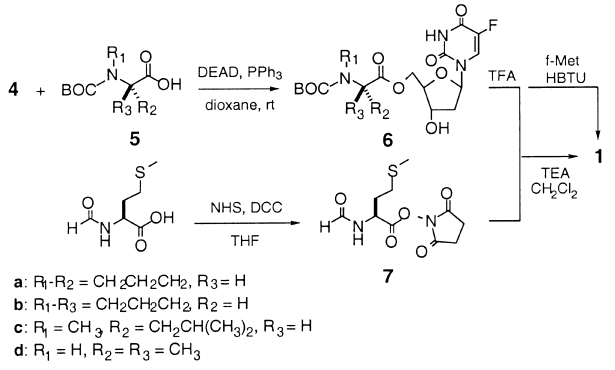


Scheme 1.

L-proline. Previous studies have shown that PDF exhibits broad substrate specificity at the penultimate position and can tolerate D-amino acids.[9,10] Unfortunately, while **1b** and **1c** are much more resistant to enzymatic degradation than the parent compound (**1a**), their formyl group is susceptible to chemical hydrolysis. Incubation in the 20 mM phosphate buffer for 18 h at 37 °C resulted in 64 and 54% degradation for **1b** and **1c**, respectively. Prodrug **1d**, which contains an α-methylalanine at the penultimate position, shows dramatically improved stability against both chemical and enzymatic hydrolysis (Fig. 1, c and d). After 18 h of incubation at 37 °C in the presence or absence of 5% fetal bovine serum, only a trace amount of FdU (< 1%) was observed. Also, when **1d** was treated with a crude *Escherichia coli* cell lysate, deformylation and cyclization were the only reactions observed (vide infra).

Compounds **1a–d** were then tested for activation by purified PDF in vitro. In the presence of *E. coli* PDF, **1a–d** were rapidly converted into deformylated intermediates **2a–d** (Fig. 2A), with k_{cat}/K_M values ranging from 750 to 2.9×10^4 M^{-1} s^{-1} (Table 1). These activities are comparable to those reported for short *N*-formylmethionyl peptide substrates.[8–10] Since in vivo deformylation is believed to be co-translational and therefore the physiological substrates of PDF are likely short unfolded peptides,[12] prodrugs **1a–d** should compete favorably with the endogenous PDF substrates in bacterial cells. Following the PDF reaction, the intermediates **2a–d** underwent facile, spontaneous intramolecular aminolysis to release FdU (retention time $t = 7.3$ min) and diketopiperazines **3a–d** ($t = 17.0$ min for **3a**) (Fig. 2A). The release of FdU displayed first-order



Scheme 2.

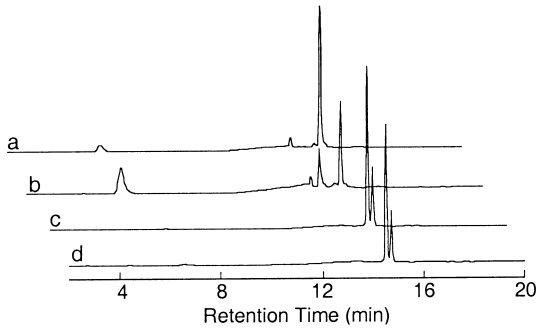


Figure 1. HPLC analysis (monitored at 214 nm) of prodrug degradation (18 h at 37 °C). a, **1a** + 20 mM phosphate buffer (pH 7.4); b, **1a** + phosphate buffer + 5% fetal bovine serum; c, **1d** + phosphate buffer; d, **1d** + phosphate buffer + 5% serum. The column (C18) was eluted with a linear gradient of 0–40% acetonitrile in water over 20 min. Retention times for FdU, formyl-Met-Pro, **1a** and **1d** were 6.5, 13.9, 14.3, and 14.5 min, respectively. The smaller peak with retention time of 14.7 min (in c and d) was due to racemization of **1d** at the methionyl residue during synthesis.

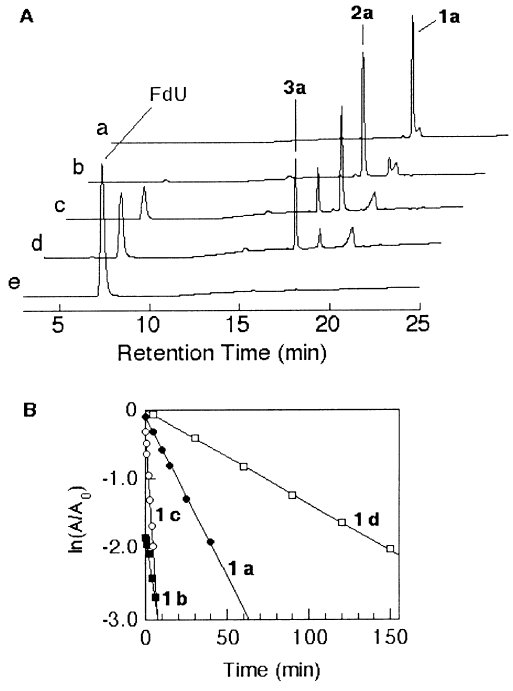


Figure 2. (A) HPLC analysis of activation of prodrug **1a**. At time 0, 67 μg of *E. coli* PDF was added to ~1 mM **1a** in 100 μL of 20 mM sodium phosphate (pH 7.4). At various times, 10-μL aliquots were withdrawn, quenched by the addition of an equal volume of 100 mM TFA, and loaded onto an HPLC instrument (C_{18} column; monitored at 214 nm). a, $t = 60$ min control without PDF; b, $t = 1$ min; c, $t = 15$ min; d, $t = 60$ min; and e, FdU standard. The retention times of **1a**, **2a**, **3a**, and FdU were 19.7, 18.2, 17.0, and 7.3 min, respectively. The small peak resistant to deformylation ($t = 20.1$ min) is f-(D-Met)-Pro-FdU. (B) First-order kinetics of cyclization of **2a–d**. A0, concentration of **2a–d** at time zero (before cyclization); A, concentration of **2a–d** at time t.

Table 1. Kinetic data of prodrug activation (pH 7.4)

Prodrug	Deformylation k_{cat}/K_M ($\times 10^3$ M^{-1} s^{-1})	Cyclization $t_{1/2}$ (min)
1a	12.1±0.4	15.2
1b	0.75±0.03	4.68
1c	1.65±0.06	2.07
1d	28.9±1.4	51.1

kinetics ($t_{1/2}$ = 2–51 min), consistent with an intramolecular reaction (Fig. 2B and Table 1). Diketopiperazine **3a** was collected from HPLC runs and its identity was confirmed by mass spectrometry.

Incorporation of unnatural amino acids into **1b–d** exerted some interesting effects on the kinetics of deformylation and cyclization reactions. The use of D-proline (**1b**) reduced the deformylation rate by 16-fold (relative to **1a**) but increased the cyclization rate by 3.2-fold ($t_{1/2}$ = 4.7 min).[6a] Similarly, *N*-methylleucine (**1c**) slowed down the PDF reaction by 7.3-fold, while increasing the cyclization rate by 7.3-fold ($t_{1/2}$ = 2.1 min).[6b] On the other hand, the use of α-aminoisobutyrate (**1d**) increased the PDF reaction rate by 2.4-fold, but reduced the cyclization rate by 3.4-fold ($t_{1/2}$ = 51 min). These results suggest that it may be possible to fine tune the rate of active drug release by varying the structure of the penultimate amino acid.

In order to test their ability to inhibit bacterial growth, compounds **1a–d** were added directly to a culture of *E. coli* BL21(DE3) cells in Luria broth. They resulted in only modest inhibition of cell growth at high concentrations (IC_{50} > 100 μM). This poor antibacterial activity could be caused by several factors. First, the prodrugs may be poorly transported into the bacterial cells. Second, the prodrugs may not be efficiently deformylated by PDF due to competition with the endogenous substrates. Third, the prodrugs may be rapidly degraded by intracellular enzymes other than PDF. We therefore tested the prodrugs with BL21(DE3) cells transformed with the overproducing plasmid pET-22b-def.[9] In the presence of 100 μM inducer, isopropyl-β-D-thiogalactopyranoside, these cells produce PDF as ~50% of their total cellular protein; any prodrugs that have entered these cells should be rapidly and completely deformylated. However, the growth inhibition profile of these overproducing cells was similar to that of the control BL21(DE3) cells. Prodrug **1d** was also tested against *Staphylococcus epidermidis*, a Gram-positive bacterium which is generally more sensitive to antibiotic treatment. Indeed, prodrug **1d** resulted in stronger growth inhibition with an IC_{50} value of ~50 μM. Since **1d** is stable against both chemical and enzymatic degradation (other than PDF), these results suggest that inefficient drug transport across the cell membrane(s) is likely the cause of poor antibacterial activity.

To further examine this issue, **1a–d** were treated with a crude bacterial cell lysate prepared from wild-type *E. coli* BL21(DE3) cells that had been grown to the mid-log phase. The cleavage products were analyzed by reversed-phase HPLC. All four compounds were rapidly deformylated by the endogenous PDF followed by cyclization and FdU release. Since **1d** was most stable against undesired chemical and enzymatic degradation, it was characterized in more detail. As shown in Figure 3, treatment of **1d** with the crude cell lysate for 1 h resulted in complete disappearance of the **1d** peak (t = 19.0 min). At the mean time, a new peak appeared at t = 15.1 min when monitored at 214 nm, which was invisible when monitored at 260 nm. This species was assigned as diketopiperazine **3d**, as it was not derived from the cell lysate, co-eluted with **3d** derived from treatment of **1d** with purified PDF, and had a molecular mass of 216.09 Da (determined by mass spectrometry after HPLC purification). Interestingly, the peak corresponding to free FdU (retention time t = 7.3 min under the conditions) was barely detectable (Fig. 3, tracing b). Examination of the HPLC chromatograms revealed an increase in intensity for a peak at t = 2.8 min (peak 3) as **1d** was hydrolyzed by PDF. Analysis of peak 3 by fast atom bombardment mass spectrometry gave two peaks at m/z = 327.17 (MH^+) and 371.22 Da ($M^+ + 2Na$), respectively, consistent with the structure of FdU 5′-phosphate. Peak 3 and its neighboring peaks (peaks 1, 2, 4, and 5) were then individually collected, treated with alkaline phosphatase, and again analyzed by HPLC. While phosphatase treatment of peaks 1, 2, 4, and 5 resulted in no change in the unknown species, treatment of peak 3 led to a peak at t = 7.3 min, the characteristic retention time of FdU under the conditions (the four natural nucleosides all have different retention times) (Fig. 3, tracing c). Thus, as FdU was generated from the cyclization reaction, it was converted by thymidine kinase in the crude lysate into the more hydrophilic FdU 5′-monophosphate, the active thymidylate synthetase inhibitor. Note that there was no degradation of **1d** by any other cellular enzymes. Taken together, the above results demonstrate that the endogenous PDF activity is adequate for the activation of prodrugs **1a–d** and inefficient deformylation is unlikely the reason for the low potency of **1a–d** for cell growth inhibition. Rather, it is the poor transport of prodrugs **1a–d** across the bacterial cell membrane(s)

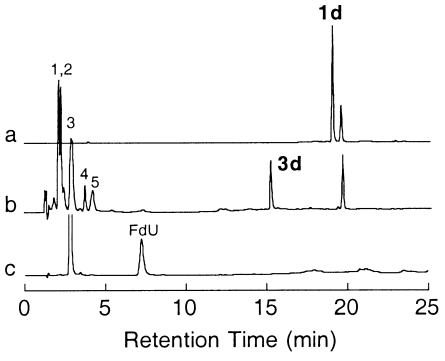


Figure 3. HPLC analysis of activation of prodrug **1d** by crude *E. coli* lysate (C_{18}; 214 nm). a. **1d** only; b. **1d** treated with cell lysate for 1 h, followed by 3 h incubation at room temperature (for cyclization to complete); and c. treatment of peak 3 derived from b with alkaline phosphatase. The small peak with retention t = 19.5 min (in a and b) is due to racemization of **1d** at the methionyl residue; the resulting isomer is resistant to PDF action.

that has limited the intracellular concentration of the prodrugs.

The prodrug approach offers several advantages over the inhibitor approach. First, the active drug is generated only inside a bacterial pathogen where active PDF is present. Such drugs should have lower toxicity to a eukaryotic host. Second, the prodrug approach is extremely versatile. In principle, any agent that blocks cell growth and requires a free nucleophile (e.g., a hydroxyl group) for toxicity can be masked by an acyl group to eliminate or reduce its toxicity. Once taken up by a bacterial pathogen, the acyl group is removed to release the cytotoxic agent, which may target any one of the bacterial proteins essential for survival. Third, prodrugs of this type are more likely to be broad-spectrum antibiotics relative to PDF inhibitors. Molecular cloning and genomic sequencing have revealed a few dozen PDF genes; their protein sequences show a wide range of homologies (20% to 65%).[13] Although the active-site structure seems to be well conserved, a subtle difference at or near the active site could render a potent inhibitor for PDF from one organism totally inactive against a different pathogen. The sequence diversity should be less of a concern for the prodrugs, because PDF is a broad-specificity enzyme that deformylates thousands of bacterial proteins and the prodrugs are close mimics of these natural substrates. Finally, bacterial cells are less likely to develop resistance to the prodrugs than to a PDF inhibitor. The sequence diversity of PDF predicts that a mutation in the substrate binding site (not the active site) may be tolerated, although such a mutation might change the substrate specificity. Such a mutation will likely affect or even abolish the inhibitor binding, causing drug resistance. Cells with such a mutation would still, however, be sensitive to the prodrugs, which are substrates of the deformylase. If a cell encounters a mutation that prevents prodrug activation, the mutant enzyme would likely fail to deformylate the cell's own proteins, resulting in a fatal consequence.

In conclusion, we have conceptually demonstrated a novel approach to antibacterial drug design which takes advantage of the unique bacterial PDF to convert a biologically inactive prodrug into an active antibacterial agent. Although the compounds described here lack potent in vivo antibacterial activity due to poor membrane permeability, redesign with more hydrophobic drugs or those that can be actively taken up by the transport systems will likely provide a new class of potent antibiotics. Such studies are currently under way.

Acknowledgement

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