Improving Specificity vs Bacterial Thymidylate Synthases through *N*-Dansyl Modulation of Didansyltyrosine

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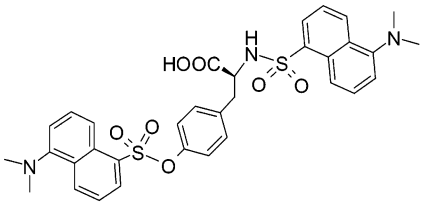
Abstract: *N*,*O*-Didansyl-L-tyrosine (DDT) represented the starting lead for further development of novel non-substrate-like inhibitors of bacterial thymidylate synthase. The *N*-dansyl structure modulation led to a submicromolar inhibitor of *Lactobacillus casei* TS (*Lc*TS), which is highly specific with respect to human TS (hTS). Using molecular dynamics simulation, a binding mode for DDT vs *Lc*TS was predicted, explaining activity and species-specificity along the series.

Thymidylate synthase (TS) (EC 2.1.1.45) is an essential enzyme for most living organisms. It catalyzes the formation of the DNA precursor, 2′-deoxythymidine-5′-monophosphate, from the substrate 2′-deoxyuridine-5′-monophosphate (dUMP) and the cofactor 5,10-methylene-5,6,7,8-tetrahydrofolate (mTHF). Because of its critical function, efforts have been focused on the design of TS inhibitors with anticancer or antimicrobial activity. However, the design of species-specific inhibitors that are able to discriminate among the different species of this highly conserved enzyme has been challenging.[2−4]

During previous research, a strategy to design novel TS inhibitors successfully combined structure-based virtual screening and in-parallel synthetic elaboration of a small, focused library. The most potent inhibitor in the library was *N*,*O*-didansyl-L-tyrosine (DDT) showing the same affinity toward *Lactobacillus casei* TS (*Lc*TS) and *Escherichia coli* TS (*Ec*TS) (K_{iLcTS} 1.4 μM; K_{iEcTS} 1.8 μM), as a result being 35-fold more specific against these two bacterial species compared to human TS (K_{ihTS} 49 μM).



DDT

In particular, *Lc*TS does not come from a pathogen itself but represents a good model for TS from pathogens such as *Staphylococcus aureus*, *Enterococcus faecalis*, and *Bacillus anthracis*, in that they all share the small domain region of 50 amino acids (residues lc-90-lc-139), which are missing in *Ec*TS and hTS.[5−8]

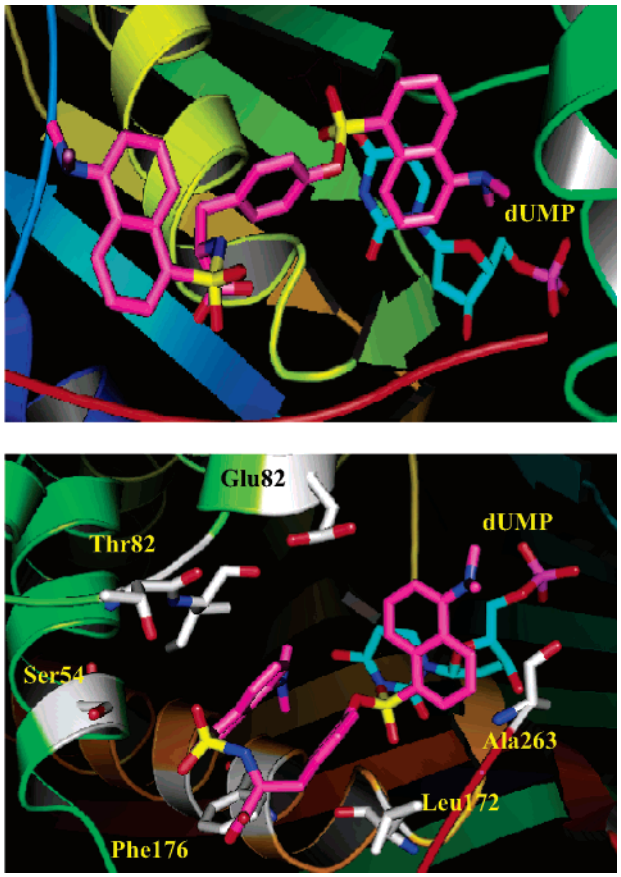


Figure 1. A: DDT−*Lc*TS predicted orientation. Carbon atoms are colored magenta for ligand and cyan for dUMP; oxygen atoms, red; nitrogen atoms, blue; sulfur atoms, yellow. B: DDT−*Ec*TS X-ray orientation. Key residues are shown. Carbon atoms are colored gray for protein, cyan for dUMP and magenta for ligand; oxygen atoms, red; nitrogen atoms, blue; sulfur atoms, yellow.

Using the docking program DOCK 3.5, an interaction model of DDT with *Lc*TS was predicted (Figure 1A).[1] The X-ray structure of the ternary complex dUMP−*Ec*TS−DDT has recently been solved, showing that the binding orientation of DDT to *Ec*TS is quite unique and different from the one DOCK predicted vs *Lc*TS (Figure 1B).[9] Upon binding to *Ec*TS, as seen in the X-ray structure, DDT induces large rearrangements of residues both within and remote from the active site, promoting the binding of DDT in an intramolecular sandwiched conformation (folded conformation), which is very different from the linear one DOCK predicted (Figure 1A).

The observed crystallographic binding orientation for DDT compared to the predicted one raises the question of the reliability of our model vs *Lc*TS and its ability to support the design of new derivatives that are highly specific for bacterial TS with respect to hTS. In the effort to answer this question and study the molecular basis for the recognition of DDT in *Lc*TS, *Ec*TS, and hTS binding pockets, a new set of dansyl derivatives stemming from the lead compound was designed.

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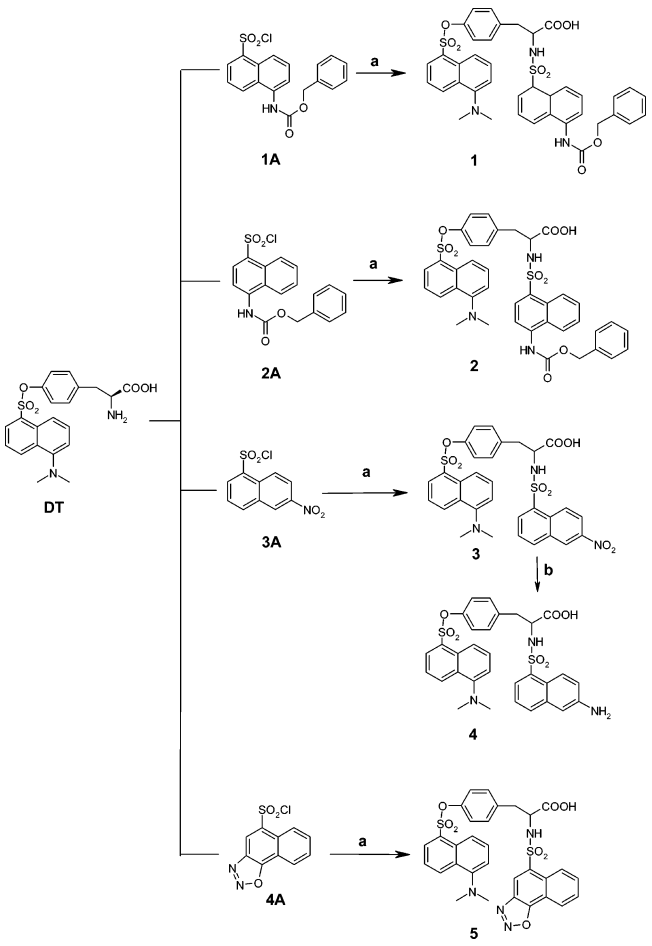
Scheme 1[a]



[a] Reagents and conditions: a: $NaHCO_3$, NaOH pH 10, r.t.; b: H_2, Pd/C.

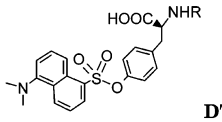
Substituting the *N*-dansyl fragment with different moieties provided the opportunity to span the active site of TS: the introduction of different chemical groups allowed the improvement and modulation of DDT activity and specificity toward bacterial species rather than hTS. Using molecular dynamics simulation, a new binding mode vs *Lc*TS was calculated to provide a better explanation of activity and specificity along the series, in comparison with hTS.

The DDT derivatives were synthesized as depicted in Scheme 1. The dansyl derivatives were prepared by reacting the dansyl-L-tyrosine (DT) with the appropriate sulfonyl chloride prepared in-house, in $NaHCO_3$ at room temperature.

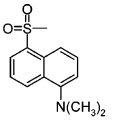
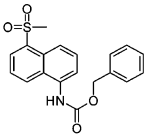
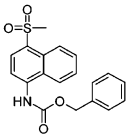
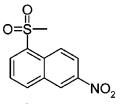
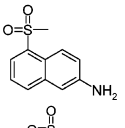
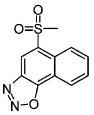
The compounds were screened for their activity and specificity against *Lc*TS, *Ec*TS, and hTS. Apparent inhibition constants (K_i) were determined, and the specificity index (SI) against hTS (Table 1) was calculated.

*Lc*TS, *Ec*TS, and hTS were purified as described.[10−12] Enzyme kinetic experiments were conducted under standard conditions.[7,13] The concentration of mTHF was kept 10 times the K_m (K_m values of *Ec*TS, *Lc*TS, and hTS are 5.3, 6.9, and 4.4 μM, respectively), and the dUMP concentration was 100 μM. Stock solutions of the inhibitors were freshly prepared in dimethyl sulfoxide (DMSO). The DMSO never exceeded the concentration of 5% in the reaction mixture. All the compounds showed a competitive pattern with respect to the folate cofactor. Each experiment was repeated at least three times and individual measurements did not differ from the mean by more than 20%.

Table 1. DDT Derivatives and Their Biological Activity Profile (SI = specificity index)



DT

Entry	R	LcTS Ki μM	EcTS Ki μM	hTS Ki μM	SI[a]	SI[b]
DDT		1.4[c]	1.8[c]	49	35	27
1		0.1	9.4	13	130	1.3
2		1.7	1.5	11	6.5	7.3
3		0.3	0.3	4.1	14	14
4		0.5	0.3	8.5	17	28
5		1.6[c]	15	1.5	0.9	0.1

[a] K_i hTS/K_i *Lc*TS. [b] K_i hTS/K_i *Ec*TS. [c] See ref 1.

Molecular dynamics studies were undertaken for the *Lc*TS−dUMP−DDT complex. Two possible ternary complexes of *Lc*TS−dUMP−DDT were built: in each of them a different binding conformation of the inhibitor was considered. The protein coordinates were taken from the 1LCA structure (*Lc*TS−dUMP−CB3717) and some modifications were introduced into the protein conformation.[5] Since the crystal structure of the *Ec*TS−dUMP−DDT complex shows that DDT induces essential and unique rearrangements in *Ec*TS, these conformational changes were included in our model of the *Lc*TS−dUMP−DDT complex, assuming that this compound could induce in *Lc*TS the same rearrangements as observed in *Ec*TS.[9] Therefore, based on the 1JG0 structure (*Ec*TS−dUMP−DDT), the conformation of two regions of *Lc*TS (lc-Gly17-lc-Try29 and lc-Phe294-lc-Val316) were modified to properly fit DDT into two binding conformations: in the first one, DDT is in the X-ray folded conformation; in the second one, the *N*-dansyl-moiety orientation was taken from the docking binding model.[1] dUMP coordinates were taken from the 1JG0 structure. The complexes were solvated with 24 Å spherical caps of TIP3P (AMBER6.0). The complexes were initially energy-minimized and then subjected to 400 ps of molecular dynamics (Sander_classic/AMBER

6.0).[14] All protein residues within 10 Å from the inhibitor, and all the water molecules were considered in the calculations. *Ec*TS[9] and hTS[15] crystal structures were used for comparative analysis. InsightII was used for visual analysis.[16]

In a small subset of DDT derivatives the *N*-dansyl moiety was substituted with chemical groups that have different molecular properties; the biological profile of the synthesized molecules was evaluated against *Lc*TS, *Ec*TS, and hTS enzymes and the structural requirements necessary for the specific recognition of DDT and derivatives vs *Lc*TS compared to *Ec*TS and hTS were analyzed.

Molecular dynamics studies were undertaken and two different models of *Lc*TS−dUMP−DDT were calculated. In the first binding mode, DDT assumes a "folded" conformation, showing a pattern of interactions similar to that observed in the *Ec*TS crystal structure, except for two hydrogen bonds formed with ec-Thr78 and ec-Ser54. These residues correspond to lc-His80 and lc-Leu56 in *Lc*TS. lc-His80 interacts with the ligand but with poor hydrogen bond geometry, whereas the apolar side-chain of lc-Leu56 cannot form any hydrogen bonds. In the second binding mode, DDT assumes a "semifolded" conformation and forms ring−ring interactions with lc-His80 and lc-Phe228, and hydrophobic interactions with lc-Ile81, lc-Trp82, lc-Leu195, and lc-Leu224. The carboxyl group interacts with the side chain of lc-Lys50, and hydrogen bonds are formed between the ligand and water molecules present in the active site (Figure 2A).

The residue ec-Thr78 is the key difference between *Lc*TS and *Ec*TS. In the folded X-ray conformation, ec-Thr78 forms a hydrogen bond with the O69 ligand atom, whereas the corresponding residue lc-His80 does not. In the case of the semifolded bound conformation, lc-His80 forms a ring−ring interaction with the tyrosine ring of DDT, whereas ec-Thr78 cannot.

These results led us to the conclusion that the folded conformation is the one preferred by *Ec*TS, while the semifolded conformation is preferred by *Lc*TS. On the basis of these results, the activity and specificity data for some of the dansyl derivatives under study were evaluated.

In hTS, in the folded conformation, DDT loses two hydrogen bonds compared to *Ec*TS. In fact, h-Gly83 and h-Lys107 (corresponding to ec-Ser54 and ec-Thr78) do not form hydrogen bonds with DDT. In the semifolded conformation, h-Lys107 (corresponding to lc-His80) does not form a ring−ring interaction with DDT. Moreover, it is reasonable that there is a small change in the enzyme conformation because of the bulkier residue h-Met311 (lc-Val314). Therefore, in hTS, both of these two binding conformations are less favored, in agreement with the differences in K_i.

Compound **1** showed the most interesting profile, being very specific vs *Lc*TS with respect to *Ec*TS and hTS, improving the specificity already shown by DDT for *Lc*TS against hTS (SI from 35-fold to 130-fold, respectively) (Table 1).

Because of the bulky substituent in position 5 of the *N*-dansyl ring, we consider that compound **1** binds to all the enzymes in the semifolded conformation. The substitution in position 5 causes the benzyloxycarboxyl group ("Z" group) of **1** to interact with the small domain region in *Lc*TS, whereas it is exposed to the solvent in the case of *Ec*TS and hTS, thus making **1** highly specific toward *Lc*TS. Compound **1** can orient the "Z" group toward lc-Glu84, lc-Phe104, lc-Gly105, and lc-Tyr118 (Figure 2B). These residues are missing in *Ec*TS and hTS, except for lc-Glu84 corresponding to ec-Glu82 and to h-Ala111. Therefore, in these enzymes the "Z" group of **1** lies on the protein surface.

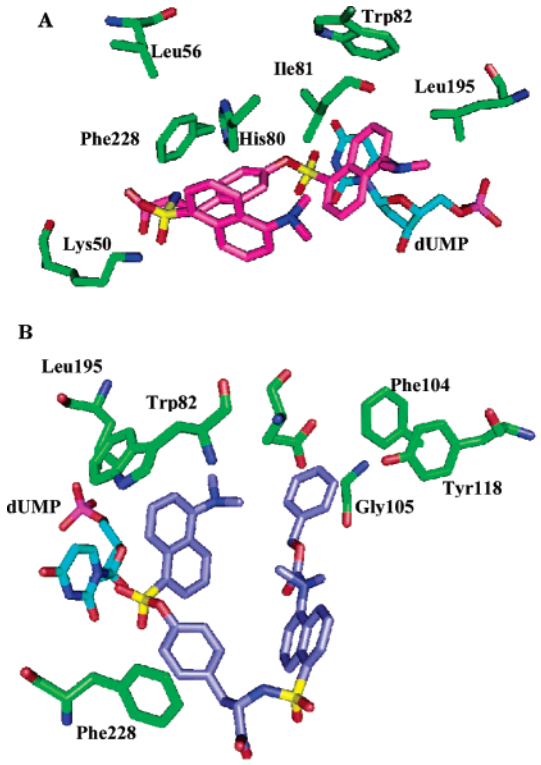


Figure 2. DDT (A) and compound **1** (B) in their semifolded conformation in *Lc*TS. Carbon atoms are colored green for protein, cyan for dUMP, and magenta for ligand; oxygen atoms, red; nitrogen atoms, blue; sulfur atoms, yellow. Figure B is rotated compared to image A for a better representation of the "Z" group interacting with the protein. Carbon atoms are colored green for protein, cyan for dUMP, and blue for ligand; oxygen atoms, red; nitrogen atoms, blue; sulfur atoms, yellow.

However, compound **2**, a close derivative of **1**, showed a completely different activity profile. Moving the functionality from position 5 to 4 removed the specificity shown by compound **1** *vs* *Lc*TS. Moreover, compound **2** (K_{ihTS} 11 μM) lost specificity vs bacterial species TS if compared to DDT and compound **1** (Table 1).

Compounds **3**, **4**, and **5** were synthesized to evaluate the possible role of different functionalities on the *N*-dansyl and how these could interfere with activity and specificity. Compounds **3** and **4** showed moderate specificity against bacterial TS with respect to hTS (SI within 14−28). Nevertheless, they showed sub-micromolar affinity for *Lc*TS and *Ec*TS. They showed the same enzyme inhibition profiles even if the N-group carries a nitro, lipophilic group in **3** and an amino, hydrophilic one in **4** (Table 1).

For compound **4,** we considered the same binding modes as for DDT. Visual inspection of *Lc*TS and *Ec*TS binding sites suggests that the amino group in position 6 of the *N*-dansyl ring allows the formation of additional hydrogen bonds with the enzyme residues

and water molecules present in the binding sites, in this way stabilizing the hydrogen bond networks. This explains the increase in the affinity of **4** compared to DDT.

Compound **5** seems able to discriminate between *Lc*TS and *Ec*TS (K_{iLcTS} 1.6 μM; K_{iEcTS} 15 μM) showing nine times lower affinity compared to *Ec*TS (Table 1).

Preliminary evaluation of the antimicrobial activity of DDT derivatives was performed: compound **1** showed improved antibacterial activity toward *Staphylococcus aureus* K23 with respect to DDT.[19]

We have described the synthesis, biological evaluation, and molecular dynamics studies of a new series of non-substrate-like antifolate inhibitors of TS. Among the synthesized molecules, compound **1** showed high specificity vs *Lc*TS with respect to *Ec*TS and hTS. It binds to the bacterial enzyme *Lc*TS at sub-micromolar concentrations and has 130-fold lower activity toward hTS. Molecular modeling studies for **1** vs *Lc*TS, *Ec*TS, and hTS suggest that the *N*-dansyl ring interacts directly with residues of TS that are peculiar of *Lc*TS. This inhibitor may lead to antimicrobial drugs targeting TSs enzymes that resemble *Lc*TS in their sequence.[7,8]

Furthermore, studies involving the design and synthesis of structurally constrained dansyl derivatives in a forthcoming paper will clarify conformation-mediated enzyme specificity mechanisms in TS enzymes.[20]

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Supporting Information Available: Mono- and bidimensional ^{1}H NMR data for all compounds. More detailed information on the modeling methodologies applied and a detailed description of the calculated model are included. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Tondi, D.; Slomczynska, U.; Costi, M. P.; Watterson, D. M.; Ghelli, S.; Shoichet, B. K. Structure-based discovery and in-parallel optimization of novel competitive inhibitors of thymidylate synthase. *Chem. Biol.* **1999**, *6*, 319−331.

(2) Jackson, R. C. Contributions of protein structure-based drug design to cancer chemotherapy. *Semin. Oncol.* **1997**, *24*, 164−172.

(3) Costi, M. P.; Tondi, D.; Rinaldi, M.; Barlocco, D.; Pecorari, P. G.; Soragni, F.; Venturelli, A.; Stroud, R. M. Structure-based studies on species-specific inhibition of thymidylate synthase. *Biochim. Biophys. Acta (BBA) − Mol. Basis Dis.* **2002**, *1587*, 206−214.

(4) Costi, P. M.; Rinaldi, M.; Tondi, D.; Pecorari, P.; Barlocco, D.; Ghelli, S.; Stroud, R. M.; Santi, D. V.; Stout, T. J.; Musiu, C.; Marangiu, E. M.; Pani, A.; Congiu, D.; Loi, G. A.; La Colla, P. Phthalein derivatives as a new tool for selectivity in thymidylate synthase inhibition. *J. Med. Chem.* **1999**, *42* (12), 2112−2124.

(5) Finer-Moore, J.; Fauman, E. B.; Foster, P. G.; Perry, K. M.; Santi, D. V.; Stroud, R. M. Refined structures of substrate-bound and phosphate-bound thymidylate synthase from Lactobacillus casei. *J. Mol. Biol.* **1993**, *20*, 1101−1116.

(6) Hardy, L. W.; Finer-Moore, J.; Montfort, W. R.; Jones, M. O.; Santi, D. V.; Stroud, R. M. Atomic structure of thymidylate synthase: target for rational drug design. *Science* **1987**, *235*, 448−455.

(7) Carreras, C. W.; Santi, D. V. The catalytic mechanism and structure of thymidylate synthase. *Annu. Rev. Biochem.* **1995**, *64*, 721−762.

(8) Coque, T. M.; Singh, K. V.; Weinstock, G. M.; Murray, B. E. Characterization of dihydrofolate reductase genes from trimethoprim-susceptible and trimethoprim-resistant strains of Enterococcus faecalis. *Antimicrob. Agents Chemother.* **1999**, *43*, 141−147.

(9) Fritz, T. A.; Tondi, D.; Finer-Moore, J. S.; Costi, M. P.; Stroud, R. M. Predicting and harnessing protein flexibility in the design of species-specific inhibitors of thymidylate synthase. *Chem. Biol.* **2001**, *8*, 981−995.

(10) Climie, S.; Santi, D. V. Chemical synthesis of the thymidylate synthase gene. *Proc. Natl. Acad. Sci.* **1990**, *87*, 633−637.

(11) Davisson, V. J.; Sirawaraporn, W.; Santi, D. V. Expression of human thymidylate synthase in Escherichia coli. *J. Biol. Chem.* **1989**, *264*, 9145−9148.

(12) Davisson, V. J.; Sirawaraporn, W.; Santi, D. V. Expression of human thymidylate synthase in Escherichia coli. *J. Biol. Chem*. **1994**, *269*, 30740.

(13) Pogolotti, A. L.; Danenberg, P. V.; Santi, D. V. Kinetics and mechanism of interaction of 10-propargyl-5,8-dideazafolate with thymidylate synthase. *J. Med. Chem.* **1986**, *29*, 478−482.

(14) Case, D. A. P., D. A.; Caldwell, J. W.; Cheatham, T. E., III.; Ross, W. S.; Simmerling, C. L.; Darden, T. A.; Merz, K. M.; Stanton, R. V.; Cheng, A. L.; Vincent, J. J.; Crowley, M.; Tsui, V.; Radmer, R. J.; Duan, Y.; Pitera, J.; Massova, I.; Seibel,; Singh, G. L.; Weiner, U. C.; Kollman, P. K. AMBER 6. 1999: University of California, San Francisco.

(15) Phan, J.; Koli, S.; Minor, W.; Dunlap, R. B.; Berger, S. H.; Lebioda, L. Human thymidylate synthase is in the closed conformation when complexed with dUMP and raltitrexed, an antifolate drug. *Biochemistry* **2001**, *40*, 1897−1902

(16) InsightII, InsighII, Modeling Environment. 2000; http://www.accelrys.com/.

(17) Shoichet B. K.; Stroud, R. M.; Santi, D. V.; Kuntz, I. D.; Perry, K. M. Structure-based discovery of inhibitors of thymidylate synthase. *Science* **1993**, *5*, 1445−1450.

(18) Strynadka, N. C.; Eisenstein, M.; Katchalski-Katzir, E.; Shoichet, B. K.; Kuntz, I. D.; Abagyan, R.; Totrov, M.; Janin, J.; Cherfils, J.; Zimmerman, F.; Olson, A.; Duncan, B.; Rao, M.; Jackson, R.; Sternberg, M.; James, M. N. Molecular docking programs successfully predict the binding of a beta-lactamase inhibitory protein to TEM-1 beta-lactamase. *Nat. Struct. Biol.* **1996**, *3*, 233−239.

(19) Compound 1, MIC 10 μg/mL. Rossi T. Personal communication.

(20) Tondi, D.; Calò, S.; Ferrari, S.; Ghelli, S.; Venturelli A.; Costi, M. P. Constrained dansyl derivatives probing molecular recognition *vs* bacterial Thymidylate Synthases. In preparation.

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