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Syntheses and Studies of Multiwarhead Siderophore-5-fluorouridine Conjugates

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Abstract—Siderophores are microbial iron chelating agents that sequester physiologically essential iron for microbes. Conjugation of drugs to siderophores allows use of active iron transport for microbially directed drug delivery. Syntheses and biological studies are described of the first multidrug isocyanurate-based siderophore analogues separately containing one, two, and three 5-fluorouridine (5-FU) derivatives as the drug component. The results indicate that a single siderophore can be used to deliver multiple drugs to target pathogenic microorganisms. © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

Microbial resistance to antibiotics is becoming a major health care concern especially among people with compromised immune systems.[1–6] Of several modes of resistance, the inability of some antibiotics to enter the cell by the usual passive diffusion process is especially significant. Thus, the development of methods to facilitate active transport of antibiotics into microbial cells is an important therapeutic goal. One attractive approach to facilitate active transport of drugs into microbes is through conjugation to known molecules which the cell must assimilate for survival.[7] Siderophores are natural iron chelators utilized by many microbes for sequestering and cellular transport of physiologically essential iron. In principle, siderophore-mediated active transport may overcome the permeability problem by smuggling the drugs into microbial cells via microbial iron transport systems. Selective recognition and delivery of the drugs to target microbes may serve to improve the efficacy of the antimicrobial agents. The therapeutic potential of siderophore–drug conjugates (Fig. 1)[8–10] has been reviewed before.[11–15]

We have previously reported the syntheses and studies of isocyanurate-based siderophores.[16,17] Trihydroxamate derivative **1** was initially designed in our laboratory to mimic the natural dihyroxamate siderophore rhodotorolic acid (**2**), but with the ability to bind ferric ion stoichiometrically. Previous biological studies indicated that artificial siderophore analogue **1** could facilitate iron assimilation for a number of microorganisms including *Escherichia coli* and *Candida albicans*. In addition, isocyanurate analogue **1** was not toxic to mammals.[16,17] Moreover, a series of isocyanurate-based siderophore conjugates with antimicrobial agents were synthesized. Preliminary biological studies revealed the ability of this synthetic siderophore to act as a drug carrier.[17,18] This prompted further investigation into potential uses of the isocyanurate-based siderophore analogue (Fig. 2).

Results and Discussion

Design of conjugates

The synthetic siderophore **1** has an isocyanurate platform with three attached iron-chelating hydroxamates. Replacement of the acetyl groups on the three hydroxamates with succinoyl groups should provide compound **3**, which has the potential for attachment of three drugs to the same siderophore component. The three drugs can be the same or even different creating a multiwarhead siderophore–drug conjugate. For a drug delivery system, multiple drug conjugates might increase the delivery efficiency since every single carrier is envisioned to carry more than one drug. On the other hand, the multiwarhead conjugates are relatively large compared to the parent siderophore analogue **1**. Thus, a potential concern was whether the multiwarhead

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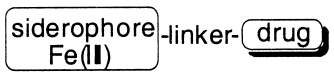
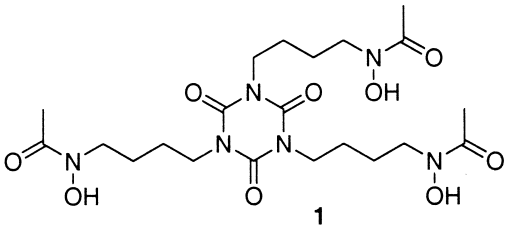


Figure 1. General siderophore–drug conjugate.



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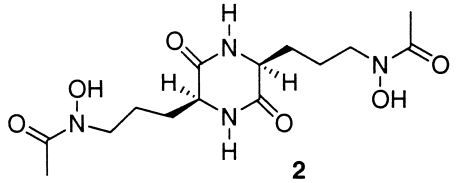

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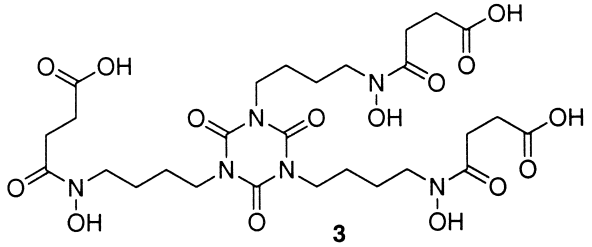
Figure 2.

conjugates could still be recognized and actively transported by microbes. However, previous studies of siderophore–drug conjugates showed that the siderophore component appears to still be recognizable and utilized despite the fact that, in some cases, the drug component of the conjugate is much larger than the siderophore component[19] (Fig. 3).

Our first choice for drug attachment was the antimicrobial agent 5-fluorouridine (5-FU), an active metabolite of the commonly used active antifungal agent 5-fluorocytosine (5-FC).[20] Valine was chosen as a spacer between the isocyanurate core and 5-FU for two reasons: (1) Val-5-FU is not only more active against fungi than 5-FU itself, but also shows strong antibacterial activity. (2) In earlier studies with ornithine-based siderophore–5-FU conjugates, use of different amino acid spacers was studied and valine-containing conjugates were found to be less prone to ester hydrolysis (half-life ~241 h) than other amino acid conjugates under assay conditions[18] (Fig. 4).



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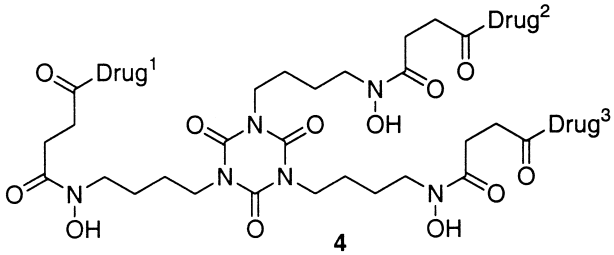

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Figure 3.

Synthesis of conjugates

The synthesis of the isocyanurate-based siderophore analogues was initiated by the preparation of a protected hydroxamate side chain precursor as outlined in Scheme 1. After treatment of *N*-trichloroethoxycarbonyl (*troc*) *O*-benzyl hydroxylamine (**5**) with NaH, the resulting sodium salt was added dropwise to an excess of 1,4-dibromobutane (**6**). This method provided the desired product **7** and bisalkylated product **8** in a ratio of 11:1. The following steps in the sequence paralleled our earlier synthesis of **10**.[17] Construction of the core structure of the isocyanurate-based siderophore analogue **10** was completed by treatment of cyanuric acid (**9**) with NaH followed by addition of bromide **7** (Scheme 1).

With tris-*troc*-protected analogue **10** in hand, sequential removal of *troc* protecting groups was explored in the hopes of obtaining the isocyanurate core with differentially functionalized handles. Complete acylation of **10** in the presence of an excess of freshly activated Zn dust and succinic anhydride provided tris-succinate **11** in 95% yield (Scheme 2). In order to sequentially remove the *troc* protecting groups, the reaction rate needed to be carefully controlled. Several different reductive methods for removal of the *troc* protecting group are reported in the literature.[21,22] With Zn/AcOH, the reaction rate is highly dependent on the acidity of the reaction medium.[22] A method involving fast initiation of the reaction followed by an immediate quench was investigated previously in our laboratory.[17] It was found that the concentration of Zn dust in the solution greatly influenced the reaction rate. When a large excess of Zn was used in the reaction, all three *troc* groups were removed quickly and almost simultaneously. When Zn was quite dilute in the reaction mixture, the *troc* groups were removed sequentially. Under carefully optimized conditions, the mono-, bis- and tris-succinate (**11**, **12** and **13**) were obtained as major products, respectively. Careful control of Zn concentration, reaction temperature, reaction time and the acidity of reaction media were all important factors. As shown in Scheme 2, treatment of **10** with three equivalents of Zn and succinic anhydride in THF containing 10% AcOH at 55–60°C followed by quenching of the reaction within 1 h provided optimal distribution of mono- and bis-succinate siderophore analogues. The unreacted

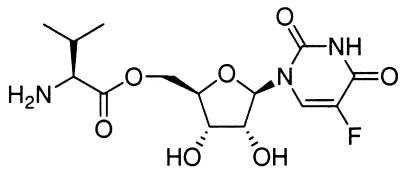
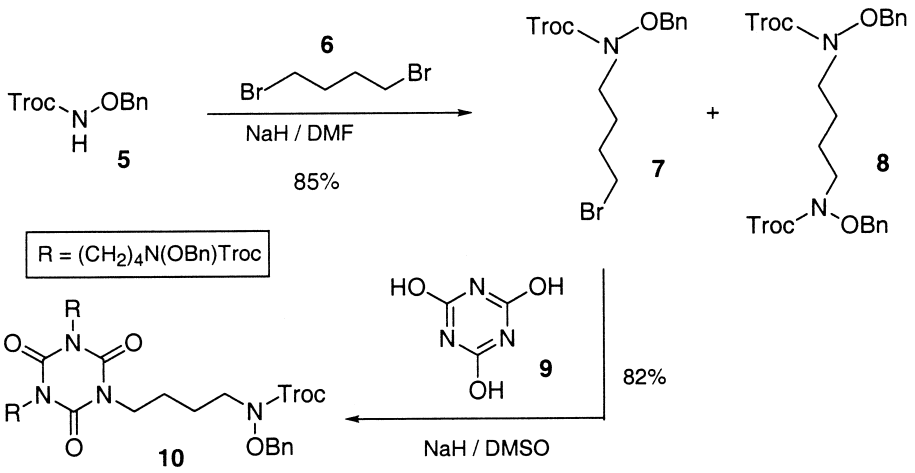
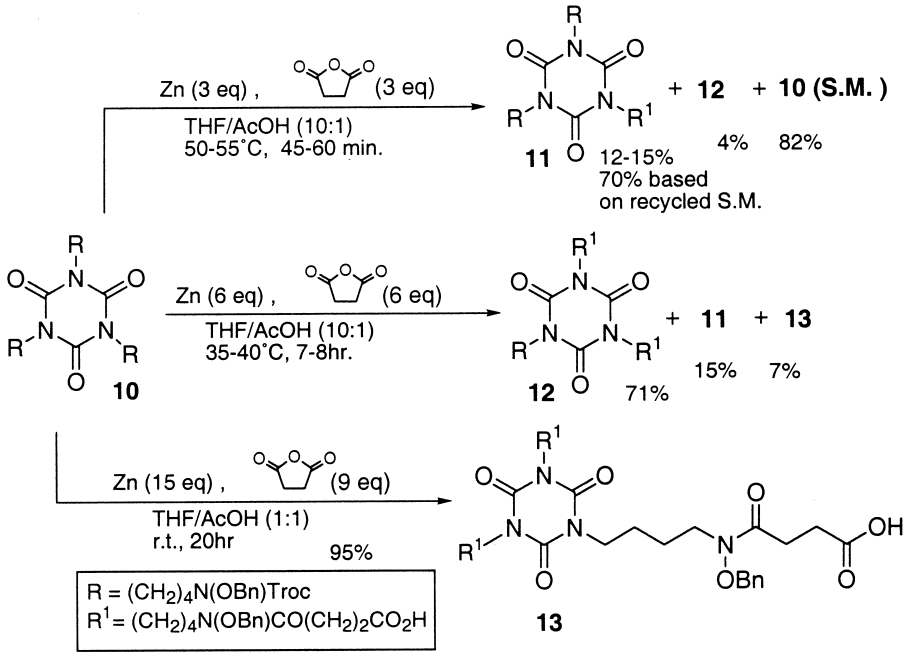


Figure 4. Valine-5-flurouridine (val-5-FU).



Scheme 1.

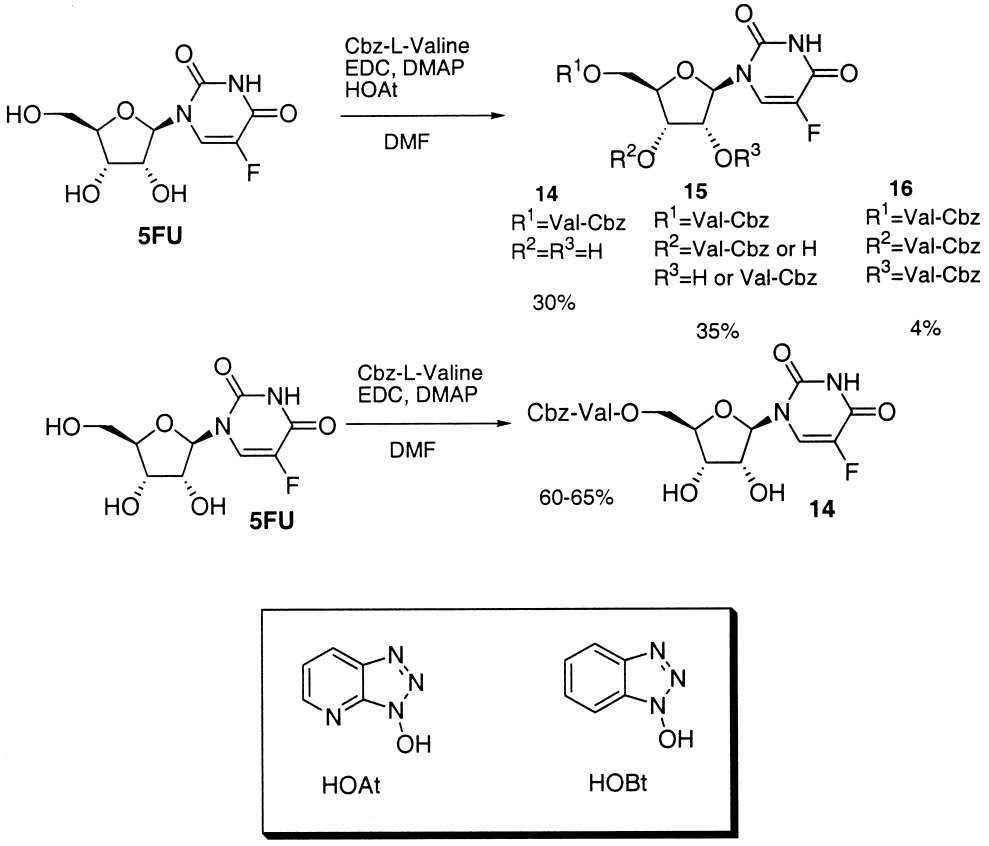


Scheme 2.

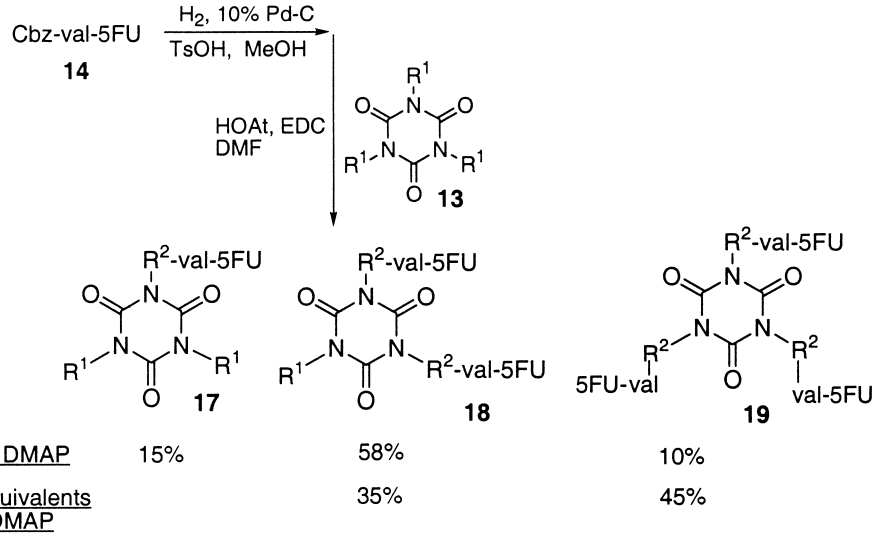
starting material could then be recycled, adding to the utility of this modified procedure. By increasing the amount of Zn to six equivalents and stirring the reaction mixture at 35–40°C for 7–8 h, bis-succinate **12** was obtained in a 71% yield. The three different products and the two starting materials were easily separated by radial chromatography. The ability to sequentially remove the *troc* protecting groups allowed for the potential introduction of three different drugs to a single iron complex. With these siderophore analogues in hand, the preparation of isocyanurate-based siderophore–multidrug conjugates was initiated.

To synthesize the val-5FU component **14**, EDC-mediated esterfication of 5-FU with Cbz-L-valine with different coupling additives was explored (Scheme 3). HOAt (1-hydroxy-7-azabenzotriazole) was found to be overly effective as a coupling additive. When HOAt was used, the interesting bis-val-5-FU adduct **15** was obtained as the major product. According to the literature precedent,[23] HOAt esters are more reactive than the esters of commonly used HOBt (1-hydroxy-7-benzotriazole). Although precise mechanistic details have not been established, the reactivity increase is presumably due to assistance from internal hydrogen bonding of the incoming nucleophiles by the nitrogen atom of pyridine residue in HOAt. The optimal coupling efficiency was found to be use of EDC alone.

The synthesis of a tris-5-FU conjugate **19** was explored next (Scheme 4). Deprotection of the Cbz group of **14** in the presence of one equivalent of *p*-TsOH provided the corresponding amine salt, which was immediately acylated with tris-succinate siderophore **13** using HOAt and EDC. The reaction was sluggish and problematic without the use of *p*-TsOH in the hydrogenation step, therefore one equivalent of *p*-TsOH was required for efficient deprotection. The coupling reaction was found



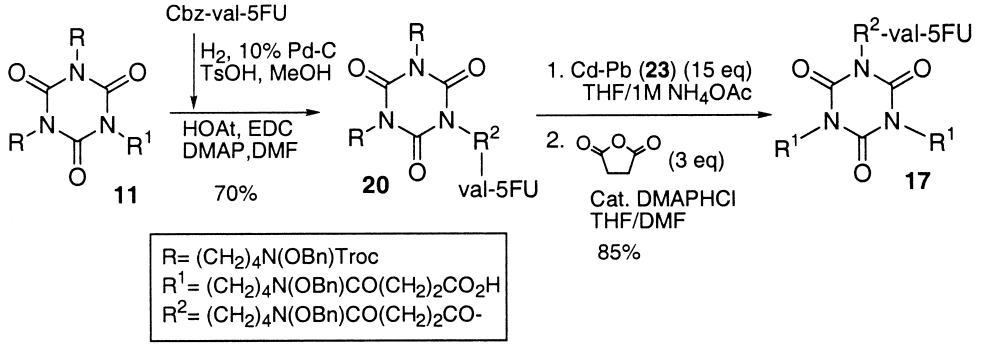
Scheme 3.



Scheme 4.

to be dependent on the acidity of the reaction media. When a catalytic amount of DMAP was used, the major product was bis-conjugate **18**, however, when a full equivalent of DMAP was used, *tris*-conjugate **19** was obtained as the major product.

Synthesis of mono-val-5FU conjugate (**17**) was attempted by an alternative route (Scheme 5). The first target was the mono-val-5FU conjugate as the protected bis-*troc* analogue **20**. It was then discovered that the Zn/AcOH conditions employed previously for *troc* protecting group removal caused decomposition of the conjugate. To overcome this problem, alternative methodology was utilized for *troc* removal.[24,25] Fortunately, 10% Cd–Pb couple was found to be an efficient reducing reagent in a pH 7, 1 M aq NH_4OAc buffer solution providing **17** in 85% yield after acylation with succinic anhydride (Scheme 5).



Scheme 5.

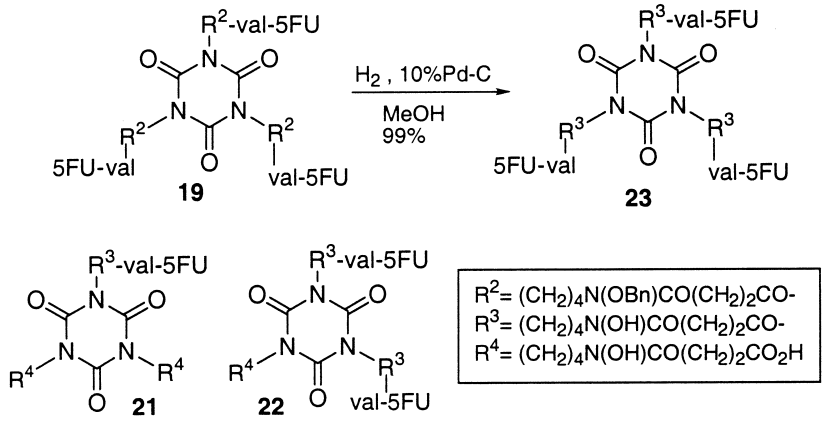
The benzyl protecting groups of all of the coupled products were removed by hydrogenolysis to obtain the desired conjugates in their free hydroxamate forms (Scheme 6).

Biological studies

To determine the antimicrobial activities of siderophore–drug conjugates, different assay methods have been employed. Previous studies using only the standard broth or agar dilution assays to determine the minimum inhibitory concentration (MIC) were found to be misleading.[17] Thus, kinetic growth assays were performed on compounds **21**, **22** and **23**. Both ferri (with stoichiometric Fe^{3+} added) and deferri forms of each of the conjugates were used for this study. All of the conjugates were tested in regular Luria broth and also in an iron-deficient medium. If the regular Luria broth was pretreated with ethylenediamine bis(*O*-hydroxyphenylacetic acid) (EDDA) before the test, it was considered to be an iron-deficient environment for the microbes.[17]

Synthetic conjugates **21**, **22** and **23** were subjected to studies of antibacterial activity against *E. coli* X580 at 10, 5 and 2.5 μM. At these concentrations, all of the val-5-FU conjugates displayed certain activity against *E. coli* X580 (Figs 5–7). All the tested conjugates showed much stronger activity under iron-deficient conditions indicating the bacteria will more actively utilize siderophores under these conditions, as expected. Thus, the bacteria recognized each conjugate as a source of nutritional iron, but in the process of actively assimilating it, the bacteria also assimilated the antibiotic and in effect committed suicide. The multiwarhead conjugates were not only active against the bacteria, but also more potent than mono conjugate **21** (Fig. 8). In fact, the tris-val-5-FU conjugate **23** was actually more potent than val-5-FU itself (Fig. 8). This may indicate that *E. coli* X580 still can recognize and actively transport these multiwarhead conjugates as planned.

To compare the delivery efficiency of multiwarhead conjugates **22** and **23** with mono conjugate **21**, further biological assays were performed (Figs 9 and 10). Tris-conjugate **23**, bis-conjugate **22**, and mono-conjugate **21** were tested at 1.25, 1.88, and 3.75 μM, respectively, in the high concentration assay. However, the total concentration of val-5-FU from all conjugates was equal to 3.75 μM since one molecule of **26** possesses three molecules of val-5-FU. As shown in Figure 9, the antibacterial activity of all three conjugates are very close to each other under iron sufficient conditions with tris-conjugate **23** being only slightly more potent than other conjugates. However, tris-conjugate **23** was more active than the mono- (**21**) and bis-conjugates (**22**) under iron-deficient conditions (Fig. 10). The concentration of mono-conjugate **21** (3.75 μM) was as much as three times that of tris-conjugates (1.25 μM). Although the concentration of val-5FU in each case



Scheme 6.

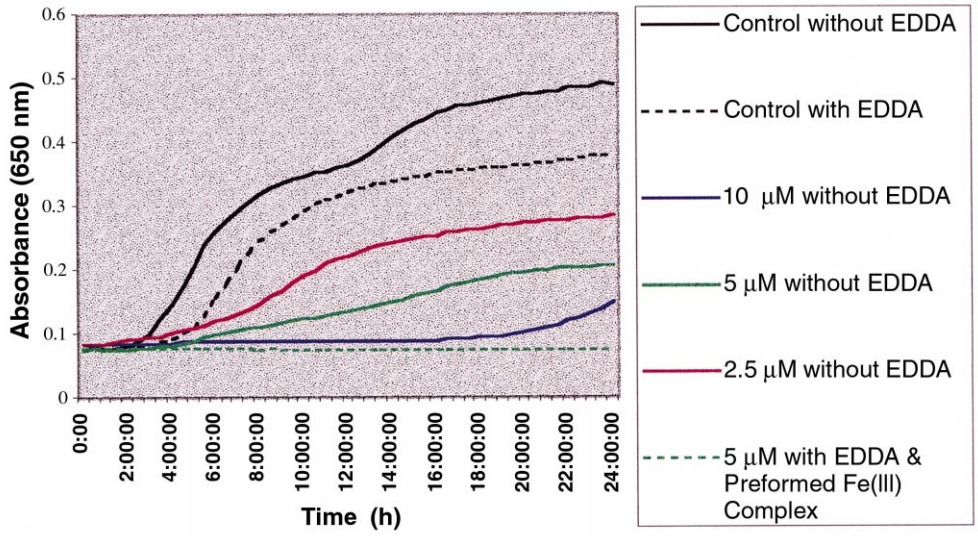


Figure 5. Growth curves of *E. coli* X580 in Luria broth in the presence of tris-val-5FU conjugate **23**.

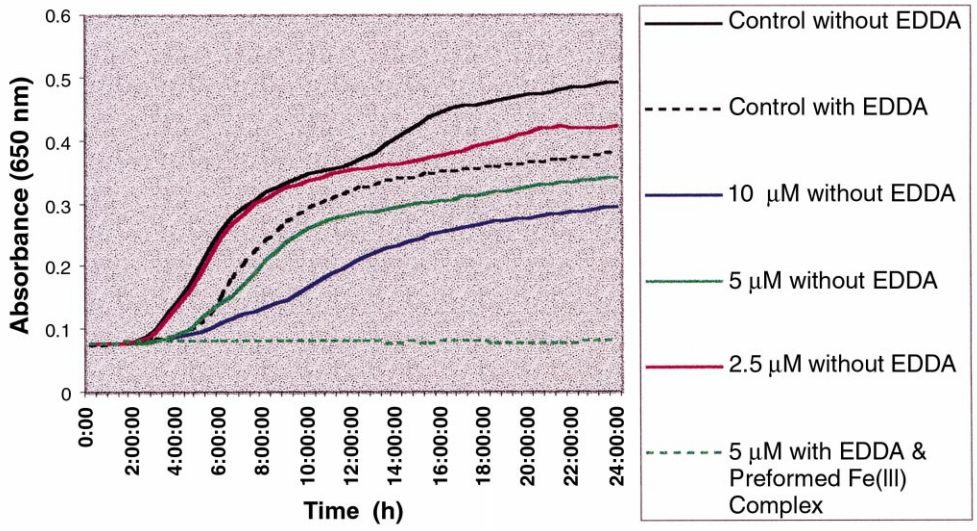


Figure 6. Growth curves of *E. coli* X580 in Luria broth in the presence of bis-val-5FU conjugate **22**.

could be considered the same (3.75 μM), higher activity of the tris-conjugate suggests that multiwarhead siderophore–drug conjugate **23** is a more efficient drug delivery system than the mono siderophore–drug conjugate **21**, or intracellular release is more effective.

The antifungal activity of conjugates **21**, **22** and **23** against *C. albicans* was also determined by kinetic growth assays in Luria broth cultures. Results similar to the antibacterial test were obtained and are summarized in Figures 11–14. All of the conjugates showed some delay of growth of fungi and multiwarhead siderophore–val-5FU conjugates were more potent than the mono siderophore–val-5-FU conjugate **21**.

The antifungal activity of the conjugates was also further investigated. The conjugates were tested at three concentrations respectively, each of which was considered to have equal effective concentrations of val-5-FU. The results are summarized in Figures 15 and 16. The multiwarhead conjugates (**22** and **23**) showed stronger activity under all conditions. Under iron-deficient conditions, the multiwarhead conjugates were much more potent than mono-conjugate (**21**), which was consistent with previous results.

The stability of siderophore-amino acid-5-FU conjugates under the assay conditions was studied previously in our laboratory.[18] As in related studies with isocyanurate-based siderophore–val-5-FU conjugate **24** (Fig. 17), a hydrolysis study employing ^{19}F NMR spectroscopy was used to determine the relative stability of conjugate **24** under bioassay conditions. Conjugate **24** showed less than 5% hydrolysis even after 19 h, corresponding to a half-life on the order of 296 h. Apparently, this result reveals that the test compounds are

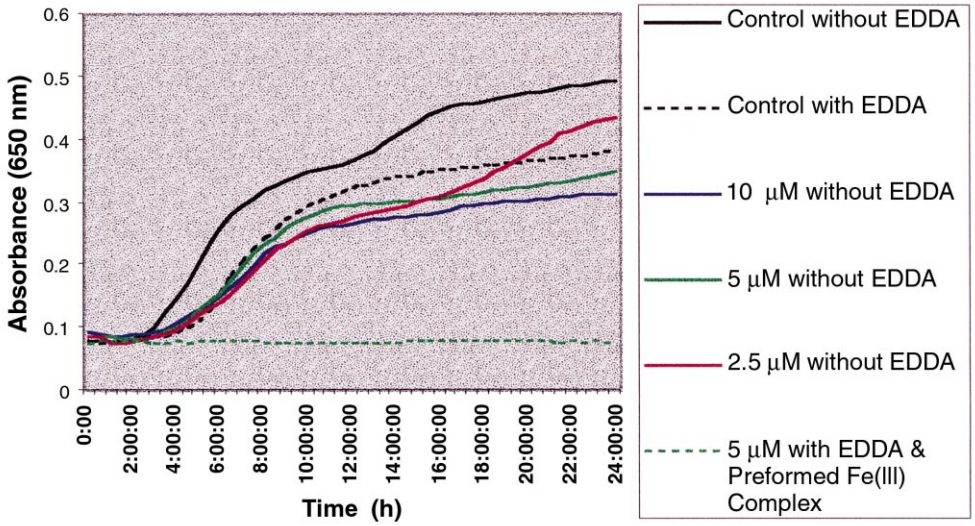


Figure 7. Growth curves of *E. coli* X580 in Luria broth in the presence of mono-val-5FU conjugate **21**.

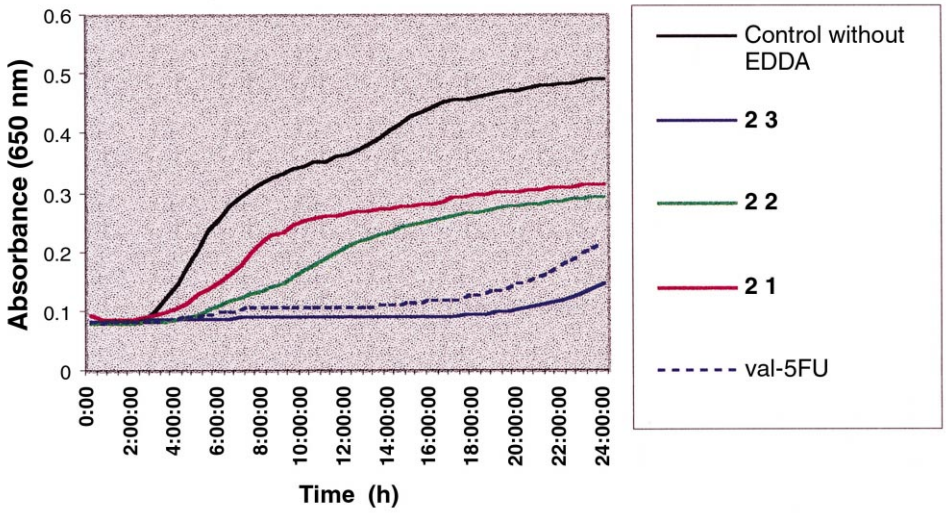


Figure 8. Growth curves of *E. coli* X580 in the presence of **23**, **22** and **21** and val-5FU at 10 μM under regular conditions.

stable under the assay conditions, indicating that the observed results are not due to spontaneous decomposition of the conjugates in the culture broth.

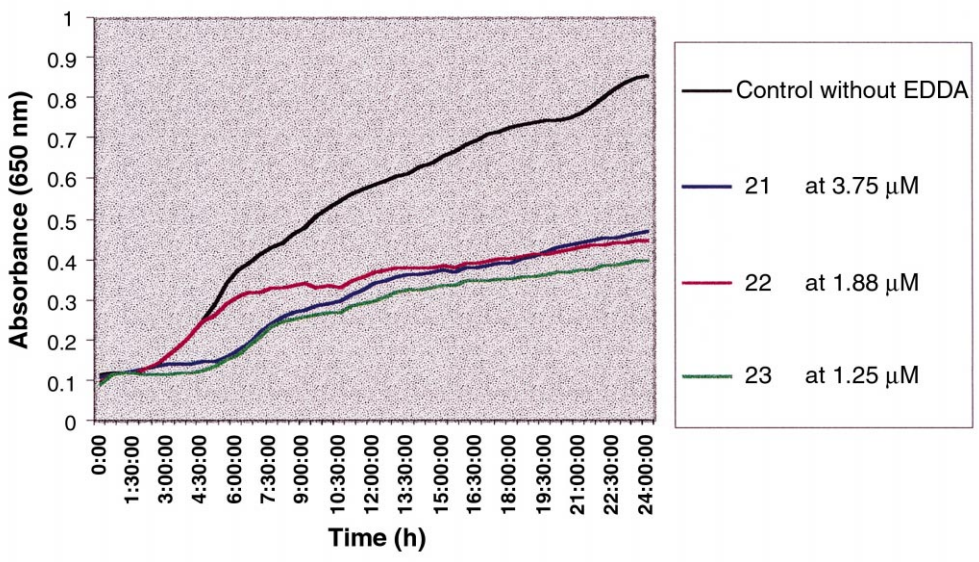
In conclusion, the isocyanurate-based siderophore–val-5-FU conjugates again displayed promising antimicrobial activities demonstrating the therapeutic potential of siderophore–drug conjugates in combating pathogenic infections. The multiwarhead conjugates were more potent against microbes than the regular mono conjugate supporting the assumption that the multiwarhead siderophore–drug conjugate is a more efficient drug delivery system, at least in the preliminary studies completed. The ability of sequential introduction of the linkage functionality may lead to the synthesis of multiwarhead conjugates with three different drugs. The biological behavior of such conjugates should be very interesting. Further synthetic and biological studies of the isocyanurate-based multiwarhead siderophore–drug conjugates are being carried out in our laboratory and will be reported in due course.

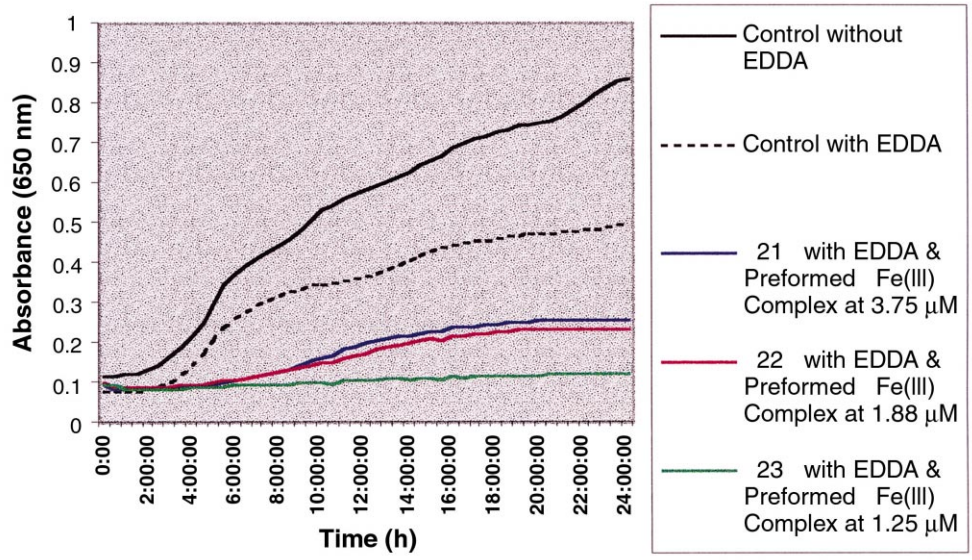
Experimental

General methods

General methods and instruments used have been described previously.[17] The term 'dried' refers to the drying of an organic layer over anhydrous sodium sulfate. All reactions were performed under nitrogen atmosphere. Compounds **7**, **10** and **13** have been previously described.[17]

[Mono[*N*-succinoyl]bis[*N*-(trichloroethoxy)] (benzyloxy)-amino]butyl] isocyanurate (11). Compound **10**[17] (0.5 g, 0.421 mmol) was dissolved in 11 mL of a 1:10 mixture of AcOH and THF. Freshly activated zinc dust (82.1 mg,





1.263 mmol) and succinic anhydride (126.3 mg, 1.263 mmol) were added to the resulting solution. The reaction mixture was stirred 40 min–1 h (monitored by TLC) at 45–50°C (water bath). Then it was diluted with cold EtOAc and residual Zn dust was removed by filtration. The solvent was evaporated and the residue was taken up into EtOAc. The EtOAc solution was washed with water several times and dried. After filtration and evaporation of the solvent, the residue was chromatographed over a Chromatotron® silica gel plate. Elution with a mixture of CH_2Cl_2, *i*-PrOH, and AcOH (100:2:1, 100:5:1, 100:10:1 gradient) provided **11** (70.2 mg, 15%), **12** (17.4 mg, 4%) and **10** (410 mg, 82%). **11**: ^{1}H NMR ($CDCl_3$) δ 1.45–1.75 (m, 12H), 2.55–2.75 (m, 4H), 3.62–3.72 (m, 6H), 3.75–3.87 (m, 6H), 4.82 (s, 6H), 4.92 (s, 4H), 7.36 (m, 15H), 9.6 (br s, 3H); ^{13}C NMR ($CDCl_3$) δ 24.14, 24.87, 27.12, 28.62, 42.46, 49.11, 75.08, 77.20, 128.49, 128.71, 128.79, 129.00, 129.17, 129.47, 134.73, 148.80, 173.12, 177.15; IR (neat) 1460, 1690, 1700, 1720 cm^{-1}; MS (FAB) *m/e* 1112.67 (MH^+), 1134.54 (MNa^+).

[[Bis[*N*-succinoyl]mono[*N*-(trichloroethoxy)] *N*-(benzyloxy)-amino]butyl] isocyanurate (12). Compound **10** (0.5 g, 0.421 mmol) was dissolved in 10 mL of a 1:1 mixture of AcOH and THF. Freshly activated zinc dust (164.2 mg, 2.526 mmol) and succinic anhydride (252.6 mg, 2.526 mmol) were added to the resulting solution. The reaction mixture was stirred 7–10 h (monitored by TLC) at 35–40°C. Then it was diluted with cold EtOAc and residual Zn dust was removed by filtration. The solvent was evaporated and the residue was taken up into EtOAc. The EtOAc solution was washed with water several times and dried. After filtration and evaporation of the solvent, the residue was chromatographed over a Chromatotron® silica gel plate. Elution with a mixture

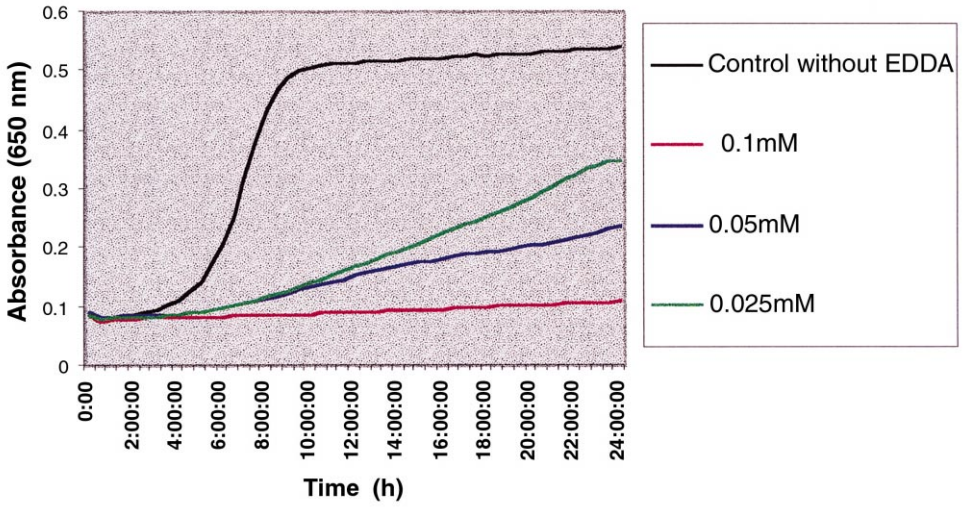


Figure 11. Growth curves of *C. albicans* in the presence of tris-val-5FU conjugate **23**.

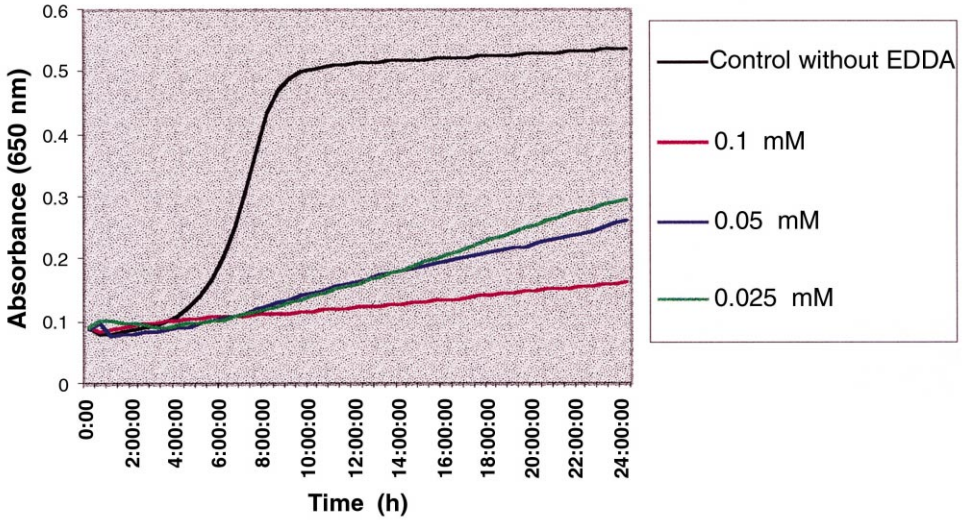


Figure 12. Growth curves of *C. albicans* in the presence of bis-val-5FU conjugate **22**.

of CH_2Cl_2, *i*-PrOH, and AcOH (100:2:1, 100:5:1, 100:10:1 gradient) provided **11** (70.2 mg, 15%), **12** (309.1 mg, 71%), **13** (28.32 mg, 7%) as colorless clear oils. **12**: ^{1}H NMR ($CDCl_3$) δ 1.45–1.75 (m, 12H), 2.55–2.75 (m, 8H), 3.48–3.72 (m, 6H), 3.75–3.87 (m, 6H), 4.83 (s, 6H), 4.92 (s, 3H), 7.34–7.39 (m, 15H); ^{13}C NMR ($CDCl_3$) δ 23.63, 24.51, 26.92, 28.31, 42.19, 44.58, 75.98, 77.19, 128.44, 128.71, 128.95, 133.98, 148.64, 173.12, 177.15; IR (neat) 1460, 1690, 1700, 1720 cm^{-1}; MS (FAB) *m/e* 1035.30 (M^+), 1057.37 (MNa^+).

5′-O-(*N*-(Benxyloxycarbonyl)-L-valinyl)-5-fluorouridine (14). Cbz-valine (50 mg, 0.2 mmol) was dissolved in 4 mL of THF, then 5-FU (52.1 mg, 0.2 mmol) was added to the flask, followed by 4 mL of DMF to dissolve the 5-FU. Then EDC (38.1 mg, 0.2 mmol) and DMAP (24.2 mg, 0.2 mmol) were added. The reaction mixture was stirred at rt overnight. The reaction mixture was diluted with EtOAc (75 mL). The EtOAc solution was washed with 0.5 M HCl, 0.3 M $NaHCO_3$, water and brine. The organic layer was then dried, filtered and evaporated. The residue was chromatographed over silica gel using a Chromatotron® with CH_2Cl_2: *i*-PrOH (95:5). This afforded **14** (59.51 mg, 62%) as a white solid. Mp: 64–67°C ^{1}H NMR ($CDCl_3$) δ 0.91 (d, 3H), 0.98 (d, 3H), 2.03–2.23 (m, 1H), 3.85–4.55 (m, 7H), 5.01–5.12 (m, 2H), 5.46 (d, 1H), 5.98 (d, 1H), 7.26–7.38 (m, 5H), 8.02 (d, 1H); ^{13}C NMR ($CDCl_3$): δ 17.33, 17.54, 19.01, 30.95, 59.48, 63.94, 67.22, 69.88, 74.39, 77.22, 81.74, 89.87, 128.05, 128.19, 128.25, 128.51, 135.91, 142.51, 149.71, 156.55, 157.53, 171.88, 176.18; IR 1580, 1700 (br), 3450 (br) cm^{-1}; MS (FAB) *m/e* calcd 496.1712 (MH^+), found 496.1738 (MH^+).

[Bis[*N*-(trichloroethoxy)carbonyl]mono[*N*-succinoyl] (benzyloxy)amino butyl] isocyanurate (20). To a solution of Cbz-valine-5-FU (31.2 mg, 0.063 mmol) in 5 mL of

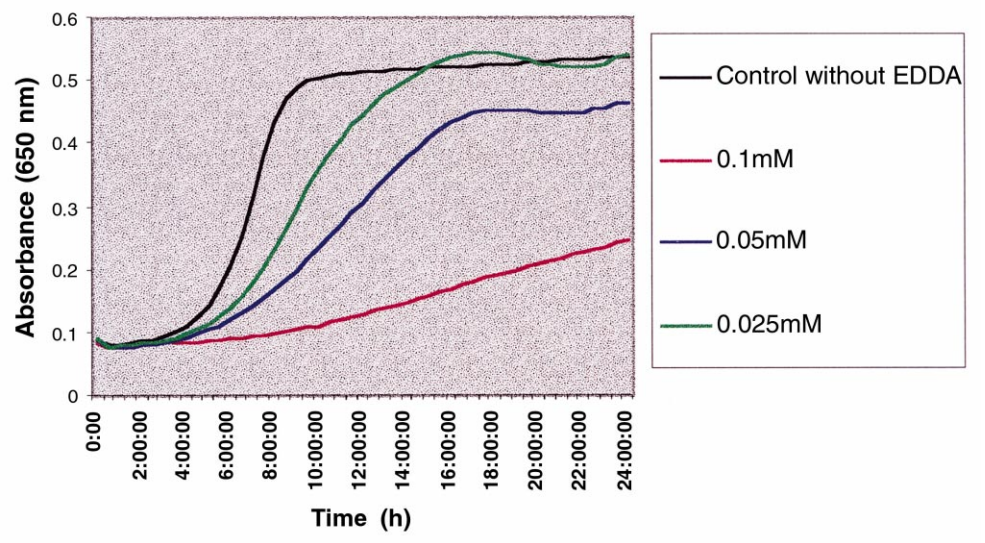


Figure 13. Growth curves of *C. albicans* in the presence of mono-val-5FU conjugate **21**.

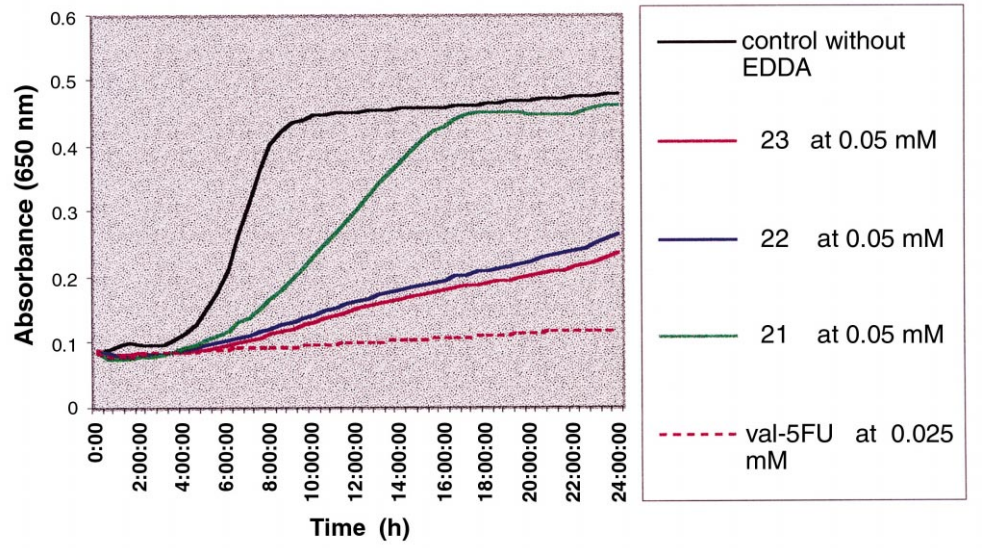


Figure 14. Growth curves of *C. albicans* in the presence of **21**, **22** and **23** and val-5FU.

deoxygenated MeOH was added *p*-TsOH (12 mg, 0.063 mmol) and 10% Pd–C (6 mg, 20% w/w). The suspension was stirred under 1 atm of H_2 for 50 min after which TLC analysis indicated that the reaction was complete. The catalyst was removed by filtration and the solvent was evaporated to afford the *p*-TsOH salt of the amine. The crude salt was dissolved in 5 mL of anhydrous DMF. A solution of **11** (70 mg, 0.063 mmol) in 5 mL of DMF was added, quickly followed by HOAt (8.6 mg, 0.063 mmol), EDC (12 mg, 0.063 mmol), and DMAP (7.6 mg, 0.063 mmol). The reaction mixture was stirred at rt overnight, then diluted with EtOAc (75 mL). The EtOAc solution was washed with 0.5 M HCl, 0.3 M $NaHCO_3$ water and brine. Then the solvent was dried, filtered and evaporated. The residue was chromatographed over a Chromatotron® silica gel plate eluting with CH_2Cl_2: *i*-PrOH (95:5). This afforded compound **20** (64.2 mg, 70%) as a clear oil. ^{1}H NMR ($CDCl_3$) δ 0.92–0.99 (d, 6H), 1.45–1.75 (m, 12H), 2.01–2.23 (m, 1H), 2.55–2.75 (m, 4H), 3.62–3.86 (m, 12H), 4.15–4.48 (m, 7H), 4.81 (s, 6H), 4.89 (s, 4H), 5.18 (d, 1H), 5.91 (d, 1H), 7.32–7.42 (m, 15H), 8.08 (d, 1H); ^{13}C NMR ($CDCl_3$) δ 17.98, 18.97, 20.38, 23.88, 24.68, 24.87, 27.64, 29.92, 30.69, 42.46, 44.73, 58.07, 61.78, 69.42, 74.15, 76.19, 76.25, 77.20, 81.63, 91.05, 125.3, 128.64, 128.92, 129.13, 134.09, 134.21, 139.12, 142.08, 148.90, 149.13, 157.05, 171.20, 172.43, 172.83, 176.82; IR 1460, 1580, 1720 (br) cm^{-1}; MS (ES) *m*/*e* 1478.01 (MNa^+).

10% Cadmium-lead couple. Lead oxide (PbO, 108 mg, 0.49 mmol) was dissolved in 5 mL of 50% aq AcOH, and the solution was slowly added to a vigorously stirred suspension of Cd dust (100 mesh, 546 mg, 4.9 mmol) in deionized water (10 mL). The Cd darkened as Pb deposited on its surface, and formed clumps that were broken up with a glass rod. The Cd–Pb couple was

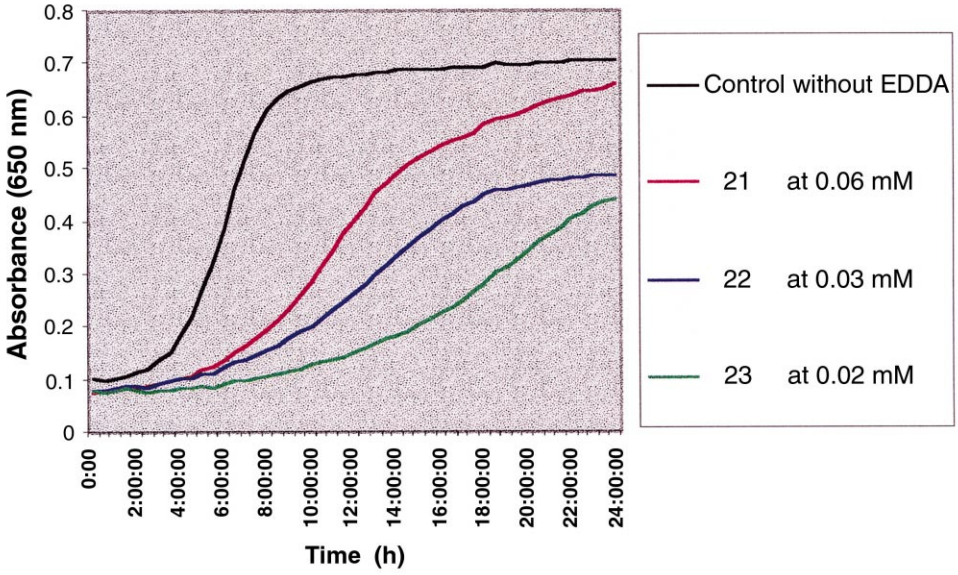


Figure 15. Growth curves of *C. albicans* in the presence of **21**, **22** and **23** at regular condition.

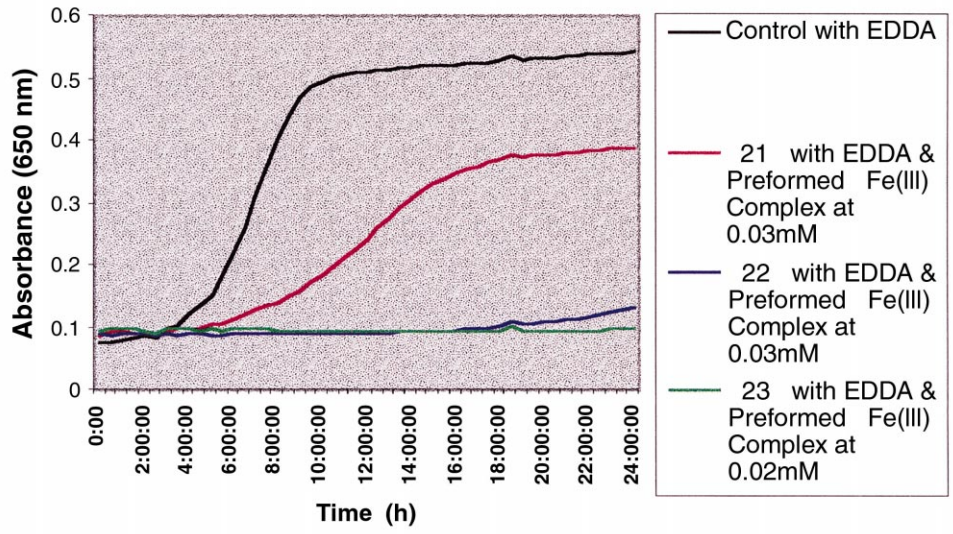


Figure 16. Growth curves of *C. albicans* in the presence of **21**, **22** and **23** at iron deficient conditions.

filtered, washed with water, then acetone, vacuum dried, ground, and stored in a closed vessel.

[Mono[*N*-succinoyl [(valinyl)-5-fluorouridine]-bis[*N*-succinoyl] (benzyloxy)amino butyl] isocyanurate (17). Cd–Pb couple (24.8 mg, 0.206 mmol) was added to a vigorously stirring mixture of compound **20** (20 mg, 0.0137 mmol), THF (4 mL) and 1 M NH_4OAc (1 mL). Cloudiness slowly developed. When the reaction was complete (TLC monitored), the solids were filtered and washed with water and THF. The filtrate was diluted with EtOAc. Then the EtOAc layer was washed with brine, dried, filtered and evaporated to afford a slight-yellow oil. The oil was dissolved in THF (4 mL) and DMF (4 mL). Then succinic anhydride (4.11 mg, 0.0411 mmol) and a catalytic amount of DMAP were added. The reaction mixture was stirred at rt overnight, then diluted with EtOAc. The EtOAc solution was washed with water, brine, then dried, filtered and concentrated. The crude oil was purified using a Chromatotron® silica gel plate eluting with CH_2Cl_2: *i*-PrOH:MeOH:AcOH (90:5:5:1). This gave compound **17** as a clear oil (15.2 mg, 85%). ^1H NMR ($CDCl_3$) δ 0.92–0.99 (d, 6H), 1.45–1.75 (m, 6H), 2.01–2.23 (m, 1H), 2.55–2.75 (m, 12H), 3.62–3.86 (m, 12H), 4.15–4.48 (m, 7H), 4.81 (s, 6H), 5.18 (d, 1H), 5.91 (d, 1H), 7.32–7.42 (m, 15H), 8.08 (d, 1H); ^{13}C NMR ($CDCl_3$) δ 17.98, 18.97, 20.38, 23.88, 24.68, 24.87, 27.64, 29.92, 30.69, 42.46, 44.73, 58.07, 61.78, 69.42, 74.15, 76.19, 76.25, 77.20, 81.63, 91.05, 125.3, 128.64, 128.92, 129.13, 134.09, 134.21, 139.12, 142.08, 148.90, 149.13, 157.05, 171.20, 172.43, 172.83, 176.82; IR 1580, 1700 (br), 3450 (br) cm^{-1}; MS (ES) *m/e* 1327.34 (MNa^+).

[Bis[*N*-succinoyl [(valinyl)-5-fluorouridine]mono[*N*-succinoyl] (benzyloxy)amino butyl] isocyanurate (18). To a

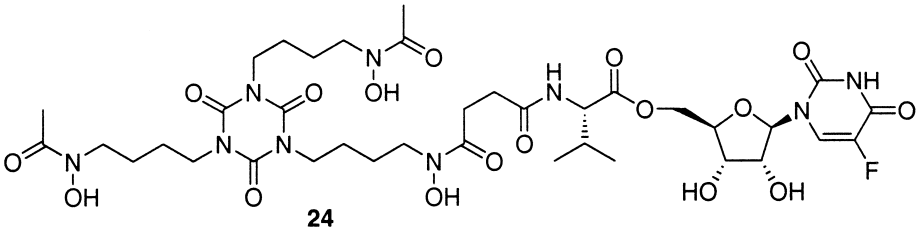


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Figure 17.

solution of Cbz-valine-5-FU (31.2 mg, 0.063 mmol) in 5 mL of deoxygenated MeOH was added *p*-TsOH (12 mg, 0.063 mmol) and 10% Pd–C (6 mg, 20% w/w). The suspension was stirred under 1 atm of H_2 for 40 min after which time TLC analysis indicated that the reaction was complete. The catalyst was removed by filtration and the solvent was evaporated to afford the *p*-TsOH salt of the amine. The crude salt was dissolved in 5 mL of anhydrous DMF. A solution of **13** (20.2 mg, 0.021 mmol) in 5 mL of DMF was added, quickly followed by HOAt (8.6 mg, 0.063 mmol), EDC (12 mg, 0.063 mmol), and a catalytic amount of DMAP (< 1 mg). The reaction mixture was stirred at rt overnight, then diluted with EtOAc (75 mL). The EtOAc solution was washed with 0.5 M HCl, 0.3 M $NaHCO_3$, water and brine. Then the solvent was dried, filtered, and evaporated. The residue was chromatographed though a Biotage Flash 40 silica gel cartridge eluting with CH_2Cl_2: *i*-PrOH:AcOH (95:5:1 to 90:10:1 gradient) to afford compound **17** (4.1 mg, 15%), **18** (20 mg, 58%) and **19** (4.2 mg, 10%) as clear oils. **18**: ^{1}H NMR ($CDCl_3$) δ 0.92–0.99 (d, 12H), 1.43–1.72 (m, 12H), 2.01–2.23 (m, 2H), 2.55–2.75 (m, 12H), 3.62–3.86 (m, 12H), 4.15–4.48 (m, 14H), 4.82 (s, 6H), 5.18 (d, 2H), 5.91 (d, 2H), 7.32–7.42 (m, 15H), 8.08 (d, 2H); ^{13}C NMR ($CDCl_3$) δ 17.98, 18.97, 20.38, 23.88, 24.68, 24.87, 27.64, 29.92, 30.69, 42.46, 44.73, 58.07, 61.78, 69.42, 74.15, 76.19, 76.25, 77.20, 81.63, 91.05, 125.3, 128.64, 128.92, 129.13, 134.09, 134.21, 139.12, 142.08, 148.90, 149.13, 157.05, 171.20, 172.43, 172.83, 176.82; IR 1460, 1580, 1700 (br), 3450 (br) cm^{-1}, MS (ES) *m*/*e* 1670.66 (MNa^+).

Tris[*N*-succinoyl [(valinyl)-5-fluorouridine] (benxyloxy)-amino]butyl] isocyanurate (19). To a solution of Cbz-valine-5FU (31.2 mg, 0.063 mmol) in 5 mL of deoxygenated MeOH was added *p*-TsOH (12 mg, 0.063 mmol) and 10% Pd–C (6 mg, 20% w/w). The suspension was stirred under 1 atm of H_2 for 40 min after which TLC analysis indicated that the reaction was complete. The catalyst was removed by filtration and the solvent was evaporated to afford the *p*-TsOH salt of the amine. The crude salt was dissolved in 5 mL of anhydrous DMF. A solution of **13** (20 mg, 0.021 mmol) in 5 mL DMF was added, quickly followed by HOAt (8.6 mg, 0.063 mmol), EDC (12 mg, 0.063 mmol) and DMAP (15.3 mg, 0.126 mmol). The reaction mixture was stirred at rt overnight, then diluted with EtOAc (75 mL). The EtOAc was washed with 0.5 M HCl, 0.3 M $NaHCO_3$, water and brine. Then the solvent was dried, filtered and evaporated. The residue was chromatographed through a Biotage Flash 40 silica gel cartridge eluting with CH_2Cl_2:*i*-PrOH:AcOH (95:5:1 to 90:10:1 gradient) to afford compound **19** (18.8 mg, 45%) and compound **18** (12.11 mg, 35%) as clear oils. **19**: ^{1}H NMR ($CDCl_3$) δ 0.92–0.99 (d, 18H), 1.45–1.75 (m, 12H), 2.01–2.23 (m, 3H), 2.55–2.75 (m, 12H), 3.62–3.86 (m, 12H), 4.15–4.48 (m, 21H), 4.81 (s, 6H), 5.18 (d, 3H), 5.91 (d, 3H), 7.32–7.42 (m, 15H), 8.08 (d, 3H); ^{13}C NMR ($CDCl_3$) δ 17.98, 18.97, 20.38, 23.88, 24.68, 24.87, 27.64, 29.92, 30.69, 42.46, 44.73, 58.07, 61.78, 69.42, 74.15, 76.19, 76.25, 77.20, 81.63, 91.05, 125.3, 128.64, 128.92, 129.13, 134.09, 134.21, 139.12, 142.08, 148.90, 149.13, 157.05, 171.20, 172.43, 172.83, 176.82; IR 1460, 1580, 1700 (br), 3450 (br) cm^{-1}, MS (ES) *m*/*e* 2014.88 (MNa^+).

[Mono[*N*-succinoyl [(valinyl)-5-fluorouridine]bis[*N*-succinoyl] hydroxyamino] butyl] isocyanurate (21). To a solution of **17** (35 mg, 0.027 mmol) in 6 mL of MeOH was added 7 mg of 10% Pd–C. This mixture was exposed to H_2 at atmospheric pressure for 4 h (monitored by TLC). The catalyst was removed by filtration and the solvent was removed by evaporation under vacuum. This afforded pure **21** (27.9 mg, 99%) as a foamy solid. ^{1}H NMR ($CDCl_3$) δ 0.92–0.99 (d, 6H), 1.45–1.75 (m, 6H), 2.01–2.23 (m, 1H), 2.55–2.75 (m, 12H), 3.62–3.86 (m, 12H), 4.15–4.48 (m, 7H), 5.18 (d, 1H), 5.91 (d, 1H), 8.08 (d, 1H); ^{1}C NMR ($CDCl_3$) δ 17.98, 18.97, 20.38, 23.88, 24.68, 24.87, 27.64, 29.92, 30.69, 42.46, 44.73, 58.07, 61.78, 69.42, 74.15, 76.19, 76.25, 77.20, 81.63, 91.05, 125.3, 134.21, 139.12, 142.08, 148.90, 149.13, 157.05, 171.20, 172.43, 172.83, 176.82; IR (neat) 1460, 1700 (br), 3450 (br) cm^{-1}; MS (ES) *m*/*e* 1057.21 (MNa^+).

[Bis[*N*-succinoyl [(valinyl)-5-fluorouridine]mono[*N*-succinoyl] hydroxyamino] butyl] isocyanurate (22). To a solution of **18** (40 mg, 0.024 mmol) in 6 mL of MeOH was added 8 mg of 10% Pd–C. This mixture was exposed to H_2 at atmospheric pressure for 4 h (monitored by TLC). The catalyst was removed by filtration and the solvent was removed by evaporation under vacuum. This afforded pure **22** (23.4 mg, 99%) as a foamy solid. ^{1}H NMR ($CDCl_3$) δ 0.92–0.99 (d, 12H), 1.45–1.75 (m, 12H), 2.01–2.23 (m, 2H), 2.55–2.75 (m, 12H), 3.62–3.86 (m, 12H), 4.15–4.48 (m, 14H), 5.18 (d, 2H), 5.91 (d, 2H), 8.08 (d, 2H); ^{13}C NMR ($CDCl_3$) δ 17.98, 18.97, 20.38, 23.88, 24.68, 24.87, 27.64, 29.92, 30.69, 42.46, 44.73, 58.07, 61.78, 69.42, 74.15, 76.19, 76.25, 77.20, 81.63, 91.05, 125.3, 134.21, 139.12, 142.08, 148.90, 149.13, 157.05, 171.20, 172.43, 172.83, 176.82; IR 1460, 1700 (br), 3450 (br) cm^{-1}; MS (ES) *m*/*e* 1378.37 (MNa^+), 1400.27 (MNa^+).

[Tris[*N*-succinoyl [(valinyl)-5-fluorouridine] hydroxyamino butyl] isocyanurate (23). To a solution of **19** (40 mg,

0.02 mmol) in 6 mL of MeOH was added 8 mg of 10% Pd–C. This mixture was exposed to H_2 at atmospheric pressure for 4 h (monitored by TLC). The catalyst was removed by filtration and the solvent was removed by evaporation under vacuum. This afforded pure **23** (34.4 mg, 99%) as a clear oil. ^{1}H NMR ($CDCl_3$) δ 0.92–0.99 (d, 18H), 1.45–1.75 (m, 18H), 2.01–2.23 (m, 3H), 2.55–2.75 (m, 12H), 3.62–3.86 (m, 12H), 4.15–4.48 (m, 21H), 5.18 (d, 3H), 5.91 (d, 3H), 8.08 (d, 3H); ^{13}C NMR ($CDCl_3$) δ 17.98, 18.97, 20.38, 23.88, 24.68, 24.87, 27.64, 29.92, 30.69, 42.46, 44.73, 58.07, 61.78, 69.42, 74.15, 76.19, 76.25, 77.20, 81.63, 91.05, 125.3, 134.21, 139.12, 142.08, 148.90, 149.13, 157.05, 171.20, 172.43, 172.83, 176.82; IR 1460, 1700 (br), 3450 (br) cm^{-1}; MS (ES) *m*/*e* 1721.41 (MH^+), 1743.58 (MNa^+).

Biological procedures for antibacterial and antifungal testing

The assay used to determine the antifungal and antibacterial activity of conjugates **21**, **22** and **23** was performed in Luria broth. The yeast strain *C. albicans* (ATCC 48130) was obtained from the American Type Culture Collection, Rockville, MD, and the parent strain *E. coli* X580 was the generous gift of Eli Lilly & Co., Indianapolis, IN. Both strains were stored at −78°C in culture tubes in a 1:1 mixture of Luria broth culture and sterile D-lactose-glycerol solution.

All inorganic compounds were obtained from Fisher. EDDA, the ferric iron chelator used to deferrate the culture medium, was obtained from Sigma and was deferrated using Rogers' procedure,[26] and a stock solution[27] (50 mg/mL) was prepared immediately prior to use. Dehydrated tryptone and yeast extract were all purchased from Fisher. Luria broth was prepared by the procedure of Miller.[28] The ferric iron chelator EDDA was added by sterile filtration through an Acrodisc 4192 sterile assembly (pore size, 0.2 μm; Fisher) to a slightly concentrated culture broth. The final culture volume was typically 100 mL.

Seed cultures were prepared by adding 0.20 mL aliquot of thawed bacteria or fungi in a culture tube containing sterile lactose-glycerol to 100 mL of sterile Luria broth. After overnight incubation at 37°C and 300 rpm, two 10 μL aliquots of the turbid seed culture were transferred to corresponding control and test cultures. The control and test cultures were distributed to a sterile 96-well plate. Stock solutions of conjugates **21**, **22** and **23** were prepared in distilled and deionized water. Then test solutions at different concentrations were made by proper dilution of the stock solution. Preformed Fe(III) complexes of **21**, **22** and **23** were made by addition of an equal volume of test stock solutions and aqueous $FeCl_3$ solution and from those, other test solutions were made by proper dilution with DI water. Solutions of the test compounds were added to the 96-well microplate, which contained sterile Luria broth or sterile Luria broth with EDDA (100 μg/mL), to give the final concentration of the conjugates, respectively.

The 96-well plate containing the cultures was then inoculated at 37°C for 24 h by the Molecular Devices Thermomax® microplate reader. The culture turbity was read at 650 nm automatically by the reader every 30 min.

Acknowledgements

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