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Synthesis and evaluation of thymine-derived carboxamides against mitochondrial thymidine kinase (TK-2) and related enzymes

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Abstract—Based on the structure of our previously identified mitochondrial thymidine kinase (TK-2) inhibitors, three series of thymine-derived carboxamides have been synthesized and tested against TK-2 and related enzymes. The methodology employed has been a solution-phase parallel synthesis based on the coupling of three thymine-derived acids [4-(thymin-1-yl)butyric acid (**I**), [4-(thymin-1-yl)-butyrylamino]acetic acid (**II**) and 6-(thymin-1-yl)hexanoic acid (**III**)] with different commercially available primary amines that carry cyano and/or phenyl groups. The couplings were performed in good yields (from 60% to 90%), with the exception of those that incorporate the highly crowded triphenylmethylamine (**e**). From the new synthesized compounds, the *N*-trityl-6-(thymin-1-yl)hexanamide (**IIIe**) was the most active TK-2 inhibitor ($IC_{50} = 19 \pm 2\,\mu M$).

1. Introduction

Deoxynucleoside kinases catalyze the phosphorylation of deoxynucleosides to their corresponding deoxynucleoside monophosphates by γ-phosphoryl transfer of ATP (or other deoxynucleoside triphosphates), and are, in general, the rate-limiting step in the salvage of deoxynucleosides.[1,2] In mammalian cells, there are four different deoxynucleoside kinases: thymidine kinase 1 (TK-1) and deoxycytidine kinase (dCK), that are located in the cytosol; and deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK-2), that have a mitochondrial localization.[1,2] Both thymidine kinases, TK-1 and TK-2, show important differences in their primary amino acid sequence, substrate specificity and level of expression in the different phases of the cell cycle.[2] TK-1 has the highest level of expression in S phase cells, with very low or no activity in resting cells. By contrast, TK-2 is constitutively expressed throughout the cell cycle, and therefore it is virtually the only pyrimidine nucleoside kinase that is physiologically active in nonproliferating and resting cells. In primary sequence and substrate specificity, TK-2 resembles other deoxynucleoside kinases from different organisms like the thymidine kinase of Herpes simplex virus type 1 TK (HSV-1 TK), and, in particular, the multisubstrate nucleoside kinase from *Drosophila melanogaster* (Dm dNK).[2,3]

In the chemotherapeutic treatment of cancer and viral diseases based on nucleoside analogues, deoxynucleoside kinases play a fundamental role, since they catalyze the first and often rate-limiting step of nucleoside analogue activation.[4] For example, the well established anti-AIDS agent AZT is mostly activated by TK-1, although TK-2 has been suggested to be important in the activation of AZT in nondividing cells that express poor if any TK-1 activity. Moreover, TK-2 has been involved in the mitochondrial toxicity observed under prolonged treatments with AZT, although this is somewhat under debate.[5,6] Similarly, TK-2 has also been involved in the mitochondrial toxicity associated to FIAU treatment of hepatitis B virus-infected individuals.[7]

Very recently, point mutations in the TK-2 gene have been associated with mitochondrial DNA depletion that resulted in a severe skeletal myopathy.[8] This and other observations have increased the interest on the impact

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of the levels of deoxynucleoside triphosphate (dNTP) pools in mitochondrial DNA synthesis,[9,10] that is now a very active area of research, and where TK-2 is considered to play a key role.

In this scenario, TK-2 inhibitors could be a valuable tool to enlighten the role of TK-2 in mitochondrial dNTP pool and homeostasis, and may also help to clarify the contribution of TK-2-catalyzed phosphorylation of antiviral drugs and their mitochondrial toxicity.

We have recently reported on the first acyclic nucleoside analogues that shown potent inhibition of TK-2 in a cell free assay, the prototype compound being 1-[(*Z*)-4-(triphenylmethoxy)-2-butenyl]thymine (**1**)[11] (Fig. 1). We have explored alternatives to replace the triphenylmethoxy moiety,[12] and have found that both the triphenylmethyl amine derivative (**2**) as well as the dibenzyl amine derivative (**3**) showed similar inhibition potencies as the prototype *O*-triphenylmethyl analogue (**1**) (Fig. 1). Although the (*Z*)-butenyl moiety is mostly responsible for the selectivity against TK-2 with respect to other thymidine kinases like HSV-1 TK,[12] it should be mentioned that the butanyl analogue (**4**) is equally potent to inhibit TK-2.[11] On the other hand, evaluation of our own collection of thymine derivatives against TK-2 have shown that 1-(ω-cyanoalkyl)thymines, in particular, 1-(8-cyanooctyl)thymine (**5**) showed significant inhibition of TK-2, in the same order of magnitude as the previously mentioned derivatives. Therefore, there is a certain structural diversity among our previously identified TK-2 thymine-derived inhibitors. However, looking for common structural features among these inhibitors, it could be proposed that they all share the presence of a thymine base, an alkyl or alkenyl spacer, and a distal electron-rich substituent (cyano and/or phenyl rings). Detailed kinetic studies performed with compound **1** and related analogues have revealed that these compounds are reversible nonnucleoside, nonsubstrate inhibitors of TK-2 that are competitive with respect to thymidine and uncompetitive with respect to ATP,[13] stressing the importance of the thymine base in these molecules to qualify as TK-2 inhibitors.

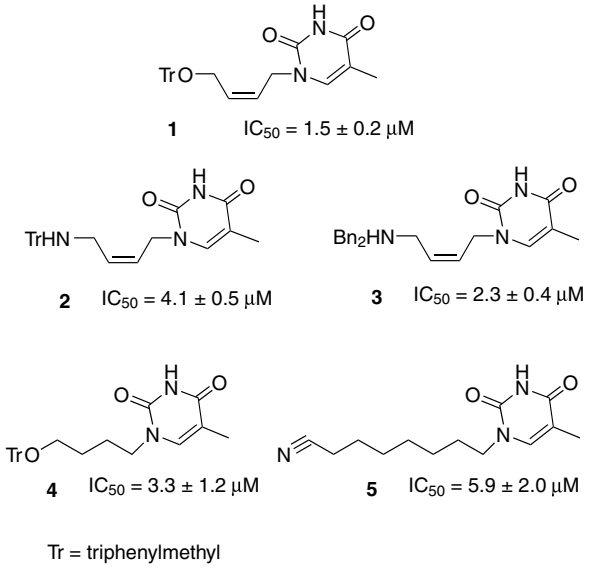


Figure 1.

In the present paper we describe the solution-phase parallel synthesis of a small library of thymine-derived carboxamides designed to be tested against TK-2 and related enzymes. The components of this library are, on one hand, thymine derivatives functionalized at N-1 through a spacer with a distal carboxylic acid (compounds **I**–**III**) and, on the other hand, commercially available primary amines (amines **a**–**e**), that carry cyano and/or phenyl rings. (Fig. 2). The coupling between the acid derivatives and the amines have resulted in the carboxamide derivatives, of general formula **X**(**a**–**e**), where **X** represents the acid used in the coupling. The introduction of the amide function(s) in the spacer may allow potential additional interactions of the inhibitor with either functional amino acid groups or the peptide backbone in the enzyme, thereby strengthening the interaction of the inhibitors with the enzyme. Moreover, the incorporation of the amide moiety would improve the log *P* values of these compounds with respect of our previous inhibitors (calculated log *P* values for compounds **1** and **2** are 5.40 and 5.37, respectively, while calculated log *P* values for the new compounds are included in Table 1).[14]

Although most of the strategies in chemical libraries make use of a central scaffold to introduce diversity at two or more of the substituents, there are also recent

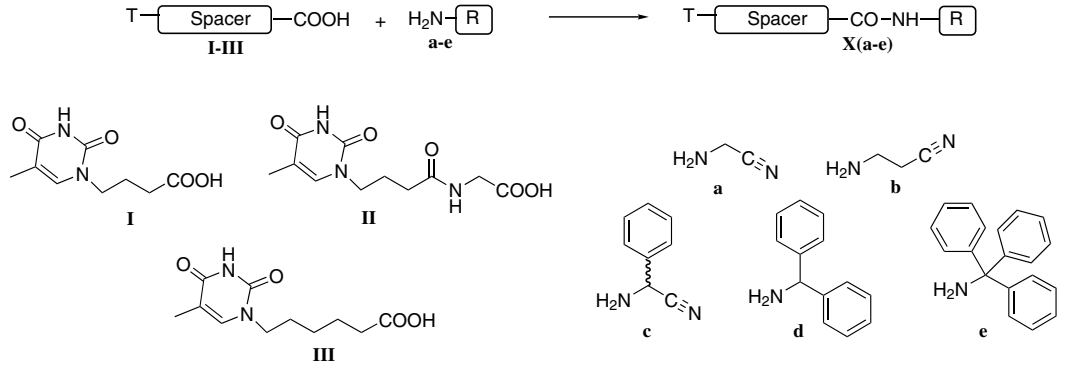


Figure 2.

Table 1. Formulae, molecular peak determined by mass spectrometry (MS), yield and calculated log P values of the thymine-derived carboxamides of general formula **X(a–e)**

Compound	Spacer	R	MS	Yield (%)	*c*Log *P*
Ia	$(CH_2)_3$	CH_2CN	251[a]	60	−1.13
Ib	$(CH_2)_3$	CH_2CH_2CN	265[a]	70	−1.14
Ic	$(CH_2)_3$	CH(Ph)CN	327[a]	90	0.50
Id	$(CH_2)_3$	$CHPh_2$	400[b]	88	2.69
Ie	$(CH_2)_3$	CPh_3	454[a]	47	4.36
IIa	$(CH_2)_3CONHCH_2$	CH_2CN	308[a]	89	−1.96
IIb	$(CH_2)_3CONHCH_2$	CH_2CH_2CN	322[a]	67	−1.97
IIc	$(CH_2)_3CONHCH_2$	CH(Ph)CN	384[a]	44	−0.33
IId	$(CH_2)_3CONHCH_2$	$CHPh_2$	435[a]	79	1.86
IIe	$(CH_2)_3CONHCH_2$	CPh_3	533[b]	11	3.53
IIIa	$(CH_2)_5$	CH_2CN	279[a]	76	−0.15
IIIb	$(CH_2)_5$	CH_2CH_2CN	293[a]	78	−0.16
IIIc	$(CH_2)_5$	CH(Ph)CN	355[a]	80	1.49
IIId	$(CH_2)_5$	$CHPh_2$	406[a]	82	3.67
IIIe	$(CH_2)_5$	CPh_3	504[b]	45	5.34
IVc	(*E*)-CH_2—CH=CH—CH_2CH_2	CH(Ph)CN	353[a]	91	1.27
IVd	(*E*)-CH_2—CH=CH—CH_2CH_2	$CHPh_2$	404[a]	88	3.45

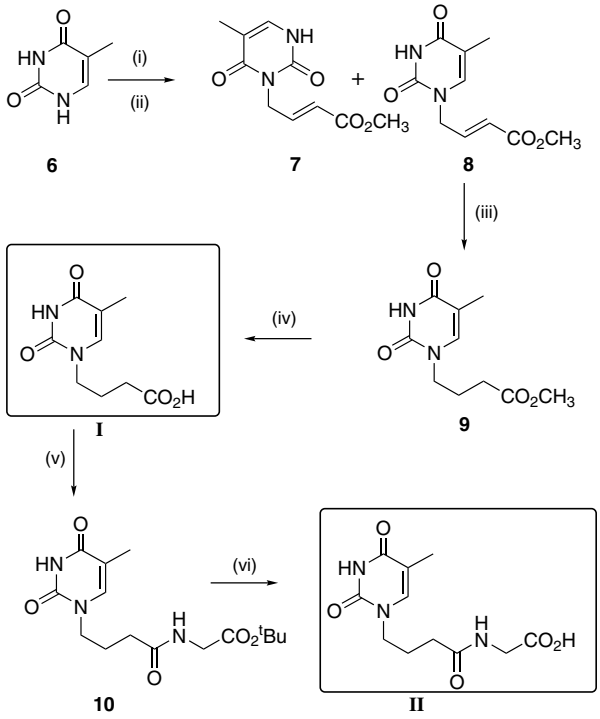
[a] $(M+1)^+$.
[b] $(M+Na)^+$.

examples of designed libraries based on the scheme 'head-spacer-tail'.[15] In our particular case, we have kept the head (thymine) intact to allow optimal interaction at the thymidine binding site of the enzyme, while a certain degree of diversity was incorporated in the spacer and/or the tail of the molecules.

2. Chemistry

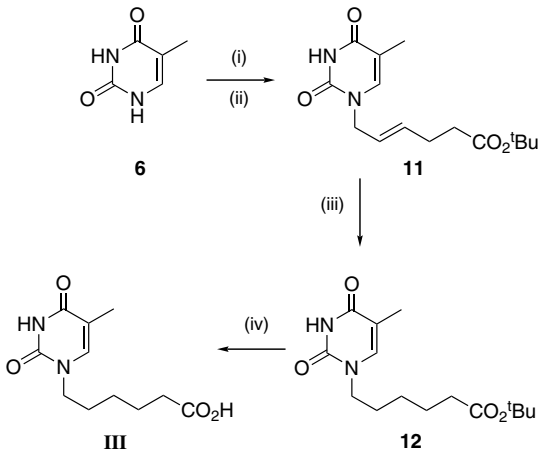
Our approach required the synthesis of the thymine derivatives functionalized with a distal carboxylic acid (acids **I**–**III**). 4-(Thymin-1-yl)butyric acid (**I**) was obtained as depicted in Scheme 1. Treatment of silylated thymine with methyl 4-bromocrotonate yielded the N-1 derivative **7** in 91% yield, together with a very small portion of the N-3 isomer (**8**, 4%). Reaction of **7** with ammonium formiate in refluxing methanol and in the presence of Pd–C as catalyst afforded the saturated analogue **9** (98% yield). Saponification of the methyl ester **9** by treatment with 1 N NaOH in dioxane, followed by neutralization by addition of a Dowex (50 W × 4, H^+ form) resin lead to the target acid **I** in a 87% yield. It should be mentioned that the employment of an allylic bromide in the initial alkylation step allows a higher yield of the desired acid **I** (78% global yield for the three steps) compared to the reported 39% yield described for the acid **I** when ethyl 4-bromobutyrate was used in the initial alkylation step.[16]

[4-(Thymin-1-yl)-butyrylamino]acetic acid (**II**) was obtained from acid **I** in two additional steps (Scheme 1). Thus, coupling of acid **I** with glycine *tert*-butyl ester employing BOP as the coupling reagent, and in the presence of Et_3N, afforded the *tert*-butyl ester **10** in 93% yield. Acid hydrolysis of the ester moiety in **10** by treatment with TFA gave the acid **II** in 96% yield.



Scheme 1. Reagents and conditions: (i) *N*,*O*-Bis(trimethylsilyl)acetamide, CH_3CN, 80 °C; (ii) methyl 4-bromocrotonate, 80 °C, 12 h (**7** (4%) and **8** (91%)); (iii) HCO_2NH_4; Pd–C (10%), refluxing MeOH, 1 h, **9** (98%); (iv) 1 N NaOH, dioxane, rt, 3 h, **I** (87%); (v) H-Gly-O^{t}Bu·HCl, BOP, Et_3N, CH_2Cl_2, 18 h, **10** (93%); (vi) TFA, $CHCl_3$, rt, 18 h, **II** (96%).

The synthesis of 6-(thymin-1-yl)hexanoic acid (**III**) is shown in Scheme 2. Treatment of silylated thymine with *tert*-butyl 6-bromo-(*E*)-4-hexenoate, synthesized according to a published procedure,[17] afforded the N-1

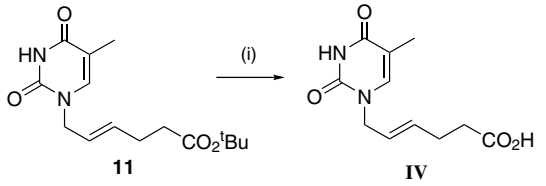


Scheme 2. Reagents and conditions: (i) *N*,*O*-Bis(trimethylsilyl)acetamide, CH_3CN, 80 °C; (ii) *tert*-butyl 6-bromo-(*E*)-4-hexenoate, 80 °C, 2 h, **11** (62%); (iii) HCO_2NH_4; Pd–C (10%), refluxing MeOH, 1 h, **12** (93%); (iv) TFA, $CHCl_3$, rt, 18 h, **III** (88%).

derivative **11** in 62% yield. Reaction of **11** with ammonium formiate in refluxing methanol and in the presence of Pd–C lead to the saturated ester **12** in 93% yield. Finally, hydrolysis of the *tert*-butyl ester by treatment with TFA in $CHCl_3$ afforded the target acid **III** in 88% yield.

The coupling between the thymine-derived acids (**I**–**III**) and the amines (**a**–**e**) was performed using BOP as coupling reagent, and in the presence of Et_3N. The structures of the resulting amides **I**–**III**(**a**–**e**) and yields are shown in Table 1. Yields go from 60% to 90% with the exception of those that incorporate the highly crowded triphenylmethylamine (**e**). When convenient, the excess of the primary amine was removed in the purification step by treatment with an acid resin (Dowex 50 W × 4, H^+ form) that worked as a scavenger.[18] The structure of all the newly synthesized compounds were confirmed by analytical and spectroscopic methods.

To further evaluate if the presence of a double bond in the spacer connecting the thymine base with the tail could influence the binding of these compounds to the target enzyme, two additional compounds were synthesized by coupling the thymine-derived acid **IV** to the amines **c** and **d** to yield the carboxamides **IVc** and **IVd** in 91% and 88% yields, respectively. The acid **IV** had been obtained by acid hydrolysis of the *tert*-butyl ester **11** (78% yield) (Scheme 3).



Scheme 3. Reagents and conditions: (i) TFA, $CHCl_3$, rt, 18 h, **IV** (78%).

3. Results and discussion

Compounds **I**–**III**(**a**–**e**) were evaluated for their inhibitory activity against phosphorylation of dThd by recombinant TK-2 and related enzymes (HSV-1 TK and Dm dNK), following described procedures.[11] The results are shown in Table 2. Our previous inhibitors **1**, **2**, **4** and **5** are also included in the table for comparison.

Based on the nature of the spacer, it is clear that those compounds that incorporate two amide moieties in the spacer (compounds **II**(**a**–**e**)) were almost devoid of inhibitory activity against the three enzymes tested. On the other hand, the carboxamides derived from acid **III** are among the most active against TK-2. Concerning the substituents at the tail, it is quite surprising to see that the compounds that carry a cyano group, compounds **I**–**III**(**a**–**b**), independent of the length and/or the nature of the spacer, are virtually inactive. When compared to the significant TK-2 inhibition of the parent cyano compound **5**, as well as the inhibition of other 1-(ω-cyanoalkyl)thymines previously tested (data not shown), we may conclude that the incorporation of one or two amide moieties in the spacer connecting the thymine and the cyano group is detrimental for activity. It could be argued that the incorporation of the amide(s) functionality(ies) provoke unfavourable interactions with the enzyme, and/or that the planarity of the amide function and the conformational restriction

Table 2. Inhibitory effect of the synthesized compounds on the phosphorylation of [methyl-^{3}H]dThd by TK-2, HSV-1 TK and Dm dNK

Compound	IC_{50} (μM)[a]		
	TK-2	HSV-1 TK	Dm dNK
Ia	>500	>500	>500
Ib	460 ± 56	>500	>500
Ic	208 ± 32	495 ± 7	218 ± 91
Id	238 ± 134	>500	346 ± 41
Ie	44 ± 7	175 ± 81	21 ± 0
IIa	>500	>500	>500
IIb	>500	>500	>500
IIc	318 ± 18	>500	375 ± 14
IId	287 ± 35	>500	292 ± 112
IIe	194 ± 48	191 ± 132	84 ± 33
IIIa	268 ± 46	>500	475 ± 35
IIIb	353 ± 56	>500	>500
IIIc	75 ± 36	275 ± 8	141 ± 110
IIId	38 ± 3	43 ± 2	103 ± 59
IIIe	19 ± 2	3.4 ± 0.4	19 ± 13
IVc	371 ± 36	>500	276 ± 25
IVd	265 ± 44	⩾ 500	232 ± 44
1[b]	1.5 ± 0.16	45 ± 1	33 ± 0.9
2[c]	2.3 ± 0.4	26 ± 4	4.4 ± 0.4
4[b]	3.3 ± 1.2	10 ± 1	19 ± 0.1
5[d]	5.9 ± 2.0	290 ± 1	44 ± 0

[a] 50% Inhibitory concentration or compound concentration (expressed in μM) required to inhibit dThd phosphorylation by 50%. Data are mean value (±SD) of at least two or three independent experiments.
[b] Described in Ref. 11.
[c] Described in Ref. 12.
[d] Described in this paper.

that it imposes, induces a wrong disposition of the head (thymine) and the tail (cyano group) so that the compounds do not optimally fit in the enzyme anymore.

When analyzing the compounds that carry one or more phenyl rings in the tail, compounds **I**–**III**(**c**–**e**), it is clear that increasing the number of the phenyl rings did also increase the potency of inhibition. In each series, the triphenyl derivative (**Ie**, **IIe** and **IIIe**) is the most active. In particular, compound **IIIe** is the most inhibitory against TK-2 among the newly synthesized compounds, but it is approximately one order of magnitude less active that the parent compounds **1**, **2** or **4**. Interestingly, the inclusion of a double bond in the spacer as shown for compounds **IVc** and **IVd** diminished the inhibitory potency when compared to the corresponding saturated analogues **IIIc** and **IIId**.

The inhibitory activity of the synthesized compounds against the other 'TK-2 like' enzymes, that is, HSV-1 TK and Dm dNK, is quite comparable to the TK-2 inhibition. Indeed, the correlation coefficients (r) between the $\log IC_{50}$ values for TK-2 and HSV-1 TK, and for TK-2 and Dm dNK were 0.847 and 0.867, respectively (Fig. 3). However it is interesting to mention that compound **IIIe** is 6-fold more active against the HSV-1 TK enzyme than against TK-2, which represents an exception compared to the prototype compounds.

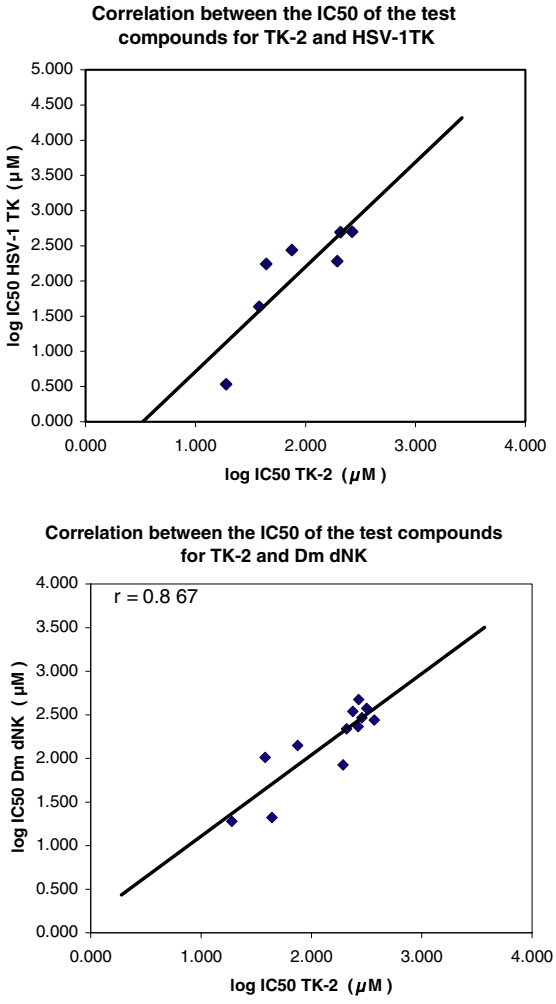


Figure 3. Correlation between the $\log IC_{50}$ values of the test compounds for TK-2 and HSV-1 TK (upper panel) and for TK-2 and Dm dNK (lower panel). Only those compounds that had a well-value for the enzymes were used in the calculations and shown in the figure. Data was taken from Table 2.

By exploring the structural requirements for the interaction of these acyclic analogues with the different nucleoside kinases, the similarities and the differences among these kinases should become more clear and, therefore, the design of more selective ligands directed against one of them (i.e., TK-2, HSV-1 TK) should become more feasible.

4. Conclusions

Three series of thymine-derived carboxamides have been prepared in a straightforward and successful solution-phase parallel synthesis by coupling thymine-derived acids [4-(thymin-1-yl)butyric acid (**I**), [4-(thymin-1-yl)-butyrylamino]acetic acid (**II**) and 6-(thymin-1-yl)hexanoic acid (**III**)] with different commercially available primary amines that carry cyano and/or phenyl groups. Compared to our previously identified TK-2 inhibitors, the incorporation of one or two amide functionalities in the spacer connecting the thymine base with the distal substituent (cyano and/or phenyl rings) abolish or diminish the inhibitory potency against TK-2. The most active compounds in each series are those that contain a triphenylmethyl moiety, in particular, the *N*-trityl-6-(thymin-1-yl)hexanamide (**IIIe**) that is endowed with an $IC_{50} = 19 \pm 2\,\mu M$ against TK-2 and an $IC_{50} = 3.4 \pm 0.4\,\mu M$ against the HSV-1 TK. Although the here described compounds are less active than our previously identified TK-2 inhibitors,[11,12] they represent an important contribution in the armamentarium of TK-2 inhibitors, that also include previously described compounds as 2′-*O*-alkylether derivatives of arabinofuranosyl nucleosides,[19,20] 5-substituted ribonucleosides as well as 3′-highly functionalized nucleosides,[21] and 3′-hexanoylamino-3′-deoxythymidine.[22] At this moment, there is no evidence that these different families of inhibitors share a same mechanism of inhibition.

However, to become efficient inhibitors of TK-2 in vivo, the compounds should be able to pass both the cell membrane and the mitochondrial double membrane in order to efficiently reach their target. From previous work, we could show that our lead compound **1** is able to cross the cell membrane and inhibit its target enzyme when present in the cytosol.[11] Still, at this stage of research, it is unclear whether these compounds can enter the mitochondrial compartment. If efficient targeting of such TK-2 inhibitors to the mitochondria become an achievable goal, the role of TK-2 in mitochondrial toxicity of antiviral and anticancer compounds, as well as other important issues such as the homeostasis and integrity of the mitochondria, the dynamics of mitochondrial dNTP pools, mitochondrial DNA repair and the existence of a substrate cycle between TK-2 and

mitochondrial nucleotidase(s) or the communication between mitochondria and the cytosol/nucleus can be better investigated.

5. Experimental

Melting points were obtained on a Reichert–Jung Kofler apparatus and are uncorrected. Microanalyses were obtained with a Heraeus CHN-O-RAPID instrument. 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 Gemini operating at 200 MHz (^{1}H) and 50 MHz (^{13}C), respectively, on a Varian INNOVA 300 operating at 299 MHz (^{1}H) and 75 MHz (^{13}C), respectively, and Varian INNOVA-400 operating at 399 MHz (^{1}H) and 99 MHz (^{13}C), respectively. Monodimensional ^{1}H and ^{13}C spectra were obtained using standard conditions. 2D inverse proton detected heteronuclear one-bond shift correlation spectra were obtained using the Pulsed Field Gradient HSQC pulse sequence. Data were collected in a 2048×512 matrix with a spectral width of 3460 Hz in the proton domain and 22,500 Hz in the carbon domain, and processed in a 2048×1024 matrix. The experiment was optimized for one bond heteronuclear coupling constant of 150 Hz. 2D Inverse proton detected heteronuclear long range shift correlation spectra were obtained using the Pulsed Field Gradient HMBC pulse sequence. The HMBC experiment was acquired in the same conditions that HSQC experiment and optimized for long range coupling constants of 7 Hz. Parallel reactions were carried out in a 12 place heated carousel reaction station from Radleys Discovery Technologies mounted on a carousel temperature controller stirring plate. 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 phosphomolibdic acid or ninhydrin. Separations on silica gel were performed by preparative centrifugal circular thin layer chromatography (CCTLC) on a ChromatotronR (Kiesegel 60 PF_{254} gipshaltig (Merck)), layer thickness (1 or 2 mm), flow rate (4 or 8 mL/min, respectively). Flash column chromatography was performed with silica gel 60 (230–400 mesh) (Merck).

All experiments involving water-sensitive compounds were conducted under scrupulously dry conditions. Acetonitrile and dichloromethane were dried by refluxing over calcium hydride. Triethylamine was dried by refluxing over potassium hydroxide. Anhydrous *N*,*N*′-dimethylformamide was purchased from Aldrich.

5.1. 1-(8-Cyanooctyl)thymine (5)

A mixture of 1-(8-bromooctyl)thymine[23,24] (175 mg, 0.55 mmol) and KCN (108 mg, 1.66 mmol) in anhydrous DMF (10 mL) was stirred at room temperature overnight. Volatiles were removed and the residue was dissolved in EtOAc (30 mL) and filtered through Celite. The filtrate was evaporated and purified by CCTLC on the Chromatotron using hexane–EtOAc (1:2) to afford 116 mg (80%) of **5** as a white solid. Mp (hexane–EtOAc): 136–138 °C. ^{1}H NMR ($CDCl_3$) δ 1.30–1.47 (m, 8H, CH_2), 1.59–1.67 (m, 4H, CH_2), 1.94 (s, 3H, CH_3-5), 2.33 (t, $J = 7.0$ Hz, 2H, CH_2CN), 3.69 (t, $J = 7.4$ Hz, 2H, NCH_2), 6.98 (s, 1H, H-6), 8.56 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ 12.26 (CH_3-5), 17.01 (CH_2CN), 25.16, 26.16, 28.41, 28.45, 28.92 (CH_2), 48.35 (NCH_2), 110.55 (C-5), 119.74 (CN), 140.31 (C-6), 150.98 (C-2), 164.41 (C-4). Anal. Calcd for $C_{14}H_{21}N_3O_2$: C, 63.85; H, 8.04; N, 15.96. Found: C, 63.90; H, 8.10; N, 16.00. MS (ES, positive mode): 264 $[M+H]^+$, 286 $[M+Na]^+$.

5.2. Methyl (*E*)-4-(thymin-1-yl)-2-butenoate (8)

To a suspension of thymine (**6**) (1.5 g, 11.90 mmol) in dry CH_3CN (39 mL), *N*,*O*-bis(trimethylsilyl)acetamide (4.4 mL) was added and the mixture was heated at 80 °C until total solubilization of thymine. Then, methyl 4-bromocrotonate (1.37 mL, 9.91 mmol) was added, and the reaction mixture was kept at 80 °C for 12 h. The mixture was allowed to reach room temperature. EtOAc (80 mL) was added and the resulting mixture was poured onto a cooled $NaHCO_3$ solution (100 mL), separated and extracted with EtOAc (2×50 mL). The combined organic phases were dried on $MgSO_4$, filtered and evaporated. The residue was purified by column chromatography [hexane–EtOAc (1:2)] to afford 100 mg (4%) of methyl (*E*)-4-(thymin-3-yl)-2-butenoate (**7**) as a white solid from the fastest moving fractions; the slowest moving fractions afforded 2.02 g (91%) of the title compound **8** as a white solid.

Data for **7**: Mp (hexane–EtOAc): 123–125 °C. ^{1}H NMR ($CDCl_3$) δ 1.90 (s, 3H, CH_3-5), 3.76 (s, 3H, OCH_3), 4.91 (dd, $J = 6.6$, 1.7 Hz, 2H, NCH_2), 6.00 (dt, $J = 11.4$, 1.7 Hz, 1H, CH_2CH=), 6.25 (dt, $J = 11.4$, 6.6 Hz, 1H, =CHCO), 7.10 (d, $J = 1.2$ Hz, 1H, H-6), 8.63 (br s, 1H, NH-3). ^{13}C NMR ($CDCl_3$) δ 12.24 (CH_3-5), 45.93 (NCH_2), 51.65 (OCH_3), 111.11 (C-5), 123.44 (CH_2 CH=), 140.32 (C-6), 142.72 (=CHCO), 151.17 (C-2), 164.62 (C-4), 166.16 (CO). MS (ES, positive mode): 225 $[M+H]^+$, 247 $[M+Na]^+$.

Data for **8**: Mp (hexane–EtOAc): 138–140 °C. ^{1}H NMR ($CDCl_3$) δ 1.95 (d, $J = 1.3$ Hz, 3H, CH_3-5), 3.78 (s, 3H, OCH_3), 4.51 (dd, $J = 5.2$, 1.7 Hz, 2H, NCH_2), 5.93 (dt, $J = 15.6$, 1.7 Hz, 1H, CH_2CH=), 6.92 (d, $J = 1.3$ Hz, 1H, H-6), 6.94 (dt, $J = 15.8$, 5.3 Hz, 1H, =CHCO), 9.04 (br s, 1H, NH-3). ^{13}C NMR ($CDCl_3$,) δ 12.35 (CH_3-5), 48.02 (NCH_2), 51.91 (OCH_3), 111.70 (C-5), 123.50 (CH_2CH=), 139.41 (C-6), 140.90 (=CHCO), 150.76 (C-2), 164.24 (C-4), 165.72 (CO). Anal. Calcd for C_{10} $H_{12}N_4O_2$: C, 53.57; H, 5.39; N, 12.49. Found: C, 53.58; H, 5.60; N, 12.60. MS (ES, positive mode): 225 $[M+H]^+$, 247 $[M+Na]^+$.

5.3. Methyl 4-(thymin-1-yl)butyrate (9)

A solution of **8** (1.63 g, 7.23 mmol) in MeOH (10 mL) was cooled at 0 °C, and then Pd–C (10%) (800 mg) and ammonium formate (3.64 g, 57.84 mmol) were added. The resulting mixture was refluxed during 1 h. After

reaching room temperature, the mixture was filtered and the filtrate evaporated under reduced pressure to afford 1.60 g (98%) of **9** as a white solid. Mp (hexane–EtOAc): 126–128 °C. ^{1}H NMR (acetone-d_6) δ 1.84 (s, 3H, CH_3-5), 1.98 (m, J = 7.0 Hz, 2H, CH_2), 2.41 (t, J = 7.5 Hz, 2H, CH_2CO), 3.63 (s, 3H, OCH_3), 3.79 (t, J = 7.0 Hz, 2H, NCH_2), 7.43 (s, 1H, H-6), 8.63 (br s, 1H, NH-3). ^{13}C NMR (acetone-d_6) δ 12.22 (CH_3-5), 31.22 (CH_2CO), 47.84 (NCH_2), 51.67 (OCH_3), 110.18 (C-5), 141.77 (C-6), 151.86 (C-2), 164.81 (C-4), 173.45 (CO). Anal. Calcd for $C_{10}H_{14}N_4O_2$: C, 53.09; H, 6.24; N, 12.38. Found: C, 52.89; H, 6.40; N, 12.14. MS (ES, positive mode): 227 $[M+H]^+$, 249 $[M+Na]^+$.

5.4. 4-(Thymin-1-yl)butyric acid (I)

A solution of **9** (1.53 g, 6.76 mmol) in dioxane (104 mL) and 1 N NaOH (20.3 mL) was stirred at room temperature for 3 h and then diluted with H_2O (50 mL). The mixture was allowed to reach pH 5 by the addition of Dowex 50 W × 4 (H^+ form), filtered, washed with H_2O–dioxane (1:1) (30 mL) and evaporated. The residue was treated with EtOH (30 mL), and the resulting precipitate was collected by filtration to yield 1.32 g (87%) of **I** as a white solid. Mp (EtOH): 205–207 °C. ^{1}H NMR (DMSO-d_6) δ 1.74 (d, J = 1.2 Hz, 3H, CH_3-5), 1.78 (m, 2H, CH_2), 2.22 (t, J = 7.3 Hz, 2H, CH_2CO), 3.63 (t, J = 7.0 Hz, 2H, NCH_2), 7.49 (d, J = 1.2 Hz, 1H, H-6), 11.20 (br s, 1H, NH-3), 12.12 (br s, 1H, COOH). ^{13}C NMR (DMSO-d_6) δ 11.97 (CH_3-5), 23.93 (CH_2), 30.61 (CH_2CO), 46.62 (NCH_2), 108.52 (C-5), 141.38 (C-6), 150.95 (C-2), 164.33 (C-4), 173.85 (CO). Anal. Calcd for C_9 $H_{12}N_4O_2$: C, 50.94; H, 5.70; N, 13.20. Found: C, 50.69; H, 5.91; N, 12.98. MS (ES, positive mode): 213 $[M+H]^+$, 235 $[M+Na]^+$.

5.5. *tert*-Butyl [4-(thymin-1-yl)-butyrylamino]acetate (10)

To a suspension of **I** (818 mg, 3.86 mmol) in dry CH_2Cl_2 (41 mL), BOP (1.71 g, 3.86 mmol) and H-Gly-O^{t}Bu·HCl (539 mg, 3.21 mmol) were added. After a few minutes, freshly distilled Et_3N (0.98 mL, 7.06 mmol) was added and the reaction mixture was stirred at room temperature for 18 h. The mixture was then diluted with $CHCl_3$ (200 mL) and washed with 0.1 N HCl (150 mL), saturated $NaHCO_3$ solution (150 mL) and H_2O (150 mL). The organic phase was dried on Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by two consecutive column chromatographies using CH_2Cl_2–MeOH (30:1) and EtOAc–acetone (2:1), as eluents, to afford 967 mg (93%) of **10** as a white solid. Mp (hexane–EtOAc): 145–147 °C. ^{1}H NMR ($CDCl_3$) δ 1.45 [s, 9H, $C(CH_3)_3$)], 1.89 (s, 3H, CH_3-5), 2.01 (m, J = 6.8 Hz, 2H, CH_2), 2.29 (t, J = 7.0 Hz, 2H, CH_2CO), 3.78 (t, J = 6.8 Hz, 2H, NCH_2), 3.91 (d, J = 5.4 Hz, 2H, $NHCH_2$), 6.72 (t, J = 5.1 Hz, 1H, $NHCH_2$), 7.11 (d, J = 1.0 Hz, 1H, H-6), 9.37 (br s, 1H, NH-3). ^{13}C NMR ($CDCl_3$) δ 12.19 (CH_3-5), 24.83 (CH_2), 27.98 [$C(CH_3)_3$], 32.16 (CH_2CO), 41.95 ($NHCH_2$), 47.40 (NCH_2), 82.30 [$C(CH_3)_3$], 110.95 (C-5), 140.65 (C-6), 151.46 (C-2), 164.35 (C-4), 169.25, 171.95 (CO). Anal. Calcd for $C_{15}H_{23}N_3O_5$: C, 55.37; H, 7.13; N, 12.91. Found: C, 55.09; H, 7.45; N, 12.65. MS (ES, positive mode): 326 $[M+H]^+$, 348 $[M+Na]^+$.

5.6. [4-(Thymin-1-yl)-butyrylamino]acetic acid (II)

A solution of **10** (950 mg, 2.92 mmol) in $CHCl_3$ (28 mL) at 0 °C was treated with TFA (14 mL). The mixture was stirred at room temperature for 18 h. Volatiles were then removed and the residue was co-evaporated with toluene and CH_2Cl_2 to afford 768 mg (96%) of **II** as a white solid. Mp (EtOH): 194–196 °C. ^{1}H NMR (DMSO-d_6) δ 1.75 (s, 3H, CH_3-5), 1.81 (m, J = 7.3 Hz, 2H, CH_2), 2.14 (t, J = 7.3 Hz, 2H, CH_2CO), 3.62 (t, J = 6.8 Hz, 2H, NCH_2), 3.72 (d, J = 5.9 Hz, 2H, $NHCH_2$), 7.49 (d, J = 1.1 Hz, 1H, H-6), 8.22 (t, J = 5.7 Hz, 1H, $NHCH_2$), 11.22 (br s, 1H, NH-3). ^{13}C NMR (DMSO-d_6) δ 11.95 (CH_3-5), 24.56 (CH_2), 31.79 (CH_2CO), 40.56 ($NHCH_2$), 46.89 (NCH_2), 108.41 (C-5), 141.53 (C-6), 150.88 (C-2), 164.03 (C-4), 171.48, 171.70 (CO). Anal. Calcd for $C_{11}H_{14}N_2O_4$: C, 49.07; H, 5.62; N, 15.61. Found: C, 48.77; H, 5.68; N, 15.35. MS (ES, positive mode): 270 $[M+H]^+$, 292 $[M+Na]^+$.

5.7. *tert*-Butyl (*E*)-6-(thymin-1-yl)-4-hexenoate (11)

To a suspension of thymine (**6**) (987 mg, 7.82 mmol) in CH_3CN (26 mL), *N*,*O*-bis(trimethylsilyl)acetamide (2.89 mL) was added and the mixture was heated at 80 °C until total solution of thymine. Then, *tert*-butyl 6-bromo-(*E*)-4-hexenoate[17] (1.62 g, 6.52 mmol) was added and the resulting mixture was kept at 80 °C during 2 h. The reaction was worked up as described for the synthesis of **8**. The residue thus obtained was purified by column chromatography using hexane–EtOAc (1:2) as eluent to yield 1.19 g (62%) of **11** as a white solid. Mp (hexane–EtOAc): 114–116 °C. ^{1}H NMR ($CDCl_3$) δ 1.43 [s, 9H, $C(CH_3)_3$], 1.92 (s, 3H, CH_3-5), 2.33–2.37 (m, 4H, CH_2CO, $=CHCH_2$), 4.27 (d, J = 6.4 Hz, 2H, NCH_2), 5.52 (dt, J = 15.4, 5.6 Hz, 1H, $NCH_2CH=$), 5.76 (dt, J = 15.4, 6.4 Hz, 1H,$=CHCH_2$), 6.98 (s, 1H, H-6), 8.42 (br s, 1H, NH-3). ^{13}C NMR ($CDCl_3$) δ 12.25 (CH_3-5), 27.50 (CH_2), 28.01 [$C(CH_3)_3$], 34.51 (CH_2CO), 49.04 (NCH_2), 110.78 (C-5), 124.33 ($NCH_2CH=$), 134.70 ($=CHCH_2$), 139.51 (C-6), 150.89 (C-2), 164.28 (C-4), 171.97 (CO). Anal. Calcd for C_{15} $H_{22}N_2O_4$: C, 61.21; H, 7.53; N, 9.52. Found: C, 61.40; H, 7.80; N, 9.55. MS (ES, positive mode): 295 $[M+H]^+$, 317 $[M+Na]^+$.

5.8. *tert*-Butyl 6-(thymin-1-yl)hexanoate (12)

Following an analogous procedure to that described for the synthesis of **9**, compound **11** (800 mg, 2.72 mmol) was reacted with ammonium formate (1.37 g, 21.74 mmol), Pd–C (10%) (400 mg) in refluxing MeOH (79 mL), to yield 749 mg (93%) of **12** as a white solid. Mp (hexane–EtOAc): 106–108 °C. ^{1}H NMR ($CDCl_3$) δ 1.34–1.40 (m, 2H, CH_2), 1.43 [s, 9H, $C(CH_3)_3$], 1.54–1.78 (m, 4H, CH_2), 1.92 (d, J = 1.1 Hz, 3H, CH_3-5), 2.22 (t, J = 7.1 Hz, 2H, CH_2CO), 3.69 (t, J = 7.1 Hz, 2H, NCH_2), 7.00 (d, J = 1.3 Hz, 1H, H-6), 9.09 (br s, 1H, NH-3). ^{13}C NMR ($CDCl_3$) δ 12.27 (CH_3-5), 24.41, 25.76 (CH_2), 28.07 [$C(CH_3)_3$], 28.73 (CH_2),

35.11 (CH_2CO), 48.24 (NCH_2), 80.19 [$C(CH_3)_3$], 110.53 (C-5), 140.41 (C-6), 150.83 (C-2), 164.24 (C-4), 172.81 (CO). Anal. Calcd for $C_{15}H_{24}N_2O_4$: C, 60.79; H, 8.16; N, 9.45. Found: C, 60.57; H, 8.40; N, 9.61. MS (ES, positive mode): 319 $[M+Na]^+$.

5.9. 6-(Thymin-1-yl)hexanoic acid (III)

Following an analogous procedure to that described for the synthesis of **II**, compound **12** (720 mg, 2.43 mmol) was reacted with TFA (12 mL) in $CHCl_3$ (24 mL) to yield 513 mg (88%) of **III** as a white solid. Mp (EtOH): 185–187 °C. ^1H NMR (DMSO-d_6) δ 1.20–1.28 (m, 2H, CH_2), 1.44–1.59 (m, 4H, CH_2), 1.72 (d, $J = 0.9$ Hz, 3H, CH_3-5), 2.19 (t, $J = 7.1$ Hz, 2H, CH_2CO), 3.58 (t, $J = 7.1$ Hz, 2H, NCH_2), 7.00 (d, $J = 1.1$ Hz, 1H, H-6), 11.18 (br s, 1H, NH-3), 11.98 (br s, 1H, COOH). ^{13}C NMR (DMSO-d_6) δ 12.62 (CH_3-5), 24.74, 26.01, 28.86 (CH_2), 34.13 (CH_2CO), 47.63 (NCH_2), 109.04 (C-5), 142.16 (C-6), 151.54 (C-2), 164.98 (C-4), 175.10 (CO). Anal. Calcd for $C_{11}H_{16}N_2O_4$: C, 54.99; H, 6.71; N, 11.66. Found: C, 54.79; H, 6.91; N, 11.52. MS (ES, positive mode): 241 $[M+H]^+$, 263 $[M+Na]^+$.

5.10. (*E*)-6-(Thymin-1-yl)-4-hexenoic acid (IV)

Compound **11** (310 mg, 1.05 mmol) was reacted with TFA (5 mL) in $CHCl_3$ (10 mL) during 18 h as described for the synthesis of **II**. Volatiles were removed and the residue was purified by column chromatography using CH_2Cl_2–MeOH (20:1) as eluent to yield 195 mg (78%) of **IV** as a white solid. Mp (EtOH): 170–172 °C. ^1H NMR (DMSO-d_6) δ 1.73 (d, $J = 1.0$ Hz, 3H, CH_3-5), 2.20–2.31 (m, 4H, CH_2CO, CH_2), 4.16 (d, $J = 5.9$ Hz, 2H, NCH_2), 5.51 (dt, $J = 15.4$, 5.9 Hz, 1H, NCH_2CH=), 5.66 (dt, $J = 15.4$, 6.0 Hz, 1H, =$CHCH_2$), 7.41 (s, 1H, H-6), 11.22 (br s, 1H, NH-3), 12.10 (br s, 1H, COOH). ^{13}C NMR (DMSO-d_6) δ 11.95 (CH_3-5), 26.96 (CH_2), 33.00 (CH_2CO), 48.23 (NCH_2), 108.68 (C-5), 124.98 (CH_2CH=), 130.02 (=$CHCH_2$), 140.87 (C-6), 150.69 (C-2), 164.27 (C-4), 171.84 (CO). Anal. Calcd for $C_{11}H_{14}N_2O_4$: C, 55.46; H, 5.92; N, 11.76. Found: C, 55.75; H, 6.10; N, 11.48. MS (ES, positive mode): 239 $[M+H]^+$, 261 $[M+Na]^+$.

5.11. General procedure for the synthesis of the carboxamide derivatives X(a–e)

To a solution of the corresponding acid derivative **I**–**III** (1.2 equiv) in dry CH_2Cl_2 (4–5 mL), BOP (1.2 equiv) and the corresponding primary amine (**a**–**e**) (1 equiv) were added. After a few minutes, freshly distilled Et_3N (1.2–3.2 equiv) was added and the reaction mixture was stirred at room temperature for 18 h. Except where specified, the mixture was then diluted with $CHCl_3$ (20 mL) and washed with 0.1 N HCl (20 mL), saturated $NaHCO_3$ solution (20 mL) and H_2O (20 mL). The organic phase was dried on Na_2SO_4 and evaporated. The residue was purified as specified.

5.12. *N*-Cyanomethyl-4-(thymin-1-yl)butyramide (Ia)

Obtained by reaction of acid **I** (80 mg, 0.38 mmol) with amine **a** (48 mg, 0.31 mmol) using BOP (167 mg, 0.38 mmol) and Et_3N (140 μL, 0.99 mmol) in CH_2Cl_2 (5 mL). The resulting precipitate, which contains the target compound, was filtered and washed with CH_2Cl_2. The solid obtained was dissolved in a mixture of H_2O–CH_3CN (2:1) (36 mL), Dowex 50 W × 4 (2 g, H^+ form) was added and the mixture was stirred at room temperature for 30 min. Then, it was filtered, the filtrate was evaporated and treated with EtOH (10 mL). The new precipitate was collected by filtration to afford 47 mg (60%) of **Ia** as a white solid. Mp (EtOH): 247–249 °C. ^1H NMR (DMSO-d_6) δ 1.73 (s, 3H, CH_3-5), 1.79 (m, $J = 7.3$ Hz, 2H, CH_2), 2.14 (t, $J = 7.3$ Hz, 2H, CH_2CO), 3.61 (t, $J = 7.0$ Hz, 2H, NCH_2), 4.09 (d, $J = 5.6$ Hz, 2H, $NHCH_2$) 7.46 (s, 1H, H-6), 8.57 (t, $J = 5.4$ Hz, 1H, $NHCH_2$), 11.20 (br s, 1H, NH-3). ^{13}C NMR (DMSO-d_6) δ 11.96 (CH_3-5), 24.28 (CH_2), 26.93 ($NHCH_2$), 31.52 (CH_2CO), 46.68 (NCH_2), 108.48 (C-5), 117.72 (CN), 141.36 (C-6), 150.91 (C-2), 164.31 (C-4), 171.99 (CO). Anal. Calcd for $C_{11}H_{14}N_4O_3$: C, 52.79; H, 5.64; N, 22.39. Found: C, 52.54; H, 5.46; N, 22.18. MS (ES, positive mode): 251 $[M+H]^+$, 273 $[M+Na]^+$.

5.13. *N*-Cyanoethyl-4-(thymin-1-yl)butyramide (Ib)

Obtained by reaction of acid **I** (80 mg, 0.38 mmol) with amine **b** (40 mg, 0.31 mmol) using BOP (167 mg, 0.38 mmol) and Et_3N (140 μL, 0.99 mmol) in CH_2Cl_2 (5 mL). The resulting precipitate, which contains the target compound, was collected by filtration and washed with CH_2Cl_2 to yield 58 mg (70%) of **Ib** as a white solid. Mp (EtOH): 179–181 °C. ^1H NMR (DMSO-d_6) δ 1.74 (s, 3H, CH_3-5), 1.82 (m, $J = 7.3$ Hz, 2H, CH_2), 2.09 (t, $J = 7.1$ Hz, 2H, CH_2CO), 2.61 (t, $J = 6.6$ Hz, 2H, CH_2CN), 3.24 (m, $J = 6.2$ Hz, 2H, $NHCH_2$), 3.62 (t, $J = 7.1$ Hz, 2H, NCH_2), 7.48 (s, 1H, H-6), 8.24 (t, $J = 6.0$ Hz, 1H, $NHCH_2$), 11.21 (br s, 1H, NH-3). ^{13}C NMR (DMSO-d_6) δ 11.93 (CH_3-5), 17.58 (CH_2CN), 24.53 (CH_2), 32.00 (CH_2CO), 34.70 ($NHCH_2$), 46.81 (NCH_2), 108.41 (C-5), 119.33 (CN), 141.43 (C-6), 150.87 (C-2), 164.30 (C-4), 171.70 (CO). Anal. Calcd for $C_{12}H_{16}N_4O_3$: C, 54.54; H, 6.10; N, 21.20. Found: C, 54.70; H, 5.88; N, 21.36. MS (ES, positive mode): 265 $[M+H]^+$, 287 $[M+Na]^+$.

5.14. (±)*N*-(Cyano-phenyl-methyl)-4-(thymin-1-yl)butyramide (Ic)

Obtained by reaction of acid **I** (80 mg, 0.38 mmol) with amine **c** (53 mg, 0.31 mmol) using BOP (167 mg, 0.38 mmol) and Et_3N (93 μL, 0.69 mmol) in CH_2Cl_2 (5 mL). The residue was purified by column chromatography [CH_2Cl_2–acetone (2:1)] and lyophilized to give 92 mg (90%) of **Ic** as a white lyophilate. ^1H NMR ($CDCl_3$) δ 1.78 (s, 3H, CH_3-5), 2.01 (m, $J = 6.8$ Hz, 2H, CH_2), 2.35 (t, $J = 6.8$ Hz, 2H, CH_2CO), 3.74 (m, 2H, NCH_2), 6.05 (d, $J = 8.1$ Hz, 1H, $NHCH$), 7.00 (s, 1H, H-6), 7.34–7.46 (m, 5H, Ph), 7.90 (d, $J = 8.1$ Hz, 1H, $NHCH$), 10.14 (br s, 1H, NH-3). ^{13}C NMR ($CDCl_3$) δ 12.14 (CH_3-5), 24.71 (CH_2), 32.03 (CH_2CO), 43.94 (NHCH), 47.40 (NCH_2), 111.56 (C-5), 117.56 (CN), 127.03, 129.10, 129.21, 133.34 (Ph), 140.47 (C-6), 152.28 (C-2), 164.44 (C-4), 171.52 (CO). Anal. Calcd

for $C_{17}H_{18}N_4O_3$: C, 62.57; H, 5.56; N, 14.17. Found: C, 62.34; H, 5.65; N, 13.98. MS (ES, positive mode): 327 $[M+H]^+$, 349 $[M+Na]^+$.

5.15. *N*-Diphenylmethyl-4-(thymin-1-yl)butyramide (Id)

Obtained by reaction of acid **I** (80 mg, 0.38 mmol) with amine **d** (58 mg, 0.31 mmol) using BOP (167 mg, 0.38 mmol) and Et_3N (53 μL, 0.38 mmol) in CH_2Cl_2 (5 mL). Without any workup, the reaction mixture was adsorbed on silica gel and purified by column chromatography [CH_2Cl_2–MeOH (20:1)] to afford 104 mg (88%) of **Id** as a white solid. Mp (EtOH): 132–134 °C. ^{1}H NMR (DMSO-d_6) δ 1.71 (s, 3H, CH_3-5), 1.80 (m, J = 6.8 Hz, 2H, CH_2), 2.21 (t, J = 7.3 Hz, 2H, CH_2CO), 3.62 (t, J = 7.3 Hz, 2H, NCH_2), 6.09 (d, J = 8.1 Hz, 1H, NHC*H*), 7.20–7.34 (m, 10H, Ph), 7.46 (s, 1H, H-6), 8.79 (d, J = 8.5 Hz, 1H, N*H*CH), 11.22 (br s, 1H, NH-3). Anal. Calcd for $C_{22}H_{23}N_3O_3$: C, 70.01; H, 6.14; N, 11.13. Found: C, 69.90; H, 6.30; N, 10.95. MS (ES, positive mode): 400 $[M+Na]^+$.

5.16. *N*-Trityl-4-(thymin-1-yl)butyramide (Ie)

Obtained by reaction of acid **I** (80 mg, 0.38 mmol) with amine **e** (82 mg, 0.31 mmol) using BOP (167 mg, 0.38 mmol) and Et_3N (53 μL, 0.38 mmol) in CH_2Cl_2 (5 mL). The residue was purified by column chromatography [EtOAc–acetone (10:1)] to afford 67 mg (47%) of **Ie** as a white solid. Mp (EtOH): 195–197 °C. ^{1}H NMR (DMSO-d_6) δ 1.69–1.73 (m, 5H, CH_3-5, CH_2), 2.26 (t, J = 7.0 Hz, 2H, CH_2CO), 3.55 (t, J = 7.0 Hz, 2H, NCH_2), 7.14-7.31 (m, 15H, Ph), 7.38 (s, 1H, H-6), 8.64 (br s, 1H, $NHCPh_3$), 11.22 (br s, 1H, NH-3). Anal. Calcd for C_{28} $H_{27}N_3$ O_3: C, 74.15; H, 6.00; N, 9.27. Found: C, 73.99; H, 6.18; N, 9.05. MS (ES, positive mode): 454 $[M+H]^+$, 476 $[M+Na]^+$.

5.17. *N*-[(Cyanomethylcarbamoyl)-methyl]-4-(thymin-1-yl)butyramide (IIa)

Obtained by reaction of acid **II** (100 mg, 0.37 mmol) with amine **a** (48 mg, 0.31 mmol) using BOP (164 mg, 0.37 mmol) and Et_3N (138 μL, 0.99 mmol) in CH_2Cl_2 (5 mL). The resulting precipitate, which contains the target compound, was collected by filtration and washed with CH_2Cl_2 to afford 85 mg (89%) of **IIa** as a white solid. Mp (EtOH): 184–186 °C. ^{1}H NMR (DMSO-d_6) δ 1.74 (s, 3H, CH_3-5), 1.74–1.85 (m, J = 7.0 Hz, 2H, CH_2), 2.14 (t, J = 7.5 Hz, 2H, CH_2CO), 3.62 (t, J = 6.6 Hz, 2H, NCH_2), 3.69 (d, J = 5.9 Hz, 2H, $NHCH_2$), 4.12 (d, J = 5.6 Hz, 2H, $NHCH_2CN$), 7.50 (s, 1H, H-6), 8.21 (t, J = 6.0 Hz, 1H, $NHCH_2$), 8.52 (t, J = 5.6 Hz, 1H, $NHCH_2CN$), 11.22 (br s, 1H, NH-3). ^{13}C NMR (DMSO-d_6) δ 11.98 (CH_3-5), 24.41 (CH_2), 27.01 (CH_2CN), 31.77 (CH_2CO), 41.74 ($NHCH_2$), 46.71 (NCH_2), 108.51 (C-5), 117.60 (CN), 141.48 (C-6), 150.99 (C-2), 164.32 (C-4), 169.95, 171.85 (CO). Anal. Calcd for $C_{13}H_{17}N_5O_4$: C, 50.81; H, 5.58; N, 22.79. Found: C, 50.59; H, 5.43; N, 22.63. MS (ES, positive mode): 308 $[M+H]^+$, 330 $[M+Na]^+$.

5.18. *N*-[(Cyanoethylcarbamoyl)-methyl]-4-(thymin-1-yl)-butyramide (IIb)

Obtained by reaction of acid **II** (100 mg, 0.37 mmol) with amine **b** (115 mg, 0.31 mmol) using BOP (164 mg, 0.37 mmol) and Et_3N (138 μL, 0.99 mmol) in CH_2Cl_2 (5 mL). Without any workup, the reaction mixture was adsorbed on silica gel and purified by two consecutive column chromatographies using CH_2Cl_2–MeOH (10:1) and EtOAc–acetone (1:1), as eluents, to yield 67 mg (67%) of **IIb** as a white solid. Mp (EtOH): 192–194 °C. ^{1}H NMR (DMSO-d_6) δ 1.74 (s, 3H, CH_3-5), 1.79 (m, J = 7.3 Hz, 2H, CH_2), 2.13 (t, J = 7.5 Hz, 2H, CH_2CO), 2.62 (t, J = 6.6 Hz, 2H, CH_2CN), 3.24–3.33 (m, J = 5.6 Hz, 2H, CH_2CH_2CN), 3.58–3.66 (m, 4H, NCH_2, $NHCH_2$), 7.51 (s, 1H, H-6), 8.17 [m, 2H, N*H*-CH_2, $NH(CH_2)_2CN$], 11.23 (br s, 1H, NH-3). ^{13}C NMR (DMSO-d_6) δ 11.96 (CH_3-5), 17.54 (CH_2CN), 24.44 (CH_2), 31.75 (CH_2CO), 34.80 (CH_2CH_2CN), 41.91 ($NHCH_2$), 46.68 (NCH_2), 108.53 (C-5), 119.24 (CN), 141.49 (C-6), 151.01 (C-2), 164.34 (C-4), 169.52, 171.74 (CO). Anal. Calcd for $C_{14}H_{19}N_5O_4$: C, 52.33; H, 5.96; N, 21.79. Found: C, 52.11; H, 5.73; N, 21.68. MS (ES, positive mode): 322 $[M+H]^+$, 344 $[M+Na]^+$.

5.19. (±)*N*-[((Cyano-phenyl-methyl)carbamoyl)-methyl]-4-(thymin-1-yl)butyramide (IIc)

Obtained by reaction of acid **II** (100 mg, 0.37 mmol) with amine **c** (58 mg, 0.31 mmol) using BOP (164 mg, 0.37 mmol) and Et_3N (95 μL, 0.68 mmol) in CH_2Cl_2 (5 mL). The residue was purified by column chromatography [CH_2Cl_2–MeOH (15:1)] to afford 52 mg (44%) of **IIc** as a white solid. Mp (hexane–EtOAc): 145–147 °C. ^{1}H NMR ($CDCl_3$) δ 1.89 (s, 3H, CH_3-5), 2.02 (m, 2H, CH_2), 2.14 (t, J = 7.5 Hz, 2H, CH_2CO), 2.27 (t, J = 6.8 Hz, 2H, CH_2CO), 3.42–3.59 (m, 2H, NCH_2), 3.92 (m, J = 17.1, 5.6 Hz, 1H, $NHCH_2$), 4.13–4.20 (m, J = 17.1, 6.6 Hz, 1H, $NHCH_2$), 6.06 (d, J = 8.1 Hz, 1H, NHC*H*), 6.95 (s, 1H, H-6), 7.29–7.44 (m, 6 H, Ph, N*H*CH), 8.30 (t, J = 8.3 Hz, 1H, $NHCH_2$), 10.40 (br s, 1H, NH-3). ^{13}C NMR (DMSO-d_6) δ 11.93 (CH_3-5), 24.50 (CH_2), 31.83 (CH_2CO), 41.70 ($NHCH_2$), 43.29 (NHCH), 46.78 (NCH_2), 108.43 (C-5), 118.47 (CN), 126.96, 128.81, 128.90, 134.21 (Ph), 141.49 (C-6), 150.90 (C-2), 164.30 (C-4), 169.13, 171.88 (CO). Anal. Calcd for $C_{19}H_{21}N_5O_4$: C, 59.52; H, 5.52; N, 18.27. Found: C, 59.70; H, 5.35; N, 18.40. MS (ES, positive mode): 384 $[M+H]^+$, 406 $[M+Na]^+$.

5.20. *N*-[(Diphenylmethylcarbamoyl)-methyl]-4-(thymin-1-yl)butyramide (IId)

Obtained by reaction of acid **II** (100 mg, 0.37 mmol) with amine **d** (57 mg, 0.31 mmol) using BOP (164 mg, 0.37 mmol) and Et_3N (52 μL, 0.37 mmol) in CH_2Cl_2 (5 mL). The residue was purified by two consecutive column chromatographies using CH_2Cl_2–MeOH (20:1), and EtOAc–acetone (1:2), as eluents, to afford 106 mg (79%) of **IId** as a white solid. Mp (EtOH): 199–201 °C. ^{1}H NMR (DMSO-d_6) δ 1.71 (s, 3H, CH_3-5), 1.73–1.82 (m, J = 7.1 Hz, 2H, CH_2), 2.12 (t, J = 7.6 Hz, 2H, CH_2CO), 3.59 (t, J = 6.8 Hz, 2H, NCH_2), 3.79 (d,

J = 5.9 Hz, NHCH_2), 6.10 (d, J = 8.8 Hz, 1H, NHCH), 7.20–7.34 (m, 10H, Ph), 7.48 (s, 1H, H-6), 8.12 (t, J = 5.6 Hz, NHCH_2), 8.80 (d, J = 8.6 Hz, 1H, NHCH), 11.21 (br s, 1H, NH-3). Anal. Calcd for $C_{24}H_{26}N_4O_4$: C, 66.34; H, 6.03; N, 12.89. Found: C, 65.96; H, 6.29; N, 12.66. MS (ES, positive mode): 435 $[M+H]^+$, 457 $[M+Na]^+$.

5.21. *N*-[(Tritylcarbamoyl)-methyl]-4-(thymin-1-yl)butyramide (IIe)

Obtained by reaction of acid **II** (100 mg, 0.37 mmol) with amine **e** (80 mg, 0.31 mmol) using BOP (164 mg, 0.37 mmol) and Et_3N (52 μL, 0.37 mmol) in CH_2Cl_2 (5 mL). The residue was purified by column chromatography [EtOAc–acetone (2:1)] to yield 17 mg (11%) of **IIe** as a white solid. Mp (EtOH): 243–245 °C. ^{1}H NMR (DMSO-d_6) δ 1.67 (s, 3H, CH_3-5), 1.72–1.79 (m, J = 7.1 Hz, 2H, CH_2), 2.14 (t, J = 8.1 Hz, 2H, CH_2CO), 3.56 (t, J = 6.8 Hz, 2H, NCH_2), 3.80 (d, J = 5.9 Hz, NHCH_2), 7.13–7.30 (m, 15H, Ph), 7.40 (s, 1H, H-6), 8.08 (t, J = 5.3 Hz, NHCH_2), 8.55 (br s, 1H, NHCPh$_3$), 10.40 (br s, 1H, NH-3). Anal. Calcd for $C_{30}H_{30}N_4O_4$: C, 70.57; H, 5.92; N, 10.97. Found: C, 70.79; H, 6.10; N, 10.80. MS (ES, positive mode): 533 $[M+Na]^+$.

5.22. *N*-Cyanomethyl-6-(thymin-1-yl)hexanamide (IIIa)

Obtained by reaction of acid **III** (75 mg, 0.31 mmol) with amine **a** (40 mg, 0.26 mmol) using BOP (138 mg, 0.31 mmol) and Et_3N (116 μL, 0.83 mmol) in CH_2Cl_2 (4 mL). The resulting precipitate, which contains the target compound, was collected by filtration and washed with CH_2Cl_2 to yield 55 mg (76%) of **IIIa** as a white solid. Mp (EtOH): 184–186 °C. ^{1}H NMR (DMSO-d_6) δ 1.20 (m, 2H, CH_2), 1.46–1.59 (m, 4H, CH_2), 1.73 (s, 3H, CH_3-5), 2.12 (t, J = 7.3 Hz, 2H, CH_2CO), 3.56 (t, J = 7.3 Hz, 2H, NCH_2), 4.09 (d, J = 5.6 Hz, 2H, NHCH_2), 7.51 (d, J = 1.2 Hz, 1H, H-6), 8.51 (t, J = 5.6 Hz, 1H, NHCH_2), 11.18 (br s, 1H, NH-3). ^{13}C NMR (DMSO-d_6) δ 11.96 (CH_3-5), 24.52, 25.37, 26.93 (CH_2), 28.22 (NHCH_2), 34.58 (CH$_2$CO), 46.97 (NCH_2), 108.42 (C-5), 117.81 (CN), 141.48 (C-6), 150.88 (C-2), 164.34 (C-4), 172.77 (CO). Anal. Calcd for $C_{13}H_{18}N_4O_3$: C, 56.10; H, 6.52; N, 20.13. Found: C, 55.97; H, 6.76; N, 19.98. MS (ES, positive mode): 293 $[M+H]^+$, 315 $[M+Na]^+$.

5.23. *N*-Cyanoethyl-6-(thymin-1-yl)hexanamide (IIIb)

Obtained by reaction of acid **III** (75 mg, 0.31 mmol) with amine **b** (33 mg, 0.26 mmol) using BOP (138 mg, 0.31 mmol) and Et_3N (116 μL, 0.83 mmol) in CH_2Cl_2 (4 mL). The resulting precipitate, which contains the target compound, was collected by filtration and washed with CH_2Cl_2 to yield 59 mg (78%) de **IIIb** as a white solid. Mp (EtOH): 170–172 °C. ^{1}H NMR (DMSO-d_6) δ 1.21 (m, 2H, CH_2), 1.52 (m, 4H, CH_2), 1.73 (s, 3H, CH_3-5), 2.07 (t, J = 7.3 Hz, 2H, CH_2CO), 2.61 (t, J = 6.4 Hz, 2H, CH_2CN), 3.24 (m, 2H, NHCH_2), 3.58 (t, J = 7.3 Hz, 2H, NCH_2), 7.50 (s, 1H, H-6), 8.17 (t, J = 5.6 Hz, 1H, NHCH_2), 11.18 (br s, 1H, NH-3). ^{13}C NMR (DMSO-d_6) δ 11.94 (CH_3-5), 17.61 (CH$_2$CN), 24.74, 25.35, 28.23 (CH_2), 34.71, 35.01 (CH$_2$CO, NHCH_2), 47.02 (NCH_2), 108.39 (C-5), 119.34 (CN), 141.49 (C-6), 150.87 (C-2), 164.33 (C-4), 172.53 (CO). Anal. Calcd for $C_{14}H_{20}N_4O_3$: C, 57.52; H, 6.90; N, 19.17. Found: C, 57.35; H, 7.08; N, 18.99. MS (ES, positive mode): 293 $[M+H]^+$, 315 $[M+Na]^+$.

5.24. (±)*N*-(Cyano-phenyl-methyl)-6-(thymin-1-yl)-hexanamide (IIIc)

Obtained by reaction of acid **III** (75 mg, 0.31 mmol) with amine **c** (49 mg, 0.26 mmol) using BOP (138 mg, 0.31 mmol) and Et_3N (80 μL, 0.57 mmol) in CH_2Cl_2 (4 mL). The residue was purified by two consecutive column chromatographies using CH_2Cl_2–MeOH (25:1), and EtOAc–acetone (1:1), as eluents, and lyophilized to give 74 mg (80%) of **IIIc** as a white lyophilate. ^{1}H NMR (acetone-d_6) δ 1.40 (m, 2H, CH_2), 1.65–1.79 (m, 4H, CH_2), 1.84 (d, J = 0.9 Hz, 3H, CH_3-5), 2.36 (t, J = 7.3 Hz, 2H, CH_2CO), 3.70 (t, J = 7.1 Hz, 2H, NCH_2), 6.23 (d, J = 8.1 Hz, 1H, NHCH), 7.46–7.54 (m, 6H, Ph, H-6), 8.25 (d, J = 7.3 Hz, 1H, NHCH), 9.96 (br s, 1H, NH-3). ^{13}C NMR (DMSO-d_6) δ 11.92 (CH_3-5), 24.52, 25.33, 28.17 (CH_2), 34.52 (CH$_2$CO), 43.25 (NHCH), 46.93 (NCH_2), 108.35 (C-5), 118.66 (CN), 126.98, 128.83, 128.97, 134.35 (Ph), 141.41 (C-6), 150.83 (C-2), 164.27 (C-4), 171.97 (CO). Anal. Calcd for $C_{19}H_{22}N_4O_3$: C, 64.39; H, 6.26; N, 15.81. Found: C, 64.25; H, 6.50; N, 15.55. MS (ES, positive mode): 355 $[M+H]^+$, 377 $[M+Na]^+$.

5.25. *N*-Diphenylmethyl-6-(thymin-1-yl)hexanamide (IIId)

Obtained by reaction of acid **III** (75 mg, 0.31 mmol) with amine **d** (55 mg, 0.26 mmol) using BOP (138 mg, 0.31 mmol) and Et_3N (44 μL, 0.31 mmol) in CH_2Cl_2 (4 mL). The residue was purified by two consecutive column chromatographies using CH_2Cl_2–MeOH (20:1), and EtOAc–acetone (1:1), as eluents, to afford 86 mg (82%) of **IIId** as a white solid. Mp (hexane–EtOAc): 175–177 °C. ^{1}H NMR ($CDCl_3$) δ 1.36 (m, 2H, CH_2), 1.66–1.81 (m, 4H, CH_2), 1.88 (d, J = 0.9 Hz, 3H, CH_3-5), 2.34 (t, J = 7.1 Hz, 2H, CH_2CO), 3.67 (t, J = 7.1 Hz, 2H, NCH_2), 6.17–6.29 (m, 2H, NHCH, NHCH), 6.97 (d, J = 1.1 Hz, 1H, H-6), 7.24–7.39 (m, 10H, Ph), 8.40 (br s, 1H, NH-3). Anal. Calcd for $C_{24}H_{27}N_3O_3$: C, 71.09; H, 6.71; N, 10.36. Found: C, 70.95; H, 6.83; N, 10.10. MS (ES, positive mode): 406 $[M+H]^+$, 428 $[M+Na]^+$.

5.26. *N*-Trityl-6-(thymin-1-yl)hexanamide (IIIe)

Obtained by reaction of acid **III** (75 mg, 0.31 mmol) with amine **e** (67 mg, 0.26 mmol) using BOP (138 mg, 0.31 mmol) and Et_3N (44 μL, 0.31 mmol) in CH_2Cl_2 (4 mL). The residue was purified by two consecutive column chromatographies using CH_2Cl_2–MeOH (20:1) and EtOAc–acetone (1:1), as eluents, to afford 55 mg (45%) of **IIIe** as a white solid. Mp (hexane–EtOAc): 172–174 °C. ^{1}H NMR ($CDCl_3$) 1.32 (m, 2H, CH_2), 1.64–1.75 (m, 4H, CH_2), 1.87 (d, J = 0.9 Hz, 3H, CH_3-5), 2.31 (t, J = 7.0 Hz, 2H, CH_2CO), 3.66 (t, J = 7.0 Hz,

2H, NCH_2), 6.60 (br s, 1H, $NHCPh_3$), 6.96 (d, $J = 1.1$ Hz, 1H, H-6), 7.17–7.33 (m, 15H, Ph), 8.33 (br s, 1H, NH-3). Anal. Calcd for $C_{30}H_{31}N_3O_3$: C, 74.82; H, 6.49; N, 8.73. Found: C, 75.19; H, 6.60; N, 9.02. MS (ES, positive mode): 504 $[M+Na]^+$.

5.27. (±)(*E*)-*N*-(Cyano-phenyl-methyl)-6-(thymin-1-yl)-hex-4-enamide (IVc)

Obtained by reaction of acid **IV** (80 mg, 0.34 mmol) with amine **c** (52 mg, 0.28 mmol) using BOP (149 mg, 0.34 mmol) and Et_3N (86 μL, 0.62 mmol) in CH_2Cl_2 (4 mL). The residue was purified by two consecutive column chromatographies using CH_2Cl_2–MeOH (20:1), and EtOAc–acetone (1:1), as eluents, to afford 90 mg (91%) of **IVc** as a white solid. Mp (EtOH): 156–158 °C. ^{1}H NMR (DMSO-d_6) δ 1.73 (s, 3H, CH_3-5), 2.19–2.40 (m, 4H, CH_2CO, CH_2), 4.16 (d, $J = 5.3$ Hz, 2H, NCH_2), 5.48 (dt, $J = 15.4$, 5.5 Hz, 1H, NCH_2CH=), 5.65 (m, $J = 15.4$ Hz, 1H, =$CHCH_2$), 6.13 (d, $J = 7.7$ Hz, 1H, NHC*H*), 7.37–7.44 (m, 6H, Ph, H-6), 9.13 (d, $J = 8.1$ Hz, 1H, N*H*CH), 11.22 (br s, 1H, NH-3). ^{13}C NMR (DMSO-d_6) δ 11.91 (CH_3-5), 27.32 (CH_2), 33.97 (CH_2CO), 43.26 (NHCH), 48.16 (NCH_2), 108.68 (C-5), 118.57 (CN), 125.01 (NCH_2 *C*H=), 126.94, 128.81, 128.94, 134.27 (Ph), 132.96 (=*C*HCH_2), 140.74 (C-6), 150.65 (C-2), 164.21 (C-4), 171.29 (CO). Anal. Calcd for $C_{19}H_{20}N_4O_3$: C, 64.76; H, 5.72; N, 15.90. Found: C, 64.90; H, 5.65; N, 15.75. MS (ES, positive mode): 353 $[M+H]^+$, 375 $[M+Na]^+$.

5.28. (*E*)-*N*-Diphenylmethyl-6-(thymin-1-yl)hex-4-enamide (IVd)

Obtained by reaction of acid **IV** (80 mg, 0.34 mmol) with amine **d** (51 mg, 0.28 mmol) using BOP (149 mg, 0.34 mmol) and Et_3N (47 μL, 0.34 mmol) in CH_2Cl_2 (4 mL). The residue was purified by column chromatography [CH_2Cl_2–MeOH (20:1)] to yield 99 mg (88%) de **IVd** as a white solid. Mp (EtOH): 216–218 °C. ^{1}H NMR (DMSO-d_6) δ 1.72 (s, 3H, CH_3-5), 2.24–2.31 (m, 4H, CH_2CO, CH_2), 4.14 (d, $J = 5.9$ Hz, 2H, NCH_2), 5.48 (dt, $J = 15.4$, 5.9 Hz, 1H, NCH_2CH=), 5.66 (dt, $J = 15.4$, 5.9 Hz, 1H, =$CHCH_2$), 6.08 (d, $J = 8.6$ Hz, 1H, NHC*H*), 7.22–7.37 (m, 11H, Ph, H-6), 8.73 (d, $J = 8.6$ Hz, 1H, N*H*CH), 11.23 (br s, 1H, NH-3). Anal. Calcd for $C_{24}H_{25}N_3O_3$: C, 71.44; H, 6.25; N, 10.41. Found: C, 71.32; H, 6.44; N, 10.30. MS (ES, positive mode): *m*/*z* 404 $[M+H]^+$, 426 $[M+Na]^+$.

5.29. TK assay using [methyl-^{3}H]dThd as the substrate

Briefly, the activity of recombinant TK-2, HSV-1 TK and Dm dNK, and the 50% inhibitory concentration of the test compounds were assayed in a 50 μL reaction mixture containing 50 mM Tris/HCl, pH 8.0, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM CHAPS, 3 mg/mL bovine serum albumin, 2.5 mM ATP, 1 μM [*methyl*^{3}H]dThd and enzyme. The samples were incubated at 37 °C for 30 min in the presence or absence of different concentrations (5-fold dilutions) of the test compounds. Aliquots of 45 μL of the reaction mixtures were spotted on Whatman DE-81 filter paper disks. The filters were washed three times for 5 min each in 1 mM ammonium formate, once for 1 min in water, and once for 5 min in ethanol. The radioactivity retained on the filter discs was determined by scintillation counting.

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