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Exploring the Ribose Sub-Pocket of the Substrate-Binding Site in *Escherichia coli* IspE: Structure-Based Design, Synthesis, and Biological Evaluation of Cytosines and Cytosine Analogues

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The enzymes of the non-mevalonate pathway for the isoprenoid biosynthesis are promising targets for the development of selective drugs for the treatment of important infectious diseases. This pathway is used by plants, many eubacteria, and apicomplexan protozoa, including major human pathogens such as *Plasmodium falciparum* and *Mycobacterium tuberculosis*, but not by humans who use the mevalonate pathway. In this work, we report on the design, synthesis, and biological evaluation of new ligands for the *E. coli* enzyme IspE. The focus of the study lies in the analysis of the ribose sub-pocket of the CDP-ME binding site. Therefore, we synthesized cytosine- and 2-aminopyridine-based inhibitors with various substituents targeting this sub-pocket at the enzyme active site. As cytosines display unexpectedly low solubilities in aqueous solution, special efforts were made to increase the water solubility of some compounds while maintaining the good binding affinities measured in earlier studies. In vitro studies showed IC_{50} values in the low micromolar to submicromolar range against *E. coli* IspE.

Introduction

Protozoan parasites belonging to the genus *Plasmodium* are the causative agents of malaria. Half of the world's human population lives in malaria-endangered regions, causing each year 300–500 million infections and more than one million deaths.[1] Although different drug combinations[2] are used in the treatment of malaria, an increasing number of multi-drug-resistant *Plasmodium* strains have been reported,[1a,2a,3] clearly showing the urgency for the development of new antimalarial drugs.

A promising strategy for developing new compounds against parasitic diseases involves targeting enzymes that are only present in the pathogens and not in the human host. In plasmodial parasites, the biosynthesis of the two essential isoprenoid precursor molecules, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), proceeds by the non-mevalonate pathway (Scheme 1), whereas in humans it proceeds exclusively by the mevalonate pathway. In 1999, Jomaa et al. reported curing malaria in rodents with fosmidomycin,[4] thereby validating the enzymes of the non-mevalonate pathway as highly attractive drug targets. Fosmidomycin is a potent inhibitor of the second enzyme in the pathway, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (IspC). Clinical trials with fosmidomycin are ongoing[5] as is the active search for more potent derivatives.[6]

A rapidly increasing number of X-ray crystal structures of the seven enzymes of the non-mevalonate pathway have recently been deposited in the PDB (RCSB Protein Data Bank).[7] On the basis of this structural information, our research group has designed and prepared the first families of ligands for the kinase IspE [4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME) kinase, EC 2.7.1.148], the fourth enzyme in the pathway.[8] Kinase IspE requires ATP and Mg^{2+} for the phosphorylation of the 2-hydroxy group of CDP-ME, which affords 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate (CDP-ME2P; Scheme 1).[9]

We chose *E. coli* IspE as a surrogate for *Plasmodium falciparum* and *Mycobacterium tuberculosis* IspE.[8c–8e] Until today, no IspE X-ray crystal structure of the *Plasmodium* enzyme could be solved as expression and purification of these enzymes has been rather difficult. The structural data on *M. tuberculosis* IspE were published only after the completion of this work. Interestingly, its rather conserved CDP-ME binding domain shows a high structural homology with the CDP-ME site of *E. coli* IspE.[8g] Earlier research yielded the 1-thiolanylated 5-ethynylated cytosine

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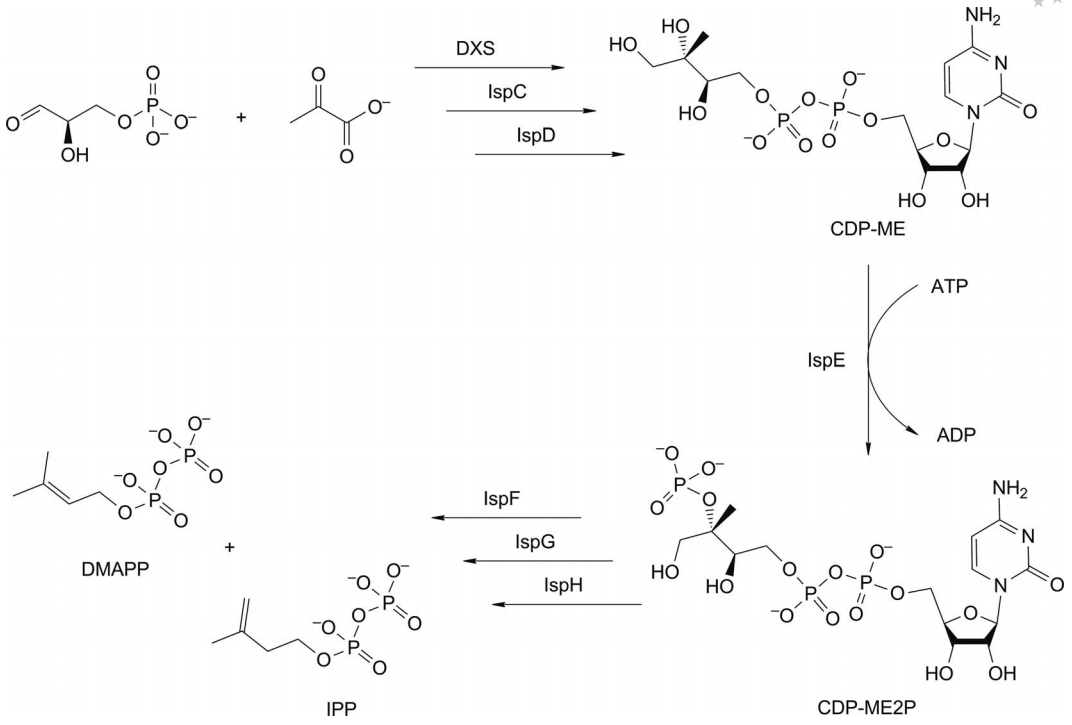


Scheme 1. Non-mevalonate pathway for the biosynthesis of the isoprenoid precursors IPP and DMAPP. DXS: 1-deoxy-D-xylulose 5-phosphate synthase (EC 2.2.1.7); IspC: 1-deoxy-D-xylulose 5-phosphate reductoisomerase (EC 1.1.1.267); IspD: 4-diphosphocytidyl-2-*C*-methyl-D-erythritol transferase (EC 2.7.7.60); IspF: 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (EC 4.6.1.12); IspG: 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate reductase (EC 1.17.4.3); IspH: 1-hydroxy-2-methyl-(2*E*)-butenyl 4-diphosphate reductase (EC 1.17.1.2).

(±)-**1** as the most active inhibitor of *E. coli* IspE (median inhibitory concentration IC_{50} = 1.7 ± 0.2 μM, K_i = 0.29 ± 0.10 μM; Figure 1). The corresponding 1-cyclopentylated cytosine **2** is also a good inhibitor of *E. coli* IspE (IC_{50} = 16 ± 0.5 μM, K_i = 1.5 ± 0.2 μM).[8d] These inhibitors bind to the cytidine pocket and the ME/phosphate binding site (see below), leaving the adenosine binding site open for its natural substrate.

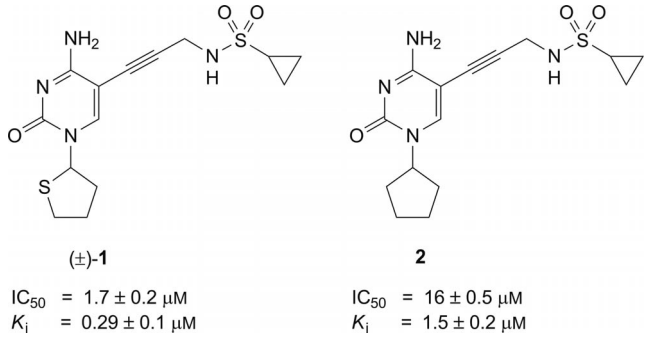


Figure 1. Structures and inhibition data of thiolane (±)-**1** and cyclopentane **2**.

Herein we report the design, synthesis, and biological activity of more polar and more water-soluble *O*-containing analogues of (±)-**1**, namely 2′-deoxycytidine **3** with a very similar structure, oxolane (±)-**4**, 4-ethoxybutanol (±)-**5**, and thietanedimethanol (±)-**6**. Earlier work on inhibitors of IspF[10] showed that 2-aminopyridines are a reasonable substitute for cytosines (for cytosine substitutes, see ref.[11]). Therefore, we also prepared the 2-aminopyridines **7a**–**i** bearing aryl moieties to fill the ribose sub-pocket.

The biological studies performed with these ligands have further enhanced the understanding of the molecular recognition properties of the IspE enzyme and cytosine-binding proteins in general. Cytosine replacements[11] are far less abundant and documented than adenine replacements,[12] for example, in kinase inhibitors,[13] or thymine surrogates, for example, in antitumor drugs.[14] Understanding cytosine recognition will become of growing importance in view of the interest in cytosine methylation/demethylation at the 5-position,[15] which is increasingly recognized as a major epigenetic mechanism in switching on (demethylated) or off (methylated) genes.[16]

Results and Discussion

Design

Molecular modeling (MOLOC[17]) of the co-crystal structure of *E. coli* IspE with bound CDP-ME and the ATP analogue ADPNP [adenosine-5′-(γ-imino)triphosphate] (2.0 Å resolution, PDB code 1OJ4)[8a] suggested that the central cytosine scaffold of the *E. coli* IspE inhibitor is

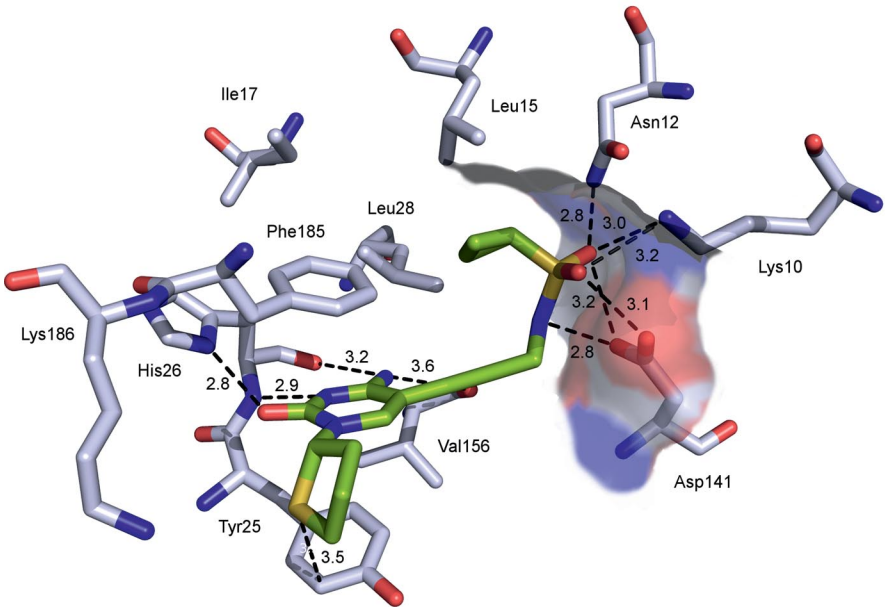


Figure 2. Complexation of inhibitor (*S*)-**1** in the cytidine binding pocket of the active site of *E. coli* IspE (PDB code: 1OJ4) modeled with MOLOC.[17] Both enantiomers of (±)-**1** are expected to bind with similar strength. Color code: carbon: green; nitrogen: blue; oxygen: red; sulfur: yellow. Heteroatom distances are given in Å. This color code is maintained throughout the article, unless otherwise stated.

sandwiched by Phe185 and partially by Tyr25 and forms three hydrogen bonds with the backbone and the side-chain of His26.[8d] This binding mode is depicted in Figure 2 for ligand (±)-**1** and is strongly supported by the X-ray structure of *Aquifex aeolicus* IspE co-crystallized with a closely related inhibitor.[8d] The small and rather lipophilic cyclopropylsulfonamido moiety appears to contribute significantly to the good binding affinity of (±)-**1**. The sulfonamido moiety, in the favorable staggered conformation with the N lone pair bisecting the O=S=O angle,[8d] can form hydrogen bonds with the catalytically important residues Lys10 and Asp141. The cyclopropyl ring fills a small, highly preorganized hydrophobic pocket lined by the side-chains of Leu15, Leu28, and Phe185, which is not occupied by the substrate CDP-ME.

Keeping the C-5 substituent of (±)-**1** unchanged, we wished to investigate the interactions of different N-1 substituents with the amino acids Tyr25, His26, and Lys186 in the ribose sub-pocket of the CDP-ME binding site. Given that the cyclopentane **2** shows a 10-fold lower binding affinity than thiolane (±)-**1**, an S···π interaction[18] with the phenol ring of Tyr25 had previously been postulated to explain the better binding of the latter (Figure 2).[8d] We hoped to test this hypothesis by evaluating the affinity of the tetrahydrofuranyl derivative (±)-**4**, the O atom of which is not expected to be directed into the π cloud of the phenol ring.[19] A co-crystal structure of bound (±)-**1** could not be obtained to prove the S···π interaction due to the low solubility of the ligand in pure aqueous buffer and because the co-crystallization protocol is intolerant to even small amounts of organic co-solvents.

In further attempts to obtain the degree of water solubility required for co-crystallization, we targeted the 2′-deoxyribose derivative **3**, for which modeling with MOLOC predicted a hydrogen bond from 3′-OH to the side-chain of Lys186 (Figure S1). The 2′-deoxyribose ring was expected to adopt a similar geometry to the one seen for the ribose of CDP-ME in the co-crystal structure (1OJ4). A further increase in water solubility was expected for 4-ethoxybutanol (±)-**5**, the ethoxy group of which, according to the molecular modeling, should engage in similar interactions with the amino acids of the ribose sub-pocket to those observed for the cyclic analogues **3** and (±)-**4** (Figure S1). With thiethane (±)-**6** (Figure S2) we hoped to gain further insight into the postulated S···π interaction of (±)-**1** while at the same time enhancing water solubility. One of the hydroxymethyl groups of (±)-**6** was expected to form a hydrogen bond with Lys186 similar to the one displayed by the bound natural substrate CDP-ME, whereas the second hydroxymethyl group has no partner to form an additional hydrogen bond and may just point into bulk water. According to the modeling, both enantiomers of (±)-**4**–**6** should fit well into the active site of *E. coli* IspE without any preference for one over the other.

In the series of 2-amino-5-arylpyridine-derived ligands, compounds **7a** and **7g**–**7i** feature a planar or nearly planar biaryl system, whereas in the *ortho*-substituted **7b**–**f** the two aromatic rings should have an increased torsional angle.[20]

Modeling suggested that the ribose binding site in between the side-chains of Pro182 and Tyr25 is actually too large to enable close van der Waals interactions with an aryl ring in the plane of the aminopyridine ring. According to

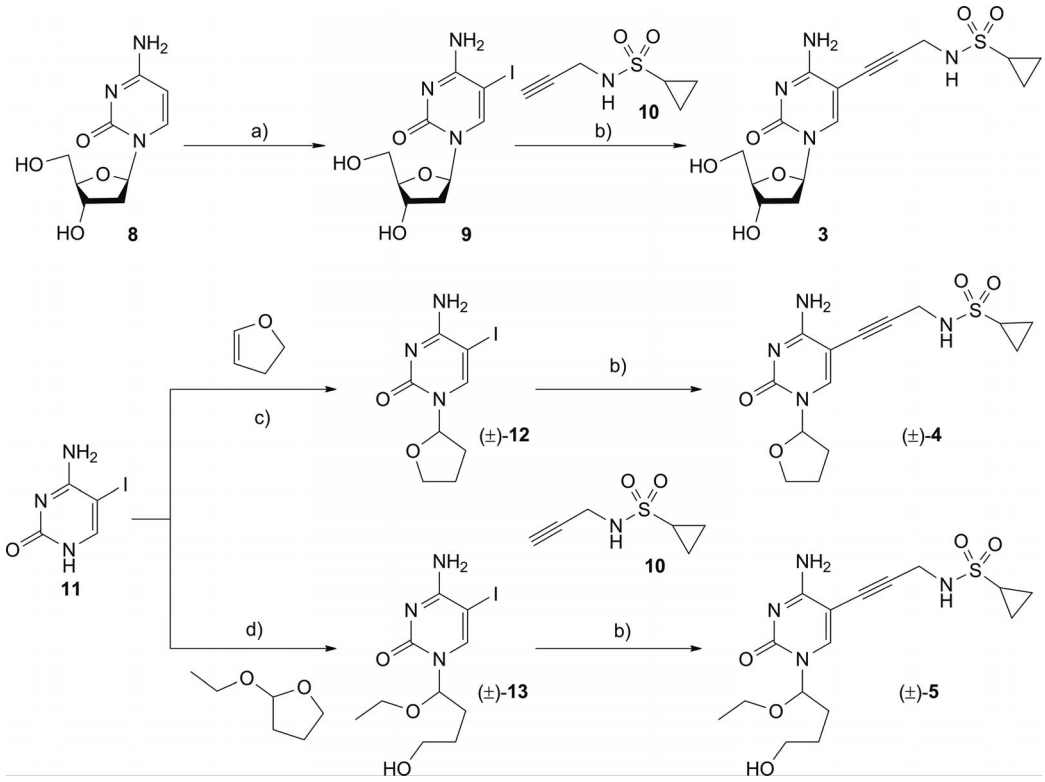
the molecular modeling (Figure S3), an aromatic ring twisted out of the plane of the aminopyridine ring could possibly establish better intermolecular contacts, and we intended to verify this hypothesis in our studies.

Synthesis

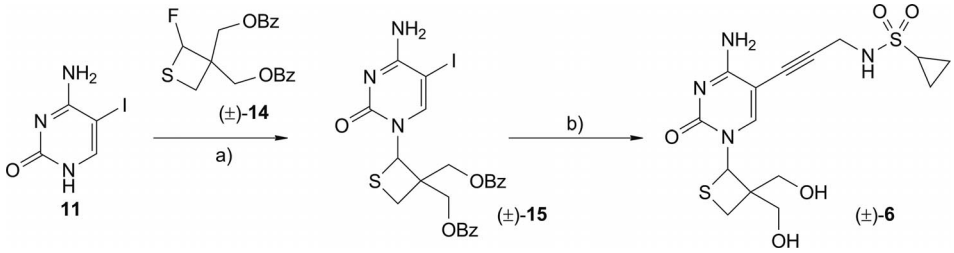
Iodination of 2′-deoxycytidine (**8**) according to the procedure of Kumar et al.,[21] but without the use of ionic liquids, gave **9**[22] in 57% yield (Scheme 2). Sonogashira coupling of **9** with the known sulfonamide **10**[8c] gave the 5-ethynylated 2′-deoxycytidine **3** in 60% yield. The syntheses of (±)-**4** and (±)-**5** started from 5-iodocytosine (**11**).[23] For the acetalization of **11** at N-1, the intermediate formation of 2-*O*-silylated 5-iodocytosine[24] is crucial for acceptable yields. The TsOH-catalyzed addition of silylated **11** to 2,3-dihydrofuran yielded (±)-**12** (19% yield), whereas the reaction with 2-ethoxyoxolane and TMSOTf led to the butanol (±)-**13** (18% yield). Sonogashira coupling of (±)-**12** and (±)-**13** with sulfonamide **10** gave the desired target compounds (±)-**4** and (±)-**5** in yields of 58 and 59%, respectively (Scheme 2).

The procedure of Nishizono et al.[25] was applied to the synthesis of the 2-fluorothietane (±)-**14** by starting from 2,2-bis(hydroxymethyl)-1,3-propanediol (five steps). Silylation under Vorbrüggen conditions[24] of iodocytosine **11** and $AgClO_4$-promoted condensation of the resulting silylated cytosine with (±)-**14** gave 35% of the thietanylated iodocytosine (±)-**15** (Scheme 3). Interestingly, a Pummerer-type reaction with the corresponding thietane 1-oxide gave only traces of the desired product, whereas a similar reaction with uracil provided acceptable yields of the product.[25] Debenzoylation of (±)-**15** and subsequent Sonogashira coupling with sulfonamide **10** afforded the desired thietanylated and ethynylated cytosine (±)-**6**, albeit in a low yield (14%). The inverse sequence of reactions, coupling before debenzoylation, failed as the coupling product decomposed under the conditions of debenzoylation.

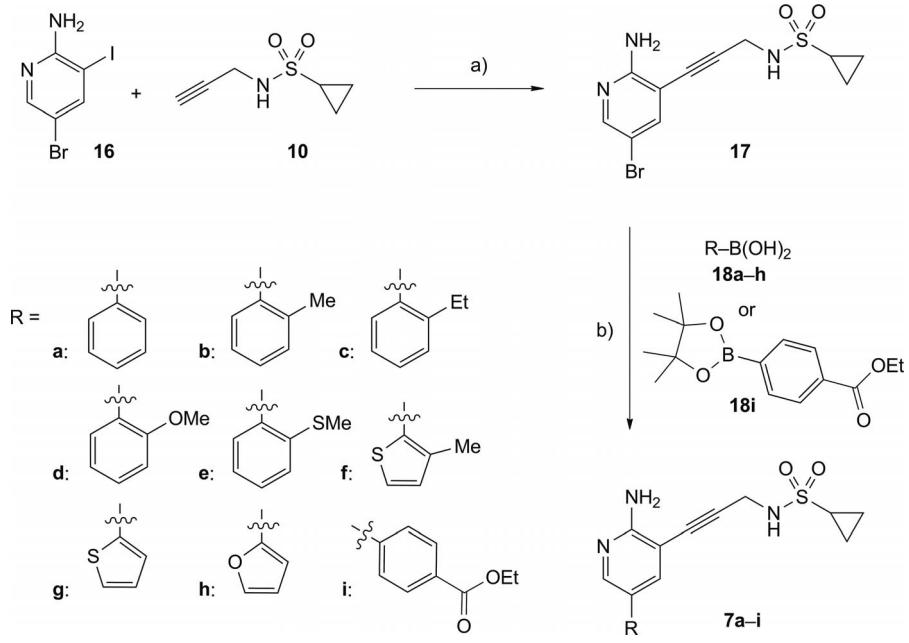
The 2-fluorothietane (±)-**14** shows characteristic J(H,F) couplings in the ^{1}H NMR spectrum. The F atom couples with the geminal 2-H [^2J(H,F) = 63.3 Hz], the *cis*-oriented 4-H [^4J(H,F) = 9.0 Hz], and the *cis*-oriented methylene group [^4J(H,F) = 3.0 Hz and < 1.5 Hz (line-broadening)]. Nishizono et al.[25] only reported the geminal ^2J(H,F) coupling. The coupling of the pseudoequatorial F atom of (±)-**14** with 4-H_{cis} is a *W* coupling transmitted through S and C-2 of the slightly puckered thietane ring, whereas a close contact is responsible for the couplings with the H atoms of the *cis*-methylene group. A *W* coupling of 6 Hz between F and 4-H_{cis} of a more strongly puckered thietane *S*-oxide has previously been observed.[26] Calculations with Spartan 10[27] (B3LYP/6-31* level of theory) correlated with the weak puckering of (±)-**14** and the strong puckering of thietane *S*-oxide, as indicated by the S–C1–C2–C3 torsion angles of 8 and 28°, respectively (Scheme 3).



Scheme 2. Synthesis of inhibitors **3**, (±)-**4**, and (±)-**5**. Reagents and conditions: (a) *N*-iodosuccinimide (NIS), CH_2Cl_2, 22 °C, 57%; (b) **10**, NEt_3, [$Pd(PPh_3)_2Cl_2$], CuI, DMF, 22 °C, 60% of **3**, 58% of (±)-**4**, and 59% of (±)-**5**; (c) hexamethyldisilazane (HMDS), 75 °C, 2,3-dihydrofuran, $TsOH·H_2O$ (cat.), CH_2Cl_2, 22 °C, 19%; (d) HMDS, 60 °C, 2-ethoxyoxolane, TMSOTf, $TsOH·H_2O$ (cat.), CH_2Cl_2, 22 °C, 18%.



Scheme 3. Synthesis of inhibitor (±)-**6**. Reagents and conditions: (a) HMDS, reflux; (±)-**14**, $AgClO_4$, $SnCl_2$, CH_2Cl_2, 22 °C, 35%; (b) NaOMe, MeOH/CH_2Cl_2, 22 °C; **10**, NEt_3, $[Pd(PPh_3)_2Cl_2]$, CuI, DMF, 22 °C, 14%.



Scheme 4. Synthesis of inhibitors **7a**–**7i**. Reagents and conditions: (a) **10**, $[Pd(PPh_3)_2Cl_2]$, CuI, NEt_3, THF, 25 °C, 87%; (b) **18a**–**i**, $[Pd(dppf)Cl_2]\cdot CH_2Cl_2$, 2 M aq. Na_2CO_3, 1,2-dimethoxyethane (DME), reflux; 59% of **7a**, 54% of **7b**, 43% of **7c**, 58% of **7d**, 56% of **7e**, 40% of **7f**, 60% of **7g**, 69% of **7h**, and 25% of **7i**.

Compounds **7a**–**i** were synthesized by two consecutive cross-coupling reactions. Sonogashira coupling of 2-amino-5-bromo-3-iodopyridine (**16**), which has previously been prepared by Beard et al.,[28] with sulfonamide **10** yielded the ethynylated 5-bromopyridine **17** (Scheme 4), the common precursor for the desired biaryls. Suzuki coupling reactions of **17** with the commercially available boronic acids **18a**–**g**, the known boronic acid **18h**,[29] and the pinacol boronate **18i**[30] led to the targeted compounds **7a**–**i** in moderate to good yields (**7a**–**h**: 40–69%; **7i**: 25%), (Scheme 4).

Biological Results

The IC_{50} values for ligands **3**, (±)-**4**–**6**, and **7a**–**i** were determined in an enzyme-coupled photometric assay[31] by using *E. coli* IspE, pyruvate kinase, and lactate dehydrogenase. Per catalytic turnover, 1 equiv. of ATP was used for the phosphorylation of CDP-ME to CDP-ME2P, which was restored by the pyruvate kinase under the conversion of phospoenol pyruvate (PEP) to pyruvate. Lactate dehydrogenase reduces pyruvate to lactate with 1 equiv. of NADH. The NADH consumption was monitored at a wavelength of 340 nm as NAD^+ does not absorb light at this wavelength. The photometric assay has previously been proven to work effectively for similar compounds by using a direct NMR spectroscopic assay.[31]

According to the biological data in Table 1, the previously reported ligand (±)-**1** remains the best inhibitor of *E. coli* IspE. Besides providing an optimal volume occupancy for the ribose binding site, inhibitor (±)-**1** has also been proposed to benefit from an S···π interaction with Tyr25. The results presented herein apparently disprove this proposal. Oxolane (±)-**4** shows nearly the same binding affinity as thiolane (±)-**1**, which would not have been expected if the latter can undergo a particular interaction that is absent in the complex of (±)-**4**. According to the molecular modeling, both five-membered rings can adopt favorable conformations with their heteroatom either approaching the tyrosine ring or turning away from it. We propose that the oxolanyl ring of (±)-**4** adopts a conformation similar to

Table 1. Inhibitory activities of inhibitors (±)-**1**, **2**, **3**, (±)-**4**–**6**, and **7a**–**i** for *E. coli* IspE and calculated physicochemical properties.

	*c*log *P*[a]	*c*log *D*[a] at pH 8.0	IC_{50} [μM]	K_i[b] (calcd.) [μM]	K_i[c] (exptl.) [μM]
(±)-**1**	1.46 ± 0.76	1.32	1.7 ± 0.2	0.6	0.29 ± 0.1
2	2.02 ± 0.63	1.89	16 ± 0.5	5.1	1.5 ± 0.2
3	–2.41 ± 0.81	–2.27	36 ± 2	13.6	–
(±)-**4**	–0.27 ± 0.73	–0.41	2.0 ± 0.2	0.7	–
(±)-**5**	0.17 ± 0.73	0.04	152 ± 0.9	57	–
(±)-**6**	–0.19 ± 0.86	–0.33	103 ± 7	38.6	–
7a	2.02 ± 1.06	2.01	≥ 500	–	128 ± 12
7b	2.57 ± 1.06	2.56	480 ± 44	180	–
7c	3.08 ± 1.06	3.07	> 500	–	–
7d	1.47 ± 1.09	1.46	242 ± 24	91	–
7e	1.72 ± 1.09	1.71	341 ± 24	128	–
7f	2.18 ± 1.08	2.17	273 ± 35	102	–
7g	1.83 ± 1.07	1.82	193 ± 9	138	–
7h	1.73 ± 1.07	1.72	> 500	–	–
7i	2.61 ± 1.07	2.60	> 500	–	–

[a] *c*log *P* and *c*log *D* values were calculated by using Advanced Chemistry Development (ACD/Labs) Software V12.5 (© 1994–2012 ACD/Labs). [b] K_i values were calculated from IC_{50} values and K_m = 150 μM (*E. coli* IspE) from the Cheng–Prusoff equation.[33] [c] K_i values were measured in a spectrophotometric assay by varying the substrate concentration from 0 to 400 μM.

the furanosyl ring of the natural substrate in which the O atom does not point into the π cloud of the phenol side-chain. The cyclopentyl derivative **2**[8d] binds more weakly than (±)-**1** and (±)-**4** by approximately a factor of eight. The heteroatoms of the alicyclic heterocycles induce local dipoles, which could provide a better interaction with the protein than the cyclopentyl substituent.[32]

Polar, water-solubility-enhancing substituents are not well tolerated near the ribose binding site (Table 1). 2′-Deoxycytidine **3**, the only enantiomerically pure compound of this series, shows an approximately 20-fold larger IC_{50} value than (±)-**1** or (±)-**4**. The 4-ethoxybutanol derivative (±)-**5** binds more weakly than (±)-**1** and (±)-**4** by almost a factor of 100. Inhibitors **3**, (±)-**5**, and (±)-**6** are moderately water-soluble, and co-crystallization experiments are ongoing to provide a deeper insight into the molecular recognition modes in the cytidine binding pocket.

The planar biaryl ligand **7g** exhibits a weak binding affinity (IC_{50} = 193 μM), whereas the other planar derivatives (**7a**, **7h**, and **7i**) show no inhibition within the measurement limits of the biological assay (IC_{50} ≥ 500 μM, Table 1). The twisted biaryls **7b**–**7f**, with the exception of **7c**, show measurable inhibition of *E. coli* IspE. Methyl ether **7d**, 3-methylated thiophene **7f**, and thioether **7e** display similar complexation strengths, which rules out any contributions of an S···π interaction to the binding of **7e** and **7f**. Overall, there is no substantial difference in the binding affinities of planar and twisted biaryls. A direct comparison between the binding data for ligands with cycloalkyl or alicyclic heterocycles filling the ribose sub-pocket and those with aromatic rings is not possible as the change from cytosine to its substitute aminopyridine lowers the overall binding affinity. Nevertheless, the data seem to suggest that the ribose sub-pocket in the substrate binding site of *E. coli* IspE is better filled by cycloalkyl and alicyclic heterocyclic residues than by aromatic rings.

Conclusions

We have described herein the design, synthesis, and in vitro evaluation of a series of inhibitors of the kinase IspE (*E. coli*). Valuable insights into the molecular recognition properties of the substrate binding site of *E. coli* IspE have been obtained. (1) Low-molecular-weight inhibitors with IC_{50} values of ca. 1 μM are obtained when appropriately functionalized cytosines direct a five-membered alicyclic heterocycle into the ribose sub-pocket. (2) Direct interactions of heterocyclic S atoms with the phenolic ring of Tyr25 are not effective; however, the local dipoles induced by the O or S atoms in the alicyclic heterocycles enhance their binding relative to cyclopentyl-substituted ligands by nearly an order of magnitude. (3) Substituted aminopyridines are potential cytosine substitutes, although the aryl-substituted derivatives prepared in this study are rather modest binders; the ribose sub-pocket does not seem to be an effective aryl-binding site. (4) Although flexible polar functional groups do not enhance but rather reduce binding affinity, water-soluble ligands are obtained that should now enable successful co-crystallization with IspE.

Experimental Section

Materials and General Methods: Reagents were purchased reagent-grade or, if necessary, purified by distillation. Dry solvents (DMF, CH_2Cl_2, MeOH, and THF) were purified by a solvent-drying system from LC Technology Solutions Inc. S-105 under nitrogen (H_2O content < 10 ppm as determined by Karl-Fischer titration). All reactions were performed in oven-dried glassware under Ar or N_2. TLC: Glass sheets coated with SiO_2 60 F_{254} from Merck, visualization by UV light at 254 nm, and staining with a solution of $KMnO_4$ (1.5 g) and K_2CO_3 (10 g) in H_2O (150 mL). Column chromatography: SiO_2 (60) from Fluka. Melting points were determined in an open capillary by using a Büchi-510 apparatus. IR spectra were recorded with a Perkin–Elmer BX FT-IR spectrophotometer (ATR

unit, Golden Gate) neat or as a film. NMR spectra were recorded at 22 °C with a Varian Gemini 300 or Bruker DRX 400 spectrometer with an automated sample changer by using the solvent peak as internal reference. Coupling constants (*J*) are given in Hz. Resonance multiplicities are described as s (singlet), br. s (broad singlet), d (doublet), t (triplet), q (quartet), quint (quintet), and m (multiplet). High-resolution mass spectra (HRMS) were recorded with a Bruker maXis-ESI-Q-TOF (ESI) or Micromass (Waters) AutoSpec Ultima-EI-EBE-MS (EI) spectrometer. The masses are given as *m*/*z* with $[M]^+$ representing the molecular ion. Elemental analyses were performed by the Mikrolabor at the Laboratorium für Organische Chemie, ETH Zürich. Nomenclature is given in accord with nucleic acid nomenclature or proposals of the computer program ACD/Name. Experimental procedures for the synthesis of compounds **7b**–**i** are available in the Supporting Information.

Enzyme-Coupled Photometric Assay for IC_{50} Determination: Assay mixtures were prepared as previously described[31] with some minor modifications: A solution (60 μL) containing 100 mM Tris-HCl (pH = 8.0), 10 mM $MgCl_2$, 2 mM dithiothreitol, 2.5 mM phosphoenolpyruvate potassium salt, 2 mM ATP, 0.46 mM NADH, 1 U of lactate dehydrogenase, 1 U of pyruvate kinase, and 1 U of IspE protein were added to the inhibitor solution (60 μL, final concentration of 8–1000 μM). The reaction was started by addition of CDP-ME (60 μL, final concentration 250 μM) and monitored at a wavelength of 340 nm at 27 °C.

5-[3-(Cyclopropylsulfonylamino)prop-1-yn-1-yl]-2′-deoxycytidine (3): A solution of **9** (100 mg, 0.29 mmol), **10** (100 mg, 0.62 mmol), and NEt_3 (0.2 mL, 1.4 mmol) in DMF (5 mL) was degassed by freeze–pump–thaw cycles performed under Ar. $[Pd(PPh_3)_2Cl_2]$ (42 mg, 0.06 mmol) and CuI (27 mg, 0.14 mmol) were added, and the mixture was stirred at 25 °C for 4 h. Concentration and chromatography (SiO_2; CH_2Cl_2/MeOH, 93:7) yielded **3** (64 mg, 60%) as a brown solid (containing triethylammonium salt). R_f = 0.32 (SiO_2; CH_2Cl_2/MeOH, 92:8). $[\alpha]_D^{22}$ = +15.0 (*c* = 0.07, CH_2Cl_2/MeOH, 1:1). M.p. 154–157 °C. ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD, 5:1): δ = 0.75–0.86 (m, 2 H, 2′′,3′′-H), 0.89–0.97 (m, 2 H, 2′′,3′′-H), 1.88–1.99 (m, 1 H, 2′-H), 2.15–2.25 (m, 1 H, 2′-H), 2.31 (tt, $J \approx$ 8.0, 3.9 Hz, 1 H, 1′′-H), 3.54 (br. d, *J* = 12.7 Hz, 1 H, 5′-H), 3.64 (br. d, *J* = 11.9 Hz, 1 H, 5′-H), 3.75 (br. s, 1 H, 4′-H), 3.88 (s, 2 H, NCH_2), 4.15 (br. s, 1 H, 3′-H), 5.96 (br. s, 1 H, 1′-H), 8.07 (s, 1 H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD, 5:1): δ = 5.01 (2 C), 29.86, 33.11, 41.04, 60.97, 69.82, 74.41, 86.60, 87.31, 90.72, 91.77, 144.41, 154.97, 164.67 ppm. IR (ATR): $\tilde{\nu}$ = 3332 (w, br.), 3208 (w, br.), 2923 (w), 2233 (w), 1642 (s), 1601 (m), 1533 (m), 1497 (m), 1436 (m), 1323 (s), 1299 (m), 1260 (m), 1190 (m), 1141 (s), 1089 (s), 1055 (s), 950 (m), 889 (m), 836 (m), 782 (m), 726 (s), 646 (m) cm^{-1}. HRMS (ESI): *m*/*z* (%) = 385.1177 (100) $[M + H]^+$, 269.0699 (52) $[M - C_5H_9O_3]^+$. HRMS (ESI): calcd. for $C_{15}H_{21}N_4O_6S^+$ $[M + H]^+$ 385.1176; found 385.1177.

(±)-*N*-{3-[1-(Oxolan-2-yl)cytosin-5-yl]prop-2-yn-1-yl}cyclopropanesulfonamide [(±)-4]: A solution of (±)-**12** (100 mg, 0.32 mmol), **10** (100 mg, 0.62 mmol), and NEt_3 (0.2 mL, 1.44 mmol) in DMF (4 mL) was degassed by freeze–pump–thaw cycles performed under Ar. $[Pd(PPh_3)_2Cl_2]$ (52 mg, 0.04 mmol) and CuI (32 mg, 0.07 mmol) were added, and the mixture was stirred at 25 °C for 6 h. Concentration and chromatography (SiO_2; CH_2Cl_2/MeOH, 97:3) yielded (±)-**4** (64 mg, 58%) as an off-white solid. R_f = 0.42 (SiO_2; CH_2Cl_2/MeOH, 95:5). M.p. 118–122 °C. ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD, 6:1): δ = 0.90–0.99 (m, 2 H, 2,3-H), 1.11 (td, *J* = 6.8, 4.8 Hz, 2 H, 2,3-H), 1.70–1.88 (m, 1 H, 4′′-H), 1.90–2.05 (m, 2 H, 3′′,4′′-H), 2.26–2.42 (m, 1 H, 3′′-H), 2.49 (tt, *J* = 8.0, 4.9 Hz, 1 H, 1-H), 3.92 (td, *J* = 8.5, 6.7 Hz, 1 H, 5′′-H), 4.08 (s, 2 H, CH_2NH), 4.20 (td, *J* = 8.2, 4.2 Hz, 1 H, 5′′-H), 5.86 (dd, *J* = 6.2, 2.6 Hz, 1 H, 2′′-H), 7.61 (s, 1 H, 6′-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD, 6:1): δ = 5.36 (2 C), 23.33, 24.60, 30.12, 33.10, 63.63, 70.22, 74.81, 88.29, 91.39, 142.79, 154.62, 164.61 ppm. IR (ATR): $\tilde{\nu}$ = 3426 (w), 3324 (w), 2961 (w), 2874 (w), 2235 (w), 1631 (s), 1611 (s), 1572 (s), 1471 (s), 1276 (m), 1249 (m), 1180 (m), 1121 (w), 1066 (s), 1032 (m), 950 (m), 922 (m), 897 (m), 779 (s), 736 (m), 619 (s) cm^{-1}. HRMS (ESI): *m*/*z* (%) = 339.1121 (100) $[M + H]^+$, 269.0703 (99) $[M - C_4H_6O + H]^+$. HRMS (ESI): calcd. for $C_{14}H_{19}N_4O_4S^+$ $[M + H]^+$ 339.1122; found 339.1121; calcd. for $C_{10}H_{13}N_4O_3S^+$ $[M - C_4H_6O + H]^+$ 269.0703; found 269.0703.

(±)-*N*-{3-[1-(1-Ethoxy-4-hydroxybutyl)cytosin-5-yl]prop-2-yn-1-yl}cyclopropanesulfonamide [(±)-5]: A solution of (±)-**13** (100 mg, 0.28 mmol), **10** (100 mg, 0.62 mmol), and NEt_3 (0.2 mL, 1.44 mmol) in DMF (4 mL) was degassed by freeze–pump–thaw cycles performed under Ar. $[Pd(PPh_3)_2Cl_2]$ (52 mg, 0.04 mmol) and CuI (32 mg, 0.07 mmol) were added, and the mixture was stirred at 25 °C for 6 h. Concentration and chromatography (SiO_2; CH_2Cl_2/MeOH, 97:3) yielded (±)-**5** (64 mg, 59%) as an orange, viscous oil. R_f = 0.38 (SiO_2; CH_2Cl_2/MeOH, 95:5). ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD, 6:1): δ = 0.90–0.99 (m, 2 H, 2,3-H), 1.06–1.18 (m, 2 H, 2,3-H), 1.11 (t, *J* = 7.1 Hz, 3 H, OCH_2CH_3), 1.44–1.60 (m, 2 H, 2′′-H), 1.61–1.72 (m, 2 H, 3′′-H), 3.39 (q, *J* = 7.0 Hz, 2 H, OCH_2CH_3), 3.50–3.56 (m, 2 H, 4′′-H), 4.08 (s, 2 H, CH_2NH), 5.63 (t, *J* = 6.1 Hz, 1 H, 1′′-H), 7.59 (s, 1 H, 6′-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD, 6:1): δ = 5.50 (2 C), 8.62, 27.35, 30.24, 32.29, 33.27, 61.11, 65.05, 68.55, 74.55, 86.37, 92.04, 142.68, 155.05, 164.07 ppm. IR (ATR): $\tilde{\nu}$ = 3411 (w), 3244 (w), 3049 (w), 2974 (w), 2914 (w), 2277 (w), 1661 (m), 1631 (s), 1614 (s), 1536 (m), 1481 (s), 1444 (m), 1387 (m), 1367 (m), 1303 (m), 1275 (m), 1244 (m), 1201 (m), 1150 (m), 1061 (s), 989 (m), 912 (m), 838 (m), 803 (m) 779 (s), 661 (m), 640 (s) cm^{-1}. HRMS (ESI): *m*/*z* (%) = 407.1360 (30) $[M + Na]^+$, 269.0692 (100) $[M - C_6H_{12}O_2 + 2H]^+$. HRMS (ESI): calcd. for $C_{16}H_{24}N_4NaO_5S^+$ $[M + Na]^+$ 407.1352; found 407.1360; calcd. for $C_{10}H_{13}N_4O_3S^+$ $[M - C_6H_{12}O_2 + 2H]^+$ 269.0703; found 269.0692.

(±)-*N*-(3-{1-[3,3-Bis(hydroxymethyl)thietan-2-yl]cytosin-5-yl}prop-2-yn-1-yl)cyclopropanesulfonamide [(±)-6]: A solution of (±)-**15** (200 mg, 0.34 mmol) in 1 M NaOMe in MeOH (7.5 mL) and CH_2Cl_2 (7.5 mL) was stirred at 22 °C for 3 h. After the addition of a satd. aq. solution of $NaHCO_3$ (10 mL), the mixture was diluted with CH_2Cl_2/*i*PrOH (3:1) (3 × 20 mL). The organic phase was separated, washed with brine, dried with Na_2SO_4, filtered, and concentrated under reduced pressure. A solution of the residue, **10** (90 mg, 0.6 mmol), and NEt_3 (0.25 mL, 1.8 mmol) in DMF (8 mL) in a Schlenk flask was degassed by freeze–pump–thaw cycles performed under Ar and then treated with $[Pd(PPh_3)_2Cl_2]$ (42 mg, 0.06 mmol) and CuI (28 mg, 0.07 mmol). The mixture was stirred at 25 °C for 18 h, diluted with CH_2Cl_2/*i*PrOH (3:1), and washed with a satd. aq. solution of $NaHCO_3$ and brine. The organic layer was dried with Na_2SO_4, filtered, and concentrated under reduced pressure. Chromatography (SiO_2; CH_2Cl_2/MeOH, 93:7) afforded (±)-**6** (18 mg, 14%) as an off-white solid. R_f = 0.38 (SiO_2; CH_2Cl_2/MeOH, 93:7). M.p. 131–134 °C. ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD, 6:1): δ = 0.89–0.97 (m, 2 H, 2,3-H), 1.05–1.12 (m, 2 H, 2,3-H), 2.42 (tt, *J* = 8.0, 4.9 Hz, 1 H, 1-H), 2.72 and 2.76 (2 d, *J* = 9.3 Hz, 2 H, 4′′-H), 3.39 and 3.45 (2 d, *J* = 11.4 Hz, 2 H, CH_2OH), 3.52 and 3.79 (2 d, *J* = 11.6 Hz, 2 H, CH_2OH), 4.02 (s, 2 H, CH_2N), 5.78 (s, 1 H, 2′′-H), 8.36 (s, 1 H, 6′-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD, 6:1): δ = 5.27 (2 C), 23.83, 30.04, 32.99, 55.49, 59.52, 60.70, 65.37, 74.42, 90.69, 91.69, 146.02, 156.09, 164.53 ppm. IR (ATR): $\tilde{\nu}$ = 3329 (w), 3200 (w), 2926 (w), 2868 (w), 2235 (w),

1637 (s), 1595 (m), 1496 (s), 1404 (m), 1322 (s), 1297 (s), 1239 (m), 1190 (w), 1140 (s), 1040 (s), 888 (s), 827 (m), 784 (s), 704 (s) cm^{-1}. HRMS (ESI): *m*/*z* (%) = 401.0945 (100) $[M + H]^+$. HRMS (ESI): calcd. for $C_{15}H_{21}N_4O_5S_2^+$ $[M + H]^+$ 401.0948; found 401.0945.

Typical Example of the Suzuki Coupling of Pyridyl Bromide 17. Synthesis of *N*-[3-(2-Amino-5-phenyl-3-pyridyl)prop-2-yn-1-yl]cyclopropanesulfonamide (7a): Pyridyl bromide **17** (170 mg, 0.50 mmol) and phenylboronic acid (**18a**; 120 mg, 1.0 mmol) were added to a mixture of DME (18 mL) and 2 M aq. Na_2CO_3 (0.5 mL) in a Schlenk flask. After degassing and addition of $[Pd(dppf)Cl_2]\cdot CH_2Cl_2$ (41 mg, 0.05 mmol), the mixture was heated at reflux for 18 h and concentrated under reduced pressure. Chromatography (SiO_2; hexane/EtOAc, 1:1) afforded **7a** (100 mg, 59%) as an orange oil. ^1H NMR (400 MHz, $CDCl_3$): δ = 1.01–1.08 (m, 2 H, 2,3-H_{trans}), 1.22–1.28 (m, 2 H, 2,3-H_{cis}), 2.56 (tt, *J* = 8.0, 4.8 Hz, 1 H, 1-H), 4.24 (d, *J* = 5.5 Hz, 2 H, NCH_2), 4.81 (s, 1 H, NH), 5.26 (br. s, 2 H, NH_2), 7.28–7.45 (m, 5 H, arom. H), 7.69 (d, *J* = 2.3 Hz, 1 H, 4′-H), 8.27 (d, *J* = 2.3 Hz, 1 H, 6′-H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 5.94 (2 C), 30.7, 33.7, 79.6, 91.4, 102.1, 125.9 (2 C), 126.5, 127.0, 128.8 (2 C), 137.0, 138.5, 146.0, 158.1 ppm. IR (ATR): $\tilde{\nu}$ = 3460 (w), 3360 (w), 3061 (w), 2228 (w), 1618 (m), 1460 (s), 1402 (m), 1326 (s), 1303 (s), 1242(m), 1142 (s), 1068 (s), 985 (m), 891 (s), 828 (m), 758 (s), 690 (s), 668 (m) cm^{-1}. HRMS (ESI): *m*/*z* (%) = 328.1115 (11) $[M + H]^+$. HRMS (ESI): calcd. for $C_{17}H_{17}N_3O_2S^+$ $[M + H]^+$ 328.1114; found 328.1115. $C_{17}H_{17}N_3O_2S$ (327.40): C 62.37, H 5.23, N 12.83; found C 61.87, H 5.35, N 12.83.

(±)-5-Iodo-1-(oxolan-2-yl)cytosine [(±)-12]: A solution of **11**[23] (500 mg, 2.11 mmol) in hexamethyldisilazane (HMDS, 4 mL) was stirred at 75 °C for 30 min, and then the solution was concentrated under reduced pressure. A solution of the residue in CH_2Cl_2 (10 mL) was treated with 2,3-dihydrofuran (160 mg, 2.2 mmol) and $TsOH\cdot H_2O$ (4 mg), stirred at 22 °C for 48 h, and the solvents were evaporated. Chromatography (SiO_2; CH_2Cl_2/MeOH, 97:3) yielded (±)-**12** (120 mg, 19%) as a white solid. R_f = 0.37 (SiO_2; CH_2Cl_2/MeOH, 96:4). M.p. > 180 °C (decomp.). ^1H NMR (300 MHz, $CDCl_3/CD_3OD$, 6:1, assignment based on the HSQC spectrum): δ = 1.68–1.85 (m, 1 H, 3′-H), 1.88–2.05 (m, 2 H, 3′,4′-H), 2.24–2.42 (m, 1 H, 4′-H), 3.91 (td, *J* = 8.5, 6.5 Hz, 1 H, 5′-H), 4.15 (td, *J* = 8.1, 4.2 Hz, 1 H, 5′-H), 5.84 (dd, *J* = 6.2, 2.8 Hz, 1 H, 2′-H), 7.66 (s, 1 H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$, 6:1): δ = 27.39, 37.12, 59.39, 74.22, 92.41, 150.11, 159.30, 167.79 ppm. IR (ATR): $\tilde{\nu}$ = 3426 (w), 3324 (w), 2961 (w), 2874 (w), 1631 (s), 1611 (s), 1572 (s), 1471 (s), 1276 (m), 1249 (m), 1180 (m), 1121 (w), 1066 (s), 1032 (m), 950 (m), 922 (m), 897 (m), 779 (s), 736 (s) cm^{-1}. HRMS (ESI): *m*/*z* (%) = 307.9886 (100) $[M + H]^+$, 237.9466 (86) $[M - C_4H_6O + H]^+$. HRMS (ESI): calcd. for $C_8H_{11}IN_3O_2^+$ $[M + H]^+$ 307.9890; found 307.9886; calcd. for $C_4H_5IN_3O^+$ $[M - C_4H_6O + H]^+$ 237.9477; found 237.9466.

(±)-1-(1-Ethoxy-4-hydroxybutyl)-5-iodocytosine [(±)-13]: A solution of **11** (1.0 g, 4.2 mmol) in HMDS (4 mL) was stirred at 60 °C for 30 min. Excess HMDS was evaporated under reduced pressure. A solution of the residue in CH_2Cl_2 (25 mL) was treated with TMSOTf (1.86 g, 8.4 mmol), 2-ethoxyoxolane (320 mg, 4.4 mmol), and $TsOH\cdot H_2O$ (20 mg), stirred at 22 °C for 48 h, and the solvents were evaporated. Chromatography (SiO_2; CH_2Cl_2/MeOH, 97:3) yielded (±)-**13** (260 mg, 18%) as a light-orange solid. R_f = 0.52 (SiO_2; CH_2Cl_2/MeOH, 96:4). M.p. > 200 °C (decomp.). ^1H NMR (300 MHz, $CDCl_3/CD_3OD$, 6:1): δ = 0.97 (t, *J* = 7.0 Hz, 3 H, OCH_2CH_3), 1.29–1.46 (m, 2 H, 3′-H), 1.46–1.61 (m, 2 H, 2′-H), 3.19–3.33 (m, 2 H, OCH_2CH_3), 3.36 (t, *J* = 6.2 Hz, 2 H, 4′-H), 5.47 (dd, *J* = 7.2, 5.1 Hz, 1 H, 1′-H), 7.58 (s, 1 H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$, 6:1): δ = 18.57, 31.31, 36.30, 60.97, 65.13, 69.00, 90.30, 150.19, 160.03, 167.53 ppm. IR (ATR): $\tilde{\nu}$ = 3411 (w), 3244 (w, br.), 3049 (w), 2974 (w), 2914 (w), 2878 (w), 2227 (w), 1661 (m), 1614 (s), 1536 (m), 1481 (s), 1444 (m), 1387 (m), 1367 (m), 1303 (m), 1275 (m), 1244 (m), 1201 (m), 1150 (w), 1061 (s), 989 (w), 966 (m), 912 (w), 838 (m), 803 (m) 779 (s) cm^{-1}. HRMS (ESI): *m*/*z* (%) = 376.0127 (31) $[M + Na]^+$, 237.9471 (100) $[C_4H_5IN_3O]^+$. HRMS (ESI): calcd. for $C_{10}H_{16}IN_3NaO_3^+$ $[M + Na]^+$ 376.0129; found 376.0127.

(±)-[2-(5-Iodocytosin-1-yl)thietane-3,3-diyl]dimethanediyl Dibenzoate [(±)-15]: A suspension of **11** (130 mg, 0.55 mmol) in HMDS (4.0 mL) was heated at reflux for 50 min. Excess HMDS was evaporated under reduced pressure. A solution of the residue in CH_2Cl_2 (4.0 mL) was cooled to 0 °C, treated with a solution of (±)-**14**[25] (200 mg, 0.55 mmol) in CH_2Cl_2 (3.0 mL), $AgClO_4$ (114 mg, 0.55 mmol), and $SnCl_2$ (104 mg, 0.55 mmol), and stirred at 22 °C for 70 h. The suspension was diluted with EtOAc and CH_2Cl_2 and washed with a satd. solution of $NaHCO_3$, water, and brine. The combined aqueous phases were extracted with CH_2Cl_2/*i*PrOH (3:1). The combined organic phases were dried with Na_2SO_4, filtered, and the solvents evaporated. Chromatography (SiO_2; CH_2Cl_2 → CH_2Cl_2/MeOH, 95:5) yielded (±)-**15** (106 mg, 35%) as a yellow solid. R_f = 0.24 (SiO_2; CH_2Cl_2/MeOH, 95:5). ^1H NMR (400 MHz, $CDCl_3/CD_3OD$, 6:1): δ = 2.90 and 3.18 (2 d, *J* = 12.0 Hz, 2 H, 4-H), 4.27 and 4.39 (2 d, *J* = 12.3 Hz, 2 H, CH_2O), 4.52 and 4.72 (2 d, *J* = 12.3 Hz, 2 H, CH_2O), 6.18 (s, 1 H, 2-H), 7.32–7.37 (m, 4 H, arom. H), 7.44–7.50 (m, 2 H, arom. H), 7.83–7.85 (m, 2 H, arom. H), 7.96–7.99 (m, 2 H, arom. H), 8.40 (s, 1 H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$, 6:1): δ = 24.80, 53.49, 56.78, 58.01, 63.19, 66.75, 128.54 (2 C), 128.63 (2 C), 128.86, 129.55, 129.76 (2 C), 129.81 (2 C), 133.35, 133.49, 148.27, 155.29, 163.56, 165.79, 166.00 ppm. IR (ATR): $\tilde{\nu}$ = 3300 (w), 3063 (w), 2947 (w), 2696 (w), 1718 (s), 1693 (m), 1631 (m), 1601 (m), 1584 (w), 1491 (w), 1450 (m), 1428 (w), 1398 (w), 1369 (w), 1337 (w), 1314 (m), 1262 (s), 1176 (m), 1094 (s), 1069 (m), 1051 (w), 1025 (m), 978 (w), 917 (w), 846 (w), 803 (w), 763 (w), 705 (s), 685 (m), 675 (m), 660 (w) cm^{-1}. HRMS (ESI): calcd. for $C_{23}H_{20}IN_3NaO_5S^+$ $[M + Na]^+$ 600.0061; found 600.0059.

***N*-[3-(2-Amino-5-bromo-3-pyridyl)prop-2-yn-1-yl]cyclopropanesulfonamide (17):** A solution of **16** (300 mg, 1 mmol), **10** (190 mg, 1.2 mmol), and NEt_3 (0.42 mL, 3 mmol) in THF (23 mL) in a Schlenk flask was degassed and treated with $[Pd(PPh_3)_2Cl_2]$ (70 mg, 0.1 mmol) and CuI (38 mg, 0.1 mmol). The mixture was stirred at 25 °C for 18 h, diluted with CH_2Cl_2, and washed with a satd. aq. solution of $NaHCO_3$ and brine. Concentration under reduced pressure and chromatography (SiO_2; hexane/EtOAc, 1:1) afforded **17** (290 mg, 87%) as a brown solid. M.p. 141–144 °C. ^1H NMR (400 MHz, $CDCl_3$): δ = 1.04–1.07 (m, 2 H, 2,3-H_{trans}), 1.23–1.25 (m, 2 H, 2,3-H_{cis}), 2.53 (tt, *J* = 8.0, 4.8 Hz, 1 H, 1-H), 4.21 (d, *J* = 3.5 Hz, 2 H, NCH_2), 4.84 (br. s, 1 H, NH), 5.15 (br. s, 2 H, NH_2), 7.95 (d, *J* = 2.3 Hz, 1 H, 4′-H), 8.05 (d, *J* = 2.3 Hz, 1 H, 6′-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 5.91 (2 C), 30.85, 33.85, 79.06, 92.20, 103.76, 141.93, 149.24, 158.09 ppm.

Supporting Information (see footnote on the first page of this article): Modeling figures referred to in the article, synthesis of aminopyridine ligands with aromatic rings, NMR spectra of new compounds.

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