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Modified 2,4-diaminopyrimidine-based dihydrofolate reductase inhibitors as potential drug scaffolds against *Bacillus anthracis*

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ABSTRACT

The current Letter describes the synthesis and biological evaluation of dihydrophthalazine-appended 2,4-diaminopyrimidine (DAP) inhibitors (1) oxidized at the methylene bridge linking the DAP ring to the central aromatic ring and (2) modified at the central ring ether groups. Structures **4a–b** incorporating an oxidized methylene bridge showed a decrease in activity, while slightly larger alkyl groups (CH_2CH_3 vs CH_3) on the central ring oxygen atoms (R^2 and R^3) had a minimal impact on the inhibition. Comparison of the potency data for previously reported RAB1 and BN-53 with the most potent of the new derivatives (**19b** and **20a–b**) showed similar values for inhibition of cellular growth and direct enzymatic inhibition (MICs 0.5–2 μg/mL). Compounds **29–34** with larger ester and ether groups containing substituted aromatic rings at R^3 exhibited slightly reduced activity (MICs 2–16 μg/mL). One explanation for this attenuated activity could be encroachment of the extended R^3 into the neighboring NADPH co-factor. These results indicate that modest additions to the central ring oxygen atoms are well tolerated, while larger modifications have the potential to act as dual-site inhibitors of dihydrofolate reductase (DHFR).

1. Introduction

Bacillus anthracis, a Gram-positive bacterium, is the etiological agent responsible for the acute infectious disease anthrax.[1] The Center for Disease Control and Prevention (CDC) classifies *B. anthracis* as a Category A potential high-priority bioterror threat agent, and it is well documented that certain strains of these bacteria have been modified to produce weapons of mass destruction to humans and animals.[2] It is also well known that these engineered strains have innate resistance to current commercial drugs.[3–6] Thus, there is a compelling and imminent need to develop new therapeutic agents to treat these resistant bacteria.

Previous studies from our research group have identified dihydrophthalazine-appended 2,4-diaminopyrimidine (DAP) derivatives as inhibitors of *B. anthracis*.[7–9] These DAP inhibitors target the key enzyme dihydrofolate reductase (DHFR) in the folate pathway, which is intended to respond to intrinsic anti-folate antibiotic resistance found in this organism.[10] Apart from our research group, there are a number of programs throughout the world developing antifolates. Targets similar to those reported herein have been studied as broad-spectrum antibiotics by Basilea Pharmaceutica.[11–14] Other examples include Iclaprim by Acino Pharma,[15] AR-709 by Evolva,[16] and 7-aryl-2,4-diaminoquinazolines by Trius Therapeutics.[17] In the current study, potential DAP inhibitors, incorporating modifications from our potent lead structures RAB1 and BN-53 (Fig. 1), were prepared and evaluated for their biological activity against *B. anthracis*.

The basic structural motif of the target is composed of three ring systems: a 2,4-diaminopyrimidine ring linked via a methylene bridge to a central 3,4-dimethoxybenzene ring, which is, in turn, tethered through an acryloyl chain to a substituted dihydrophthalazine (Fig. 1). Based on previous work by Basilea Pharmaceutica[11–14] and Barrow et al.,[4] a number of DAP inhibitors were designed, synthesized, and evaluated for their biological activities. The initial modifications were made at R^1 on the dihydrophthalazine ring. A series of substrates were prepared with different R^1 substituents,[7,9] and from this set, RAB1 (minimum inhibitory concentration, MIC 1–4 μg/mL) and BN-53 (MIC 0.5 μg/mL) were identified as potential drugs.[7,10] The second alterations were carried out at R^4 on the DAP ring, but increased activity was not observed.[8] In a further quest for improved potency over RAB1 and BN-53, the current study investigated analogs resulting from oxidation of the methylene bridge linking the DAP fragment to the central ring, as well as

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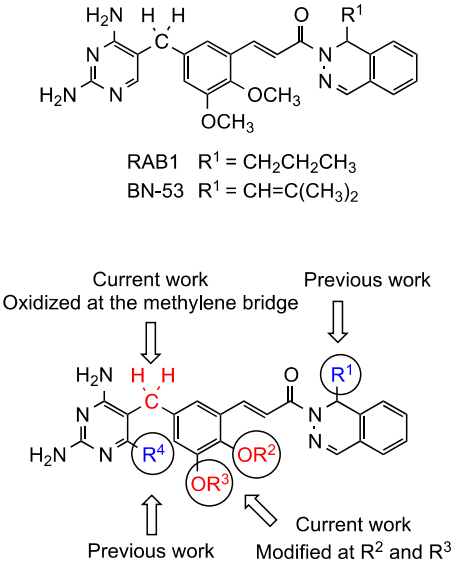


Figure 1. Structural modifications of the DAP-based inhibitor and lead compounds.

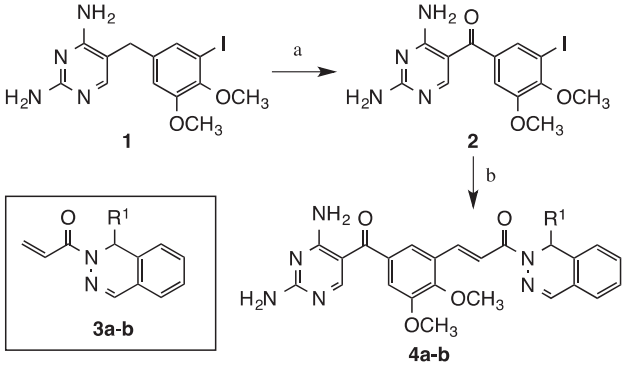
changes at R^2 and R^3, and assessed the biological activities of the resulting compounds.

2. Results and discussion

2.1. Chemistry

The molecular targets in Scheme 1 incorporate a ketone function in the methylene bridge between the DAP moiety and the central dimethoxybenzene ring, but retain the propyl and isobutenyl groups at R^1 on the dihydrophthalazine. The preparation of these structures was a two-step process involving benzylic oxidation of **1** to ketone **2**, followed a by Heck coupling to the acrylamide derivative **3** to yield **4**. Benzylic oxidation of **1** was performed in 78% yield using potassium dichromate in acetic acid at reflux. This was followed by a palladium(II) acetate-mediated Heck reaction of the resulting ketone **2** with (±)-1-(1-alkyl-2(1*H*)-phthalazinyl)-2-propen-1-ones **3a** and **3b** in DMF at 140 °C to give the target molecules **4a** (79%) and **4b** (75%), respectively.[7,8,18,19]

The synthesis of drug candidates, modified at the central ring ether groups (R^2 and R^3), while retaining the propyl and isobutenyl substitution on the dihydrophthalazine, are shown in Scheme 2. Benzaldehydes **6–9**, derived from 5-iodovanillin, were prepared using methodology previously described.[20] Condensation of **6–9** with 3-morpholinopropionitrile (**10**) using sodium methoxide in dry DMSO gave adducts **11–14**, which were treated sequentially in dry EtOH with aniline hydrochloride and guanidine hydrochloride, both in the presence of sodium methoxide, to afford the substituted 2,4-diamino-5-benzyl-1,3-pyrimidines **15–18** in 72–80% yields. Heck coupling of **15–18** with **3a** and **3b** then generated the target compounds **19a–b**, **20a–b**, **21a** and **22a** in 70–78% yields.



Scheme 1. Synthesis of structures incorporating an oxidized methylene bridge. Reagents and conditions: (a) $K_2Cr_2O_7$, AcOH, 118 °C; (b) **3a–b** [a: $R^1 = CH_2CH_2CH_3$; b: $R^1 = CH=C(CH_3)_2$], $Pd(OAc)_2$, 1-ethylpiperidine, DMF, 140 °C.

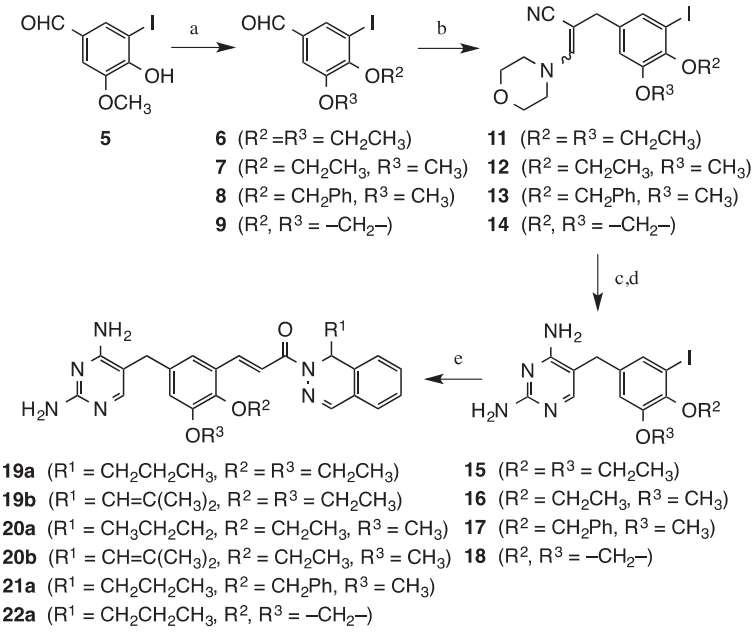
Phenol **27**, which would allow variation of groups at R^3 on the central ring of RAB1, was synthesized (Scheme 3) from the 3-methoxymethyl (MOM)-substituted benzaldehyde **23**, prepared by modification of our previously reported procedure.[20] Sodium methoxide promoted condensation of **10** with **23** produced **24**, which was cyclocondensed with aniline hydrochloride and guanidine hydrochloride to give the substituted 2,4-diamino-5-benzyl-1,3-pyrimidine **25** in 83% yield. Heck coupling of **25** with **3a** gave the MOM-protected catechol **26**, which was deprotected using methanolic hydrogen chloride to give phenol **27** (58%). Finally, treatment of **27** with DBU at room temperature in dichloromethane, followed by addition of propyl iodide, benzoyl chloride, 4-methoxybenzoyl chloride, 4-nitrobenzoyl chloride, 4-(trifluoromethoxy)benzoyl chloride, 4-fluorobenzoyl chloride, or 4-(trifluoromethyl)benzoyl chloride, afforded targets **28–34** in 62–92% yields.

2.2. Biology

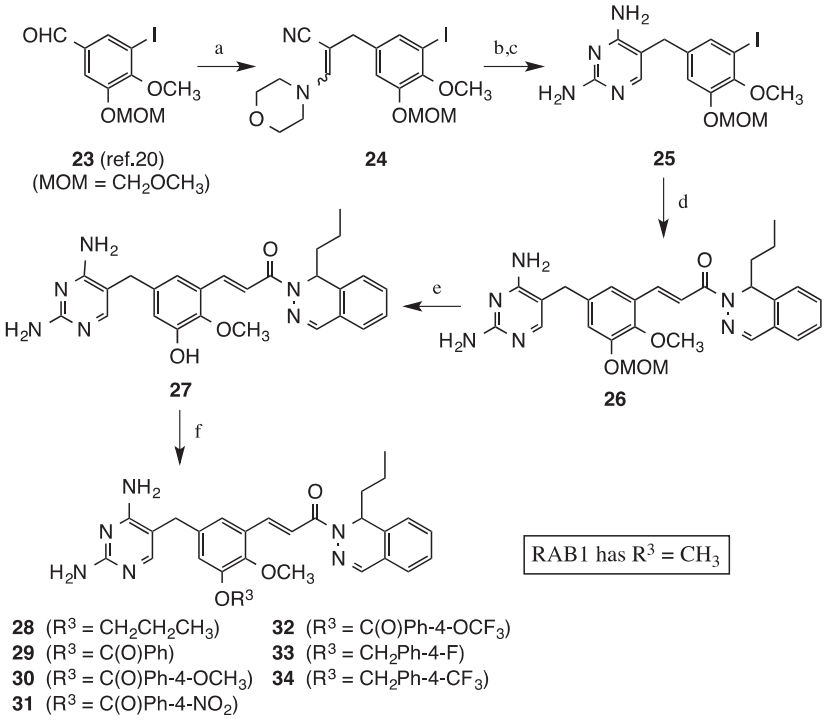
The biological activities of our new dihydrophthalazine-appended DAP derivatives are summarized in Table 1. Compounds incorporating a ketone function between the DAP ring and the central dimethoxybenzene ring, as found in **4a** (R^1 = propyl, $R^2 = R^3 = CH_3$) and **4b** (R^1 = isobutenyl, $R^2 = R^3 = CH_3$), exhibited low activity. The impact on direct inhibition of the DHFR enzyme did display a preference for the isobutenyl moiety at R^1, but this change did not rescue the lack of inhibition of bacterial cell growth.

Alterations of the central-ring ether groups to incorporate slightly larger moieties at either R^2 alone, or in addition to R^3, had little impact on the potency. For example, when compared with unmodified RAB1 (R^1 = propyl, $R^2 = R^3 = CH_3$), **19a** (R^1 = propyl, $R^2 = R^3 = CH_2CH_3$) and **20a** (R^1 = propyl, $R^2 = CH_2CH_3$, $R^3 = CH_3$) showed similar activity. Substitution of larger groups, such as a benzyl at R^2 in **21a** (R^1 = propyl, R^2 = benzyl, $R^3 = CH_3$) or a propyl at R^3 in **28** (R^1 = propyl, $R^2 = CH_3$, R^3 = propyl), exerted a small, but measurable, affect on the growth of the bacterial cells and on the purified enzyme. In contrast, compounds having a smaller methylene moiety bridging R^2 and R^3, as in **22a** (R^1 = propyl, $R^2 = R^3 = -CH_2-$), showed reduced activity. Comparison of the activity data for unmodified BN-53 (R^1 = isobutenyl, $R^2 = R^3 = CH_3$) with that for **19b** (R^1 = isobutenyl, $R^2 = R^3 = CH_2CH_3$) and **20b** (R^1 = isobutenyl, $R^2 = CH_2CH_3$, $R^3 = CH_3$) revealed no measurable change in either inhibition of cellular growth or direct enzymatic inhibition. When contextualized within the binding pocket of the DHFR enzyme from *B. anthracis*, small extensions (replacing CH_3 with CH_2CH_3) at R^2 and R^3 would be readily accommodated without unfavorable steric interaction with any protein atoms. Finally, the comparable reduction in activity for the much larger benzyl at R^2 in **21a** versus the smaller propyl at R^3 in **28** suggests that the R^3 position has a greater impact on potency.

Further modification in the central ring installed larger groups at the R^3 position to give compounds **29–34** (R^1 = propyl, $R^2 = CH_3$, R^3 = variable). These compounds exhibited lower efficacy, and this was revealed more dramatically in the enzyme inhibition assay. In reactions with purified DHFR protein, four of the six R^3 derivatives were unable to achieve at least 50% inhibition at the limit of compound solubility if the compound was added after the NADPH. Only two derivatives, **29** and **31**, effectively inhibited the enzyme with this order of addition. Structure **29** contained the minimum addition of a benzoyl group at R^3, although the K_i



Scheme 2. Synthesis of structures modified at R^2 and R^3. Reagents and conditions: (a) Ref. 20; (b) NaOMe, DMSO, 65–90 °C; (c) $PhNH_2$·HCl, NaOMe, EtOH, 78 °C; (d) $(H_2N_2)C$ = NH·HCl, NaOMe, EtOH, 78 °C; (e) **3a–b** [a: $R^1 = CH_2CH_2CH_3$; b: $R^1 = CH = C(CH_3)_2$], $Pd(OAc)_2$, 1-ethylpiperidine, DMF, 140 °C.



Scheme 3. Synthesis of analogs of RAB1 modified at R^3. Reagents and conditions: (a) NaOMe, DMSO, 65–90 °C; (b) $PhNH_2$·HCl, NaOMe, EtOH, 78 °C; (c) $(H_2N_2)C$ = NH·HCl, NaOMe, EtOH, 78 °C; (d) **3a** $Pd(OAc)_2$, 1-ethylpiperidine, DMF, 140 °C; (e) HCl, MeOH, 23 °C; (f) R^3X, DBU, CH_2Cl_2, 23 °C.

was barely measurable. When compounds were added prior to the NADPH co-factor, the inhibition improved remarkably such that all but one compound had measurable K_i values. Compound **31** (R^3 = 4-nitrobenzoyl), the only polarized structure tested, stood out as remarkably better than the others in this series. Nevertheless, it was not as efficacious as RAB1 or BN-53, and the MIC value did not indicate the same remarkable gain in potency that the K_i value revealed.

The compounds containing the larger extensions from R^3 present an interesting picture when viewed in the context of the DHFR substrate site. These inhibitors are known to dock with the DAP moiety, which closely mimics the natural folate substrate[10,22] Based on our structural data to date, it is likely that each compound within the inhibitor series binds with a relatively conserved orientation.[10] We hypothesize that these larger extensions from R^3 are approaching the neighboring NADPH co-factor site. This hypothesis is supported, in part, by experiments of enzyme inhibition in which the compounds were instead added prior to the NADPH co-factor. In this situation, the measurable K_i values decreased and three additional compounds showed inhibition. It

Table 1
Biological activity of new DAP inhibitors relative to earlier compounds

Compound	MIC (μg/mL) *B. anthracis* Sterne	K_i (nM) ± SEM *B. anthracis* DHFR	K_i (nM) ± SEM *B. anthracis* NADPH first
TMP[a]	>2048	77233	—
RAB1[b]	1–4	9.4 ± 0.2	—
BN-53[b]	0.5	8.3 ± 0.2	—
4a	>128	83.4 ± 2.3	—
4b	>128	15.1 ± 0.6	—
19a	4	20.2 ± 0.2	—
19b	1–2	9.3 ± 0.2	—
20a	2	9.9 ± 0.2	—
20b	0.5	8.8 ± 0.4	—
21a	4	38.8 ± 0.6	14.6 ± 0.4
22a	32	1300 ± 11	1090 ± 167
28	4	13.8 ± 0.2	14.6 ± 0.4
29	4–16	2200 ± 20	720 ± 20
30	8–16	>2300	2200 ± 30
31	8	310 ± 3	260 ± 6
32	8	>2100	220 ± 6
33	8–16	>2400	>2400
34	8–16	>2200	700 ± 10

[a] TMP (trimethoprim) data from Barrow et al.[21]
[b] RAB1 and BN-53 data from Bunce et al.[7]

is of note that compound **28** was relatively unchanged by the order of addition experiment, as it was predicted to not encroach on the co-factor site. If our hypothesis of dual-site binding is correct, the K_i values would no longer be reflective of the enzymatic inhibition as it would now be a double-competitive reaction with both the folate substrate and with the co-factor. This may contribute to the immeasurable K_i values while retaining inhibitory activity at the whole cell level.

3. Conclusions

The current investigation describes the synthesis and biological evaluation of dihydrophthalazine-appended DAP inhibitors oxidized at the methylene bridge linking the DAP ring to the structure and modified at the ether groups of the central aromatic ring. The indication from activity studies of **4a** and **4b** is a requirement for flexibility in the methylene linkage between the DAP group and the central dialkoxy-substituted ring. Alteration of this tetrahedral geometry to a trigonal planar arrangement, as found in the ketone-derivatized structures, abolished all cellular growth inhibition (Table 1). Alterations at R^2 and R^3 are well tolerated when the added group is small and conservative, such as the addition of ethyl groups in compounds **19a**–**b** and **20a**–**b**. This is also true when a larger and hydrophobic benzyl moiety is added at R^2, as in **21a**, or propyl at R^3, as in **22a** (Fig. 2). This is particularly striking when viewed versus the ketone modifications in compounds **4a** and **4b**, and strongly suggests locations in the inhibitory compounds that can accept substitutions while maintaining potency. However, larger additions at R^3 (viz. **29**–**34**) are less well tolerated at the level of enzymatic inhibition, while those modifications have a more tempered effect on the inhibition of whole cell growth. Further experimentation is warranted to completely explore the reason for the reduced potency and possible mechanisms for dual-site inhibition. Our working hypothesis is that this effect results from extension of the compound beyond the targeted folate substrate pocket. If this occurs, it is sub-optimal due to the strained conformation the compound would have to adopt to fill this pocket. It is reasonable that this happens in only a fraction of the interactions, and in the remaining binding region, this added compound bulk would promote unfavorable interplay with the solvent surrounding the substrate-binding site. While we cannot rule out cross-reaction with bacterial targets aside from DHFR in this setting, the concept of creating a dual-pocket inhibitor is a promising area in antifolate development[23–25] Future design of DHFR inhibitors involving this important class of 2,4-diaminopyrimidines should incorporate more restrained linkages and/or more spatially targeted modifications to assess this possibility.

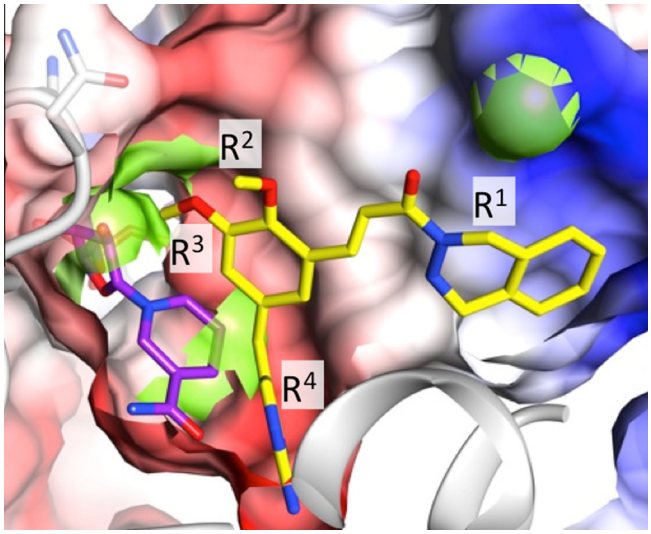


Figure 2. Extension from the R^3 position of the DAP inhibitor is predicted to clash with co-factor binding. The DHFR binding site accommodates an NADPH co-factor, shown with purple carbon atoms, as well as a folate substrate or inhibitor (the dihydrophthalazine-appended DAP inhibitor is shown with yellow carbon atoms). The protein is displayed as a Coulombic-shaded van der Waals surface (red is acidic, blue is basic) and with a grey backbone ribbon. Modifications of the inhibitor scaffold are labeled as discussed in the text. Clashes between the protein and modified inhibitors were calculated and are visualized by green surface shading. Of note are green shadings proximal to the R^3 modification site, the methylene bridge and the R^1 site. The green sphere is Arg-53 of the DHFR enzyme, which can interact with R^1 of the inhibitor.

4. Experimental section

Commercial anhydrous *N*,*N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were stored under dry nitrogen and transferred by syringe into reactions when needed. Tetrahydrofuran (THF) was dried over potassium hydroxide pellets and distilled from lithium aluminum hydride prior to use. All other commercial reagents were used as received.

Unless otherwise specified, all reactions were run under dry nitrogen in oven-dried glassware. Reactions were monitored by thin layer chromatography on silica gel GF plates (Analtech, No. 21521). Preparative separations were performed by column chromatography on silica gel (Davisil,® grade 62, 60–200 mesh) mixed with UV-active phosphor (Sorbent Technologies, No. UV-05); band elution was monitored using a hand held UV lamp. Saturated $NaHCO_3$, NaCl and NH_4Cl used in work-up procedures refer to aqueous solutions. Melting points were uncorrected. FT-IR spectra were run as thin films on sodium chloride disks. ^1H and ^{13}C NMR spectra were measured on a Varian GEMINI 300 instrument at 300 MHz and 75 MHz, respectively, and referenced to internal tetramethylsilane. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA 30071.

4.1. Synthesis of structures incorporating an oxidized methylene bridge (Scheme 1)

4.1.1. (2,4-Diaminopyrimidin-5-yl)(3-iodo-4,5-dimethoxyphenyl)methanone (2)

To a stirred solution of **1** (10.0 g, 25.9 mmol) in AcOH (80 mL) was added $K_2Cr_2O_7$ (30.5 g, 0.104 mol, 4 equiv), and the solution was placed in a preheated oil bath at 120 °C and refluxed for a

period of 6 h. The reaction mixture was cooled to 0 °C and poured into ice cold water with stirring. The mixture was then extracted with EtOAc (3 × 200 mL). The organic extracts were washed with saturated $NaHCO_3$ (3 × 100 mL), water, saturated NaCl, dried ($MgSO_4$), filtered, and concentrated under vacuum to give a thick brown liquid. The compound was purified using a silica gel column and eluted with CH_2Cl_2/MeOH/Et_3N (10:4:1). Evaporation of the solvent yielded product **2** (8.08 g, 78%) as an off-white solid, mp 206–208 °C. IR: 3448, 3382, 3326, 1598 cm^{-1}; ^1H NMR (DMSO-d_6): δ 8.28 (br s, 1H), 8.14 (s, 1H), 7.54 (br s, 1H), 7.45 (d, J = 1.6 Hz, 1H), 7.20 (d, J = 1.6 Hz, 1H), 7.13 (br s, 2H), 3.85 (s, 3H), 3.78 (s, 3H); ^{13}C NMR (DMSO-d_6): δ 191.5, 164.3, 163.8, 163.6, 152.0, 150.1, 137.2, 129.8, 113.4, 103.3, 92.4, 60.0, 56.1.

4.1.2. (±)-(*E*)-3-[5-(2,4-Diaminopyrimidine-5-carbonyl)-2,3-dimethoxyphenyl]-1-(1-propyl-2(1*H*)-phthalazinyl)-2-propen-1-one (4a)

To a stirred solution of **2** (1.00 g, 2.50 mmol) in dry DMF (7 mL) was added a solution of (±)-**3a** (657 mg, 2.88 mmol, 1.15 equiv)[7] in DMF (1 mL), followed by *N*-ethylpiperidine (340 mg, 0.41 mL, 3.00 mmol 1.2 equiv) and $Pd(OAc)_2$ (20 mg, 0.089 mmol). The reaction mixture was heated at 140 °C for 20 h and then gradually cooled to 0 °C. Isolation of the product was achieved by pouring the cooled reaction mixture directly onto a 50-cm × 2.5-cm silica gel flash chromatography column packed in CH_2Cl_2. Impurities were eluted using CH_2Cl_2, and the final product was collected using CH_2Cl_2/MeOH/Et_3N (95:5:1). Evaporation of the solvent gave a pale yellow solid, which was further purified using a 15-cm × 2-cm silica gel column, packed with CH_2Cl_2/MeOH/Et_3N (95:5:1). This second chromatography removed colored impurities as well as minor contaminants. Evaporation of the solvent gave **4a** as an off-white solid. The compound was further purified by recrystallization from MeOH to give a white solid (990 mg, 79%), mp 212–214 °C. IR: 3377, 3143, 1660, 1593 cm^{-1}; ^1H NMR (DMSO-d_6): δ 8.33 (br s, 2H), 8.18 (s, 1H), 7.93 (s, 1H), 7.89 (d, J = 16.5 Hz, 1H), 7.68 (d, J = 16.5 Hz, 1H), 7.58–7.37 (complex m, 5H), 7.24 (s, 1H), 7.12 (br s, 2H), 5.84 (t, J = 6.6 Hz, 1H), 3.88 (s, 3H), 3.87 (s, 3H), 1.54 (m, 2H), 1.17 (sextet, J = 7.1 Hz, 2H), 0.82 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 192.8, 165.4, 164.4, 163.9, 163.6, 152.5, 149.3, 143.0, 135.9, 135.5, 133.6, 131.7, 128.3, 128.0, 126.5, 126.1, 123.6, 119.2, 119.0, 11.7, 103.5, 60.9, 56.1, 50.4, 36.9, 17.8, 13.7. Anal. Calcd for $C_{27}H_{28}N_6O_4$; C, 64.79; H, 5.64; N, 16.79. Found: C, 64.63; H, 5.75; N, 16.51.

4.1.3. (±)-(*E*)-3-[5-(2,4-Diaminopyrimidine-5-carbonyl)-2,3-dimethoxyphenyl]-1-(1-isobutenyl-2(1*H*)-phthalazinyl)-2-propen-1-one (4b)

This compound was prepared as above using **2** (1.00 g, 2.50 mmol), (±)-**3b** (691 mg, 2.88 mmol, 1.15 equiv),[7] *N*-ethylpiperidine (340 mg, 0.41 mL, 3.00 mmol 1.2 equiv), and $Pd(OAc)_2$ (20 mg, 0.089 mmol) in dry DMF (8 mL) under N_2 to give **4b** (960 mg, 75%) as an off-white solid, mp 138–140 °C. IR: 3320, 3179, 1655, 1594, cm^{-1}; ^1H NMR (DMSO-d_6): δ 8.65 (br s, 1H), 8.39 (br s, 1H), 8.11 (d, J = 16.5 Hz, 1H), 7.67 (d, J = 16.5 Hz, 1H), 7.62 (s, 1H), 7.49 (d, J = 1.6 Hz, 1H), 7.43 (td, J = 7.1, 1.6 Hz, 1H), 7.33 (t, J = 7.1 Hz, 1H), 7.27 (d, J = 6.6 Hz, 1H), 7.16 (d, J = 6.6 Hz, 1H), 7.15 (d, J = 1.6 Hz, 1H), 6.58 (d, J = 9.9 Hz, 1H), 5.67 (br s, 1H), 5.47 (br s, 2H), 5.30 (d, J = 9.9 Hz, 1H), 3.93 (s, 3H), 3.91 (s, 3H), 2.05 (s, 3H), 1.65 (s, 3H); ^{13}C NMR (DMSO-d_6): δ 194.3, 166.2, 164.6, 164.4, 163.4, 153.1, 150.7, 141.5, 136.3, 134.6, 134.5, 134.2, 131.9, 129.5, 127.9, 126.2, 125.8, 123.3, 122.1, 120.1, 119.7, 113.2, 105.2, 61.5, 56.0, 50.0, 25.7, 18.6. Anal. Calcd for $C_{28}H_{28}N_6O_4 \cdot 0.4\ H_2O$: C, 64.70; H, 5.58; N, 16.17. Found: C, 64.67; H, 5.63; N, 15.83.

4.2. Synthesis of structures modified at R^2 and R^3 (Scheme 2)

4.2.1. Benzaldehydes 6–9 and 23

These compounds were prepared on a 54-mmol scale via a two-step literature procedure[20] in the following yields: **6** (80%), **7** (92%), **8** (92%), **9** (78%) and **23** (88%). The spectral data matched those reported.[20]

4.2.2. 3-Morpholinopropionitrile (10)

This compound was prepared on a 0.28-mol scale according to a literature procedure.[26] The crude product was vacuum distilled to give **10** (37.2 g, 95%) as a colorless liquid, bp 88–90 °C (0.5 mmHg) [lit.[26] bp 149 °C (20 mmHg)].

4.2.3. 5-(3,4-Diethoxy-5-iodobenzyl)pyrimidine-2,4-diamine (15)

To a stirred solution of NaOMe (1.69 g, 31.3 mmol, 1.0 equiv) in DMSO (25 mL) was added dropwise 3-morpholinopropionitrile **10** (5.25 g, 37.5 mmol, 1.2 equiv) at 65 °C. The reaction mixture was heated to 90 °C, followed by the addition of a warm solution of **6** (10.0 g, 31.3 mmol) in DMSO (15 mL) over 20 min. Stirring was continued at 90 °C for 45 min. The crude reaction mixture was then cooled in an ice bath, poured into ice-cold water, and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic extracts were washed with saturated NaCl (1 × 100 mL), dried ($MgSO_4$), filtered, and concentrated under vacuum to give crude **11** as dark red oil.

The crude product **11** was re-dissolved in dry ethanol (150 mL), aniline hydrochloride (4.46 g, 34.4 mol, 1.1 equiv) was added, and the reaction mixture was heated under reflux for 1 h. Guanidine hydrochloride (7.16 g, 75.0 mmol, 2.4 equiv), followed by NaOMe (6.75 g, 125 mmol, 4 equiv), was then added to the reaction mixture under hot conditions, and refluxing was continued for 4 h. The reaction mixture was concentrated to 1/4 of the volume under vacuum, and ice-cold water was added, resulting in the formation of a pale yellow solid. The solid was collected, washed with ice-cold ethanol, water, and finally with Et_2O to give an off-white solid. The product was recrystallized using ethanol/water (4:1) to give **15** (10.4 g, 80%) as a white solid, mp 173–175 °C. IR: 3472, 3327, 3176 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.57 (s, 1H), 7.15 (s, 1H), 6.95 (s, 1H), 6.19 (br s, 2H), 5.82 (br s, 2H), 4.01 (q, J = 7.1 Hz, 2H), 3.93 (q, J = 7.1 Hz, 2H), 3.53 (s, 2H), 1.32 (coincident t, J = 7.1 Hz, 6H); ^{13}C NMR (DMSO-d_6): δ 162.4, 162.2, 156.0, 151.1, 145.8, 138.5, 129.1, 114.6, 105.3, 93.2, 68.2, 63.9, 31.7, 15.7, 14.6.

4.2.4. 5-(4-Ethoxy-3-iodo-5-methoxybenzyl)pyrimidine-2,4-diamine (16)

This compound was prepared as described above for compound **15** using **7** (10.0 g, 32.7 mmol), **10** (5.49 g, 39.2 mmol, 1.2 equiv), and NaOMe (1.77 g, 32.7 mmol, 1.0 equiv) in DMSO (40 mL) to give crude **12** as a dark red oil. This oil in ethanol (150 mL) was further treated with $PhNH_2$·HCl (5.08 g, 39.2 mmol, 1.2 equiv), guanidine·HCl (7.50 g, 78.5 mmol, 2.4 equiv), and NaOMe (7.06 g, 130.7 mmol, 4.0 equiv) to give **16** (10.2 g, 78%) as a white solid, mp 170–172 °C. IR: 3477, 3323, 3174 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.57 (s, 1H); 7.14 (d, J = 1.8 Hz, 1H), 6.97 (d, J = 1.8 Hz, 1H), 6.16 (br s, 2H), 5.77 (br s, 2H), 3.90 (q, J = 7.0 Hz, 2H), 3.76 (s, 3H), 3.53 (s, 2H), 1.31 (t, J = 7.0 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 162.4, 162.1, 156.1, 152.0, 145.5, 138.7, 129.1, 113.7, 105.3, 93.2, 68.2, 55.8, 31.7, 15.6.

4.2.5. 5-[4-(Benzyloxy)-3-iodo-5-methoxybenzyl]pyrimidine-2,4-diamine (17)

This compound was prepared as described above for compound **15** using **8** (10.0 g, 27.2 mmol), **10** (4.57 g, 32.6 mmol, 1.2 equiv), and NaOMe (1.47 g, 27.2 mmol, 1.0 equiv) in DMSO (40 mL) to give crude **13** as a dark brown oil. This oil in ethanol (150 mL) was

further treated with $PhNH_2$·HCl (4.22 g, 32.6 mmol, 1.2 equiv), guanidine·HCl (6.23 g, 65.2 mmol, 2.4 equiv), and NaOMe (5.87 g, 109 mol, 4.0 equiv) to afford the product. The work-up procedure was altered for this compound. After completion, the reaction mixture was concentrated to dryness under vacuum, 100 mL of ice-cold water was added, and the compound was extracted with EtOAc (3 × 150 mL). The combined organic extracts were washed with saturated NaCl (1 × 100 mL), dried ($MgSO_4$), filtered, and concentrated under vacuum. The crude mixture was then purified by column chromatography using a 30-cm × 2.5-cm silica gel column eluted with CH_2Cl_2/MeOH/Et_3N (98:2:1) to afford **17** (9.03 g, 72%) as a yellow solid, mp 158–160 °C. IR: 3330, 3178 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.59 (s, 1H), 7.53 (m, 2H), 7.38 (m, 3H), 7.19 (s, 1H), 7.03 (s, 1H), 6.21 (br s, 2H), 5.82 (br s, 2H), 4.89 (s, 2H), 3.82 (s, 3H), 3.57 (s, 2H); ^{13}C NMR (DMSO-d_6): δ 162.4, 162.2, 156.0, 152.1, 145.1, 139.0, 137.1, 129.3, 128.2 (2C), 128.0, 113.8, 105.3, 92.9, 73.7, 55.9, 31.8.

4.2.6. 5-(3-Iodo-4,5-methylenedioxybenzyl)pyrimidine-2,4-diamine (18)

This compound was prepared as described above for compound **15** using **9** (9.03 g, 32.7 mmol), **10** (5.49 g, 39.2 mmol, 1.2 equiv), and NaOMe (1.77 g, 32.7 mmol, 1.0 equiv) in DMSO (40 mL) to give crude **14** as a dark red oil. This oil in ethanol (150 mL) was further treated with $PhNH_2$·HCl (5.08 g, 39.2 mmol, 1.2 equiv), guanidine·HCl (7.49 g, 78.5 mmol, 2.4 equiv), and NaOMe (7.06 g, 130.7 mmol, 4.0 equiv) to give **18** (9.07 g, 75%) as a white solid, mp 218–220 °C. IR: 3440, 3322, 3214 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.54 (s, 1H), 7.02 (s, 1H), 6.78 (s, 1H), 6.14 (br s, 2H), 6.03 (s, 2H), 5.76 (br s, 2H), 3.49 (s, 2H); ^{13}C NMR ($CDCl_3$): δ 162.2, 162.1, 155.9, 147.3, 146.2, 136.5, 128.9, 108.9, 105.6, 100.5, 71.3, 31.7.

4.2.7. (±)-(*E*)-3-{5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2,3-diethoxyphenyl}-1-(1-propyl-2(1*H*)-phthalazinyl)-2-propen-1-one (19a)

This compound was prepared as described above for **4a** using **15** (1.00 g, 2.42 mmol), (±)-**3a** (634 mg, 2.78 mmol, 1.15 equiv), *N*-ethylpiperidine (328 mg, 0.40 mL, 2.90 mmol 1.2 equiv), and $Pd(OAc)_2$ (20 mg, 0.089 mmol) in dry DMF (8 mL) under N_2 to give **19a** (968 mg, 78%) as an off-white solid, mp 118–120 °C. IR: 3334, 1652, 1630 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.93 (s, 1H), 7.89 (d, J = 15.9 Hz, 1H), 7.64 (d, J = 15.9 Hz, 1H), 7.57 (s, 1H), 7.52 (m, 2H), 7.45 (d, J = 6.8 Hz, 1H), 7.39 (t, J = 7.1 Hz, 1H), 7.23 (s, 1H), 6.97 (s, 1H), 6.53 (br s, 2H), 6.06 (br s, 2H), 5.84 (t, J = 6.6 Hz, 1H), 4.01 (overlapping q, J = 7.1 Hz, 4H), 3.59 (s, 2H), 1.54 (m, 2H), 1.34 (t, J = 6.6 Hz, 3H), 1.30 (t, J = 7.1 Hz, 3H), 1.17 (sextet, J = 7.1 Hz, 2H), 0.82 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 165.6, 162.5, 161.0, 153.4, 151.8, 145.3, 142.8, 137.2, 135.9, 133.6, 131.7, 128.3, 128.2, 126.5, 126.1, 123.7, 118.7, 117.7, 115.6, 106.3, 68.7, 63.7, 50.4, 36.8, 32.3, 17.8, 15.5, 14.7, 13.7. Anal. Calcd for $C_{29}H_{34}N_6O_3$·2.5 H_2O: C, 62.24; H, 7.06; N, 15.02. Found: C, 62.46; H, 7.26; N, 15.05.

4.2.8. (±)-(*E*)-3-{5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2,3-diethoxyphenyl}-1-(1-isobutenyl-2(1*H*)-phthalazinyl)-2-propen-1-one (19b)

This compound was prepared as described above for **4a** using **15** (1.00 g, 2.42 mmol), (±)-**3b** (667 mg, 2.78 mmol, 1.15 equiv), *N*-ethylpiperidine (328 mg, 0.40 mL, 2.90 mmol 1.2 equiv), and $Pd(OAc)_2$ (20 mg, 0.089 mmol) in dry DMF (8 mL) under N_2 to give **19b** (915 mg, 72%) as an off-white solid, mp 251–252 °C. IR: 3325, 3147, 1661, 1590 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.91 (s, 1H), 7.87 (d, J = 15.9 Hz, 1H), 7.63 (d, J = 15.9 Hz, 1H), 7.54 (s, 1H), 7.51 (m, 2H), 7.42 (m, 3H), 7.31 (d, J = 7.1 Hz, 1H), 7.26 (s, 1H), 7.00 (s, 1H), 6.89 (br s, 2H), 6.50 (d, J = 9.9 Hz, 1H), 5.24 (d, J = 9.9 Hz, 1H), 4.02 (m, 4H), 3.63 (s, 2H), 1.96 (s, 3H), 1.60 (s, 3H), 1.35 (t, J = 7.1 Hz, 3H), 1.31 (t, J = 7.1, 3H); ^{13}C NMR (DMSO-d_6): δ 165.3, 163.3, 156.9, 151.9, 145.5, 142.0, 137.2, 134.4, 133.8, 133.5, 132.2, 128.3, 128.2, 126.2 (2C), 123.1, 122.1, 119.0, 117.9, 115.8, 107.8, 68.7, 63.9, 49.2, 31.8, 25.3, 18.4, 15.5, 14.7 (one aromatic C unresolved). Anal. Calcd for $C_{30}H_{34}N_6O_3$·5.7 H_2O: C, 57.26; H, 7.27; N, 13.35. Found: C, 57.12; H, 7.00; N, 13.27.

4.2.9. (±)-(*E*)-3-{5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2-ethoxy-3-methoxyphenyl}-1-(1-propyl-2(1*H*)-phthalazinyl)-2-propen-1-one (20a)

This compound was prepared as described above for **4a** using **16** (1.00 g, 2.50 mmol), (±)-**3a** (656 mg, 2.88 mmol, 1.15 equiv), *N*-ethylpiperidine (339 mg, 0.41 mL, 3.00 mmol 1.2 equiv), and $Pd(OAc)_2$ (20 mg, 0.089 mmol) in dry DMF (8 mL) under N_2 to give **20a** (925 mg, 74%) as an off-white solid, mp 145–147 °C. IR: 3334, 3196, 1650, 1603 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.94 (s, 1H), 7.89 (d, J = 15.9 Hz, 1H), 7.67 (d, J = 15.9 Hz, 1H), 7.56 (s, 1H), 7.53 (m, 2H), 7.46 (d, J = 6.8 Hz, 1H), 7.40 (t, J = 7.1 Hz, 1H), 7.27 (s, 1H), 7.07 (br s, 2H), 7.02 (s, 1H), 6.57 (br s, 2H), 5.84 (t, J = 6.6 Hz, 1H), 3.97 (q, J = 7.1 Hz, 2H), 3.79 (s, 3H), 3.63 (s, 2H), 1.54 (m, 2H), 1.30 (t, J = 7.1 Hz, 3H), 1.17 (sextet, J = 7.1 Hz, 2H), 0.82 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 165.6, 163.0, 158.4, 152.7, 148.4, 145.2, 142.7, 137.1, 135.0, 133.6, 131.9, 131.7, 128.2, 126.5, 126.1, 123.6, 118.9, 117.9, 114.8, 107.2, 68.7, 55.8, 50.3, 36.8, 32.0, 17.8, 15.4, 13.6. Anal. Calcd for $C_{28}H_{32}N_6O_3$·4.5 H_2O: C, 57.82; H, 7.10; N, 14.45. Found: C, 57.89; H, 6.94; N, 14.49.

4.2.10. (±)-(*E*)-3-{5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2-ethoxy-3-methoxyphenyl}-1-(1-isobutenyl-2(1*H*)-phthalazinyl)-2-propen-1-one (20b)

This compound was prepared as described above for **4a** using **16** (1.00 g, 2.50 mmol), (±)-**3b** (690 mg, 2.88 mmol, 1.15 equiv), *N*-ethylpiperidine (339 mg, 0.41 mL, 3.00 mmol 1.2 equiv), and $Pd(OAc)_2$ (20 mg, 0.089 mmol) in dry DMF (8 mL) under N_2 to give **20b** (896 mg, 70%) as an off-white solid, mp 140–142 °C. IR: 3354, 3146, 1662, 1591 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.93 (s, 1H), 7.89 (d, J = 15.9 Hz, 1H), 7.64 (d, J = 15.9 Hz, 1H), 7.60 (s, 1H), 7.52 (m, 2H), 7.43 (t, J = 7.1 Hz, 1H), 7.31 (d, J = 7.1 Hz, 1H), 7.25 (s, 1H), 7.01 (s, 1H), 6.66 (br s, 2H), 6.51 (d, J = 9.9 Hz, 1H), 6.19 (br s, 2H), 5.25 (d, J = 9.9 Hz, 1H), 3.97 (q, J = 7.1 Hz, 2H), 3.80 (s, 3H), 3.63 (s, 2H), 1.97 (s, 3H), 1.61 (s, 3H), 1.31 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 165.3, 162.6, 160.3, 152.6, 152.0, 145.1, 142.0, 137.2, 135.7, 133.8, 133.5, 132.2, 128.2 (2C), 126.2, 126.1, 123.1, 122.2, 118.8, 117.9, 114.7, 106.5, 68.7, 55.8, 49.2, 32.2, 25.3, 18.4, 15.4. Anal. Calcd for $C_{29}H_{32}N_6O_3$·2.4 H_2O: C, 62.65; H, 6.38; N, 15.10. Found: C, 62.52; H, 6.10; N, 14.75.

4.2.11. (±)-(*E*)-3-{2-(Benzyloxy)-5-[(2,4-diamino-5-pyrim-idinyl) methyl]-3-methoxyphenyl}-1-(1-propyl-2(1*H*)-phthalazinyl)-2-propen-1-one (21a)

This compound was prepared as described above for **4a** using **17** (1.00 g, 2.16 mmol), (±)-**3a** (568 mg, 2.49 mmol, 1.15 equiv), *N*-ethylpiperidine (294 mg, 0.36 mL, 2.60 mmol 1.2 equiv), and $Pd(OAc)_2$ (20 mg, 0.089 mmol) in dry DMF (8 mL) under N_2 to give **21a** (850 mg, 70%) as an off-white solid, mp 123–125 °C. IR: 3474, 3342, 3181, 1654, 1605 cm^{-1}; ^1H NMR (DMSO-d_6): δ 8.06 (d, J = 15.9 Hz, 1H), 7.80 (s, 1H), 7.64 (d, J = 15.9 Hz, 1H), 7.62 (s, 1H), 7.46 (m, 3H), 7.40–7.25 (complex m, 5H), 7.18 (d, J = 7.1 Hz, 1H), 7.11 (s, 1H), 6.67 (s, 1H), 5.90 (t, J = 6.6 Hz, 1H), 5.00 (s, 2H), 4.77 (br s, 2H), 4.60 (br s, 2H), 3.81 (s, 3H), 3.68 (s, 2H), 1.65 (m, 2H), 1.27 (m, 2H), 0.86 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 166.5, 162.6, 162.2, 156.7, 153.7, 145.9, 142.2, 137.4, 137.0, 134.2, 134.1, 131.4 130.2, 128.6, 128.3, 128.00, 127.95, 126.5, 125.6, 124.0, 118.9, 118.8, 112.9, 106.4, 75.4, 55.9, 51.3, 37.3, 34.4, 18.3, 13.8. Anal. Calcd for $C_{33}H_{34}N_6O_3$·0.3 H_2O: C, 69.77; H, 6.14; N, 14.79. Found: C, 69.70; H, 6.14; N, 14.44.

4.2.12. (±)-(*E*)-3-{5-[(2,4-Diaminopyrimidin-5-yl)methyl]-2,3-methylenedioxyphenyl}-1-(1-propylphthalazin-2(1*H*)-yl)-2-propen-1-one (22a)

This compound was prepared as described above for **4a** using **18** (1.00 g, 2.70 mmol), (±)-**3a** (709 mg, 3.11 mmol, 1.15 equiv), *N*-ethylpiperidine (366 mg, 0.44 mL, 3.24 mmol 1.2 equiv), and $Pd(OAc)_2$ (20 mg, 0.089 mmol) in dry DMF (8 mL) under N_2 to give **22a** (901 mg, 71%) as an off-white solid, mp 123–125 °C. IR: 3424, 3312, 3100, 1635, 1599 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.96 (s, 1H), 7.74 (d, J = 16.0 Hz, 1H), 7.55 (s, 1H), 7.54–7.41 (complex, 3H), 7.50 (d, J = 16.0 Hz, 1H), 7.38 (d, J = 7.4 Hz, 1H), 7.06 (s, 1H), 6.82 (s, 1H), 6.13 (2s, 4H), 5.85 (t, J = 6.7 Hz, 1H), 5.73 (br s, 2H), 3.53 (s, 2H), 1.52 (m, 2H), 1.17 (sextet, J = 7.4 Hz, 2H), 0.81 (t, J = 7.4 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 165.5, 162.3, 162.1, 155.7, 147.8, 144.4, 143.0, 136.8, 134.5, 133.6, 131.7, 128.3, 126.5, 126.1, 123.6, 122.0, 119.2, 116.7, 110.0, 105.8, 101.7, 50.4, 36.8, 32.2, 17.8, 13.7. Anal. Calcd for $C_{26}H_{26}N_6O_3 \cdot 0.6$ EtOH: C, 65.58; H, 5.99; N, 16.87. Found: C, 65.40; H, 5.85; N, 16.76.

4.3. Synthesis of compounds modified at R^3 (Scheme 3)

4.3.1. 5-[3-Iodo-4-methoxy-5-(methoxymethoxy)benzyl]-pyrimidine-2,4-diamine (25)

This compound was prepared as described above for compound **15** using **23**[20] (10.0 g, 24.0 mmol), **10** (4.03 g, 28.8 mmol, 1.2 equiv) and NaOMe (1.30 g, 24.0 mmol, 1.0 equiv) in DMSO (40 mL) to give intermediate **24** (9.24 g, 90%) as a dark brown oil. This oil in ethanol (150 mL) was treated with $PhNH_2 \cdot HCl$ (3.10 g, 24.0 mmol, 1.0 equiv), guanidine·HCl (5.50 g, 57.6 mmol, 2.4 equiv), and NaOMe (5.18 g, 96.0 mmol, 4.0 equiv) to afford a dark yellow solution. The work-up procedure was altered for this compound. The crude reaction mixture was concentrated to dryness under vacuum, 100 mL of ice-cold water was added, and the compound was extracted with EtOAc (3 × 150 mL). The combined organic extracts were washed with saturated NaCl (1 × 100 mL), dried ($MgSO_4$), filtered, and concentrated under vacuum. The crude mixture was then purified by column chromatography using 30-cm × 2.5-cm silica gel column eluted with CH_2Cl_2/MeOH/Et_3N (98:2:1) to afford **25** (8.29 g, 83%) as a white solid, mp 123–125 °C. IR: 3461, 3153, 1625 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.54 (s, 1H), 7.22 (d, J = 1.6 Hz, 1H), 7.05 (d, J = 1.6 Hz, 1H), 6.14 (br s, 2H), 5.76 (br s, 2H), 5.9 (s, 2H), 3.70 (s, 3H), 3.51 (s, 2H), 3.40 (s, 3H); ^{13}C NMR (DMSO-d_6): δ 162.4, 162.2, 156.1, 149.3, 147.3, 138.9, 130.7, 117.2, 105.1, 94.6, 92.6, 60.0, 56.0, 31.6.

4.3.2. (±)-(*E*)-3-{5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2-methoxy-3-(methoxymethoxy)-phenyl]-1-(1-propyl-2(1*H*)-phthalazinyl)-2-propen-1-one (26)

This compound was prepared as described above for **4a** using **25** (1.00 g, 2.40 mmol), with (±)-**3a** (630 mg, 2.76 mmol, 1.15 equiv), *N*-ethylpiperidine (326 mg, 0.40 mL, 2.88 mmol 1.2 equiv), and $Pd(OAc)_2$ (20 mg, 0.089 mmol) in dry DMF (8 mL) under N_2 to give **26** (719 mg, 58%) as a brown solid. ^1H NMR revealed the product **26** contained compound **27** (hydroxy derivative) in the ratio of (8:2) as a mixture. The product was taken to the next step without further purification or analysis.

4.3.3. (±)-(*E*)-3-{5-[(2,4-Diamino-5-pyrimidinyl)methyl]-3-hydroxy-2-methoxyphenyl}-1-(1-propyl-2(1*H*)-phthalazinyl)-2-propen-1-one (27)

To a stirred solution of crude **26** (1.00 g, 1.94 mmol) in CH_2Cl_2 (35 mL) was added MeOH·HCl (35 mL), and the reaction mixture was stirred for 24 h at room temperature. Evaporation of the solvent gave a dark brown liquid. To remove all traces of MeOH·HCl, a 50-mL portion of Et_2O/CH_2Cl_2 (1:1) was added, and the solvent was removed under vacuum. This process was repeated 5–6 times to produce an off-white solid. This solid was triturated with Et_2O, and the product was collected to give **27** (0.86 g, 94%) as a white solid, mp 135–137 °C. IR: 3481, 3345, 3204, 1652, 1617 cm^{-1}; ^1H NMR (DMSO-d_6): δ 9.50 (br s, 1H), 7.94 (s, 1H), 7.86 (d, J = 15.9 Hz, 1H), 7.63 (d, J = 15.9 Hz, 1H), 7.58–7.48 (complex m, 3H), 7.46 (d, J = 7.1 Hz, 1H), 7.39 (d, J = 7.1 Hz, 1H), 7.15 (s, 1H), 6.70 (s, 1H), 6.16 (br s, 2H), 5.85 (t, J = 6.6 Hz, 1H), 5.79 (br s, 2H), 3.73 (s, 3H), 3.54 (s, 2H), 1.54 (m, 2H), 1.17 (sextet, J = 7.1 Hz, 2H), 0.82 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 165.6, 162.3, 162.2, 155.6, 150.2, 145.0, 142.7, 137.1, 136.3, 133.6, 131.7, 128.2, 127.8, 126.5, 126.0, 123.7, 118.1, 117.3, 105,5, 60.5, 50.3, 36.8, 32.0, 17.8, 14.0, 13.7. Anal. Calcd for $C_{26}H_{28}N_6O_3 \cdot 0.5\ H_2O$: C, 64.85; H, 6.07; N, 17.45. Found: C, 64.93; H, 5.94; N, 17.15.

4.3.4. (±)-(*E*)-3-{5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2-methoxy-3-propoxyphenyl}-1-(1-propyl-2(1*H*)-phthalazinyl)-2-propen-1-one (28)

To a stirred solution of **27** (150 mg, 0.318 mmol) in CH_2Cl_2 (8 mL) was added DBU (53 mg, 0.052 mL, 0.350 mmol, 1.1 equiv), and the solution was stirred for 30 min. To the reaction mixture was added dropwise propyl bromide (43 mg, 0.032 mL, 0.350 mmol, 1.1 equiv), and stirring was continued for 20 h. The reaction mixture was evaporated to dryness and purified on a 20-cm × 20-cm silica gel, preparative thin layer chromatography plate eluted with CH_2Cl_2/MeOH/Et_3N (95:5:1). The product band was washed with CH_2Cl_2/MeOH/Et_3N (95:5:1) to afford an off-white solid. This solid was triturated with Et_2O to remove Et_3N, and the compound was filtered and dried under vacuum to afford **28** (144 mg, 88%) as a white solid, mp 194–195 °C. IR: 3342, 3157, 1659, 1637 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.94 (s, 1H), 7.87 (d, J = 15.9 Hz, 1H), 7.64 (d, J = 15.9 Hz, 1H), 7.53 (m, 3H), 7.46 (d, J = 7.1 Hz, 1H), 7.40 (d, J = 7.1 Hz, 1H), 7.28 (s, 1H), 7.09 (br s, 2H), 7.01 (s, 1H), 6.59 (br s, 2H), 5.85 (t, J = 6.3 Hz, 1H), 3.94 (t, J = 6.0 Hz, 2H), 3.78 (s, 3H), 3.62 (s, 2H), 1.77 (sextet, J = 6.6 Hz, 2H), 1.54 (m, 2H), 1.17 (sextet, J = 7.1 Hz, 2H), 1.01 (t, J = 7.1 Hz, 3H), 0.82 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 165.6, 163.0, 158.4, 151.9, 148.3, 146.3, 142.8, 136.7, 135.1, 133.6, 131.7, 128.3, 127.9, 126.5, 126.1, 123.6, 118.6, 117.9, 115.7, 107.3, 69.9, 60.7, 50.3, 36.8, 32.0, 22.1, 17.8, 13.7, 10.6. Anal. Calcd for $C_{29}H_{34}N_6O_3 \cdot 3.7\ H_2O$: C, 59.92; H, 7.18; N, 14.46. Found: C, 59.83; H, 7.19; N, 14.39.

4.3.5. (±)-(*E*)-5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2-methoxy-3-[3-oxo-3-(1-propyl-2(1*H*)-phthalazinyl)-1-propen-1-yl]phenyl benzoate (29)

This compound was prepared as described above for compound **28** using **27** (150 mg, 0.318 mmol), DBU (53 mg, 0.052 mL, 0.350 mmol, 1.1 equiv), and benzoyl chloride (49 mg, 0.041 mL, 0.350 mmol, 1.1 equiv) in CH_2Cl_2 (8 mL) to give **29** (169 mg, 92%) as an off-white solid, mp 125–127 °C. IR: 3335, 3194, 1739, 1654, 1604 cm^{-1}; ^1H NMR (DMSO-d_6): δ 8.20 (d, J = 7.7 Hz, 2H), 8.01 (d, J = 15.9 Hz, 1H), 7.78 (s, 1H), 7.71 (d, J = 15.9 Hz, 1H), 7.66 (m, 2H), 7.52 (t, J = 7.7 Hz, 2H), 7.44 (obscured, 1H), 7.42 (s, 1H), 7.36 (t, J = 7.1 Hz, 1H), 7.29 (d, J = 7.1 Hz, 1H), 7.18 (d, J = 7.1 Hz, 1H), 6.99 (s, 1H), 5.90 (t, J = 6.6 Hz, 1H), 5.05 (br s, 2H), 4.76 (br s, 2H), 3.81 (s, 3H), 3.72 (s, 2H), 1.64 (m, 2H), 1.26 (m, 2H), 0.87 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 166.3, 164.7, 163.6, 161.8, 155.9, 144.9, 144.6, 142.5, 136.4, 134.2, 134.1, 133.8, 131.4, 130.9, 130.3, 128.9, 128.7, 128.0, 126.5, 125.7, 125.0, 124.2, 123.9, 119.6, 105.8, 62.1, 51.4, 37.3, 33.6, 18.3, 13.8. Anal. Calcd for $C_{33}H_{32}N_6O_4 \cdot 5.3\ H_2O$: C, 58.97; H, 6.39; N, 12.50. Found: C, 58.98; H, 6.52; N, 12.50.

4.3.6. (±)-(*E*)-5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2-methoxy-3-[3-oxo-3-(1-propyl-2(1*H*)-phthalazinyl)-1-propen-1-yl]phenyl 4-methoxybenzoate (30)

This compound was prepared as described above for compound **28** using **27** (150 mg, 0.318 mmol), DBU (53 mg, 0.052 mL, 0.350 mmol, 1.1 equiv), and 4-methoxybenzoyl chloride (60 mg, 0.047 mL, 0.350 mmol, 1.1 equiv) in CH_2Cl_2 (8 mL) to give **30** (168 mg, 87%) as an off-white solid, mp 124–126 °C. IR: 3474, 3344, 3188, 1732, 1605 cm^{-1}; ^1H NMR (DMSO-d_6): δ 8.14 (d, J = 8.8 Hz, 2H); 8.01 (d, J = 15.9 Hz, 1H); 7.81 (s, 1H), 7.70 (d, J = 15.9 Hz, 1H), 7.67 (s, 1H), 7.48–7.40 (complex m, 2H), 7.36 (t, J = 7.1 Hz, 1H), 7.30 (d, J = 7.1 Hz, 1H), 7.18 (d, J = 7.1 Hz, 1H), 6.99 (complex m, 3H), 5.90 (t, J = 6.6 Hz, 1H), 4.77 (br s, 2H), 4.65 (br s, 2H), 3.90 (s, 3H), 3.80 (s, 3H), 3.72 (s, 2H), 1.65 (m, 2H), 1.28 (m, 2H), 0.87 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 166.4, 164.4, 164.1, 162.5, 162.3, 157.1, 150.0, 144.7, 142.5, 136.6, 134.3, 134.2, 132.0, 131.4, 130.8, 128.0, 126.5, 125.7, 124.9, 124.4, 124.0, 121.2, 119.5, 113.9, 105.8, 62.0, 55.5, 51.4, 37.3, 33.7, 18.3, 13.8. Anal. Calcd for $C_{34}H_{34}N_6O_5 \cdot H_2O$: C, 65.37; H, 5.81; N, 13.45. Found: C, 65.15; H, 5.65; N, 13.10.

4.3.7. (±)-(*E*)-5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2-methoxy-3-[3-oxo-3-(1-propyl-2(1*H*)-phthalazinyl)-1-propen-1-yl]phenyl 4-nitrobenzoate (31)

This compound was prepared as described above for compound **28** using **27** (150 mg, 0.318 mmol), DBU (53 mg, 0.052 mL, 0.350 mmol, 1.1 equiv), and 4-nitrobenzoyl chloride (65 mg, 0.350 mmol, 1.1 equiv) in CH_2Cl_2 (8 mL) to give **31** (154 mg, 78%) as a yellow solid, mp 130–132 °C. IR: 3480, 3344, 3176, 1744, 1607 cm^{-1}; ^1H NMR (DMSO-d_6): δ 8.38 (s, 2H), 8.00 (d, J = 15.9 Hz, 1H), 7.81 (s, 1H), 7.72 (d, J = 15.9 Hz, 1H), 7.68 (s, 1H), 7.46 (complex m, 4H), 7.37 (t, J = 7.1 Hz, 1H), 7.30 (d, J = 6.6 Hz, 1H), 7.29 (d, J = 7.1 Hz, 1H), 6.98 (d, J = 1.6 Hz, 1H), 5.90 (t, J = 6.6 Hz, 1H), 4.78 (br s, 2H), 4.63 (br s, 2H), 3.80 (s, 3H), 3.74 (s, 2H), 1.65 (m, 2H), 1.28 (m, 2H), 0.87 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 166.2, 162.9, 162.4, 162.3, 157.1, 151.0, 149.6, 144.3, 142.6, 136.1, 134.7, 134.3, 134.2, 131.5, 131.4, 131.1, 128.0, 126.5, 125.7, 125.6, 123.9, 123.8, 123.6, 120.0, 105.6, 62.3, 51.4, 37.3, 33.6, 18.3, 13.8. Anal. Calcd for $C_{33}H_{31}N_7O_6 \cdot 0.8\ H_2O$: C, 62.31; H, 5.17; N, 15.41. Found: C, 62.28; H, 4.86; N, 15.53.

4.3.8. (±)-(*E*)-5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2-methoxy-3-[3-oxo-3-(1-propyl-2(1*H*)-phthalazinyl)-1-propen-1-yl]phenyl 4-(trifluoromethoxy)benzoate (32)

This compound was prepared as described above for compound **28** using **27** (150 mg, 0.318 mmol), DBU (53 mg, 0.052 mL, 0.350 mmol, 1.1 equiv), and 4-(trifluoromethoxy)benzoyl chloride (79 mg, 0.055 mL, 0.350 mmol, 1.1 equiv) in CH_2Cl_2 (8 mL) to give **32** (172 mg, 82%) as an off-white solid, mp 118–120 °C. IR: 3339, 3182, 1743, 1656, 1606 cm^{-1}; ^1H NMR (DMSO-d_6): δ 8.25 (d, J = 8.8 Hz, 2H), 8.00 (d, J = 16.5 Hz, 1H), 7.81 (s, 1H), 7.70 (d, J = 16.5 Hz, 1H), 7.68 (s, 1H), 7.45 (m, 2H), 7.41–7.28 (complex m, 4H), 7.19 (d, J = 7.7 Hz, 1H), 6.97 (d, J = 1.6 Hz, 1H), 5.90 (t, J = 6.6, 1H), 4.79 (br s, 2H), 4.62 (br s, 2H), 3.80 (s, 3H), 3.73 (s, 2H), 1.65 (m, 2H), 1.28 (m, 2H), 0.87 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6): δ 166.3, 163.5, 162.5, 162.2, 156.9, 153.2, 149.8, 144.5, 142.6, 136.3, 134.4, 134.2, 132.3, 131.5, 131.0, 128.0, 127.2, 126.5, 125.7, 125.3, 124.0, 123.9, 123.7 (q, J = 258.1 Hz), 120.5, 119.8, 105.7, 62.2, 51.4, 37.3, 33.6, 18.3, 13.8. Anal. Calcd for $C_{34}H_{31}F_3N_6O_5 \cdot 5.5\ H_2O$: C 53.75; H 5.57; N 11.06. Found: C, 53.96; H, 5.57; N, 11.04.

4.3.9. (±)-(*E*)-3-{5-[(2,4-Diamino-5-pyrimidinyl)methyl]-3-[(4-fluorobenzyl)oxy]-2-methoxyphenyl}-1-(1-propyl-2(1*H*)-phthalazinyl)-2-propen-1-one (33)

This compound was prepared as described above for compound **28** using **27** (150 mg, 0.318 mmol), DBU (53 mg, 0.052 mL, 0.350 mmol, 1.1 equiv), and 4-fluorobenzyl bromide (66 mg, 0.044 mL, 0.350 mmol, 1.1 equiv) in CH_2Cl_2 (8 mL) to give **33** (160 mg, 87%) as a yellow solid, mp 115–117 °C. IR: 3473, 3338, 3183, 1649, 1605 cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.07 (d, J = 15.9 Hz, 1H), 7.78 (s, 1H), 7.67 (s, 1H), 7.66 (d, J = 15.9 Hz, 1H), 7.45 (t, J = 7.1 Hz, 1H), 7.40–7.26 (complex m, 4H), 7.19 (d, J = 7.1 Hz, 1H), 7.16 (s, 1H), 7.05 (t, J = 8.8 Hz, 2H), 6.66 (d, J = 1.1 Hz, 1H), 5.91 (t, J = 6.6 Hz, 1H), 5.00 (s, 2H), 4.78 (br s, 2H), 4.52 (br s, 2H), 3.87 (s, 3H), 3.65 (s, 2H), 1.65 (m, 2H), 1.28 (m, 2H), 0.87 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 166.5, 162.55, 162.50 (d, J = 245.0 Hz), 162.2, 156.7, 152.3, 147.9, 142.4, 137.1, 134.3, 133.9, 132.3, 131.4, 130.0, 129.3 (J = 7.7 Hz), 128.0, 126.5, 125.7, 124.0, 119.4, 118.9, 115.5 (J = 21.3 Hz), 115.1, 106.3, 70.2, 61.4, 51.3, 37.3, 34.2, 18.3, 13.8. Anal. Calcd for $C_{33}H_{33}FN_6O_3 \cdot 0.5\ H_2O$: C, 67.22; H, 5.81; N, 14.25. Found: C, 67.04; H, 5.99; N, 14.00.

4.3.10. (±)-(*E*)-3-{5-[(2,4-Diamino-5-pyrimidinyl)methyl]-2-methoxy-3-[(4-(trifluoromethyl)benzyl)oxy]phenyl}-1-(1-propyl-2(1*H*)-phthalazinyl)-2-propen-1-one (34)

This compound was prepared as described above for compound **28** using **27** (150 mg, 0.318 mmol), DBU (53 mg, 0.052 mL, 0.350 mmol, 1.1 equiv), and 4-(trifluoromethyl)benzyl bromide (84 mg, 0.054 mL, 0.350 mmol, 1.1 equiv) in CH_2Cl_2 (8 mL) to give **34** (124 mg, 62%) as a yellow solid, mp 145–147 °C. IR: 3328, 3175, 1659, 1620 cm^{-1}; ^1H NMR ($CDCl_3$): δ 8.01 (d, J = 15.9 Hz, 1H), 7,68 (d, J = 15.9 Hz, 1H), 7.66 (s, 1H), 7.60 (d, J = 8.0 Hz, 2H), 7.54 (d, J = 8.0 Hz, 2H), 7.42 (t, J = 7.1 Hz, 1H), 7.33 (m, 3H), 7.27 (s, 1H), 7.27 (d, J = 7.1 Hz, 1H), 7.14 (d, J = 7.1 Hz, 1H), 7.13 (s, 1H), 6.80 (s, 1H), 6.69 (br s, 4H), 5.86 (t, J = 6.6 Hz, 1H), 5.12 (s, 2H), 3.86 (s, 1H), 3.66 (s, 2H), 1.61 (m, 2H), 1.33 (m, 2H), 0.82 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$): δ 166.6, 163.6, 157.1, 152.3, 147.9, 142.7, 140.6, 136.8, 134.1, 132.2, 131.5, 130.2, 130.0, 129.8, 128.1, 127.5, 126.5, 125.8, 125.3, 124.1 (d, J = 270.9 Hz), 123.9, 120.2, 119.4, 115.4, 108.3, 70.1, 61.4, 51.4, 37.3, 33.5, 18.3, 13.8. Anal. Calcd for $C_{34}H_{33}F_3N_6O_3 \cdot 3.0\ H_2O$: C, 59.64; H, 5.74; N, 12.27. Found: C, 59.70; H, 5.95; N, 12.37.

4.4. Biological measurements

The methods used to assess potency have been previously published.[7,8] Briefly, the minimum inhibitory concentration (MIC) was the lowest concentration of compound needed to block growth of a standardized culture of *B. anthracis* Sterne as measured by turbidity at 600 nm and by visual inspection.[27] Enzymatic activity was reconstituted in vitro with saturating concentrations of co-factor and dihydrofolate reductase. The amount of tetrahydrofolate produced was monitored, and the concentration of compound that inhibited 50% of this reaction was then determined. This value, an IC_{50}, was converted to an inhibition constant (K_i) by incorporating the strength of binding to dihydrofolate (K_m) through the formalism of Cheng-Prusoff.[28]

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