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Exploring the Chemical Space around the Privileged Pyrazolo[3,4-*d*]pyrimidine Scaffold: Toward Novel Allosteric Inhibitors of T315I-Mutated Abl

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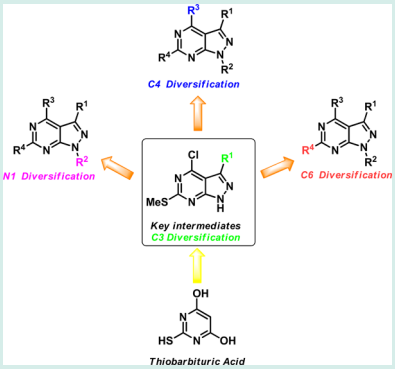
Supporting Information

ABSTRACT: A library of pyrazolo[3,4-*d*]pyrimidines, endowed with a high level of molecular diversity, has been developed applying a synthetic sequence that allowed C3, N1, C4, and C6 substitution. The enzymatic screening of this "privileged scaffold"-based compound collection, validated the use of a diversity-oriented approach in a field characteristically explored by target-oriented synthesis. In fact, several compounds showed high activity against the selected kinases (i.e., Src, Abl *wt*, and T315I mutated-form), furthermore and interestingly a new compound has emerged as an allosteric inhibitor of the T315I mutated-form of Abl, opening up new opportunities for the development of a novel class of noncompetitive inhibitors of Abl (T315I).



KEYWORDS: *pyrazolo[3,4-d]pyrimidine, diversity-oriented synthesis, dual Src/Abl inhibitors, Abl T315I, allosteric inhibitors*

INTRODUCTION

The pyrazolo[3,4-*d*]pyrimidine nucleus represents an interesting scaffold for the development of drug-candidates aiming at a wide range of biological targets[1,2] and has been exploited in the development of kinase inhibitors,[3,4] antiviral agents,[5,6] adenosine antagonists,[7,9] glutamate modulators,[10] and antitubercular agents.[11] Because of its structure, isosteric to that of purine, this nitrogen-containing heterocycle behaves as a "privileged" scaffold,[12] acting in antagonism with natural purines and showing different specificity for the biological targets depending on its chemical functionalization. In this context, our research group has long focused on the design and synthesis of 4-amino-substituted pyrazolo[3,4-*d*]pyrimidines active on different oncogenic tyrosine kinases and cancer cell lines depending on the nature and position of substituents on the heterocyclic core.[13−22]

Our target-based approach, aimed toward the improvement of the tyrosine kinase inhibitory activity, always considered the computational analysis of the target-inhibitor complexes to design and synthesize compounds with improved affinity via point-modification of the pyrazolo[3,4-*d*]pyrimidine scaffold. On the basis of this approach, the synthesis of several active 4-amino-substituted pyrazolo[3,4-*d*]pyrimidines, has been achieved (Figure 1).[13]

Recent literature showed how approaches directed toward the creation of libraries endowed with high chemical variability (such as combinatorial chemistry,[23] diversity-oriented synthesis (DOS),[24] biology-oriented synthesis (BIOS)[25]) could lead to interesting and unexpected biological results.

Fascinated by the potential results achievable by applying this kind of approach to the pyrazolo[3,4-*d*]pyrimidine privileged scaffold, in the present work we focused on the development of a divergent synthetic strategy for the construction of a highly functionalized library of pyrazolo[3,4-*d*]pyrimidines to be tested against oncogenic kinases.

Among the different synthetic strategies commonly used for the synthesis of functionalized pyrazolo[3,4-*d*]pyrimidines, the most used are those starting from the pyrimidine or, alternatively, the pyrazole core.[26] In our research group, these compounds have been synthesized starting from commercial

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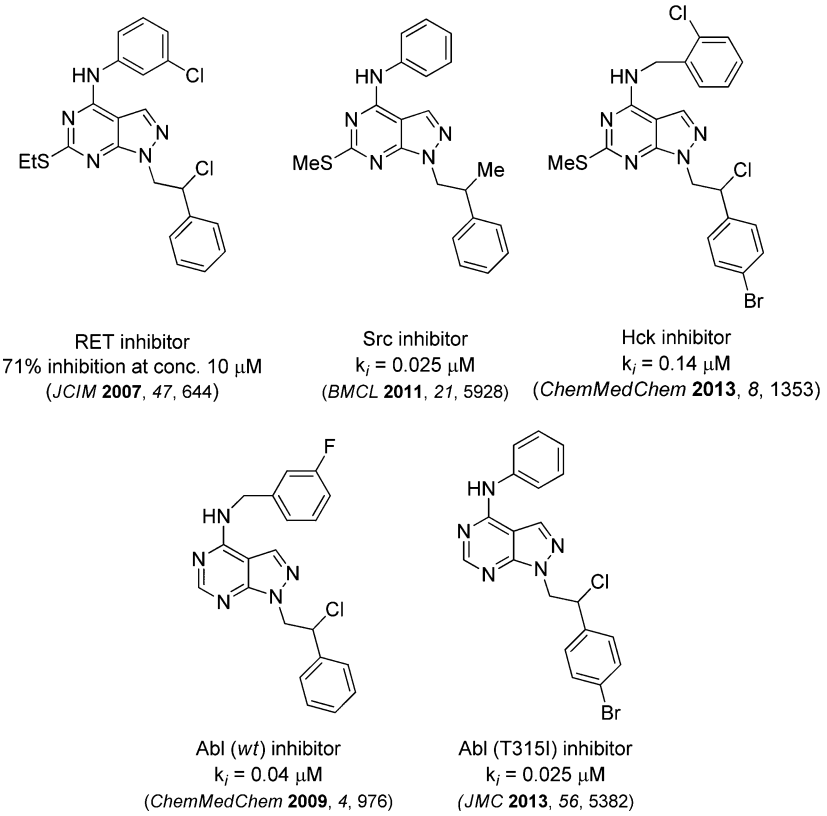

Figure 1. Selected 4-amino-substituted pyrazolo[3,4-*d*]pyrimidines synthesized in our laboratories, active toward different tyrosine kinases (RET, Src, Hck, Abl *wt*, and T315I mutated-form).

phenyloxiranes (**A**), following the route to construct the pyrazole core (**B**)[14−22] or, alternatively, from allopurinol (**D**)[13e,27] (Figure 2, routes *a* and *b*, respectively). However, the main drawback of the approach exemplified in route *a* is represented by its poor versatility in developing N1 substituted analogues: the chemical diversity in the N1 side chain is in fact limited by the commercial availability and substitution of the starting phenyloxiranes (**A**). Recently, we proposed a more versatile approach that allowed obtaining C6 unsubstituted compounds, based on the use of allopurinol (**D**) as the starting material. Although this strategy allowed to generate different 1,4-disubstituted pyrazolo[3,4-*d*]pyrimidines, C6 substituted compounds cannot be obtained, partially limiting its versatility (Figure 2, route *b*). In the present work, we focused on the development of a divergent synthetic approach (Figure 2, route *c*) for the generation of highly functionalized pyrazolo[3,4-*d*]pyrimidines: substitution of C3 and C6 position on the core, plus the diversification in the N1 and C4 position was obtained, giving access to a library of 4-amino substituted pyrazolo[3,4-*d*]pyrimidines, characterized by a high level of molecular diversity.

The synthesis and the subsequent biological evaluation of this diversified collection have been performed with the aim of understanding if a diversity-oriented approach based on a privileged scaffold could quickly lead to interesting and promising hit compounds in a field characteristically explored by the more elaborate target-oriented approach. The obtained results proved valid the application of this strategy to the privileged pyrazolo[3,4-*d*]pyrimidine nucleus. The synthesized compounds were in fact screened in cell-free assays to identify novel compounds able to inhibit selected tyrosine kinases (Src, Abl wild type and T315I mutated-form) involved in the development and progression of tumors, such as chronic myeloid leukemia (CML). As a result, potent inhibitors of the selected kinases were identified and, more interestingly, a novel

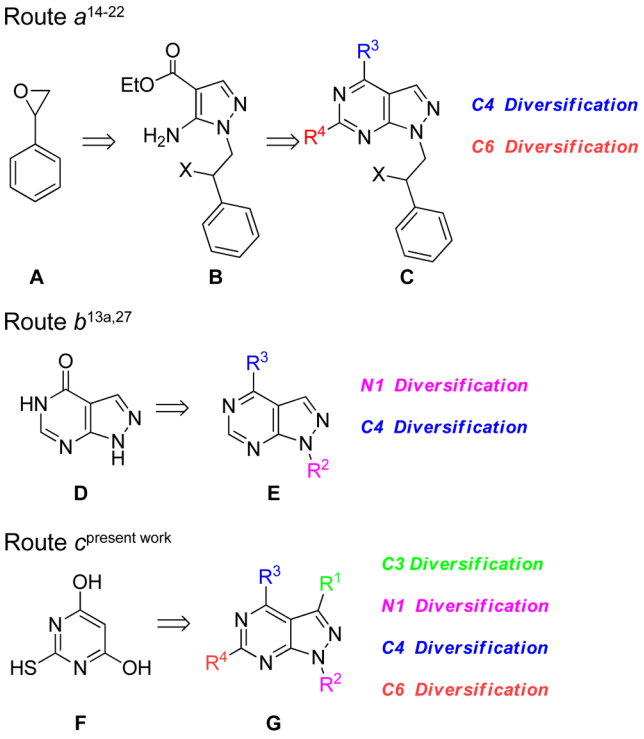

Figure 2. Three different routes developed in our laboratories for the synthesis of pyrazolo[3,4-*d*]pyrimidines.

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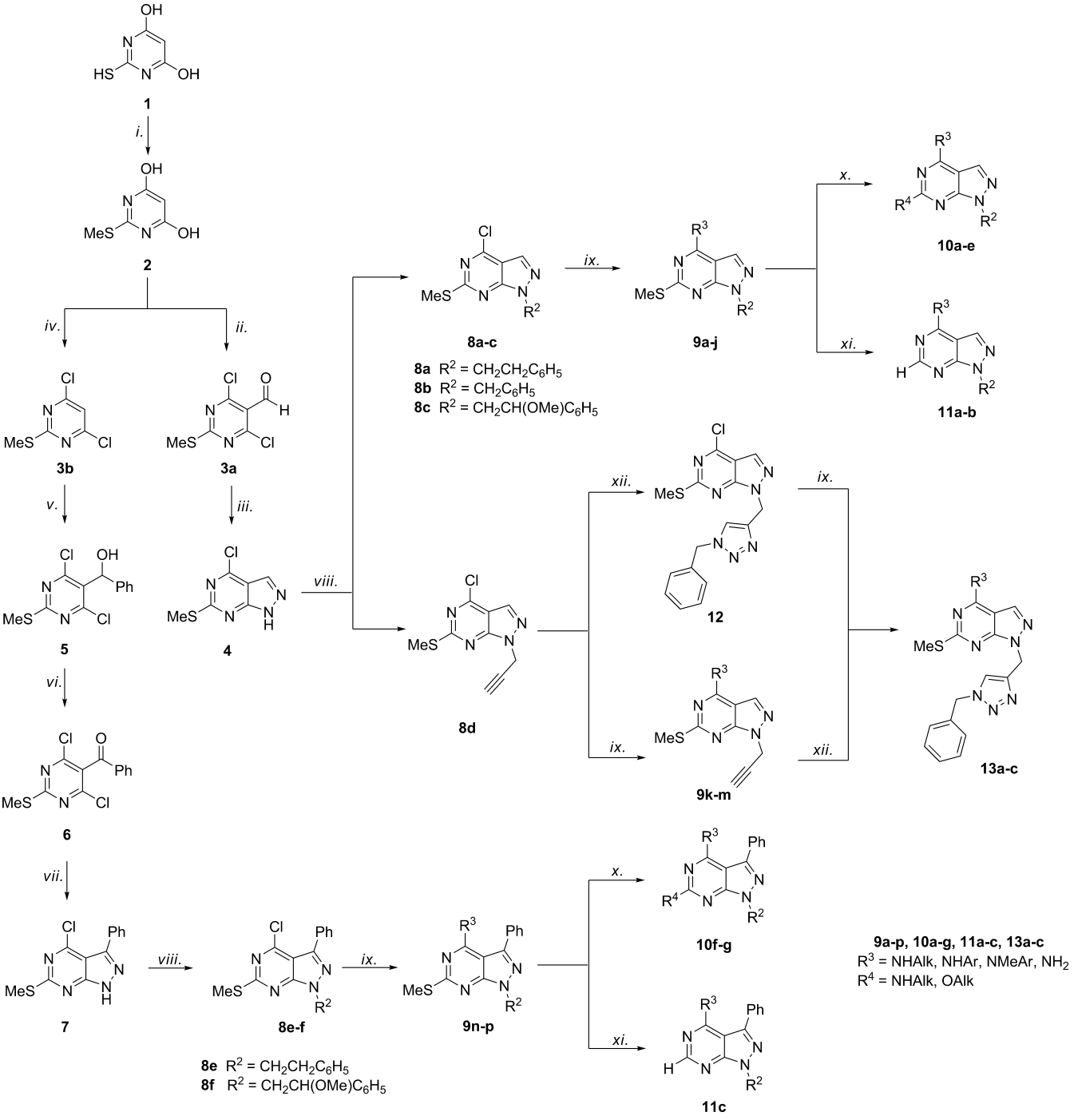
Scheme 1. Synthetic Procedure for 4-Amino-Substituted Pyrazolo[3,4-*d*]pyrimidines[a]



[a]Reagents and conditions: (i) KOH, MeI, H_2O, reflux, 3 h; (ii) $POCl_3$, DMF, reflux, 12 h; (iii) TEA, $N_2H_4{\cdot}H_2O$, dioxane, rt, 5 h; (iv) $POCl_3$, reflux, 12 h; (v) LDA, −78 °C, anhydrous THF, PhCHO; (vi) MnO_2, toluene, 1 h, Dean−Stark trap; (vii) TEA, $N_2H_4{\cdot}H_2O$, dioxane, rt, 1 h; (viii) R^2OH, Ph_3P, DIAD, anhydrous THF, μW 100 °C, 3 min; (ix) amine, EtOH, reflux, 12 h; (x) *m*-CPBA, DCM, rt, 2 h, then nucleophile, THF, 40 °C, 12 h; (xi) H_2, $Pd(OH)_2/C$, MeOH, 2 h; (xii) BnCl, NaN_3, $CuSO_4{\cdot}5H_2O$, sodium ascorbate, μW 125 °C, 10 min.

family of N1 substituted 1,2,3-triazol-4-yl derivatives turned out to act as allosteric inhibitors against T315I-mutated Abl, which confers resistance to the drugs currently available for CML treatment.[28]

RESULTS AND DISCUSSION

When planning the development of this collection of pyrazolo[3,4-*d*]pyrimidines, our aim was to generate a synthetic pathway that could allow a sequential functionalization of the C3, N1, C4, and C6 position of this core, enabling the synthesis of a wide variety of compounds, in a timely fashion, with easy purification, from cheap and commercially available starting materials, frequently using well-known and solid procedures (Scheme 1). This approach showed higher versatility in the decoration of the N1 position and gave the chance of accessing

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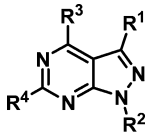
Table 1. Structure and in Vitro Src, Abl *wt*, and T315I-Mutated Form Kinase Inhibitory Activities of Compounds 9a−p, 10a−g, 11a−c, and 13a−c



					K_i [μM][a]		
	R^1	R^2	R^3	R^4	Abl *wt*	Abl T315I	Src
9a	H	$CH_2CH_2C_6H_5$	NHC_6H_4mCl	SMe	>100	ND[d]	0.09
9b	H	$CH_2CH_2C_6H_5$	NHcyclohexyl	SMe	1.45	0.003	6.84
9c	H	$CH_2CH_2C_6H_5$	NHC_6H_4mOH	SMe	0.036	0.01	1.90
9d	H	$CH_2CH_2C_6H_5$	NHC_6H_5	SMe	1.89	0.178	0.92
9e	H	$CH_2CH_2C_6H_5$	NHC_6H_4pOCF$_3$	SMe	0.552	0.056	0.01
9f	H	$CH_2C_6H_5$	NHcyclohexyl	SMe	0.145	0.165	5.1
9g	H	$CH_2C_6H_5$	NHC_6H_4mOH	SMe	0.013	1.354	0.15
9h	H	$CH_2CH(OMe)C_6H_5$	NHC_6H_4mOH	SMe	0.462	4.32	1.23
9i	H	$CH_2CH(OMe)C_6H_5$	$NHCH_2$4-pyridine	SMe	>100	>100	5.52
9j	H	$CH_2CH(OMe)C_6H_5$	NHC_6H_4pOCF$_3$	SMe	1.047	0.51	0.56
9k	H	CH_2CCH	NHC_6H_4mOH	SMe	0.92	>100	>100
9l	H	CH_2CCH	N(Me)2-pyridine	SMe	1.375	0.066	0.24
9m	H	CH_2CCH	NHC_6H_4pOCF$_3$	SMe	N.D.[d]	>100	>100
9n	C_6H_5	$CH_2CH_2C_6H_5$	NHMe	SMe	0.077	0.074	1.95
9o	C_6H_5	$CH_2CH(OMe)C_6H_5$	NHMe	SMe	0.004	0.006	1.416
9p	C_6H_5	$CH_2CH(OMe)C_6H_5$	NH_2	SMe	0.444	0.028	0.18
10a	H	$CH_2CH_2C_6H_5$	NHC_6H_4mCl	NH*n*Pr	0.019	0.004	0.04
10b	H	$CH_2CH_2C_6H_5$	NHC_6H_4mCl	$NHCH_2CH_2$morpholine	0.49	0.18	0.75
10c	H	$CH_2CH_2C_6H_5$	NHcyclohexyl	OCH_2CH_2morpholine	1.42	0.2	0.47
10d	H	$CH_2CH_2C_6H_5$	NHC_6H_4pOCF$_3$	NH*n*Pr	0.06	0.02	0.701
10e	H	$CH_2CH_2C_6H_5$	NHC_6H_4mCl	NHMe	0.003	0.004	1.07
10f	C_6H_5	$CH_2CH_2C_6H_5$	NHMe	NHMe	0.122	0.047	2.426
10g	C_6H_5	$CH_2CH(OMe)C_6H_5$	NHMe	NHMe	0.124	0.022	10.437
11a	H	$CH_2CH_2C_6H_5$	NHC_6H_4mCl	H	>100	ND[d]	0.48
11b	H	$CH_2CH_2C_6H_5$	NHC_6H_4pOCF$_3$	H	0.105	0.037	>100
11c	C_6H_5	$CH_2CH(OMe)C_6H_5$	NH_2	H	0.26	0.562	1.113
13a	H	1-benzyl-1*H*-1,2,3-triazol-4-yl) methyl	NHC_6H_4mOH	SMe	0.29 (14.7)[b]	0.067 (3.16)[b,c]	9.48 (151.7)[b]
13b	H	1-benzyl-1*H*-1,2,3-triazol-4-yl) methyl	N(Me)2-pyridine	SMe	0.29 (33.4)[b]	0.41 (3.63)[b,c]	1.19 (35.0)[b]
13c	H	1-benzyl-1*H*-1,2,3-triazol-4-yl) methyl	NHC_6H_4pOCF$_3$	SMe	0.67 (14.7)[b]	0.077 (8.66)[b,c]	2.19 (19.0)[b]

[a]Data represent the mean of three independent experiments. Standard deviation (SD) was lower than 5%. K_i values toward isolated kinases calculated according to the eq 2 (see Supporting Information). [b]ID_{50} values are reported in parentheses. [c]ID_{50} values are not dependent on the concentration of ATP and the peptide substrate. [d]ND: not determined

C6 and C3 substituted and unsubstituted compounds from the same starting material.

Compounds **4** and **7**, differing only in the functionalization of C3, represent the key intermediates, which allowed creating a wide range of derivatives through a sequence of chemical modifications. First, the R^2 at the N1 position can be introduced through *N*-alkylation or Mitsunobu reaction, using either alcohols or halogenated alkyl-chains; second, the chlorine on the C4 position can be displaced by various nucleophiles to introduce different R^3 substituents. Third, the thiomethyl moiety on C6 can be converted, via oxidation, in a leaving group that can be easily displaced by different nucleophiles to introduce various substituent (R^4) in C6 position.

The planned synthetic approach started from cheap, stable and commercially available thiobarbituric acid (**1**). As first step, an alkylation of the thiol moiety was performed using methyl iodide in aqueous media with potassium hydroxide as base, acidification with HCl 1 N allowed the recovery of pure **2** as a white precipitate.[29]

S-methyl thiobarbituric acid (**2**), was treated with the in situ generated Vilsmeier reagent to give 4,6-dichloro-2-(methylthio)pyrimidine-5-carbaldehyde (**3a**)[30] or alternatively with phosphorus(V) oxychloride to obtain the 4,6-dichloro-derivative **3b**.[31] 4,6-Dichloro-2-methylsulfanyl-pyrimidine-5-carbaldehyde (**3a**) gave access to C3 unsubstituted compounds, whereas 4,6-dichloro-2-methylsulfanyl-pyrimidine (**3b**) was used for the synthesis of C3 substituted pyrazole[3,4-*d*]pyrimidines. 4,6-Dichloro-2-(methylthio)pyrimidine-5-carbaldehyde (**3a**) was reacted with hydrazine monohydrate in dioxane, using triethylamine as base to give the C3 unsubstituted pyrazole[3,4-*d*]pyrimidine core (**4**) in quantitative yield.[32]

4,6-Dichloro-2-methylsulfanyl-pyrimidine (**3b**), characterized by an acid proton *ortho* to the chloro substituents, was treated

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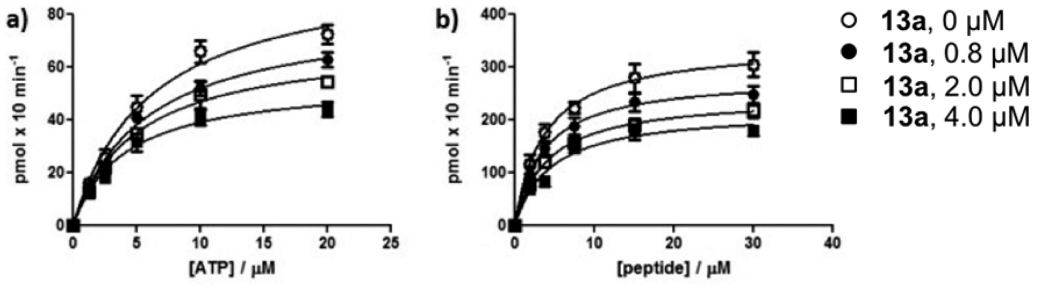

	13a concentration[a]			
	0 μM	0.8 μM	2 μM	4 μM
$V_{max\ (ATP)}$ [pmol min $^{-1}$]	97.97 ± 6.82	81.05 ± 3.89	73.91 ± 3.98	60.49 ± 3.36
$K_{m\ (ATP)}$ [μM]	6.00 ± 1.06	5.60 ± 0.77	5.49 ± 0.81	5.63 ± 0.78
$V_{max\ (peptide)}$ [pmol min $^{-1}$]	343.3 ± 13.5	281.3 ± 10.47	244.2 ± 7.04	217.9 ± 15.95
$K_{m\ (peptide)}$ [μM]	3.69 ± 0.49	3.52 ± 0.45	3.99 ± 0.41	3.76 ± 0.65

[a] Data represent the mean ± standard deviation (SD) of three independent experiments. Each reaction was performed as described in the Experimental Section.

Figure 3. Kinetic analysis of kinase reactions of T315I-mutated Abl in the presence of different concentration of compound **13a** (0, 0.8, 2.0, and 4.0 μM). (a) Variation of the reaction velocity of T315I-mutated Abl as a function of ATP concentration at different fixed concentrations of compound **13a**. (b) Variation of the reaction velocity of T315I-mutated Abl as a function of peptide substrate concentration at different fixed concentrations of compound **13a**. (c) Effect of compound **13a** at various concentrations (0−4 μM) on the K_m and V_{max} values.

with LDA at −78 °C to generate the nucleophile able to react with aromatic aldehydes, such as benzaldehyde, giving the secondary alcohol. The alcohol **5** was oxidized to the corresponding ketone **6** using manganese dioxide in toluene; the subsequent reaction with hydrazine gave access to the C3 substituted pyrazole[3,4-*d*]pyrimidine core (**7**).[33]

The pyrazolo[3,4-*d*]pyrimidine nucleus of **4** and **7** was substituted at the N1 position using a microwave irradiated Mitsunobu reaction, using commercially available alcohols (such as 2-phenyl- and 2-methoxy-2-phenyl-ethanol, benzyl- and propargyl alcohol). This approach allowed us to obtain a set of compounds (**8a−f**), with short reaction times and moderate to excellent yields, introducing different R^2 substituents. In the next step, C4 functionalization was achieved by nucleophilic substitution of the chlorine with primary and secondary amines in order to obtain a first set of potentially active pyrazolo[3,4-*d*]pyrimidines (**9a−p**).

To introduce additional variability in the C6 position, we performed an oxidation of the thiomethyl moiety of compounds **9a**, **b**, **e**, **n**, and **o** using *m*-chloroperbenzoic acid in dichloromethane to obtain a mixture of sulfone and sulfoxide. The latter mixtures were then treated with nucleophiles such as *n*-propylamine, 2-morpholinoethanol, 4-(2-aminoethyl)morpholine and methylamine to obtain derivatives **10a−g**.[32]

In addition, starting from *S*-methyl-substituted compounds **9a**, **e**, and **p**, the C6 unsubstituted products **11a−c** were obtained by applying a hydrogenation protocol using palladium hydroxide as catalyst in methanol. To add a wider variability regarding the N1 substitution, thus enhancing the structural novelty of this diversity-oriented library with comparison to the previously synthesized ones, we decided to perform a Huisgen reaction on the alkyne chain of compound **9k−m**, to synthesize a novel family of 1,2,3-triazol-4-yl derivatives **13a−c** (Scheme 1). Benzylazide, generated in situ from benzylchloride and sodium azide, was reacted with the propargylic moiety of compound **9k−m** using $CuSO_4 \cdot 5H_2O$ and sodium ascorbate as catalyst under microwave-assisted conditions to give compounds **13a−c** in high yields after only 10 min.[34] To demonstrate the versatility of the click reaction on our pyrazole[3,4-*d*]pyrimidine compounds, the same reaction was also performed on the C4-unfunctionalized compound **8d** to give the triazolo intermediate **12**, finally converted into **13a−c** by nucleophilic aromatic substitution with different amines.

Finally, the affinity of the synthesized library toward Src and Abl (*wt* and T315I mutated-form) was evaluated in cell-free assays. Many of the compounds showed interesting dual Src/Abl activity, with a general micromolar or nanomolar potency, comparable or higher to that of the analogues we previously obtained via target-oriented approaches (**9a** and **10a**).[13a] Compound **9b** showed K_i value of 3 nM, against Abl T315I mutated-form. The C3 phenyl derivative **9o**, exhibited a 4 nM K_i value against Abl *wt* and a 6 nM toward T315I mutated-form. Compound **10e** characterized by an *N*-methylamino C4 moiety showed K_i values of 3 and 4 nM against Abl *wt* and T315I mutated-form, respectively (Table 1). In analogy with previously reported results[13e] and with the data of several compounds (**9−11**) reported in Table 1, also the triazolo derivatives **13a−c** showed a better affinity toward the T315I mutated-form of Abl with respect to the wild type form.

Considering also to the peculiar structure of compounds **13a−c**, we decided to investigate the mechanism of action of these derivatives in the inhibition of Abl T315I. The most active compound **13a**, was chosen for kinetic studies in order to elucidate the mechanism of inhibition toward T315I Abl. Surprisingly, the obtained data revealed that compound **13a** inhibits the Abl T315I with a noncompetitive kinetic with respect to both the ATP and the peptide substrate, thus representing an allosteric inhibitor of the T315I Abl mutated-form (Figure 3). Furthermore, we can suggest that the structurally and activity related compounds **13b** and **c** act with similar kinetics; as a consequence these two compounds (**13b** and **c**), together with **13a**, could represent a novel class of so far unexplored allosteric T315I Abl inhibitors whose target is currently under study.

CONCLUSION

Focusing on the pyrazolo[3,4-*d*]pyrimidine scaffold, we built a diversity-oriented compound collection around this molecular framework by decorating the core in four different positions. The developed synthetic pathway allowed for C3, N1, C4, and C6 substitution, starting from cheap and stable starting material (thiobarbituric acid, **1**). On the basis of this approach, we have discovered potent inhibitors of three tyrosine kinases (Src and Abl *wt* and T315I mutated-form) whose activity is comparable or higher to that of the analogues we previously obtained via target-oriented approaches. More significantly, the same study opened up new opportunities for the development of a novel class of noncompetitive inhibitors of Abl T315I mutated-form, which proved to act with an allosteric mechanism and with a higher affinity for this mutated kinase compared to the Abl *wt*. The 1,2,3-triazol-4-yl moiety, that characterizes the N1 position of this family, represents the structural point of novelty in comparison to the previously synthesized derivatives. These results demonstrated that a diversity-oriented approach focused on a "privileged" scaffold, might be a resourceful method for the development of new drug candidates. In fact, although the field of tyrosine kinases, which represent well-characterized targets, is typically faced by a targeted-oriented approach, our work proved the validity of a diversity-oriented method for the study of these known targets. Additional studies to assess structural−activity relationships regarding selectivity toward different kinases and mechanisms of action are currently ongoing in our lab and will be reported in due course.

EXPERIMENTAL PROCEDURES

General Information. All commercially available chemicals were used as supplied without further purification. THF was dried over Na/benzophenone prior to use, $POCl_3$ was freshly distilled, while DMF was bought already anhydrous. Anhydrous reactions were run under a positive pressure of dry Argon. Microwave irradiation experiments were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC). The machine consists of a continuous focused microwave power delivery system with operator-selectable power output from 0 to 300 W. The temperature of the contents of the vessels was monitored using a calibrated infrared temperature control located under the reaction vessel.

Merck precoated TLC plates (silica gel 60 F_{254}, 0.25 mm) were used. UV detection was monitored at 254 nm. Chromatographic purifications were performed on columns packed with Merk 60 silica gel, 23−400 mesh, for flash technique. ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker Avance DPX400 (Bruker Biospin, Germany). Chemical shifts were reported in parts per million (ppm) from tetramethylsilane as internal standard. Multiplicities were indicated as follow: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet), dt (doublet of triplet), td (triplet of doublet), bs (broad singlet). Coupling constants were reported in hertz (Hz). LC-MS was performed with the UV detection at 254 nm and a single quadrupole mass spectrometer using electrospray ionization (ESI) source.

General Procedure 1 (GP1): 4 and 7. To an ice-cooled solution of the appropriate aldehyde or ketone (**3a** or **6**) (0.45 mmol, 1.00 equiv) and TEA (0.45 mmol, 1.00 equiv) in dioxane was added $N_2H_4{\cdot}H_20$ (0.50 mmol, 1.10 equiv). After 5 min the ice bath was removed and the reaction mixture was stirred 5 h at rt. After this time, 10 mL of 0.5 N HCl solution was added to the mixture, followed by extraction with EtOAc (3 × 20 mL). The collected organic phases were dried over Na_2SO_4, filtered and the solvent removed under reduced pressure; this afforded the desired product that was used in the next step without further purification.

General Procedure 2 (GP2): 8a−f. To an ice-cooled solution of Ph_3P (0.15 mmol, 1.50 equiv) and 4-chloro-6-(methylthio)-1H-pyrazolo[3,4-*d*]pyrimidine (**4** and **7**) (0.10 mmol, 1.00 equiv) in anhydrous THF (2 mL) was added the appropriate alcohol (0.15 mmol, 1.50 equiv), followed by dropwise addition of DIAD (0.15 mmol, 1.50 equiv). The reaction mixture was stirred at 4 °C for 15 min then allowed to warm to rt. The reaction was irradiated under μW at 100 °C for 3 min; after evaporation of the solvent the oily residue obtained was purified by flash chromatography using a PE/EtOAc mixture as eluent.

General Procedure 3 (GP3): 9a−p and 13a−c. The desired amine (0.45 mmol, 5.00 equiv) was added to a solution of the appropriate 1-N-substitued 4-chloro-6-(methylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**8a−f** and **12**) (0.15 mmol, 1.00 equiv) in EtOH (2 mL). The reaction solution was stirred under reflux, until the starting material spot disappeared from TLC. The solvent was removed under reduced pressure to obtain the reaction crude, that was purified as described in each section.

General Procedure 4 (GP4): 5. To a solution of 4,6-dichloro-2-(methylthio)pyrimidine (**3b**) (150.0 mg, 0.77 mmol, 1.00 equiv) in anhydrous THF (2 mL) at −78 °C was added dropwise 2 N solution of LDA (1.39 mmol, 1.8 equiv). The mixture was stirred at the same temperature for 30 min then the aldehyde (1.16 mmol, 1.5 equiv) in anhydrous THF (2 mL) was added dropwise at r.t and the resulting mixture was stirred for 1 h. NH_4Cl s.s. was added and the aqueous phase was extracted two times with EtOAc. The collected organic phases were dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The oily residue obtained was purified by flash chromatography, using PE/EtOAc = 98:2 as eluent.

General Procedure 5 (GP5): 6. In a flask equipped with a Dean−Stark trap, a mixture of the secondary alcohol (**5**) (0.50 mmol, 1.00 equiv), toluene, and MnO_2 was heated to boiling for 1 h. The mixture was filtered on Celite using EtOAc. The organic phase was dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure to obtain the product used without further purification in the next step.

General Procedure 6 (GP6): 10a−g. *m*-CPBA (0.13 mmol, 2.10 equiv) was added to a solution of the appropriate 6-(methylthio)-pyrazolo[3,4-*d*]pyrimidin-4-amine (**9a**, **b**, **e**, **n**, and **o**) (0.06 mmol, 1.00 equiv) in DCM. The reaction mixture was stirred at rt for 2 h, the solvent was evaporated under reduced pressure. The white solid residue was dissolved in EtOAc, the organic phase was washed twice with $NaHCO_3$ s.s., then dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to obtain an orange oily residue (sulfone and sulfoxide), which was solubilized in THF. The desired nucleophile (0.30 mmol, 5.00 equiv) was added and the reaction mixture was stirred at 40 °C o.n. The reaction crude, obtained after evaporation of the solvent, was purified by flash chromatography using Hex/EtOAc = 2:1 as eluent.

General Procedure 7 (GP7): 12 and 13a−c. Benzyl-chloride (0.09 mmol, 2.00 equiv) and NaN_3 (0.09 mmol, 2.00 equiv) were suspended in H_2O/*t*BuOH = 1:1 (1 mL), then the appropriate 6-(methylthio)-1-(prop-2-ynyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine (**8d** and **9k−m**) (0.05 mmol, 1.10 equiv),

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$CuSO_4 \cdot 5H_2O$ (0.01 equiv) and sodium ascorbate (0.10 equiv) were added. The reaction mixture was irradiated under μW at 125 °C for 10 min. The solvent was removed under reduced pressure and the residue purified by flash chromatography using DCM/MeOH = 99:1 as eluent.

General Procedure 8 (GP8): 11a–c. The appropriate 6-(methylthio)-pyrazolo[3,4-*d*]pyrimidin-4-amine (**9a**, **e**, and **p**) (0.05 mmol, 1.00 equiv) was dissolved in MeOH (5.0 mL) under a nitrogen atmosphere. $Pd(OH)_2/C$ (0.10 equiv) was added and the reaction mixture was stirred at r.t for 2 h under H_2 atmosphere. The mixture was filtered on Celite and the solvent removed under reduced pressure to obtain the products as a white solids.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details about instruments, synthesis and characterization of compounds, and enzymatic assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ REFERENCES

(1) Chauhan, M.; Kumar, R. Medicinal attributes of pyrazolo[3,4-d]pyrimidines: A review. *Bioorg. Med. Chem.* **2013**, *21*, 5657–5668.

(2) Schenone, S.; Bruno, O.; Radi, M.; Botta, M. New insights into small-molecule inhibitors of Bcr-Abl. *Med. Res. Rev.* **2011**, *31*, 1–41.

(3) (a) Dinér, P.; Alao, J. P.; Söderlund, J.; Sunnerhagen, P.; Grøtli, M. Preparation of 3-substituted-1-isopropyl-1*H*-pyrazolo[3,4-*d*]-pyrimidin-4-amines as RET kinase inhibitors. *J. Med. Chem.* **2012**, *55*, 4872–4876. (b) Yang, L. L.; Li, G. B.; Yan, H. X.; Sun, Q. Z.; Ma, S.; Ji, P.; Wang, Z. R.; Feng, S.; Zou, J.; Yang, S. Y. Discovery of *N*6-phenyl-1*H*-pyrazolo[3,4-d]pyrimidine-3,6-diamine derivatives as novel CK1 inhibitors using common-feature pharmacophore model based virtual screening and hit-to-lead optimization. *Eur. J. Med. Chem.* **2012**, *56*, 30–38.

(4) (a) Kumar, A.; Ahmad, I.; Chhikara, B. S.; Tiwari, R.; Mandal, D.; Parang, K. Synthesis of 3-phenylpyrazolopyrimidine-1,2,3-triazole conjugates and evaluation of their Src kinase inhibitory and anticancer activities. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 1342–1346. (b) Deng, X.; Okram, B.; Ding, Q.; Zhang, J.; Choi, Y.; Adrián, F. J.; Wojciechowski, A.; Zhang, G.; Che, J.; Bursulaya, B.; Cowan-Jacob, S. W.; Rummel, G.; Sim, T.; Gray, N. S. Expanding the diversity of allosteric Bcr-Abl inhibitors. *J. Med. Chem.* **2010**, *53*, 6934–6946.

(5) Chern, J.; Shia, K.; Hsu, T.; Tai, C.; Lee, C.; Lee, Y.; Chang, C.; Tseng, S.; Shih, S. Design, synthesis, and structure–activity relationships of pyrazolo[3,4-*d*]pyrimidines: a novel class of potent enterovirus inhibitors. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2519–2525.

(6) Bektemirov, T. A.; Chekunova, E. V.; Korbukh, I. A.; Bulychev, Y. N.; Yakunina, N. G.; Preobrazhenskaya, M. N. Antiviral activity of substituted 6-methylmercaptopyrazolo(3,4-d)pyrimidines and their ribosides. *Acta Virol.* **1981**, *25*, 326–329.

(7) Taliani, S.; La Motta, C.; Mugnaini, L.; Simorini, F.; Salerno, S.; Marini, A. M.; Da Settimo, F.; Cosconati, S.; Cosimelli, B.; Greco, G.; Limongelli, V.; Marinelli, L.; Novellino, E.; Ciampi, O.; Daniele, S.; Tricavelli, M. L.; Martini, C. Novel N^2-substituted pyrazolo[3,4-*d*]pyrimidine adenosine A_3 receptor antagonists: Inhibition of A_3-mediated human glioblastoma cell proliferation. *J. Med. Chem.* **2010**, *53*, 3954–3963.

(8) Briel, D.; Aurich, R.; Egerland, U.; Unverferth, K. Synthese substituierter 6-phenylpyrazolo[3,4-*d*]pyrimidine mit potenziell adenosin-A_{2A}-antagonistischer wirkung. *DiePharmazie* **2005**, *60*, 732–735.

(9) Poulsen, S. A.; Quinn, R. J. Synthesis and structure–activity relationship of pyrazolo[3,4-*d*]pyrimidines: Potent and selective adenosine A_1 receptor antagonists. *J. Med. Chem.* **1996**, *39*, 4156–4161.

(10) Niswender, C. M.; Lebois, E. P.; Luo, Q.; Kim, K.; Muchalski, H.; Yin, H.; Conn, P. J.; Lindsley, C. W. Positive allosteric modulators of the metabotropic glutamate receptor subtype 4 (mGluR4): Part I. Discovery of pyrazolo[3,4-*d*]pyrimidines as novel mGluR4 positive allosteric modulators. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5626–5630.

(11) Trivedi, A. R.; Dholariya, B. H.; Vakhariya, C. P.; Dodiya, D. K.; Ram, H. K.; Kataria, V. B.; Siddiqui, A. B.; Shah, V. H. Synthesis and anti-tubercular evaluation of some novel pyrazolo[3,4-*d*]pyrimidine derivatives. *Med. Chem. Res.* **2012**, *21*, 1887–1891.

(12) Welsch, M. E.; Snyder, A. S.; Stockwell, B. R. Privileged scaffolds for library design and drug discovery. *Curr. Opin. Chem. Biol.* **2010**, *14*, 347–361.

(13) (a) Radi, M.; Brullo, C.; Crespan, E.; Tintori, C.; Musumeci, F.; Biava, M.; Schenone, S.; Dreassi, E.; Zamperini, C.; Maga, G.; Pagano, D.; Angelucci, A.; Bologna, M.; Botta, M. Identification of potent c-Src inhibitors strongly affecting the proliferation of human neuroblastoma cells. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 5928–5933. (b) Tuccinardi, T.; Manetti, F.; Schenone, S.; Martinelli, A.; Botta, M. Construction and validation of a RET TK catalytic domain by homology modeling. *J. Chem. Inf. Model.* **2007**, *47*, 644–655. (c) Schenone, S.; Bruno, O.; Bondavalli, F.; Ranise, A.; Mosti, L.; Menozzi, G.; Fossa, P.; Donnini, S.; Santoro, A.; Ziche, M.; Manetti, F.; Botta, M. Antiproliferative activity of new 1-aryl-4-amino-1*H*-pyrazolo[3,4-*d*]pyrimidine derivatives toward the human epidermoid carcinoma A431 cell line. *Eur. J. Med. Chem.* **2004**, *39*, 939–946. (d) Tintori, C.; Laurenzana, I.; La Rocca, F.; Falchi, F.; Carraro, F.; Ruiz, A.; Estè, J. A.; Kissova, M.; Crespan, E.; Maga, G.; Biava, M.; Brullo, C.; Schenone, S.; Botta, M. Identification of Hck inhibitors as hits for the development of antileukemia and anti-HIV agents. *ChemMedChem* **2013**, *8*, 1353–1360. (e) Radi, M.; Tintori, C.; Musumeci, F.; Brullo, C.; Zamperini, C.; Dreassi, E.; Fallacara, A. L.; Vignaroli, G.; Crespan, E.; Zanoli, S.; Laurenzana, I.; Filippi, I.; Maga, G.; Schenone, S.; Angelucci, A.; Botta, M. Design, synthesis, and biological evaluation of pyrazolo[3,4-*d*]pyrimidines active in vivo on the Bcr-Abl T315I mutant. *J. Med. Chem.* **2013**, *56*, 5382–5394.

(14) Manetti, F.; Pucci, A.; Magnani, M.; Locatelli, G. A.; Brullo, C.; Naldini, A.; Schenone, S.; Maga, G.; Carraro, F.; Botta, M. Inhibition of Bcr-Abl phosphorylation and induction of apoptosis by pyrazolo-[3,4-*d*]pyrimidines in human leukemia cells. *ChemMedChem* **2007**, *2*, 343–353.

(15) Manetti, F.; Santucci, A.; Locatelli, G. A.; Maga, G.; Spreafico, A.; Serchi, T.; Orlandini, M.; Bernardini, G.; Caradonna, N. P.; Spallarossa, A.; Brullo, C.; Schenone, S.; Bruno, O.; Ranise, A.; Bondavalli, F.; Hoffmann, O.; Bologna, M.; Angelucci, A.; Botta, M. Identification of a novel pyrazolo[3,4-*d*]pyrimidine able to inhibit cell proliferation of a human osteogenic sarcoma in vitro and in a xenograft model in mice. *J. Med. Chem.* **2007**, *50*, 5579–5588.

(16) Radi, M.; Dreassi, E.; Brullo, C.; Crespan, E.; Tintori, C.; Bernardo, V.; Valoti, M.; Zamperini, C.; Daigl, H.; Musumeci, F.; Carraro, F.; Naldini, A.; Filippi, I.; Maga, G.; Schenone, S.; Botta, M. Design, synthesis, biological activity, and ADME properties of pyrazolo[3,4-*d*]pyrimidines active in hypoxic human leukemia cells: A lead optimization study. *J. Med. Chem.* **2011**, *54*, 2610–2626.

(17) Bondavalli, F.; Botta, M.; Bruno, O.; Ciacci, A.; Corelli, F.; Fossa, P.; Lucacchini, A.; Manetti, F.; Menozzi, G.; Mosti, L.; Ranise, A.; Schenone, S.; Tafi, A.; Trincavelli, M. L. Synthesis, molecular

modeling studies, and pharmacological activity of selective A_1 receptor antagonists. *J. Med. Chem.* **2002**, *45*, 4875−4887.
(18) Schenone, S.; Brullo, C.; Bruno, O.; Bondavalli, F.; Mosti, L.; Maga, G.; Crespan, E.; Carraro, F.; Manetti, F.; Tintori, C.; Botta, M. Synthesis, biological evaluation and docking studies of 4-amino substituted 1*H*-pyrazolo[3,4-*d*]pyrimidines. *Eur. J. Med. Chem.* **2008**, *43*, 2665−2676.
(19) Tintori, C.; Magnani, M.; Schenone, S.; Botta, M. Docking, 3D-QSAR studies and in silico ADME prediction on c-Src tyrosine kinase inhibitors. *Eur. J. Med. Chem.* **2009**, *44*, 990−1000.
(20) Manetti, F.; Locatelli, G. A.; Maga, G.; Schenone, S.; Modugno, M.; Forli, S.; Corelli, F.; Botta, M. A combination of docking/dynamics simulations and pharmacophoric modeling to discover new dual c-Src/Abl kinase inhibitors. *J. Med. Chem.* **2006**, *49*, 3278−3286.
(21) Falchi, F.; Manetti, F.; Carraro, F.; Naldini, A.; Maga, G.; Crespan, E.; Schenone, S.; Bruno, O.; Botta, M. 3D QSAR models built on structure-based alignments of Abl tyrosine kinase inhibitors. *ChemMedChem* **2009**, *4*, 976−987.
(22) Spreafico, A.; Schenone, S.; Serchi, T.; Orlandini, M.; Angelucci, A.; Magrini, D.; Bernardini, G.; Collodel, G.; Di Stefano, A.; Tintori, C.; Bologna, M.; Manetti, F.; Botta, M.; Santucci, A. Antiproliferative and proapoptotic activities of new pyrazolo[3,4-*d*]pyrimidine derivative Src kinase inhibitors in human osteosarcoma cells. *FASEB J.* **2008**, *22*, 1560−1571.
(23) Kim, J.; Lee, W. S.; Koo, J.; Lee, J.; Park, S. B. Synthesis and library construction of privileged tetra-substituted Δ^5-2-oxopiperazine as β-turn structure mimetics. *ACS Comb. Sci.* **2014**, *16*, 24−32.
(24) Galloway, W. R. J. D.; Isidro-Llobet, A.; Spring, D. R. Diversity-oriented synthesis as a tool for the discovery of novel biologically active small molecules. *Nat. Commun.* **2010**, *1*, 80.
(25) Wetzel, S.; Bon, R. S.; Kumar, K.; Waldmann, H. Biology-oriented synthesis. *Angew. Chem., Int. Ed.* **2011**, *50*, 10800−10826.
(26) Schenone, S.; Bruno, O.; Radi, M.; Botta, M. 4-Amino-substituted pyrazolo[3,4-*d*]pyrimidines: Synthesis and biological properties. *Mini-Rev. Org. Chem.* **2009**, *6*, 220−233.
(27) Radi, M.; Bernardo, V.; Vignaroli, G.; Brai, A.; Biava, M.; Schenone, S.; Botta, M. An alternative synthetic approach for the synthesis of biologically relevant 1,4-disubstituted pyrazolo[3,4-*d*]pyrimidines. *Tetrahedron Lett.* **2013**, *54*, 5204−5206.
(28) Radi, M.; Schenone, S.; Botta, M. Allosteric inhibitors of Bcr-Abl: Towards novel myristate-pocket binders. *Curr. Pharm. Biotech.* **2013**, *14*, 477−487.
(29) Arora, N.; Billedeau, R. J.; Dewdney, N. J.; Gabriel, T.; Goldstein, D. M.; O'Yang, C.; Soth, M.; Trejo-Martin, A. p38 MAP kinase inhibitors and methods for using the same. U.S. Patent 20070203160 A1, 2007.
(30) Tandon, M.; Wang, J.; Namdev, N. Substituted pyrazolo-pyrimidine compounds. U.S. Patent 20100249110 A1, 2010.
(31) Erickson, S. D.; Gillespie, P.; Guertin, K. R.; Karnachi, P. S.; Kim, K.; Ma, C.; McComas, W. W.; Pietranico-Cole, S. L.; Qi, L.; Tilley, J. W.; Zhang, Q. GPR119 Receptor Agonists. U.S. Patent 20090286812 A1, 2009.
(32) Kim, D. C.; Lee, Y. R.; Yang, B.; Shin, K. J.; Kim, D. J; Chung, B. Y.; Yoo, K. Synthesis and biological evaluations of pyrazolo[3,4-*d*]pyrimidines as cyclin-dependent kinase 2 inhibitors. *Eur. J. Med. Chem.* **2003**, *38*, 525−532.
(33) Chuaqui, C. E.; Huang, S.; Ioannidis, S.; Shi, J.; Su, M.; Su, Q. Heterocyclic jak kinase inhibitors. CA Patent 2737217 A1, 2010.
(34) Castagnolo, D.; Dessì, F.; Radi, M.; Botta, M. Synthesis of enantiomerically pure α-[4-(1-substituted)-1,2,3-triazol-4-yl]-benzylacetamides via microwave-assisted click chemistry: towards new potential antimicrobial agents. *Tetrahedron Asymmetry* **2007**, *18*, 1345−1350.