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5′-Trityl-Substituted Thymidine Derivatives as a Novel Class of Antileishmanial Agents: *Leishmania infantum* EndoG as a Potential Target

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Two series of 5′-triphenylmethyl (trityl)-substituted thymidine derivatives were synthesized and tested against *Leishmania infantum* axenic promastigotes and amastigotes. Several of these compounds show significant antileishmanial activity, with IC_{50} values in the low micromolar range. Among these, 3′-*O*-(isoleucylisoleucyl)-5′-*O*-(3,3,3-triphenylpropanoyl)thymidine displays particularly good activity against intracellular amastigotes. Assays performed to characterize the nature of parasite cell death in the presence of the tritylthymidines indicated significant alterations in mitochondrial transmembrane potential, an increase in superoxide concentrations, and also significant decreases in DNA degradation during the cell death process. Results point to the mitochondrial nuclease LiEndoG as a target for the action of this family of compounds.

Introduction

Leishmaniasis, one of the most prevalent neglected diseases, is a major health problem in tropical and subtropical countries, with more than 12 million people affected and about 300 million persons at risk of infection. The manifestation of this disease can be cutaneous, diffuse cutaneous, mucocutaneous, or visceral.[1] It is transmitted to humans by the bite of an infected insect vector, the female *Phlebotominae* sand fly. The *Leishmania* parasite is present in two different forms: the promastigote form in the vector, and the amastigote form in the mammalian host.

Unfortunately, the arsenal of available drugs to combat the various forms of leishmaniasis is very limited. Pentavalent antimonials, pentamidine, amphotericin B, and miltefosine are currently the most effective compounds against this family of diseases.[2,3] However, treatment with most of these is usually accompanied by significant side effects. These include severe acute pancreatitis and cardiac arrhythmia for the antimonials; nephrotoxicity or even anaphylaxis for amphotericin B; renal, pancreatic, and hepatic toxicities for pentamidine; and an extremely long half-life for miltefosine that favors the development of resistance, which is a serious problem common to many of these drugs.[4] Therefore, there is an urgent need for the development of new antileishmanial agents based on molecular scaffolds distinct from those explored thus far. It can be speculated that by exploring different molecular frameworks, we may obtain bioactive compounds with a mechanism of action different from those of drugs in current use.

Focusing on nucleoside metabolism, *Leishmania* species lack the metabolic pathway for de novo synthesis of purine nucleosides. Therefore, salvage of purines is an obligatory pathway and has been the focus of considerable interest.[5] In contrast, the situation is quite different for pyrimidines, the metabolism of which has generally been considered less susceptible to therapeutics than the pathway of purine salvage.[6] Indeed, to the best of our knowledge, there are very few examples of pyrimidine nucleosides such as 5-substituted pyrimidine nucleosides[7–10] with reported antileishmanial activity in vitro. Nonetheless, it is reasonable to expect that improved knowledge about the enzymes involved in pyrimidine biosynthesis may afford new targets. In this respect, a thymidine kinase in *L. major* was recently identified and shown to be involved in flagellum formation, promastigote shape and growth, and virulence.[11] Another interesting example comes from the search for inhibitors of the parasitic dUTPase, in which 5′-*O*-trityl-3′-*O*-TBDMSi-2′-deoxyuridine was identified as an inhibitor of *L. donovani* replication, with an IC_{50} value of 17 μM. However, further careful research revealed that this activity could not be ascribed to the inhibition of the related *L. major* dUTPase, as the compound was virtually inactive against the isolated enzyme, indirectly suggesting the involvement of a yet unidentified molecular target.[12]

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Based on these findings, we decided to evaluate our own collection of 5′-trimethylphenyl (trityl)-substituted pyrimidine nucleosides and analogues against *L. infantum* promastigotes. We found that 5′-tritylthymidine derivatives with a valine ester at the 3′-position (compounds **1** and **2**, Figure 1) were able to efficiently inhibit promastigote replication at a concentration of 12.5 μM. Interestingly, the corresponding 3′-hydroxy analogues **3** and **4** (Figure 1) were virtually inactive.

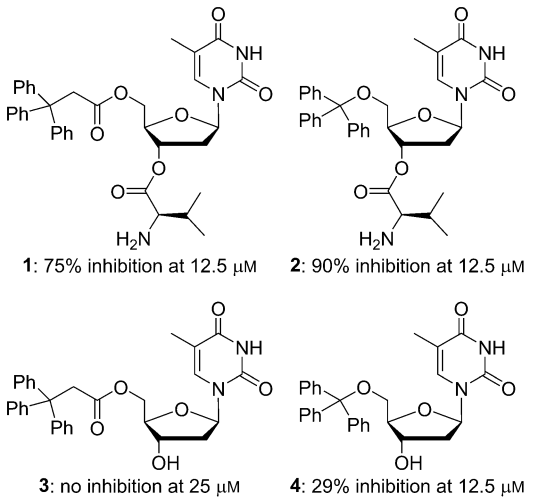


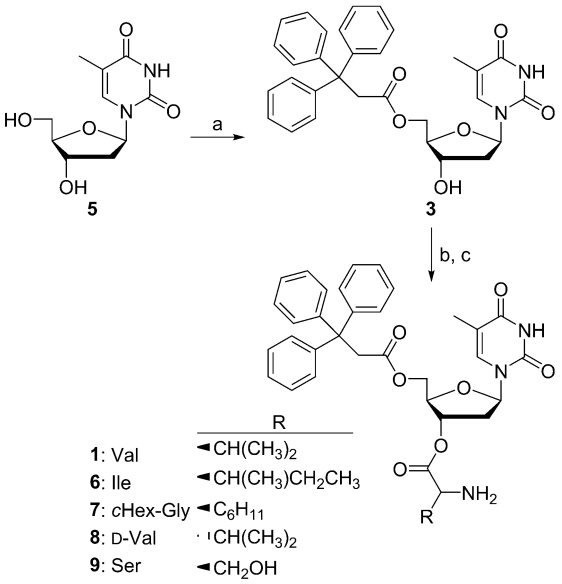
Figure 1. 5′-Trityl nucleosides initially tested against *L. infantum* promastigotes.

The work described herein includes the synthesis and biological evaluation of various analogues of our prototype compounds **1** and **2** by varying the nature of the amino acid at the 3′-position, by modifying the nexus at the 5′-position connecting the nucleoside and the trityl substituent, by introducing subtle changes at the base moiety, and by elongating the substituent at the 3′-position either by preparing dipeptidyl derivatives or by including a spacer between the 3′-hydroxy group and the amino acid residue. The observed changes in mitochondrial activity, together with the surprisingly low percentage of hypoploid cells in the population of treated parasites, led us to the identification of *L. infantum* endonuclease G (LiEndoG), a mitochondrial nuclease, as a target for these molecules.

Results and Discussion

Chemistry

The synthesis of prototype compound **1** and 3′-*O*-aminoacyl analogues **6**–**9** is illustrated in Scheme 1. Reaction of thymidine **5** with 3,3,3-triphenylpropionic acid under Mitsunobu conditions (Ph_3P and DBAB) effectively afforded the 5′-substituted compound **3** in 88% yield. The coupling of **3** with various amino acids protected as their corresponding Boc derivatives was performed in the presence of PyBOP and triethylamine. The amino acid derivatives incorporated were Boc-Val-OH, Boc-Ile-OH, Boc-*c*HexGly-OH, Boc-D-Val-OH, and Boc-Ser(OSi-



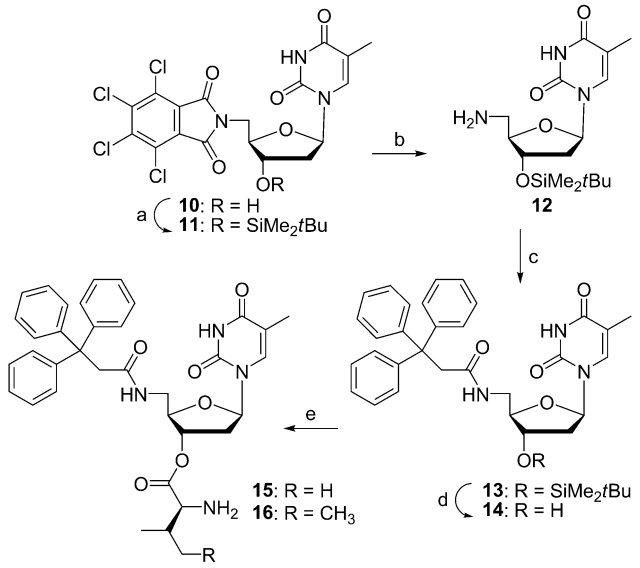
Scheme 1. *Reagents and conditions:* a) triphenylpropionic acid (2.0 equiv), PPh_3 (2.0 equiv), DBAD (2.0 equiv), DMF, 0 °C, 1.5 h, 88%; b) 1. Boc-Xaa-OH (2.0 equiv), PyBOP (1.2 equiv), Et_3N (2.5 equiv), CH_2Cl_2, 0 °C, overnight; 2. TFA (23 equiv), CH_2Cl_2, RT, 3–5 h (**1**, 63%; **6**, 63%; **7**, 52%; **8**, 62%; **9**, 52%).

Me_2tBu)-OH. After coupling, treatment with trifluoroacetic acid (TFA) removed the Boc protecting group together with the Si-Me_2tBu group in the side chain of the Ser derivative to afford the NH_2-free compounds **1** and **6**–**9**. Based on the results obtained and synthetic accessibility, the amino acids of choice for the next series of compounds were L-Val and L-Ile.

Our next aim was to modify the linker at the 5′-position connecting the nucleoside and the trityl substituent by replacement of the ester group at the 5′-position by the corresponding amide. This required the synthesis of 5′-amino-5′-deoxythymidine as the key intermediate that had been described by Telzlaff et al.[13] Thus, treatment of thymidine with tetrachlorophthalimide under Mitsunobu conditions, as described, afforded tetrachlorophthalimido compound **10** (Scheme 2). However, treatment of **10** with hydrazine hydrate to obtain 5′-amino-5′-deoxythymidine gave, in our hands, a compound difficult to isolate and handle. We therefore decided to introduce a protecting group at the 3′-position of **10** prior to its transformation into the amino derivative.

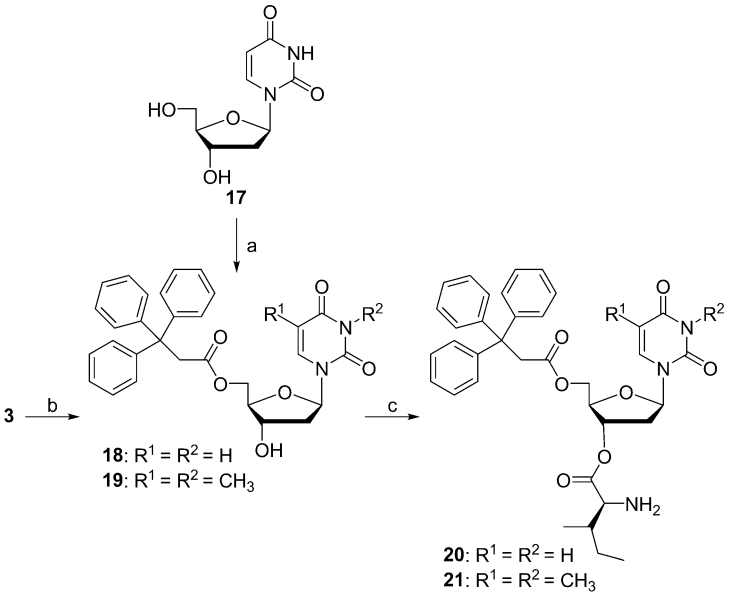
Thus, compound **10** was allowed to react with $tBuMe_2SiCl$ in the presence of imidazole to provide the 3′-protected nucleoside **11** (Scheme 2). Reaction of **11** with hydrazine hydrate led to the 5′-amino derivative **12** in 96% yield. This amino nucleoside was subjected to a BOP-mediated coupling with 3,3,3-triphenylpropionic acid to afford amide **13** in excellent yield. Removal of the 3′-*O*-silyl protecting group (compound **14**) followed by coupling of the amino acids at the 3′-position and Boc deprotection afforded the desired nucleosides **15** and **16**, with Val and Ile residues at the 3′-position, respectively (Scheme 2).

Our next goal was to evaluate how subtle modifications at the pyrimidine base of the nucleoside affects their activity

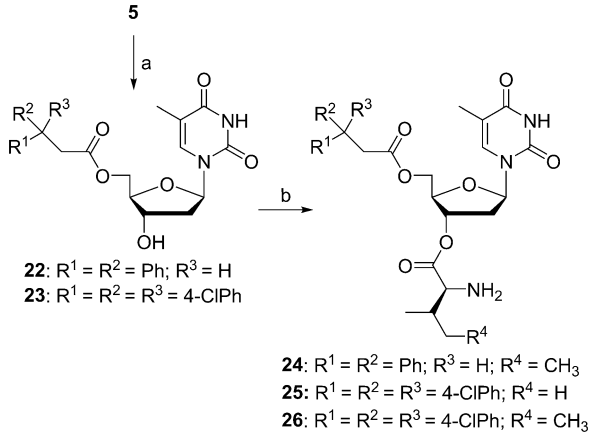


Scheme 2. *Reagents and conditions:* a) TBDMSCl (1.5 equiv), imidazole (3.0 equiv), DMF, RT, overnight, 77%; b) $H_2NNH_2H_2O$ (3.0 equiv), EtOH, reflux, 1 h, 96%; c) triphenylpropionic acid (1.5 equiv), BOP (1.5 equiv), Et_3N (1.5 equiv), CH_2Cl_2, RT, overnight 95%; d) 1 N HCl, THF, RT, overnight, 93%; e) 1. Boc-Val-OH or Boc-Ile-OH (2.0 equiv), PyBOP (1.2 equiv), Et_3N (2.5 equiv), CH_2Cl_2, 0 °C, overnight; 2. TFA (23 equiv), CH_2Cl_2, RT, 3–5 h (**15**, 60%; **16**, 70%).

against in vitro parasite growth. Reaction of 2′-deoxyuridine (**17**, Scheme 3) with 3,3,3-triphenylpropionic acid under Mitsunobu conditions afforded the 5′-substituted nucleoside **18** in 70% yield. Alternatively, reaction of the 5′-substituted thymidine derivative **3** with methyl iodide in acetone in the presence of potassium carbonate under microwave (MW) irradiation at 100 °C for 1 h led to the 3-*N*-methyl analogue **19** in 85% yield. Both nucleosides **18** and **19** were subjected to PyBOP-mediated coupling with Boc-Ile-OH followed by treatment with TFA to yield the respective Ile derivatives **20** and **21**.



Scheme 3. *Reagents and conditions:* a) triphenylpropionic acid (2.0 equiv), PPh_3 (2.0 equiv), DBAD (2.0 equiv), DMF, 0 °C, 1.5 h, 70%; b) MeI (4.0 equiv), K_2CO_3 (0.5 equiv), acetone, 100 °C, 1 h, MW, 85%; c) 1. Boc-Ile-OH (2.0 equiv), PyBOP (1.2 equiv), Et_3N (2.5 equiv), CH_2Cl_2, 0 °C, overnight; 2. TFA (23 equiv), CH_2Cl_2, RT, 3–5 h (**20**, 63%; **21**, 80%).

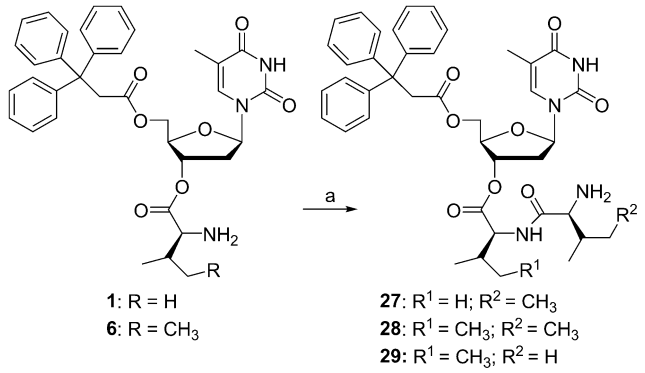


Scheme 4. *Reagents and conditions:* a) diphenylpropionic acid or 3,3,3-tris(4-chlorophenyl)propionic acid (2.0 equiv), PPh_3 (2.0 equiv), DBAD (2.0 equiv), DMF, 0 °C, 1.5 h (**22**, 51%; **23**, 61%); b) 1. Boc-Ile-OH or Boc-Val-OH (2.0 equiv), PyBOP (1.2 equiv), Et_3N (2.5 equiv), CH_2Cl_2, 0 °C, overnight; 2. TFA (23 equiv), CH_2Cl_2, RT, 3–5 h (**24**, 58%; **25**, 55%; **26**, 66%).

We also modified the ester group at the 5′-position of the nucleoside by replacing the triphenylpropanoyl group with structurally related analogues. For this purpose, we selected two commercial acids: 3,3-diphenylpropionic acid and 3,3,3-tris(4-chlorophenyl)propionic acid. Thus, reaction of thymidine **5** with the above-mentioned acids under Mitsunobu conditions afforded the 5′-substituted thymidine derivatives **22** and **23** in 51 and 61% yields, respectively (Scheme 4). Reaction of **22** with Boc-Ile-OH followed by treatment with TFA yielded **24** (58% yield). Similarly, reaction of **23** with Boc-Val-OH and Boc-Ile-OH, followed by Boc removal, led to the target compounds **25** and **26** in 55 and 66% respective yields.

We next focused our interest on the 3′-position of the nucleoside. Starting from the 3′-*O*-aminoacyl derivatives **1** and **6**, we decided to incorporate an additional amino acid to prepare dipeptidyl derivatives. Thus, reaction of **1** with Boc-Ile-OH and treatment with TFA afforded the dipeptidyl nucleoside **27** (Scheme 5). Similarly, reaction of the isoleucyl derivative **6** with Boc-Ile-OH or Boc-Val-OH, followed by Boc removal, yielded the Ile-Ile compound **28** and the Val-Ile analogue **29**.

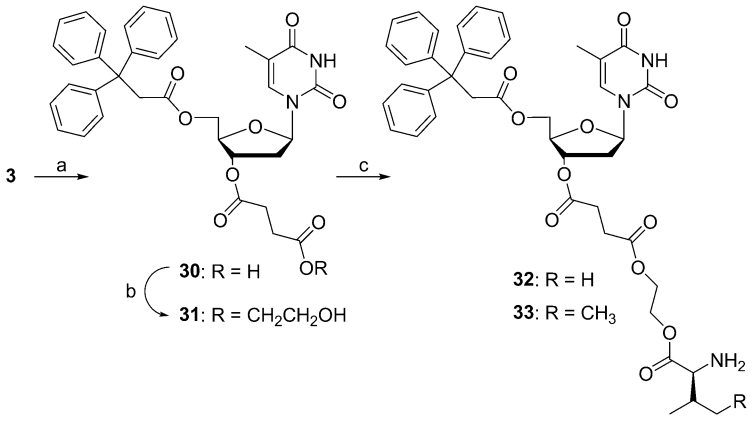
A second series of modifications at the 3′-position of the prototype compound **1** consisted of introducing a spacer between the 3′-hydroxy group of the nucleoside and the amino acid derivative. The proposed spacer incorporated a succinate and an ethylene glycol, as recently employed in the coupling of amino acids to the antiviral cidofovir.[14] Thus, reaction of **3** with succinic anhydride in pyridine in the presence of DMAP afforded the hemisuccinate **30** in



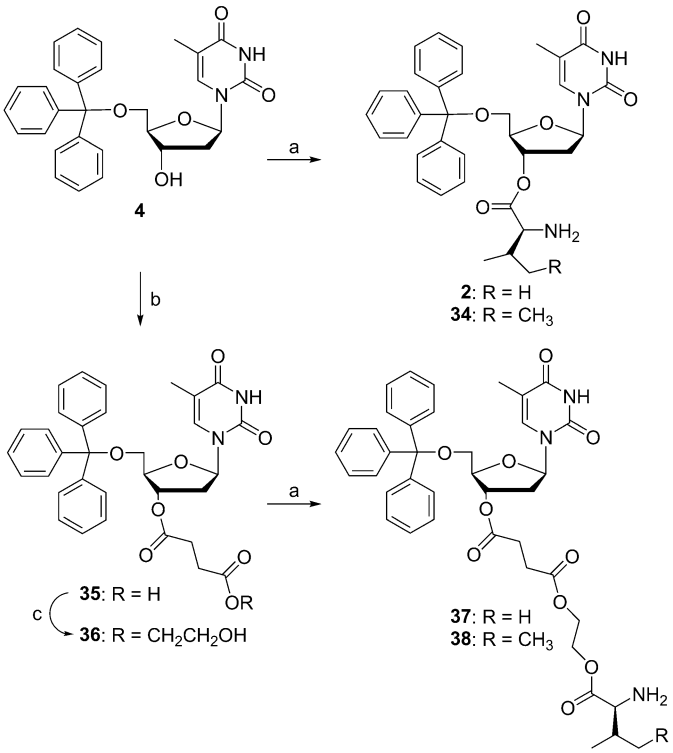
Scheme 5. *Reagents and conditions:* a) 1. Boc-Ile-OH or Boc-Val-OH (2.0 equiv), PyBOP (1.2 equiv), Et_3N (2.5 equiv), CH_2Cl_2, 0 °C, overnight; 2. TFA (23 equiv), CH_2Cl_2, RT, 3–5 h (**27**, 54%; **28**, 50%; **29**, 82%).

82% yield (Scheme 6). Further reaction of **30** with ethylene glycol using PyBOP as the condensation agent afforded alcohol **31** (53% yield). This alcohol was treated with Boc-Val-OH or Boc-Ile-OH in the presence of PyBOP, followed by removal of the Boc protecting group. In this way, conjugates **32** and **33** were obtained (36 and 35% respective yields for the two steps).

The trityl ether **2** had also been identified as an interesting hit in our initial screen of *L. infantum* promastigote replication inhibitors. Based on the significant structural similarity between ester **1** and ether **2**, in the trityl ether series we performed only those modifications that led to the most successful compounds in the ester series. Condensation of 5′-*O*-tritylthymidine[15] (**4**, Scheme 7) with Fmoc-Val-OH or Fmoc-Ile-OH was performed in the presence of PyBOP, followed by removal of the Fmoc protecting group with piperidine. In this way the 3′-*O*-Val **2** and 3′-*O*-Ile derivatives **34** were obtained (74 and 65% yield, respectively). On the other hand, reaction of 5′-*O*-tritylthymidine (**4**) with succinic anhydride in dichloromethane in the presence of triethylamine and DMAP afforded



Scheme 6. *Reagents and conditions:* a) succinic anhydride (2.0 equiv), DMAP (1.0 equiv), pyridine, RT, overnight (82%); b) ethylene glycol (10.0 equiv), PyBOP (1.3 equiv), Et_3N (1.3 equiv), CH_2Cl_2, RT, overnight (53%); c) 1. Boc-Val-OH or Boc-Ile-OH (2.0 equiv), PyBOP (1.2 equiv), Et_3N (2.5 equiv), CH_2Cl_2, 0 °C, overnight; 2. TFA (23 equiv), CH_2Cl_2, RT, 3–5 h (**32**, 36%; **33**, 35%).



Scheme 7. *Reagents and conditions:* a) 1. Fmoc-Val-OH or Fmoc-Ile-OH (2.0 equiv), PyBOP (1.2 equiv), Et_3N (2.5 equiv), CH_2Cl_2, 0 °C, overnight; 2. piperidine, CH_2Cl_2, RT, 1 h (**2**, 74%; **34**, 65%); b) succinic anhydride (2.0 equiv), DMAP (1.0 equiv), pyridine, RT, 7 h (**37**, 96%; **38**, 71%).

the hemisuccinate **35** in 82% yield. BOP-mediated condensation with ethylene glycol gave alcohol **36** (92% yield), which was coupled to Fmoc-Val-OH or Fmoc-Ile-OH, followed by piperidine treatment to afford conjugates **37** and **38** (96 and 71% respective yields).

Biological evaluation

The synthesized compounds were initially screened against the promastigote form of *L. infantum*. Those compounds showing significant activity in this assay ($IC_{50} < 25$ μM) were also tested against amastigotes and, to measure their cytotoxicity against human cell lines, proliferating Jurkat cells. The IC_{50} values obtained are listed in Table 1. Miltefosine was included as a reference compound.

In the trityl ester series and concerning the evaluation of the 3′-*O*-aminoacyl derivatives **1** and **6**–**9** against promastigotes, it is clear that the presence of an amino acid is required for effective inhibition. Moreover, the nature of the amino acid side chain has a clear impact on the antileishmanial effect. Whereas the L-Ser derivative **9** was virtually inactive ($IC_{50} > 25$ μM), moderate activity (5 μM $< IC_{50} <$ 10 μM) was observed for L- and D-Val derivatives **1** and **8**, and good activity ($IC_{50} < 5$ μM) was shown when L-Ile and L-*c*HexGly were included at the 3′ position (com-

Table 1. In vitro activity of the synthesized trityl esters, amides, and ethers against the promastigote form of *L. infantum*, the amastigote form of *L. infantum*, and Jurkat cells.[a]

Compd	IC_{50} [μM] Promastigote	Amastigote	Jurkat
1	7.67 ± 0.21	> 25	16.9 ± 0.5
2	2.88 ± 0.13	10.63 ± 0.44	17.1 ± 2.2
3	> 25	ND	ND
4	> 25	ND	ND
6	3.4 ± 0.6	7.33 ± 0.04	9.87 ± 1.8
7	3.5 ± 0.6	6.33 ± 0.01	13.9 ± 0.7
8	6.51 ± 0.03	22.60 ± 1.52	15.3 ± 0.4
9	> 25	ND	ND
15	> 25	ND	ND
16	> 25	ND	ND
20	7.31 ± 3.22	12.76 ± 0.25	9.3 ± 0.3
21	5.87 ± 0.75	9.68 ± 0.36	10.6 ± 0.9
24	17.29 ± 1.64	> 25	22.4 ± 1
25	2.59 ± 0.24	4.49 ± 0.52	6.83 ± 0.1
26	6.56 ± 0.05	7.07 ± 0.02	9.9 ± 0.3
27	6.67 ± 0.23	22.33 ± 0.31	16.2 ± 1.1
28	1.31 ± 0.09	11.75 ± 0.74	15.9 ± 0.6
29	5.99 ± 0.33	13.42 ± 0.14	8.28 ± 1.0
32	6.38 ± 1.17	14.52 ± 1.40	24.9 ± 2.3
33	3.78 ± 0.14	8.65 ± 0.51	20.9 ± 1.2
34	2.22 ± 0.09	6.11 ± 0.01	7.0 ± 0.9
37	4.33 ± 0.05	6.44 ± 0.01	17.2 ± 0.9
38	4.51 ± 0.65	7.37 ± 0.02	15.6 ± 1.5
miltefosine	46.45 ± 3.03	3.96 ± 0.53	48

[a] Values are expressed as the mean ± SEM of three independent experiments; ND: not determined.

pounds **6** and **7**, respectively). Therefore, it can be concluded that a hydrophobic side chain of the amino acid is the preferred substituent for this position. Compounds **6** and **7** maintained good activity when assayed against amastigotes.

Interestingly, the antiparasitic effect was significantly decreased by changing the linker between the trityl substituent and the 5′-position of the nucleoside from an ester to an amide (compare esters **1** and **6** with the corresponding amides **15** and **16**). On the other hand, subtle changes in the pyrimidine base (while maintaining L-Ile at the 3′-position) decreased activity against promastigotes, as evidenced by the fact that both the uracil (**20**) and 3-*N*-methylthymine (**21**) derivatives showed IC_{50} values > 5 μM. The IC_{50} value increased significantly if the triphenylmethyl ester was replaced by the corresponding diphenylmethyl derivative (compound **24**). On the other hand, the 5′-tris(4-chlorophenyl)propanoyl compounds with either Val or Ile at the 3′-position (**25** and **26**, respectively) maintained IC_{50} values close to 5 μM in promastigotes, showing a strikingly similar activity in amastigotes. IC_{50} values against Jurkat cells are twofold higher than against the parasites. Moving back to the triphenylpropanoyl compounds, coupling of dipeptides at the 3′-position (**27**–**29**), or incorporation of a succinate-glycol spacer between the nucleoside and the amino acid (**32** and **33**) caused minimal variations in IC_{50} values relative to the single amino acid derivatives **1** and **6**, except that toxicity toward Jurkat cells was notably decreased for most of them. In general, the compounds are less active against amastigotes than against promastigotes, yet a significant number of them show IC_{50} values in the low micromolar range (< 10 μM) against the amastigote form of the parasite. Among the most active compounds are Ile derivative **6**, *c*HexGly derivative **7**, 5′-tris(4-chlorophenyl)propanoyl compounds with Val or Ile at the 3′-position (**25** and **26**, respectively), those with dipeptides coupled at the 3′-position (compound **28**), and the succinate-glycol-Ile derivative **33**.

Concerning the trityl ether derivatives **2**, **4**, **34**, **37**, and **38**, all of the 3′-substituted compounds were active against *L. infantum* promastigotes, with IC_{50} values < 5 μM. Except for compound **2** ($IC_{50} \approx 10$ μM), all of them also showed significant inhibitory activity against amastigotes, with IC_{50} values in the 5 μM range. Also in this case, IC_{50} values against Jurkat cells were three- to fourfold higher than against the parasites.

Leishmanicidal activity was also assayed in amastigote-infected phorbol myristate acetate (PMA)-differentiated THP-1 cells by flow cytometry.[16] Seven compounds were selected on the basis of their high activity against axenic parasites and moderate toxicity in Jurkat cells. Accordingly, compounds **2**, **6**, **28**, **32**, **33**, **37**, and **38** were assayed over the course of 48 h in differentiated THP-1 cells infected with *L. infantum* amastigotes expressing green-fluorescent protein (eGFP); infected cells were identified by the green fluorescence emitted by their intracellular amastigotes (Figure 2).

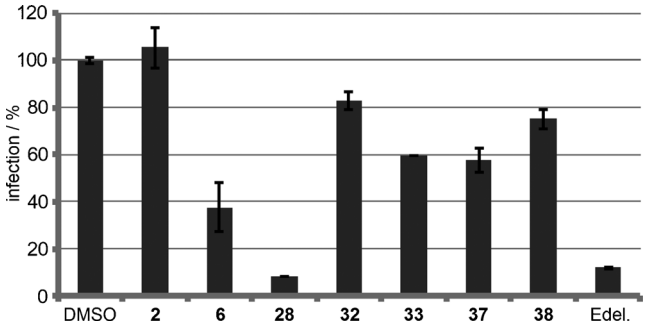


Figure 2. Inhibition of intracellular infection rates: PMA-differentiated THP-1 cells were infected with amastigotes expressing green-fluorescent protein (eGFP). Percentages of infection were evaluated by counting the number of green-fluorescent PMA-differentiated THP-1 cells by flow cytometry. All compounds were assayed at 15 μM. Edelfosine (Edel.) was assayed at 3 μM. Error bars represent standard deviation; results are representative of three independent experiments.

Most of the compounds assayed were able to decrease the number of infected cells by approximately 20–40%, but only compound **28** showed a leishmanicidal activity against intracellular parasites similar to that observed for the control drug edelfosine (87% decrease in the number of infected macrophages), with an estimated IC_{50} value of 8.0 ± 0.15 μM. According to these results, compound **28** was selected for the study of death-related processes in the parasites. Changes in the mitochondrial transmembrane potential ($\Delta\Psi_m$) of logarithmically growing *L. infantum* promastigotes were measured with the potentiometric probe TMRM.[17] The results presented in Figure 3a indicate a rapid but transient increase in the mean $\Delta\Psi_m$ when the parasites were incubated with **28** at 10 μM, as

shown by the significant increase in the TMRM-derived fluorescence observed during the first 20 min of treatment. The mean values of fluorescence are restored to that of the control parasites after 80 min exposure to the compound. The mitochondrial proton gradient uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, 100 μM) was added as a positive control of mitochondrial depolarization.

Changes in mitochondrial transmembrane potential are frequently associated with variations in superoxide ($O2^{\bullet-}$) production that can be detected by flow cytometry with specific probes such as MitoSOX™. The increase in MitoSOX-derived fluorescence over the first 20 min of treatment with compound **28**, similar to that observed during the same period in the antimycin-treated cells, is indicative of an accumulation of $O2^{\bullet-}$ inside the mitochondrion (Figure 3b). Accordingly, compound **28** induces changes in the activity of the single mitochondrion of the parasites, causing its sudden hyperpolarization and a concomitant increase in superoxide production that eventually leads to cell death.

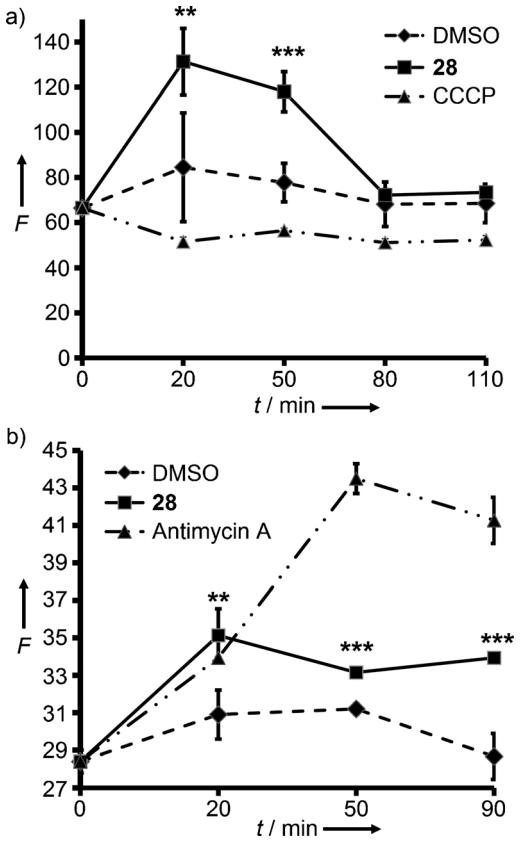


Figure 3. a) Analysis of mitochondrial transmembrane potential ($\Delta\Psi_m$): TMRM-derived fluorescence in *L. infantum* promastigotes treated with compound **28** (10 μM) or with the uncoupler CCCP (100 μM). *Y*-axis values represent the mean fluorescence (*F*) of the entire population of living cells. Parasites treated with an equivalent concentration of DMSO used as solvent are included as a control (DMSO). Fluorescence was analyzed by flow cytometry ($n=3$). Samples were collected and analyzed at 20, 50, 80, and 110 min of treatment. Error bars represent standard deviation; results are representative of three independent experiments. b) Analysis of intra-mitochondrial superoxide concentration: MitoSOX-derived fluorescence in *L. infantum* promastigotes treated with compound **28** (10 μM) or with antimycin A (20 μM). *Y*-axis values represent the mean fluorescence (*F*) of the entire population of living cells. Parasites treated with an equivalent concentration of DMSO used as solvent are included as a control (DMSO). Fluorescence was analyzed by flow cytometry ($n=3$). Samples were collected and analyzed at 20, 50, and 90 min of treatment. Error bars represent standard deviation; results are representative of three independent experiments. The mean value of the treatments differ from that of the DMSO control with p values <0.05 (**) or 0.005 (***).

Figure 4a shows bi-parametric plots of forward scatter (Fs) and propidium iodide (PI) staining of control, staurosporine-, and **28**-treated parasites. The lower right quadrant (C4) represents healthy parasites displaying no alterations in shape or plasma membrane integrity, which constitute 96% of the population of the untreated control parasites. This number is decreased to 54 and 56% as a consequence of 24 h treatment with staurosporine (17 μM) or **28** (10 μM), respectively. Events in any of the other three quadrants are representative of parasites that are either dead or in the process of dying. DNA content in the treated parasites was analyzed by flow cytometry. Surprisingly, although staurosporine and compound **28** at the indicated concentrations cause a similar deleterious effect in the population (Figure 4a), the process of DNA degradation associated with cell death is much lower in **28**-treated parasites (19% of hypoploid cells) than in staurosporine-treated parasites (39% of hypoploid cells) (Figure 4b). The decreased percentage of hypoploid parasites after treatment with **28**, together with its effect on $\Delta\Psi_m$ and $O2^{\bullet-}$ production, led us to postulate that the target for this compound could be a mitochondrial protein that mediates nuclear DNA degradation during the cell-death process. The recently characterized LiEndoG protein fulfills both requisites.[18]

Accordingly, the nuclease activity of this enzyme in the presence of increasing concentrations of **28** was evaluated in a fluorimetric assay. Purified recombinant LiEndoG was incubated with a double-stranded DNA probe (16 nucleotides long) that contains 6-carboxyfluorescein (FAM) attached to both 3′ ends and tetramethylrhodamine (TAMRA) bound to both 5′ ends. In the undigested probe, FAM-derived fluorescence is quenched by the proximity of TAMRA. Digestion of any of the two oligonucleotides causes separation of the fluorophore from the quencher, so that a fluorescent signal can then be emitted. Fluorescence generated as a consequence of the nuclease activity of LiEndoG clearly diminishes in the presence of increasing concentrations of compound **28** (Figure 5a). This inhibitory effect on LiEndoG was also assayed for compounds **3**, **4**, **6**, and **25**. Reactions in the presence of compounds **3** and **4** at 25 μM were very slightly inhibited relative to the control reaction, which indicates that the substituent at the 3′ position is important for LiEndoG inhibitory activity. Compound **6** showed moderate inhibitory activity, whereas compounds **25** and **28** displayed high capacity for LiEndoG inhibition. Thus, the ability of the compounds to inhibit LiEndoG activity strongly correlates with their leishmanicidal capacity, and the presence of substituents at the 3′-position is essential for both activities.

Based on the ability of compound **28** to inhibit LiEndoG activity and also to decrease intracellular infection rates without causing severe cell damage, we decided to gauge the evolution of this compound in PMA-differentiated THP-1 cells. Thus a sample of **28** was incubated with in cell-free extract of these

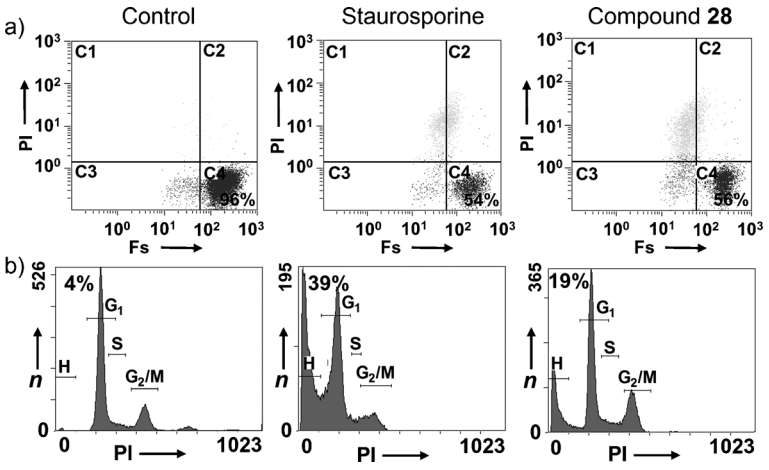


Figure 4. DNA degradation in treated parasites: a) Bi-parametric plots of forward scatter (Fs) and propidium iodide (PI) staining of DMSO (Control), 17 μM staurosporine- and 10 μM compound-**28**-treated parasites. Healthy parasites without alterations in shape or plasma membrane integrity are located in the lower right quadrant (C4). b) DNA content in the parasite populations shown in panel a). Cell-cycle distributions of the parasites along the G_1, S, or G_2/M phases are indicated. Percentages of parasites with decreased DNA content (hypoploid parasites) are indicated (H); *n* = number of events; PI = propidium iodide-derived fluorescence.

cells at 37 °C, and the stability of the compound was monitored by HPLC at various time points. Compound **28** evolved toward the Ile derivative **6** and further to the free 3′-hydroxy compound **3**. In contrast, compound **28** is perfectly stable in PBS after 24 h incubation at 37 °C, and no hydrolysis products were detected.

Therefore, it can be hypothesized that the presence of the dipeptide at the 3′-position in compound **28** favors entrance into the differentiated THP-1 cells, where it exerts its leishmanicidal effect until it is enzymatically cleaved toward the Ile derivative **6** and eventually to **3**. It must be pointed out that compound **6** also has significant leishmanicidal activity and is able to cause moderate inhibition of LiEndoG, which may extend the initial activity of compound **28** once it has been modified by endogenous esterases.

The results reported above suggest that LiEndoG is a likely target for the compounds described herein. EndoGs are members of a family of DNA/RNA nonspecific ββα metal nucleases that have been demonstrated to take part in the apoptotic process, whereby they translocate to the nucleus and contribute to the degradation of genomic DNA into oligonucleosomal fragments.[18] Apart from their involvement in the death process, these nucleases have been shown to be necessary for the proper growth of several unicellular organisms, indicating a dual (pro-life and pro-death) function, most likely due to their postulated role in mitochondrial DNA recombination.[19–22] A recent study reveals that *EndoG*$^{-/-}$ mice have impaired mitochondrial respiration and increased production of reactive oxygen species, also associated with a decrease in the ratios of mitochondrial to genomic DNA.[23] Accordingly, blockage of these pro-life activities of LiEndoG may be responsible for the antileishmanial activity of this family of compounds.

Conclusions

Two series of 5′-trityl-substituted thymidine derivatives were synthesized and assayed against *L. infantum* promastigotes and amastigotes. A significant number of these compounds showed pronounced inhibitory activity against *L. infantum* promastigotes, and had slightly higher IC_{50} values against amastigotes. Because of its good activity against intracellular amastigotes, compound **28** was selected for analysis of its cellular effects. Profound changes in mitochondrial $\Delta\Psi_m$ and $O_2^{\bullet-}$ production were observed as early as 20 min after compound addition. Because of the low percentage of hypoploid parasites observed after **28**-induced cell death, the nuclease LiEndoG was assayed as a putative target for this family of compounds. The activity of this enzyme in the presence of selected compounds was significantly inhibited. The varying activity of the derivatives against intracellular amastigotes strongly correlates with their ability to inhibit this enzyme. Taken together, LiEndoG can be considered a macromolecular target responsible for the toxic activity of these compounds on *L. infantum*.

Experimental Section

Chemistry

General procedures: Melting points were obtained on a Reichert-Jung Kofler apparatus and are uncorrected. Microanalysis was obtained with a Heraeus CHN-O-RAPID instrument. The elemental compositions of the compounds agreed to within ±0.4% of the calculated values. Electrospray mass spectra were measured on a quadrupole mass spectrometer equipped with an electrospray source (Hewlett–Packard, LC–MS HP 1100). ^{1}H and ^{13}C NMR spectra were recorded on a Varian INNOVA 300 operating at 299 (^{1}H) and 75 MHz (^{13}C), and a Varian INNOVA-400 operating at 399 (^{1}H) and 99 MHz (^{13}C).

Analytical TLC was performed on silica gel 60 F_{254} (Merck) precoated plates (0.2 mm). Spots were detected under UV light (254 nm) and/or by charring with phosphomolybdic acid. Separations on silica gel were performed by preparative centrifugal circular thin-layer chromatography (CCTLC) on a Chromatotron (Kieselgel 60 PF_{254} gipshaltig, Merck), with a layer thickness of 1 and 2 mm and flow rate of 4 or 8 mL min^{-1}, respectively. Flash chromatography was performed using a force flow Horizon HPFG system (Biotage) with Flash 25 or 40 silica gel cartridges.

All experiments involving water-sensitive compounds were conducted under scrupulously dry conditions. Et_3N and CH_3CN were dried by holding at reflux over CaH. THF was dried by reflux over sodium/benzophenone. Anhydrous *N,N*′-dimethylformamide (DMF) was purchased from Aldrich. Microwave reactions were performed

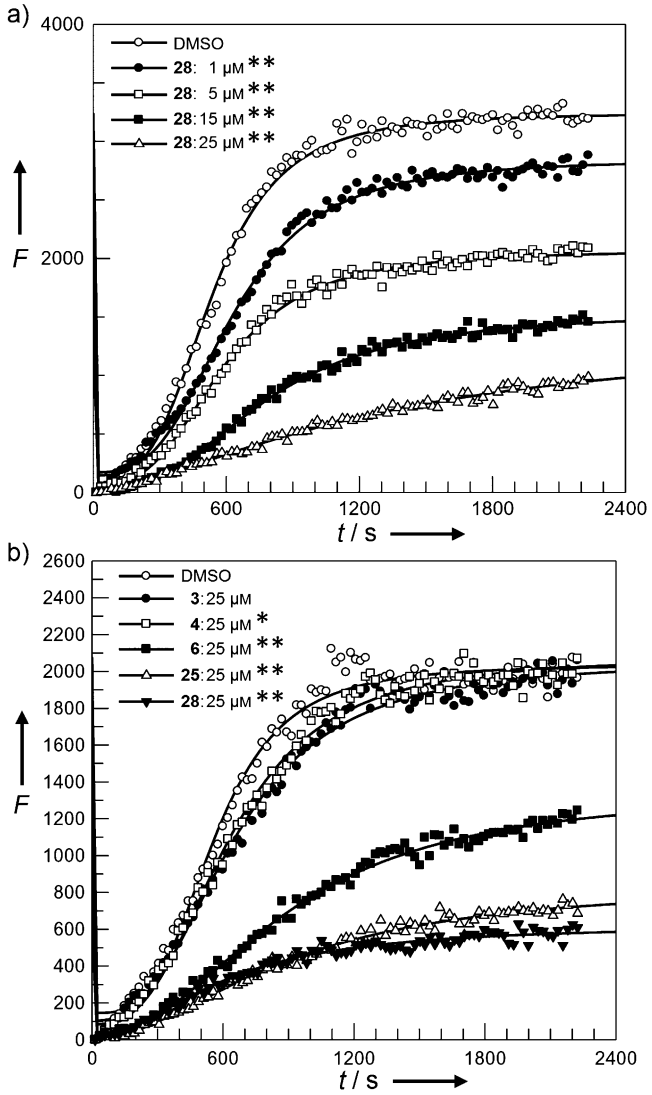


Figure 5. Inhibition of LiEndoG activity was assayed by the cleavage of a dual-labeled double-stranded probe. a) LiEndoG-mediated DNA degradation is followed by an increase in fluorescence (*F*) observed over 40 min of probe digestion in the presence of increasing amounts of compound **28**. b) LiEndoG-mediated DNA degradation in the presence of compounds **3**, **4**, **6**, **25**, and **28** at 25 μM. The slopes of the linear section of the curves differ from that of the DMSO control with *p* values $<10^{-6}$ (**) or 10^{-3} (*).

with a Biotage Initiator 2.0 single-mode cavity instrument from Biotage (Uppsala, Sweden). Experiments were carried out in sealed microwave process vials using the standard absorbance level (400 W maximum power). The temperature was measured with an IR sensor on the outside of the reaction vessel.

5′-*O*-(3,3,3-Triphenylpropanoyl)thymidine (3): A solution of dry di-*tert*-butylazodicarboxylate (DBAD; 460 mg, 2.0 mmol) in DMF (2.5 mL) was added dropwise to a solution containing thymidine (**5**; 242 mg, 1.0 mmol), triphenylpropionic acid (604 mg, 2.0 mmol), and Ph_3P (525 mg, 2.0 mmol) in dry DMF (2.5 mL) at 0 °C. The mixture was stirred at 0 °C under argon for 1.5 h. MeOH (2.5 mL) was added, and volatiles were removed. The residue was treated with 4 N HCl in dioxane (5 mL), and stirred for an additional hour. After removal of the solvent in vacuo, the residue was dissolved in CH_2Cl_2 (50 mL) and washed with 4 N HCl (30 mL) and a solution of $NaHCO_3$ (30 mL). The organic phase was dried on anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified by flash chromatography (CH_2Cl_2/MeOH, 100:0→96:4) to yield 463 mg (88%) of **3** as a white solid; mp: 94–96 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 549 $[M+Na]^+$; ^{1}H NMR ($CDCl_3$): $\delta=1.85$ (d, $J=1.0$ Hz, 3H, 5-CH_3), 1.95 (m, 1H, H-2′), 2.27 (m, 1H, H-2″), 3.67 (d, $J=15.6$ Hz, 1H, CH_2CO), 3.74 (m, 1H, H-4′), 3.82 (m, 1H, H-3′), 3.91 (d, $J=15.6$ Hz, 1H, CH_2CO), 3.94 (dd, $J=12.1$, 4.1 Hz, 1H, H-5′), 4.17 (dd, $J=12.1$, 4.3 Hz, 1H, H-5″), 6.15 (pseudo-t, $J=6.4$ Hz, 1H, H-1′), 7.15 (d, $J=1.1$ Hz, 1H, H-6), 7.20–7.29 (m, 15H, CPh_3), 8.31 ppm (br s, 1H, 3-NH); Anal. calcd for $C_{31}H_{30}N_2O_6$: C 70.71, H 5.74, N 5.32, found: C 70.54, H 5.66, N 5.19.

General procedure for the synthesis of 3′-amino acid ester derivatives: To a solution of the nucleoside derivative (1.0 mmol) in CH_2Cl_2 (5 mL) at 0 °C, the corresponding Boc-protected amino acid (2.0 mmol), PyBOP (624 mg, 1.2 mmol), and Et_3N (345 μL, 2.5 mmol) were added, and the solution was adjusted to pH 11 by the addition of DMAP. The reaction mixture was stirred overnight, diluted with CH_2Cl_2 (50 mL), and washed with a 10% aqueous solution of citric acid (3×20 mL), H_2O (3×20 mL), a 5% aqueous solution of $NaHCO_3$ (3×20 mL), and brine (3×20 mL). The organic phase was dried on anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified as specified. The fractions containing the desired compound were evaporated. A solution of the Boc-protected compound (1.0 mmol) in CH_2Cl_2 (10 mL) was treated with trifluoroacetic acid (TFA; 1.7 mL, 23 mmol). The reaction mixture was stirred at room temperature for 3–5 h, and volatiles were removed. The residue was purified by CCTLC in the Chromatotron, as specified in each case.

5′-*O*-(3,3,3-Triphenylpropanoyl)-3′-*O*-valylthymidine (1): Reaction of **3** (100 mg, 0.19 mmol) with Boc-Val-OH (82 mg, 0.38 mmol), PyBOP (118 mg, 0.23 mmol), and Et_3N (66 μL, 0.47 mmol) afforded a residue that was purified in the Chromatotron (hexane/EtOAc, 1:1) to yield 103 mg of the Boc-protected ester [MS (ES, +) *m/z* 726 $[M+1]^+$]. Treatment of this compound with TFA, as described in the general procedure, afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 75 mg (63%, two steps) of **1** as a white solid; mp: 85–86 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 626 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta=0.81$ (d, $J=6.8$ Hz, 3H, CH_3-γ), 0.86 (d, $J=6.8$ Hz, 3H, CH_3-γ), 1.72 (s, 3H, 5-CH_3), 1.85 (m, 1H, CH-β), 2.17 (m, 1H, H-2′), 2.34 (m, 1H, H-2″), 3.14 (d, $J=5.2$ Hz, 1H, CH-α), 3.84 (s, 2H, CH_2CO), 3.87–4.00 (m, 3H, H-4′, H-5′), 4.97 (m, 1H, H-3′), 6.12 (m, 1H, H-1′), 7.15–7.27 (m, 15H, CPh_3), 7.44 (s, 1H, H-6), 11.37 ppm (br s, 1H, NH); Anal. calcd for $C_{36}H_{39}N_3O_7$: C 69.10, H 6.28, N 6.72, found: C 68.97, H 6.31, N 6.80.

3′-*O*-Isoleucyl-5′-*O*-(3,3,3-triphenylpropanoyl)thymidine (6): Following the general procedure, reaction of **3** (100 mg, 0.19 mmol) with Boc-Ile-OH (87 mg, 0.38 mmol), PyBOP (118 mg, 0.23 mmol), and Et_3N (66 μL, 0.47 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield the Boc-protected ester [MS (ES, +) *m/z*: 762 $[M+Na]^+$]. Treatment of this compound with TFA afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 76 mg (63%, two steps) of **6** as a white solid; mp: 78–79 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 640 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta=0.79$–0.84 (m, 6H, CH_3-γ, CH_3-δ), 1.12 (m, 1H, CH′-γ), 1.40 (m, 1H, CH″-γ), 1.58 (m, 1H, CH-β), 1.72 (s, 3H, 5-CH_3), 2.18 (m, 1H, H-2′), 2.34 (m, 1H, H-2″), 3.18 (d, $J=5.6$ Hz, 1H, CH-α), 3.83 (s, 2H, CH_2CO), 3.88–4.00 (m, 3H, H-4′, H-5′), 4.96 (m, 1H, H-3′), 6.12 (dd, $J=6.6$, 6.3 Hz, 1H, H-1′), 7.14–7.30 (m, 15H, CPh_3), 7.44 (s, 1H, H-6), 11.36 ppm (br s, 1H, NH); Anal. calcd for $C_{37}H_{41}N_3O_7$: C 69.47, H 6.46, N 6.57, found: C 69.19, H 6.44, N 6.51.

3′-*O*-Cyclohexylglycyl-5′-*O*-(3,3,3-triphenylpropanoyl)thymidine (7): Following the general procedure, reaction of **3** (90 mg, 0.17 mmol) with Boc-*c*HexGly-OH (88 mg, 0.34 mmol), PyBOP (107 mg, 0.20 mmol) and Et_3N (59 μL, 0.43 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 20:1) to yield the corresponding ester [MS (ES, +) *m/z* 788 $[M+Na]^+$]. Treatment of this compound with TFA and purification in the Chromatotron (CH_2Cl_2/MeOH, 10:1) afforded 59 mg (52%, two steps) of **7** as a white solid; mp: 84–86 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 666 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 0.97–1.16 (m, 5H, *c*Hex), 1.48–1.70 (m, 6H, *c*Hex), 1.72 (d, *J* = 1.0 Hz, 3H, 5-CH_3), 2.18 (m, 1H, H-2′), 2.32 (m, 1H, H-2″), 3.19 (d, *J* = 5.6 Hz, 1H, CH-α), 3.83 (s, 2H, CH_2CO), 3.88 (m, 1H, H-4′), 3.95 (m, 2H, H-5′), 4.96 (m, 1H, H-3′), 6.12 (dd, *J* = 7.9, 6.4 Hz, 1H, H-1′), 7.14–7.27 (m, 15H, CPh_3), 7.44 (d, *J* = 1.0 Hz, 1H, H-6), 11.33 ppm (s, 1H, 3-NH); Anal. calcd for $C_{39}H_{43}N_3O_7$: C 70.36, H 6.76, N 4.59, found: C 70.12, H 6.91, N 4.62.

5′-*O*-(3,3,3-Triphenylpropanoyl)-3′-*O*-D-valylthymidine (8): Following the general procedure, reaction of **3** (122 mg, 0.23 mmol) with Boc-D-Val-OH (100 mg, 0.46 mmol), PyBOP (140 mg, 0.27 mmol), and Et_3N (80 μL, 0.56 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 30:1) to yield the desired ester [MS (ES, +) *m/z* 748 $[M+Na]^+$]. Treatment of the Boc-protected ester with TFA afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 89 mg (62%, two steps) of **8** as a white solid; mp: 66–68 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 626 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 0.84 (d, *J* = 6.8 Hz, 3H, CH_3-γ), 0.88 (d, *J* = 6.8 Hz, 3H, CH_3-γ), 1.72 (d, *J* = 1.2 Hz, 3H, 5-CH_3), 1.85 (m, 1H, CH-β), 2.14 (m, 1H, H-2′), 2.32 (m, 1H, H-2″), 3.16 (d, *J* = 5.4 Hz, 1H, CH-α), 3.81 (d, *J* = 15.9 Hz, 1H, CH_2CO'), 3.88 (d, *J* = 15.9 Hz, 1H, CH_2CO''), 3.93–4.00 (m, 3H, H-4′, H-5′), 4.93 (m, 1H, H-3′), 6.12 (dd, *J* = 8.5, 6.0 Hz, 1H, H-1′), 7.13–7.27 (m, 15H, CPh_3), 7.43 (d, *J* = 1.2 Hz, 1H, H-6), 11.37 ppm (s, 1H, 3-NH); Anal. calcd for $C_{36}H_{39}N_3O_7$: C 68.88, H 6.38, N 6.69, found: C 69.05, H 6.66, N 6.71.

3′-*O*-Seryl-5′-*O*-(3,3,3-triphenylpropanoyl)thymidine (9): Following the general procedure, reaction of **3** (82 mg, 0.16 mmol) with Boc-Ser(TBDMSi)-OH[24] (100 mg, 0.31 mmol), PyBOP (97 mg, 0.19 mmol), and Et_3N (55 μL, 0.40 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 30:1) to yield the protected ester [MS (ES, +) *m/z* 850 $[M+Na]^+$]. Treatment of this compound with TFA afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 51 mg (52%, two steps) of **9** as a white solid; mp: 85–87 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 614 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 1.72 (s, 3H, 5-CH_3), 2.18 (m, 1H, H-2′), 2.32 (m, 1H, H-2″), 3.39 (t, *J* = 4.6 Hz, 1H, CH-α), 3.56 (m, 2H, CH_2-β), 3.85 (s, 2H, CH_2CO), 3.94–4.00 (m, 3H, H-4′, H-5′), 4.87 (br s, 1H, OH), 4.95 (m, 1H, H-3′), 6.13 (dd, *J* = 8.2, 6.2 Hz, 1H, H-1′), 7.15–7.27 (m, 15H, CPh_3), 7.43 ppm (s, 1H, H-6); Anal. calcd for $C_{34}H_{35}N_3O_8 \cdot 0.5H_2O$: C 65.58, H 5.83, N 6.75, found: C 65.36, H 6.16, N 6.78.

5′-(1,3-Dihydro-1,3-dioxo-4,5,6,7-tetrachloroisoindol-2-yl)-3′-*O*-(*tert*-butyldimethylsilyl)-5′-deoxythymidine (11): To a solution containing *tert*-butyldimethylsilyl chloride (107 mg, 0.71 mmol) and imidazole (96 mg, 1.41 mmol) in dry DMF (2 mL), compound **10**[14] (240 mg, 0.47 mmol) was added. The reaction mixture was stirred at room temperature overnight, and volatiles were removed. The residue was purified by CCTLC in the Chromatotron (EtOAc/hexane, 1:2→1:1) to afford 225 mg (77%) of **11** as a white solid; mp: 110–112 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 622 $[M+1]^+$ with 4Cl isotopic pattern; ^{1}H NMR ([D$_6$]DMSO): δ = 0.90 (s, 9H, $(CH_3)_3$), 2.03 (s, 3H, 5-CH_3), 2.09 (m, 1H, H-2′), 2.31 (ddd, *J* = 13.5, 5.5, 2.4 Hz, 1H, H-2″), 3.86 (d, *J* = 7.0 Hz, 2H, H-5′), 4.24–4.30 (m, 2H, H-3′, H-4′), 6.19 (dd, *J* = 8.0, 5.5 Hz, 1H, H-1′), 7.39 (d, *J* = 1.0 Hz, 1H, H-6), 8.14 ppm (s, 1H, 3-NH).

5′-Amino-3′-*O*-(*tert*-butyldimethylsilyl)-5′-deoxythymidine (12): To a suspension of **11** (195 mg, 0.31 mmol) in EtOH (5 mL), $H_2NNH_2 \cdot H_2O$ (49 μL, 1.00 mmol) was added. The reaction mixture was stirred at reflux for 1 h. The white solid precipitated was filtered off. The filtrate was evaporated, and the residue was purified by CCTLC in the Chromatotron (CH_2Cl_2/MeOH, 5:1) to afford 107 mg (96%) of **12** as a colorless oil. The analytical and spectroscopic data are identical to those previously described.[25]

3′-*O*-(*tert*-Butyldimethylsilyl)-5′-(3,3,3-triphenylpropanamide)-5′-deoxythymidine (13): To a solution of **12** (109 mg, 0.31 mmol) in CH_2Cl_2 (1.5 mL), 3,3,3-triphenylpropionic acid (139 mg, 0.46 mmol), BOP (204 mg, 0.46 mmol), and Et_3N (64 μL, 0.46 mmol) were added. The reaction mixture was stirred at room temperature overnight, and volatiles were removed. The residue was purified by CCTLC in the Chromatotron (EtOAc/hexane, 1:2→1:1) to yield 186 mg (95%) of **13** as a white solid; mp: 94–96 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 641 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 0.84 (s, 9H, $(CH_3)_3$), 1.72 (s, 3H, 5-CH_3), 1.91 (m, 1H, H-2′), 2.02 (m, 1H, H-2″), 3.03 (m, 2H, H-5′), 3.49 (m, 1H, H-4′), 3.60 (s, 2H, CH_2), 4.14 (m, 1H, H-3′), 6.19 (pseudo-t, *J* = 7.1 Hz, 1H, H-1′), 7.11–7.23 (m, 15H, CPh_3), 7.41 (s, 1H, H-6), 7.33 (t, *J* = 5.6 Hz, 1H, NH), 11.30 ppm (s, 1H, 3-NH).

5′-(3,3,3-Triphenylpropanamide)-5′-deoxythymidine (14): A suspension of **13** (140 mg, 0.22 mmol) in THF (2 mL) was treated with 1 N HCl (0.8 mL). The reaction mixture was stirred at room temperature overnight. The reaction was diluted with EtOAc (25 mL) and washed with a solution of $NaHCO_3$ (2×10 mL) and H_2O (2×10 mL). The organic phase was dried on anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified by CCTLC in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to afford 106 mg (93%) of **14** as a white solid; mp: 116–118 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 526 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 1.72 (s, 3H, 5-CH_3), 1.97 (m, 2H, H-2′), 3.04 (m, 2H, H-5′), 3.49 (m, 1H, H-4′), 3.60 (s, 2H, CH_2), 3.93 (m, 1H, H-3′), 5.16 (d, *J* = 4.0 Hz, 1H, OH), 6.19 (pseudo-t, *J* = 6.7 Hz, 1H, H-1′), 7.11–7.24 (m, 15H, CPh_3), 7.39 (s, 1H, H-6), 7.78 (t, *J* = 5.7 Hz, 1H, NH), 11.28 ppm (s, 1H, 3-NH); Anal. calcd for $C_{31}H_{31}N_3O_5 \cdot 2H_2O$: C 66.30, H 6.28, N 7.48, found: C 65.94, H 6.54, N 7.58.

5′-(3,3,3-Triphenylpropanamide)-3′-*O*-valyl-5′-deoxythymidine (15): Following the general procedure for the synthesis of 3′-amino acid ester derivatives, reaction of **14** (57 mg, 0.11 mmol) with Boc-Val-OH (47 mg, 0.22 mmol), PyBOP (67 mg, 0.13 mmol), and Et_3N (37 μL, 0.27 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield the coupling derivative [MS (ES, +) *m/z* 725 $[M+1]^+$], that was treated with TFA for Boc deprotection. The final residue was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 42 mg (60%, two steps) of **15** as a white solid; mp: 124–125 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 625 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 0.93 (d, *J* = 6.8 Hz, 3H, CH_3-γ), 0.94 (d, *J* = 6.8 Hz, 3H, CH_3-γ), 1.73 (s, 3H, 5-CH_3), 2.05–2.25 (m, 3H, H-2′, CH-β), 3.09–3.17 (m, 3H, H-5′, CH-α), 3.61 (s, 2H, CH_2CO), 3.77 (m, 1H, H-4′), 5.08 (m, 1H, H-3′), 6.10 (dd, *J* = 8.2, 6.1 Hz, 1H, H-1′), 7.14–7.24 (m, 15H, CPh_3), 7.46 (s, 1H, H-6), 7.87 (t, *J* = 4.8 Hz, 1H, NH), 11.35 ppm (s, 1H, 3-NH); Anal. calcd for $C_{36}H_{40}N_4O_6$: C 69.21, H 6.45, N 8.97, found: C 69.44, H 6.69, N 9.01.

3′-*O*-Isoleucyl-5′-(3,3,3-triphenylpropanamide)-5′-deoxythymidine (16): Following the general procedure for the synthesis of 3′-amino acid ester derivatives, reaction of **14** (44 mg, 0.08 mmol) with Boc-Ile-OH (39 mg, 0.17 mmol), PyBOP (52 mg, 0.10 mmol), and Et_3N (29 μL, 0.21 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to provide the coupling

product [MS (ES, +) *m/z* 739 $[M+1]^+$]. This was treated with TFA for Boc deprotection. The final residue was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 36 mg (70%, two steps) of **16** as a white solid; mp: 83–85 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 639 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 0.80–0.84 (m, 6H, CH_3-γ, CH_3-δ), 1.15 (m, 1H, CH′-γ), 1.40 (m, 1H, CH″-γ), 1.58 (m, 1H, CH-β), 1.72 (s, 3H, 5-CH_3), 2.06–2.26 (m, 2H, H-2′), 3.11 (m, 2H, H-5′), 3.17 (d, *J* = 5.5 Hz, 1H, CH-α), 3.60 (s, 2H, CH_2CO), 3.69 (m, 1H, H-4′), 4.98 (m, 1H, H-3′), 6.06 (dd, *J* = 8.3, 6.1 Hz, 1H, H-1′), 7.11–7.23 (m, 15H, CPh_3), 7.47 (s, 1H, H-6), 7.82 (t, *J* = 5.4 Hz, 1H, NH), 11.34 ppm (s, 1H, 3-NH); Anal. calcd for $C_{37}H_{42}N_4O_6$: C 69.57, H 6.63, N 8.77, found: C 69.66, H 6.54, N 8.57.

2′-Deoxy-5′-*O*-(3,3,3-triphenylpropanoyl)uridine (18): To a solution containing 2′-deoxyuridine (**17**; 100 mg, 0.44 mmol), 3,3,3-triphenylpropionic acid (302 mg, 0.87 mmol) and Ph_3P (262 mg, 0,87 mmol) in dry DMF (1.1 mL), a solution of DBAD (230 mg, 0.87 mmol) in DMF (1.1 mL) was added dropwise. The mixture was stirred at 0 °C under argon for 1.5 h. MeOH (2.5 mL) was added, and volatiles were removed. The work-up was performed as described for **3**. The residue that was purified by HPFC (CH_2Cl_2/MeOH, 100:0→92:8) to yield 158 mg (70%) of **18** as an amorphous white solid. MS (ES, +) *m/z* 513 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 2.04 (m, 2H, H-2′), 3.69 (m, 1H, H-4′), 3.80 (br s, 2H, CH_2CO), 3.87 (m, 1H, H-3′), 3.94 (m, 2H, H-5′), 5.30 (d, *J* = 4.2 Hz, 1H, OH), 5.57 (d, *J* = 8.0 Hz, 1H, H-5), 6.07 (pseudo-t, *J* = 6.7 Hz, 1H, H-1′), 7.15–7.28 (m, 15H, CPh_3), 7.51 (d, *J* = 8.1 Hz, 1H, H-6), 11.31 ppm (br s, 1H, 3-NH).

***N*^{3}-Methyl-5′-*O*-(3,3,3-triphenylpropanoyl)thymidine (19)**: To a solution of **3** (125 mg, 0.24 mmol) in acetone (2 mL) in 5 mL Pyrex microwave process vial, K_2CO_3 (16 mg, 0.12 mmol) and MeI (60 μL, 0.95 mmol) were added. The reaction vessel was sealed, stirred, and subsequently irradiated for 60 min at 100 °C in a single-mode microwave reactor. The mixture was purified by flash chromatography (CH_2Cl_2/MeOH, 100:0→96:4) to afford 152 mg (85%) of **19** as a white solid; mp: 56–58 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 541 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 1.75 (d, *J* = 1.0 Hz, 3H, 5-CH_3), 2.05 (m, 1H, H-2′) 3.15 (s, 3H, NCH_3), 3.41 (m, 1H, H-2″), 3.72 (m, 1H, H-4′), 3.82 (s, 2H, CH_2CO), 3.90–4.01 (m, 3H, H-5′, H-3′), 5.31 (d, *J* = 4.2 Hz, 1H, OH), 6.16 (pseudo-t, *J* = 6.8 Hz, 1H, H-1′), 7.14–7.27 (m, 15H, CPh_3), 7.43 ppm (d, *J* = 1.0 Hz 1H, H-6).

2′-Deoxy-3′-*O*-isoleucyl-5′-*O*-(3,3,3-triphenylpropanoyl)uridine (20): Following the general procedure for the synthesis of 3′-amino acid ester derivatives, reaction of **18** (67 mg, 0.13 mmol) with Boc-Ile-OH (60 mg, 0.26 mmol), PyBOP (81 mg, 0.16 mmol), and Et_3N (46 μL, 0.33 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 30:1) to yield 72 mg of the coupling product [MS (ES, +) *m/z* 748 $[M+Na]^+$]. This compound was treated with TFA for removal of the Boc protecting group. After removal of volatiles, the residue was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 51 mg (63%, two steps) of **20** as a white solid; mp: 81–83 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 626 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 0.79–0.84 (m, 6H, CH_3-γ, CH_3-δ), 1.12 (m, 1H, CH′-γ), 1.38 (m, 1H, CH″-γ), 1.58 (m, 1H, CH-β), 2.17–2.36 (m, 2H, H-2′), 3.18 (d, *J* = 5.6 Hz, CH-α), 3.82 (s, 2H, CH_2CO), 3.88–4.98 (m, 3H, H-4, H-5′), 4.94 (m, 1H, H-3′), 5.62 (d, *J* = 8.0 Hz, 1H, H-5), 6.07 (m, 1H, H-1′), 7.14–7.27 (m, 15H, CPh_3), 7.56 (d, *J* = 8.1 Hz, 1H, H-6), 11.35 ppm (br s, 1H, 3-NH); Anal. calcd for $C_{36}H_{39}N_3O_7$: C 69.10, H 6.28, N 6.72, found: C 68.94, H 6.06, N 6.68.

3′-*O*-Isoleucyl-*N*^{3}-methyl-5′-*O*-(3,3,3-triphenylpropanoyl)thymidine (21): Following the general procedure for the synthesis of 3′-amino acid ester derivatives, reaction of **19** (148 mg, 0.27 mmol) with Boc-Ile-OH (127 mg, 0.55 mmol), PyBOP (171 mg, 0.33 mmol), and Et_3N (95 μL, 0.68 mmol) afforded a residue that was purified by flash chromatography (CH_2Cl_2/MeOH, 100: 96:4) to yield 174 mg of the coupling product [MS (ES, +) *m/z* 776 $[M+Na]^+$]. This was deprotected by treatment with TFA. After removal of volatiles, the residue was purified by flash chromatography (CH_2Cl_2/MeOH, 100.0→92:8) to yield 141 mg (80%, two steps) of **21** as a white solid; mp: 67–69 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 654 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 0.86–0.91 (m, 6H, CH_3-γ, CH_3-δ), 1.27 (m, 1H, CH′-γ), 1.43 (m, 1H, CH″-γ), 1.79 (s, 3H, 5-CH_3), 1.87 (m, 1H, CH-β), 2.27 (m, 1H, H-2′), 2.39 (m, 1H, H-2″), 3.16 (m, 4H, N-CH_3, CH-α), 3.81 (d, *J* = 16.3 Hz, 1H, CH_2CO'), 3.87 (d, *J* = 16.3 Hz, 1H, CH_2CO''), 3.99–4.03 (m, 3H, H-4′, H-5′), 5.07 (m, 1H, H-3′), 6.21 (pseudo-t, *J* = 7.1 Hz, 1H, H-1′), 7.14–7.27 (m, 15H, CPh_3), 7.50 (s, 1H, H-6), 8.34 ppm (br s, 2H, NH_2); Anal. calcd for $C_{38}H_{43}N_3O_7$: C 69.81, H 6.63, N 6.43, found: C 69.72, H 6.78, N 6.52.

5′-*O*-(3,3-Diphenylpropanoyl)thymidine (22): As described for the synthesis of **3**, thymidine (**5**; 100 mg, 0.41 mmol) reacted with 3,3-diphenylpropionic acid (186 mg, 0.82 mmol) in the presence of Ph_3P (215 mg, 0.82 mmol) and DBAD (189 mg, 0.82 mmol). The residue was purified by flash chromatography (CH_2Cl_2/MeOH, 100:0→96:4) to yield 93 mg (51%) of **22** as an amorphous white solid. MS (ES, +) *m/z* 451 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 1.74 (s, 3H, 5-CH_3), 1.99–2.18 (m, 2H, H-2′), 3.17 (m, 2H, CH_2CO), 3.80 (m, 1H, H-4′), 4.08–4.15 (m, 3H, H-3′, H-5′, H-5″), 4.43 (t, *J* = 8.0 Hz, 1H, C*H*Ph_2), 5.37 (d, *J* = 4.1 Hz, 1H, OH), 6.15 (pseudo-t, *J* = 6.8 Hz, 1H, H-1′), 7.14–7.32 (m, 10H, CPh_2), 7.40 (s, 1H, H-6), 11.33 ppm (br s, 1H, 3-NH).

5′-*O*-[3,3,3-Tris(4-chlorophenyl)propanoyl]thymidine (23): As described for the synthesis of **3**, thymidine (**5**; 100 mg, 0.41 mmol) reacted with 3,3,3-tris(4-chlorophenyl)propionic acid (332 mg, 0.82 mmol) in the presence of Ph_3P (215 mg, 0.82 mmol) and DBAD (189 mg, 0.82 mmol). The final residue was purified by flash chromatography (CH_2Cl_2/MeOH, 100:0→96:4) to yield 156 mg (61%) of **23** as an amorphous white solid. MS (ES, +) *m/z* 652 $[M+Na]^+$ with 3Cl isotopic pattern; ^{1}H NMR ([D$_6$]DMSO): δ = 1.70 (s, 3H, 5-CH_3), 2.01–2.15 (m, 2H, H-2′), 3.69 (m, 1H, H-4′), 3.80 (d, *J* = 17.0 Hz, 1H, CH_2CO'), 3.87 (d, *J* = 17.0 Hz, 1H, CH_2CO''), 3.92–4.04 (m, 3H, H-3′, H-5′), 5.33 (d, *J* = 4.2 Hz, 1H, OH), 6.12 (pseudo-t, *J* = 6.4 Hz, 1H, H-1′), 7.16 (d, *J* = 8.5 Hz, 6H, CPh_3), 7.32 (d, *J* = 8.5 Hz, 6H, CPh_3), 7.37 (s, 1H, H-6), 11.32 ppm (s, 1H, 3-NH).

3′-*O*-Isoleucyl-5′-*O*-(3,3-diphenylpropanoyl)thymidine (24): Following the general procedure for the synthesis of 3′-amino acid ester derivatives, reaction of **22** (80 mg, 0.18 mmol) with Boc-Ile-OH (82 mg, 0.36 mmol), PyBOP (111 mg, 0.21 mmol), and Et_3N (62 μL, 0.44 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield the coupling product [MS (ES, +) *m/z* 687 $[M+Na]^+$]. Treatment with TFA followed by removal of volatiles afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 59 mg (58%, two steps) of **24** as a white solid; mp: 58–60 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 564 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): δ = 0.80–0.84 (m, 6H, CH_3-γ, CH_3-δ), 1.12 (m, 1H, CH′-γ), 1.39 (s, m, 1H, CH″-γ), 1.58 (m, 1H, CH-β), 1.75 (d, *J* = 1.1 Hz, 3H, 5-CH_3), 2.22 (m, 1H, H-2′), 2.40 (m, 1H, H-2″), 3.18 (m, 3H, CH_2CO, CH-α), 4.01 (m, 1H, H-4′), 4.15 (m, 2H, H-5′), 4.43 (t, *J* = 8.0 Hz, 1H, C*H*Ph_2), 5.09 (m, 1H, H-3′), 6.15 (dd, *J* = 8.2, 6.2 Hz, 1H, H-1′), 7.10–7.17 (m, 2H, $CHPh_2$), 7.21–7.32 (m, 8H, $CHPh_2$), 7.47 (d, *J* = 1.1 Hz, 1H, H-6), 11.38 ppm (s, 1H, 3-NH); Anal. calcd for $C_{31}H_{37}N_3O_7 \cdot 2H_2O$: C 62.21, H 7.21, N 7.30, found: C 62.09, H 6.89, N 7.01.

3′-*O*-Valyl-5′-*O*-[3,3,3-tris(4-chlorophenyl)propanoyl]thymidine (25): Following the general procedure the synthesis of 3′-amino acid ester derivatives, reaction of **23** (93 mg, 0.15 mmol) with Boc-Val-OH (64 mg, 0.30 mmol), PyBOP (92 mg, 0.18 mmol), and Et_3N (51 μL, 0.37 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield the corresponding ester [MS (ES, +) *m/z* 852 $[M+Na]^+$ with a 3 Cl isotopic pattern]. Treatment with TFA and removal of volatiles afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 60 mg (55%, two steps) of **25** as a white solid; mp: 105–107 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 728 $[M+1]^+$ with a 3 Cl isotopic pattern; ^{1}H NMR ([D$_6$]DMSO): $\delta = 0.81$ (d, $J = 6.8$ Hz, 3H, CH_3-γ), 0.86 (d, $J = 6.8$ Hz, 3H, CH_3-γ), 1.72 (d, $J = 0.9$ Hz, 3H, 5-CH_3), 1.83 (m, 1H, CH-β), 2.21 (m, 1H, H-2′), 2.40 (m, 1H, H-2″), 3.13 (d, $J = 5.4$ Hz, 1H, CH-α), 3.79–4.09 (m, 5H, H-4′, H-5′, CH_2CO), 5.02 (m, 1H, H-3′), 6.13 (dd, $J = 7.6$, 6.2 Hz, 1H, H-1′), 7.17 (d, $J = 8.7$ Hz, 6H, CPh_3), 7.32 (d, $J = 8.7$ Hz, 6H, CPh_3), 7.45 (d, $J = 0.9$ Hz, 1H, H-6), 11.36 ppm (br s, 1H, NH); Anal. calcd for $C_{36}H_{36}Cl_3N_3O_7$: C 59.31, H 4.98 N, 5.76, Cl 14.59, found: C 59.18, H 4.91, N 5.99, Cl 14.26.

3′-*O*-Isoleucyl-5′-*O*-[3,3,3-tris(4-chlorophenyl)propanoyl]thymidine (26): Following the general procedure the synthesis of 3′-amino acid ester derivatives, reaction of **23** (115 mg, 0.18 mmol) with Boc-Ile-OH (84 mg, 0.36 mmol), PyBOP (112 mg, 0.22 mmol), and Et_3N (62 μL, 0.45 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 106 mg of the coupling product [MS (ES, +) *m/z* 866 $[M+Na]^+$ with a 3 Cl isotopic pattern]. After treatment with TFA and removal of volatiles, the residue was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 88 mg (66%, two steps) of **26** as a white solid; mp: 122–125 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 744 $[M+1]^+$ with a 3 Cl isotopic pattern; ^{1}H NMR ([D$_6$]DMSO): $\delta = 0.84$–0.89 (m, 6H, CH_3-γ, CH_3-δ), 1.20 (m, 1H, CH′-γ), 1.43 (m, 1H, CH″-γ), 1.73 (s, 3H, 5-CH_3), 1.80 (m, 1H, CH-β), 2.24 (m, 1H, H-2′), 2.41 (m, 1H, H-2″), 3.80–3.85 (m, 2H, CH_2CO′, CH-α), 3.90 (d, $J = 16.4$ Hz, 1H, CH_2CO″), 3.95–4.10 (m, 3H, H-4, H-5′), 5.10 (m, 1H, H-3′), 6.18 (dd, $J = 7.8$, 6.6 Hz, 1H, H-1′), 7.17 (d, $J = 8.7$ Hz, 6H, CPh_3), 7.32 (d, $J = 8.7$ Hz, 6H, CPh_3), 7.45 (s, 1H, H-6), 11.39 ppm (s, 1H, 3-NH); Anal. calcd for $C_{37}H_{38}Cl_3N_4O_6$: C 59.81, H 5.15, Cl 14.31, N 5.65, found: C 59.72, H 5.09, Cl 14.23, N 5.82.

3′-*O*-(Isoleucylvalyl)-5′-*O*-(3,3,3-triphenylpropanoyl)thymidine (27): Following a similar procedure to that described for the synthesis of 3′-amino acid ester derivatives, reaction of **1** (48 mg, 0.08 mmol) with Boc-Ile-OH (35 mg, 0.15 mmol), PyBOP (47 mg, 0.09 mmol), and Et_3N (27 μL, 0.19 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 30:1) to yield the coupling product [MS (ES, +) *m/z* 839 $[M+1]^+$]. Treatment with TFA and removal of volatiles afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 32 mg (54%, two steps) of **27** as a white solid; mp: 93–95 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 740 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 0.78$–0.95 (m, 12H, CH_3-γ-Ile, CH_3-γ-Val, CH_3-δ-Ile), 1.08 (m, 1H, CH′-γ-Ile), 1.43 (m, 1H, CH″-γ-Ile), 1.57 (m, 1H, CH-β-Ile), 1.72 (s, 3H, 5-CH_3), 2.03 (m, 1H, CH-β-Val), 2.17 (m, 1H, H-2′), 2.34 (m, 1H, H-2″), 3.06 (d, $J = 5.7$ Hz, 1H, CH-α-Ile), 3.78–4.00 (m, 5H, CH_2CO, H-4′, H-5′), 4.16 (pseudo-t, $J = 6.6$ Hz, 1H, CH-α-Val), 4.97 (m, 1H, H-3′), 6.12 (dd, $J = 8.0$, 6.4 Hz, 1H, H-1′), 7.14–7.27 (m, 15H, CPh_3), 7.43 (d, $J = 0.9$ Hz, 1H, H-6), 7.95 (br s, 2H, NH_2), 8.12 (d, $J = 7.4$ Hz, 1H, NH-Val), 11.25 ppm (s, 1H, 3-NH); Anal. calcd for $C_{42}H_{50}N_4O_8 \cdot 1.5\,H_2O$: C 65.86, H 6.97, N 7.32, found: C 65.93, H 6.86, N 7.39.

3′-*O*-(Isoleucylisoleucyl)-5′-*O*-(3,3,3-triphenylpropanoyl)thymidine (28): As described for **27**, reaction of **6** (90 mg, 0.14 mmol) with Boc-Ile-OH (65 mg, 0.28 mmol), PyBOP (87 mg, 0.17 mmol), and Et_3N (49 μL, 0.35 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 30:1) to yield the coupling product [MS (ES, +) *m/z* 876 $[M+Na]^+$]. Treatment with TFA and purification of the residue in the Chromatotron (CH_2Cl_2/MeOH, 10:1) afforded 58 mg (55%, two steps) of **28** as a white solid; mp: 111–113 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 754 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 0.80$–0.91 (m, 12H, CH_3-γ-Ile, CH_3-δ-Ile), 1.11 (m, 2H, CH′-γ-Ile), 1.23 (m, 2H, CH′-γ-Ile), 1.72 (s, 3H, 5-CH_3,), 1.69–1.88 (m, 2H,CH-β-Ile), 2.14 (m, 1H, H-2′), 2.23 (m, 1H, H-2″), 3.56 (d, $J = 5.8$ Hz, 1H, CH-α-Ile), 3.78–4.02 (m, 5H, CH_2CO, H-4′, H-5′), 4.25 (pseudo-t, $J = 6.5$ Hz, 1H, CH-α-Ile), 4.96 (m, 1H, H-3′), 6.13 (dd, $J = 8.1$, 6.2 Hz, 1H, H-1′), 7.13–7.27 (m, 15H, CPh_3), 7.43 (s,1H, H-6), 8.15 (d, $J = 6.3$ Hz, 1H, NH-Ile), 11.37 ppm (s, 1H, 3-NH); Anal. calcd for $C_{43}H_{52}N_4O_8$: C 68.60, H 6.90, N 7.44, found: C 68.81, H 7.05, N 7.31.

3′-*O*-(Valyisoleucyl)-5′-*O*-(3,3,3-triphenylpropanoyl)thymidine (29): As described for **27**, reaction of **6** (155 mg, 0.25 mmol) with Boc-Val-OH (108 mg, 0.50 mmol), PyBOP (155 mg, 0.30 mmol), and Et_3N (86 μL, 0.62 mmol) afforded a residue that was purified by HPFC (CH_2Cl_2/MeOH, 100:0→96:4) to yield the coupling product [MS (ES, +) *m/z* 836 $[M+1]^+$]. Treatment with TFA and removal of volatiles afforded a residue that was purified by HPFC (CH_2Cl_2/MeOH, 100:0→90:10) to yield 151 mg (82%, two steps) of **29** as a white solid: mp: 132–133 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 739 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta = 0.82$–0.96 (m, 12H, CH_3-γ-Ile, CH_3-γ-Val, CH_3-δ-Ile), 1.15 (m, 1H, CH′-γ-Ile), 1.45 (m, 1H, CH″-γ-Ile), 1.72 (s, 3H, 5-CH_3), 1.83 (m, 1H, CH-β-Ile), 2.03 (m, 1H, CH-β-Val), 2.17 (m, 1H, H-2′), 2.34 (m, 1H, H-2″), 3.65 (d, $J = 5.6$ Hz, 1H, CH-α-Val), 3.78–3.90 (m, 3H, CH_2CO, H-4′), 3.94–4.00 (m, 2H, H-5′), 4.25 (pseudo-t, $J = 6.4$ Hz, 1H, CH-α-Ile), 4.96 (m, 1H, H-3′), 6.13 (dd, $J = 8.4$, 6.1 Hz, 1H, H-1′), 7.13–7.27 (m, 15H, CPh_3), 7.43 (d, $J = 1.1$ Hz, 1H, H-6), 7.95 (br s, 2H, NH_2), 8.65 (d, $J = 6.8$ Hz, 1H, NH-Ile), 11.38 ppm (s, 1H, 3-NH); Anal. calcd for $C_{42}H_{50}N_4O_8 \cdot 2\,H_2O$: C 68.27, H 6.82, N 7.58, found: C 68.10, H 6.98, N 7.39.

4-[(2*R*,3*S*,5*R*)-5-(Thymin-1-yl)-2-(3,3,3-triphenylpropanoyloxymethyl)tetrahydrofuran-3-yloxy]-4-oxobutanoic acid (30): To a solution of **3** (132 mg, 0.25 mmol) in anhydrous pyridine (4 mL), DMAP (31 mg, 0.25 mmol), and succinic anhydride (50 mg, 0.50 mmol) were added. The reaction mixture was stirred at room temperature overnight, diluted with CH_2Cl_2 (50 mL), and washed with a 10% aqueous solution of KH_2PO_4 (2×30 mL), and H_2O (30 mL). The organic phase was dried on anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified by flash chromatography (CH_2Cl_2/MeOH, 100:0→90:10) to afford 129 mg (82%) of **30** as an amorphous solid. MS (ES, +) *m/z* 627 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta =$ 1.71 (s, 3H, 5-CH_3), 2.15 (m, 1H, H-2′), 2.30 (m, 1H, H-2″), 2.59–2.72 (m, 4H, CH_2CH_2), 3.78–4.05 (m, 5H, H-4′, H-5′, CH_2CO), 4.92 (m, 1H, H-3′), 6.11 (dd, $J = 8.1$, 6.2 Hz, 1H, H-1′), 7.17–7.27 (m, 15H, CPh_3), 7.43 (s, 1H, H-6), 11.38 ppm (s, 1H, 3-NH).

2-Hydroxyethyl-[(2*R*,3*S*,5*R*)-5-(thymin-1-yl)-2-(3,3,3-triphenylpropanoyloxymethyl)tetrahydrofuran-3-yl] succinate (31): To a solution of **30** (85 mg, 0.13 mmol) in CH_2Cl_2 (1.0 mL), ethylene glycol (76 μL, 1.35 mmol), PyBOP (88 mg, 0.17 mmol), and Et_3N (23 μL, 0.17 mmol) were added. The reaction mixture was stirred at room temperature overnight, diluted with CH_2Cl_2 (25 mL), and washed with a 5% aqueous solution of citric acid (10 mL), $NaHCO_3$ solution (10 mL), and brine. The organic phase was dried on anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified by flash chromatography (CH_2Cl_2/MeOH, 100:0→95:5) to afford 48 mg (53%) of **31** [MS (ES, +) *m/z* 671 $[M+Na]^+$]. This compound was used as such for the subsequent steps.

2-[(*S*)-2-Amino-3-methylbutanoyloxy]ethyl-[(2*R*,3*S*,5*R*)-5-(thymin-1-yl)-2-(3,3,3-triphenylpropanoyloxymethyl)tetrahydrofuran-3-yl] succinate (32): Following a similar procedure to that described for the synthesis of 3′-amino acid ester derivatives, reaction of **31** (80 mg, 0.12 mmol) with Boc-Val-OH (52 mg, 0.24 mmol), PyBOP (74 mg, 0.14 mmol), and Et_3N (42 μL, 0.30 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield the coupling product [MS (ES, +) *m/z* 893 $[M+Na]^+$]. Treatment with TFA and purification of the residue in the Chromatotron (CH_2Cl_2/MeOH, 10:1) yielded 33 mg (36%, two steps) of **32** as a white solid; mp: 63–65 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 771 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta=0.86$ (pseudo-t, $J=6.5$ Hz, 6H, CH_3-γ), 1.72 (s, 3H, 5-CH_3), 1.90 (m, 1H, CH-β), 2.15 (m, 1H, H-2′), 2.30 (m, 1H, H-2″), 2.58 (m, 4H, $OCCH_2CH_2CO$), 3.20 (d, $J=5.0$ Hz, 1H, CH-α), 3.85 (m, 2H, CH_2CO), 3.90–3.99 (m, 3H, H-4′, H-5′), 4.20–4.33 (m, 4H, OCH_2CH_2O), 4.93 (m, 1H, H-3′), 6.10 (dd, $J=8.4$, 6.1 Hz, 1H, H-1′), 7.14–7.27 (m, 15H, CPh_3), 7.43 (s, 1H, H-6), 11.36 ppm (s, 1H, 3-NH); Anal. calcd for $C_{42}H_{47}N_3O_{11}$: C 65.53, H 6.15, N 5.46, found: C 65.80, H 6.34, N 5.22.

2-[(*S*)-2-Amino-3-methylpentanoyloxy]ethyl-[(2*R*,3*S*,5*R*)-5-(thymin-1-yl)-2-(3,3,3-triphenylpropanoyloxymethyl)tetrahydrofuran-3-yl] succinate (33): As described for the synthesis of **32**, reaction of **31** (67 mg, 0.10 mmol) with Boc-Ile-OH (46 mg, 0.20 mmol), PyBOP (62 mg, 0.12 mmol) and Et_3N (34 μL, 0.25 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/EtOAc, 6:4) to yield 57 mg of the coupling product [MS (ES, +) *m/z* 884 $[M+1]^+$]. Treatment with TFA and purification of the residue in the Chromatotron (CH_2Cl_2/MeOH, 10:1) yielded 28 mg (35%, two steps) of **33** as a white solid; mp: 58–59 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 784 $[M+1]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta=0.79$–0.83 (m, 6H, CH_3-γ, CH_3-δ), 1.14 (m, 1H, CH′-γ), 1.40 (m, 1H, CH″-γ), 1.58 (m, 1H, CH-β), 1.71 (s, 3H, 5-CH_3), 2.15 (m, 1H, H-2′), 2.30 (m, 1H, H-2″), 2.58 (m, 4H, $OCCH_2CH_2CO$), 3.24 (d, $J=5.4$ Hz, 1H, CH-α), 3.78–4.00 (m, 5H, H-4′, H-5′, CH_2CO), 4.20–4.32 (m, 4H, OCH_2CH_2O), 4.92 (m, 1H, H-3′), 6.10 (dd, $J=8.2$, 6.3 Hz, 1H, H-1′), 7.14–7.27 (m, 15H, CPh_3), 7.43 (s, 1H, H-6), 11.36 ppm (s, 1H, 3-NH); Anal. calcd for $C_{43}H_{49}N_3O_{11}$: C 65.89, H 6.30, N, 5.36, found: 65.88, H 6.45, N 5.29.

5′-*O*-Trityl-3′-*O*-valylthymidine (2): Following the general procedure the synthesis of 3′-amino acid ester derivatives, reaction of 5-*O*-tritythymidine[16] (**4**; 150 mg, 0.31 mmol), with Fmoc-Val-OH (210 mg, 0.62 mmol), PyBOP (193 mg, 0.37 mmol), and Et_3N (107 μL, 0.77 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2) to yield the coupling product [MS (ES, +) *m/z* 829 $[M+Na]^+$]. Treatment with piperidine (85 μL) and removal of volatiles afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 133 mg (74%, two steps) of **2** as a white solid: mp: 88–90 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 607 $[M+Na]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta=0.74$ (d, $J=6.5$ Hz, 3H, CH_3-γ), 0.82 (d, $J=6.5$ Hz, 3H, CH_3-γ), 1.46 (s, 3H, 5-CH_3), 1.78 (m, 1H, CH-β), 2.30 (m, 2H, H-2′), 3.10 (d, $J=5.1$ Hz, CH-α), 3.25 (m, 2H, H-5′), 4.02 (m, 1H, H-4′), 5.32 (m, 1H, H-3′), 6.21 (pseudo-t, $J=6.8$ Hz, 1H, H-1′), 7.27–7.39 (m, 15H, CPh_3), 7.52 (s, 1H, H-6), 11.38 ppm (br s, 1H, 3-NH); Anal. calcd for $C_{34}H_{37}N_3O_6$: C 69.96, H 6.39, N 7.20, found: C 69.85, H 6.21, N 7.05.

3′-*O*-Isoleucyl-5′-*O*-tritylthymidine (34): Following the general procedure the synthesis of 3′-amino acid ester derivatives, reaction of 5′-*O*-tritythymidine[16] (**4**; 100 mg, 0.21 mmol), with Fmoc-Ile-OH (146 mg, 0.41 mmol), PyBOP (129 mg, 0.25 mmol), and Et_3N (72 μL, 0.52 mmol) afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 30:1) to yield the coupling product [MS (ES, +) *m/z* 842 $[M+Na]^+$]. Treatment with piperidine (72 μL) followed by purification of the residue in the Chromatotron (CH_2Cl_2/MeOH, 10:1) yielded 81 mg (65%, two steps) of **34** as a white solid; mp: 84–86 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 620 $[M+Na]^+$; ^{1}H NMR ([D$_6$]DMSO): $\delta=0.76$–0.87 (m, 6H, CH_3-γ, CH_3-δ), 1.03 (m, 1H, CH′-γ), 1.23 (m, 1H, CH″-γ), 1.47 (s, 3H, 5-CH_3), 1.53 (m, 1H, CH-β), 2.30–2.50 (m, 2H, H-2′), 3.15 (d, $J=5.5$ Hz, 1H, CH-α), 3.26 (m, 2H, H-5′), 4.03 (m, 1H, H-4′), 5.30 (m, 1H, H-3′), 6.21 (pseudo-t, $J=7.0$ Hz, 1H, H-1′), 7.27–7.38 (m, 15H, CPh_3), 7.52 (s, 1H, H-6), 11.37 ppm (br s, 1H, 3-NH); Anal. calcd for $C_{35}H_{39}N_3O_6$: C 70.33, H 6.58, N 7.03, found: C 70.12, H 6.57, N 6.98.

4-[(2*R*,3*S*,5*R*)-5-(Thymin-1-yl)-2-(trityloxymethyl)tetrahydrofuran-3-yloxy]-4-oxobutanoic acid (35): To a suspension of 5′-*O*-tritylthymidine[16] (**4**; 150 mg, 0.31 mmol) in CH_2Cl_2 (7 mL), Et_3N (86 μL, 0.62 mmol), DMAP (37 mg, 0.31 mmol), and succinic anhydride (62 mg, 0.62 mmol) were added. The reaction mixture was stirred at room temperature under argon for 7 h, diluted with CH_2Cl_2 (30 mL), and washed with a 10% aqueous solution of KH_2PO_4 (2× 15 mL), and H_2O (2×15 mL). The organic phase was dried on anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified in a SPE silica cartridge (CH_2Cl_2/MeOH, 300:1→200:1) to afford 149 mg (82%) of **35** as a white solid; mp: 97–99 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 584 $[M]^+$; ^{1}H NMR ($CDCl_3$): $\delta=1.38$ (s, 3H, 5-CH_3), 2.41 (m, 1H, H-2′), 2.51 (m, 1H, H-2″), 2.59–2.72 (m, 4H, OCH_2CH_2O), 3.42, 3.48 (dd, $J=10.6$, 2.5 Hz, 2H, H-5′), 4.17 (m, 1H, H-4′), 5.46 (m, 1H, H-3′), 6.38 (dd, $J=9.0$, 5.4 Hz, 1H, H-1′), 7.24–7.41 (m, 15H, CPh_3), 7.61 (s, 1H, H-6), 9.72 ppm (s, 1H, 3-NH).

2-Hydroxyethyl-[(2*R*,3*S*,5*R*)-5-(thymin-1-yl)-2-(trityoxymethyl)tetrahydrofuran-3-yl] succinate (36): To a solution of **35** (130 mg, 0.22 mmol) in CH_2Cl_2 (1 mL) was added ethylene glycol (50 μL, 0.89 mmol), BOP (119 mg, 0.27 mmol), and Et_3N (37 μL, 0.27 mmol). The reaction mixture was stirred at room temperature for 3 h, and diluted with EtOAc (25 mL), washed with a 5% aqueous solution of citric acid (10 mL), a $NaHCO_3$ solution (10 mL), and brine. The organic phase was dried on anhydrous $MgSO_4$, filtered, and evaporated. The residue was purified by CCTLC in the Chromatotron (CH_2Cl_2/MeOH, 20:1) to afford 128 mg (92%) of **36** as a white solid; mp: 73–74 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 651 $[M+Na]^+$; ^{1}H NMR ($CDCl_3$): $\delta=1.36$ (d, $J=0.9$ Hz, 3H, 5-CH_3), 2.47 (m, 2H, H-2′), 2.67 (m, 4H, $COCH_2CH_2CO$), 3.47 (d, $J=2.4$ Hz, 2H, H-5′), 3.82 (m, 2H, CH_2OH), 4.14 (m, 1H, H-4′), 4.24 (m, 2H, OCH_2), 5.49 (m, 1H, H-3′), 6.40 (dd, $J=8.8$, 5.7 Hz, 1H, H-1′), 7.24–7.40 (m, 15H, CPh_3), 7.60 (d, $J=1.1$ Hz, 1H, H-6), 8.24 ppm (s, 1H, 3-NH).

2-[(*S*)-2-Amino-3-methylbutanoyloxy]ethyl-[(2*R*,3*S*,5*R*)-5-(thymin-1-yl)-2-(trityloximethyl)tetrahydrofuran-3-yl] succinate (37): Following a similar procedure to that described for the synthesis of 3′-amino acid ester derivatives, compound **36** (85 mg, 0.14 mmol) reacted with Fmoc-Val-OH (130 mg, 0.38 mmol), PyBOP (187 mg, 0.30 mmol), and Et_3N (37 μL, 0.30 mmol). The crude was dissolved in DMF (1 mL) and treated with piperidine (72 μL). Removal of volatiles afforded a residue that was purified in the Chromatotron (CH_2Cl_2/MeOH, 10:1) to yield 60 mg (96%) of **37** as a white solid; mp: 62–64 °C (CH_2Cl_2/MeOH); MS (ES, +) *m/z* 728 $[M+1]^+$; ^{1}H NMR ($CDCl_3$): $\delta=0.90$ (d, $J=6.8$ Hz, 3H, CH_3-γ), 0.97 (d, $J=6.8$ Hz, 3H, CH_3-γ), 1.36 (s, 3H, 5-CH_3), 2.01 (m, 1H, CH-β), 2.45 (m, 2H, H-2′), 2.67 (m, 4H, $COCH_2CH_2CO$), 3.33 (d, $J=4.9$ Hz, 1H, CH-α), 3.47 (d, $J=2.2$ Hz, 2H, H-5′), 4.13 (m, 1H, H-4′), 4.32 (m, 4H, CH_2O), 5.50 (m, 1H, H-3′), 6.40 (dd, $J=8.2$, 6.2 Hz, 1H, H-1′), 7.25–7.43 (m, 15H, CPh_3), 7.58 ppm (s, 1H, H-6); Anal. calcd for $C_{40}H_{45}N_3O_{10}\cdot H_2O$): C 64.42, H 6.35, N 5.63, found: C 64.18, H 6.03, N 5.70.

2-[(*S*)-2-Amino-3-methylpentanoyloxy]ethyl-[(2*R*,3*S*,5*R*)-5-(thymin-1-yl)-2-(trityloximethyl)tetrahydrofuran-3-yl] succinate (38): Following a reaction sequence similar to that described for

37, alcohol **36** (110 mg, 0.17 mmol) reacted with Fmoc-Ile-OH (93 mg, 0.26 mmol), PyBOP (109 mg, 0.21 mmol), and Et_3N (30 μL, 0.21 mmol), and was deprotected with piperidine (85 μL). The residue was purified by HPFC (CH_2Cl_2/MeOH, 100:0→96:4) to yield 91 mg (71%) of 38; mp: 57–59 °C; MS (ES, +) *m/z* 742 $[M+1]^+$; ^{1}H NMR ($CDCl_3$): $\delta = 0.77$–0.85 (m, 6H, CH_3-γ, CH_3-δ), 1.08 (m, 1H, CH′-γ), 1.36 (m, 1H, CH″-γ), 1.43 (s, 3H, 5-CH_3), 1.55 (m, 1H, CH-β), 2.31–2.50 (m, 2H, H-2′), 2.57 (m, 4H, $COCH_2CH_2CO$), 3.14–3.19 (m, 2H, H-5′, CH-α), 3.30 (m, 1H, H-5″), 4.05 (m, 1H, H-4′), 4.16–4.29 (m, 4H, CH_2O), 5.30 (m, 1H, H-3′), 6.40 (dd, $J = 8.3$, 6.2 Hz, 1H, H-1′), 7.27–7.39 (m, 15H, CPh_3), 7.50 (s, 1H, H-6), 11.36 ppm (br s, 1H, NH); Anal. calcd for $C_{41}H_{47}N_3O_{10}$: C 66.38, H 6.39, N 5.66, found: C 66.09, H 6.60, N 5.91.

Biology

Cells and culture conditions: L. infantum promastigotes (MCAN/ES/89/IPZ229/1/89) were grown in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), antibiotics, and 25 mM HEPES (pH 7.2) at 26 °C. *L. infantum* axenic amastigotes were grown in M199 medium (Invitrogen, Leiden, The Netherlands) supplemented with 10% heat-inactivated FCS, 1 g L^{-1} β-alanine, 100 mg L^{-1} L-asparagine, 200 mg L^{-1} sucrose, 50 mg L^{-1} sodium pyruvate, 320 mg L^{-1} malic acid, 40 mg L^{-1} fumaric acid, 70 mg L^{-1} succinic acid, 200 mg L^{-1} α-ketoglutaric acid, 300 mg L^{-1} citric acid, 1.1 g L^{-1} sodium bicarbonate, 5 g L^{-1} MES, 0.4 mg L^{-1} hemin, and 10 mg L^{-1} gentamicine, pH 5.4 at 37 °C. Jurkat cells were grown in RPMI-1640 medium (Sigma–Aldrich) supplemented with 10% heat-inactivated FCS, antibiotics, and 10 mM HEPES, pH 7.2 at 37 °C and 5% CO_2. THP-1 cells were grown in RPMI-1640 medium (Gibco, Leiden, The Netherlands) supplemented with 10% heat-inactivated FCS, antibiotics, 1 mM HEPES, 2 mM glutamine, and 1 mM sodium pyruvate, pH 7.2 at 37 °C and 5% CO_2.

Cell proliferation assays: Drug treatment of promastigotes and amastigotes was performed during logarithmic growth phase at a concentration of 2×10^6 parasites mL^{-1} at 26 °C or 1×10^6 parasites mL^{-1} at 37 °C for 24 h, respectively. Drug treatment of Jurkat and THP-1 cells was performed during the logarithmic growth phase at a concentration of 4×10^5 cells mL^{-1} at 37 °C and 5% CO_2 for 24 h. The percentage of living cells was evaluated by flow cytometry by the propidium iodide (PI) exclusion method.[16] Briefly, treated parasites were stained for 10 min with 5 μg mL^{-1} PI. The number of PI-positive parasites was determined in a Beckman–Colulter FC500 flow cytometer.

Leishmania infection assays: THP-1 cells were seeded at 120 000 cells mL^{-1} in 24-multiwell plates (Nunc, Roskilde, Denmark) and differentiated to macrophages for 24 h in 1 mL RPMI-1640 medium containing 10 ng mL^{-1} phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich). The culture medium was removed, and *L. infantum* amastigotes (1.2×10^6 in 1 mL THP-1 medium) were added to each well; 3 h later, all medium with non-infecting amastigotes was removed, washed three times with 1× phosphate-buffered saline (PBS) and replaced with new THP-1 medium and the corresponding treatment. After 48 h of treatment, medium was removed; THP-1 cells were washed three times with 1× PBS and detached with TrypLE Express (Invitrogen, Leiden, The Netherlands) according to the manufacturer's indications. Infection was measured by flow cytometry.

Cytotoxicity assays in human cells: Drug treatment of Jurkat cells was performed during logarithmic growth phase at a concentration of 4×10^5 cells mL^{-1} at 37 °C and 5% CO_2 for 24 h. The percentage of living cells was evaluated by flow cytometry by the propidium iodide (PI) exclusion method.

Hydrolysis studies in cell extracts: Cell extracts from PMA-differentiated THP-1 were prepared according to the procedure described by Saboulard et al.[26] Hydrolysis studies of **28** were performed as follows: Aliquots of 200 μL of compound **28** (100 μM) were mixed with 10 μL cell extract, and the samples were incubated at 37 °C. At various time points (0, 0.5, 1, 2, 4, and 24 h), MeOH (100 μL) was added to inactivate the enzymes. These samples were centrifuged, and the supernatants were injected on HPLC. Identification of the hydrolysis products was based on the retention times of the reference compounds and co-elution experiments under identical analytical conditions.

HPLC analysis of hydrolysis products: Analysis of the hydrolysis products after cell extract incubation was performed by HPLC injecting 30 μL of the supernatant. HPLC spectra were recorded on an Aligent 1120 Compact LC using a diode array detector (λ 230–400 nm), and an analytical ACE5 C_{18}-300 column (150×4.6 mm, 300 Å). Solvents used were CH_3CN (0.05% TFA) for A, and H_2O (0.05% TFA) for B, and the flow rate was 1 mL min^{-1}. The gradient used was 30→60% A over 20 min. The retention times of the analytes were: 16.8 min for compound **28**, 14.2 min for compound **6**, and 13.5 min for compound **1**.

Mitochondrial transmembrane potential: Parasites were incubated in RPMI medium with 10% FCS at 1.5×10^6 mL with 50 nM tetramethylrhodamine methyl ester perchlorate (TMRM, Sigma Cat. #T5428) probe for 15 min at 26 °C and then analyzed by flow cytometry. A positive control of mitochondrial depolarization was obtained in parallel by incubating logarithmic promastigotes at 1.5×10^6 mL with the protonophore (H^+ ionophore) and uncoupler of oxidative phosphorylation in mitochondria, carbonyl cyanide 3-chlorophenylhydrazone (CCCP) during the indicated times and then stained with TMRM as described above.

Mitochondrial superoxide detection: Parasites were preloaded for 10 min with 2 μM MitoSOX, and then fluorescence emission was measured at the indicated times in a Beckman Coulter FC500 flow cytometer.

DNA content analysis by flow cytometry: Parasites (1.5×10^6) were centrifuged at 1000 *g* for 5 min, the pellet was resuspended in 100 μL PBS, cold (−20 °C) 70% EtOH (600 μL) was added, and then parasites were incubated on ice for 30 min. After incubation, the parasites were washed with 1 mL PBS/50 mM EDTA, pelleted at 1000 *g*, and then resuspended in 400 μL PBS/50 mM EDTA/50 μg mL^{-1} RNase and incubated for 30 min at 37 °C. Propidium iodide (PI) was then added to a final concentration of 5 μg mL^{-1}, and the parasites were immediately analyzed for PI fluorescence in a Beckman Coulter FC500 flow cytometer.

LiEndoG activity: The nuclease activity of LiEndoG in the presence of selected compounds was monitored by measuring the increase in fluorescence derived from the digestion of a double-stranded probe. 6-Carboxyfluorescein (FAM)-derived fluorescence is quenched by the proximity of tetramethylrhodamine (TAMRA) in the undigested probe constructed by hybridization of the oligonucleotides FAM-5′-CTG TCG CTA CCT GTG G-3′-TAMRA and FAM-5′-CCA CAG GTA GCG ACA G-3′-TAMRA. Digestion of any of the two oligonucleotides causes separation of the fluorophore and quencher, and then a fluorescent signal can be emitted; 30 pmol of the double-stranded probe were digested with LiEndoG 2.5 ng μL^{-1}. After digestion, both fluorophores separate, and FAM-derived fluorescence can be detected. Reactions were monitored in a Victor

1420 Multilabel Counter (Wallac) at an excitation wavelength of 492 nm and an emission wavelength of 517 nm.

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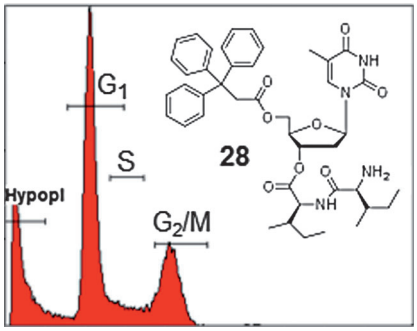

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5′-Trityl-Substituted Thymidine Derivatives as a Novel Class of Antileishmanial Agents: *Leishmania infantum* EndoG as a Potential Target