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Graphical Abstract

Synthesis and Activity of Benzimidazole-1,3-dioxide Inhibitors of Separase

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Synthesis and Activity of Benzimidazole-1,3-dioxide Inhibitors of Separase

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ABSTRACT: Due to the oncogenic activity of cohesin protease, separase in human cancer cells, modulation of separase enzymatic activity could constitute a new therapeutic strategy for targeting resistant, separase-overexpressing aneuploid tumors. Herein, we report the synthesis, structural information, and structure-activity relationship (SAR) of separase inhibitors based on modification of the lead molecule 2,2-dimethyl-5-nitro-2H-benzimidazole-1,3-dioxide, named Sepin-1, (**1**) identified from a high-throughput-screen. Replacement of $-NO_2$ at C5 with other functional groups reduce the inhibitory activity in separase enzymatic assay. Substitution of the two methyl groups with other alkyl chains at the C2 moderately improves the effects on the inhibitory activity of those compounds. Modifications on 2*H*-benzimidazole-1,3-dioxide or the skeleton have variable effect on inhibition of separase enzymatic activity. Density-functional theory (DFT) calculations suggest there may be a correlation between the charges on the oxide moieties on these compounds and their activity in inhibiting separase enzyme.

In recent years, breast cancer (BC) has become one of the most common types of cancer in humans, both in new cases and cancer death rates in females.[1] Separase, an enzyme that cleaves the chromosomal cohesin complex during mitosis, is overexpressed and mislocalized in a number of human tumors.[2] Overexpression of separase has been found in more than 60% of BC, 50% of triple-negative BC, and 65% of Luminal-B BC tumors.[2-4] Separase overexpression in animal models induces aneuploid mammary tumors. Since the structure of human separase has not been solved and structural information of a fungal separase has only recently become available,[5] high throughput screening was used to search for potential separase inhibitors.[6] The screening of 14,400-compound library provided 5 compounds with good activity, in which 2,2-dimethyl-5-nitro-2*H*-benzimidazole-1,3-dioxide (**1** Figure 1) exhibited the highest activity toward inhibiting separase with a half-maximal inhibitory concentration (IC_{50}) of 15 μM.[6] In the original paper this compound was named Sepin-1.[6] Further studies showed that the benzimidazole-1,3-dioxide **1** inhibits the growth of separase-overexpressing human triple-negative BC xenografts in mice in a dose-dependent manner, and has no appreciable effect on such tumors with low-separase expression, suggesting that the specificity and efficacy of this compound in targeting tumors is related to separase overexpression. Targeting separase by **1** also results in high levels of apoptosis. These results suggest that inhibition of separase represents a new line of therapy to treat breast and other tumors overexpressing separase. In this contribution, we report the synthesis, structural information, biological activity, and structure-activity relationship (SAR) study of separase inhibitors based on struc-

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tural modification of Sepin-1 (**1**). The study was designed to determine the sites on the molecule most relevant for activity and through their modification develop more active analogs. Three regions of **1** scaffold were modified and the resulting molecules tested for activity; the substituents at the C5 position, the heterocycle attached to the benzene ring, and the C2 position of carbon between the two nitrogen atoms (Figure 1).

The structure of Sepin-1 (1) is not typically what one would consider to be "drug like". The molecule is small, highly polar, possess a nitro group and may be redox active. However, this report is not the first time these molecules have been found to have selective and interesting biological activity. Boiani et al. has previously reported that derivatives of the 2*H*-benzimidazole-1,3-dioxide scaffold are effective anti-trypanosomatid agents. With the tested versions possessing no toxicity in BALB/c mice with dosing of 30 mg/kg/day for 10 days, with the animals observed for 60 days.[7]

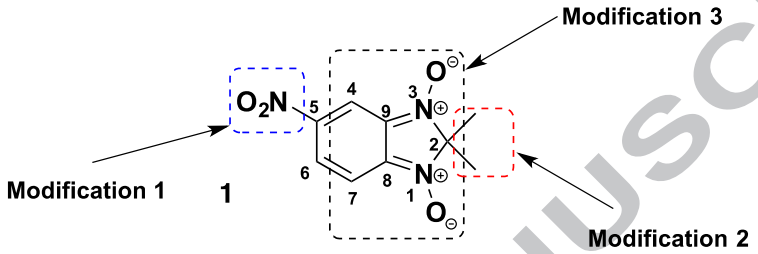
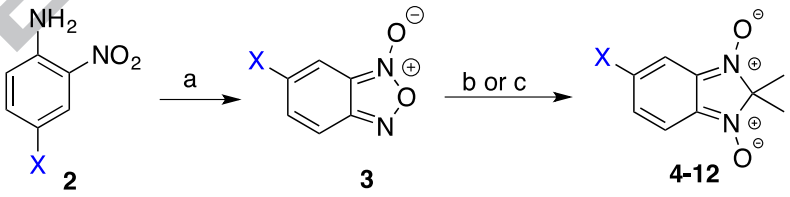


Figure 1. Structure of **1** with domains marked for structure activity study.

The general route to the benzimidazole derivatives starts with the acid catalyzed cyclization of nitroaniline (**2**) to give benzofuroxans (**3**). Reaction with an alcohol in the presence of sulfuric acid then provides the 2*H*-benzimidazole-1,3-dioxides derivatives (**4-12**).[6, 7] Versions where the nitro group was replaced by other functionality, including carboxylic acid, ester, fluorine, bromine, and ethoxyl groups, were synthesized by starting with the appropriate nitroaniline (**2**). The bromine-substituted compound (**11**) was used for further modification by the Suzuki coupling reaction. Additionally, the product bearing fluorine (**9**) was reacted with different nucleophiles such as primary amines, allowing for modification at the C5 position on the 2*H*-benzimidazole-1,3-dioxides skeleton. Scheme 1 outlines the synthetic route and the chemical structures of derivatives with the nitro group replaced by other groups to give compounds **4-12 (Table 1)**.

Scheme 1. Synthesis of Derivatives at C5 Position



a) $NaNO_2$, H_2SO_4, AcOH, NaN_3, H_2O, 0-80 °C, 2h or KOH, EtOH, NaOCl 8%. b) *i*PrOH, H_2SO_4, RT, 2 hrs. c) *i*PrNO_2, pyridine, THF, RT, 18 hrs, (**10**) followed by treatment of **9** with *n*-butyl amine, EtOH, RT, overnight, (**12**) followed by treatment of **11** with $PhB(OH)_2$, (S*i*Pr)Pd(allyl)Cl, *t*-BuONa, 1,4-dioxane, 60 °C, 3 hrs

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TABLE 1. Yield of derivatives at the nitro group

Compound	X	Yield (%) from **3**
1	NO_2	74
4	COOH	31
5	$COOCH_3$	56
6	OEt	38
7	CF_3	49
8	SO_2CF_3	42
9	F	77
10	$NH(C_4H_9)$	70
11	Br	71
12	C_6H_5	31

The modification of functionality at the C2 position of the 2*H*-benzimidazole-1,3-dioxides was obtained by using different secondary alcohols in the reaction with benzofuroxans **3** while keeping the composition of other positions, such as nitro group, the same as in **1**. Because this step in the reaction mechanism involves a carbocation, the products obtained can result from rearrangement. Due to intermediacy of a carbocation and the harsh conditions requiring sulfuric acid the scope of alcohol substrates is limited. The installation of functional groups, including alkenes, alkynes, alcohols and amines into this position, has been challenging. Six different compounds (**13**-**18**, Scheme 2, Table 2) that have alkyl chains, spiro-derivatives, and aryl chains have been synthesized and evaluated to determine the effect of this position on the biological activity of the 2*H*-benzimidazole-1,3-dioxide derivatives.

Further modification of **1** was performed in a series of individual reactions. Compounds **20** and **21** were generated from their corresponding benzofuroxan using sulfuric acid and *iso*-propanol. Structure **23** was formed by the bromination of **1** at the position *meta*- to the nitro group. The quinoxaline-1,4-dioxide (**25**) was synthesized from benzofuroxan (**24**) bearing methyl ester group and evaluated for its ability to inhibit separase due to its potential bioactivity.[8-9] The precursor to **1**, 5-nitrobenzofuroxan (**3)** and its reduced form **22** were also assayed to determine their inhibition of separase enzyme.

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Scheme 2. Synthesis of Derivatives at C2 Position

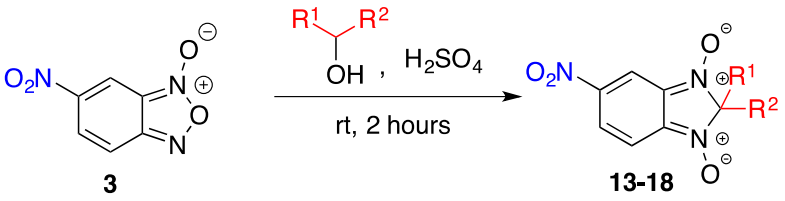
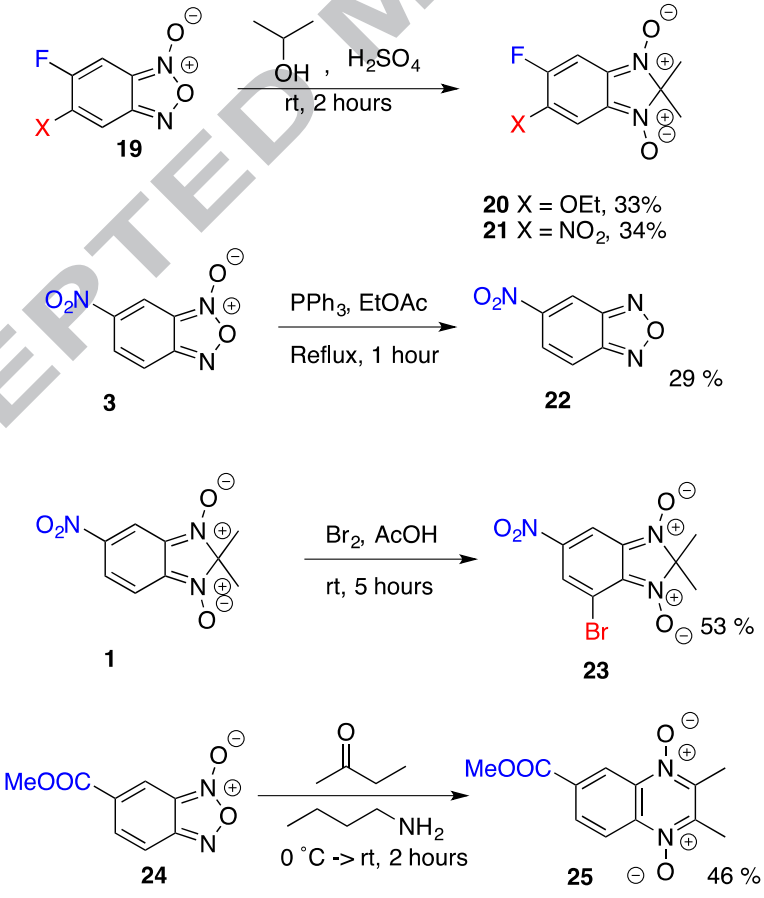


TABLE 2. Yield of derivatives at the C2 position

Compound	R^1	R^2	Yield (%) from **3**
13	CH_3	C_2H_5	63
14	C_2H_5	C_3H_7	21
15	CH_3	C_4H_9	14
16	CH_3	$PhCO_2H$	25
17	$-(CH_2)_4-$		63
18	$-(CH_2)_5-$		43

Scheme 3. Other Derivatives of Scaffold



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As mentioned earlier, **1** exhibits inhibitory activity toward separase with an IC_{50} of 15 μM. To determine what structural features are important for separase inhibition, the derivatives were evaluated for their ability to inhibit separase relative to the parent compound **1**.

Replacement of the nitro group at the C5 position by other functional groups significantly reduces the inhibitory activity of the derivatives compared to the lead compound **1**. Since, due to their reduction to nitroso functionality, aromatic nitro groups are often problematic in drug development and biological assays, the first goal was to replace that group with a pharmacophore equivalent. Unfortunately all versions in which the nitro group has been replaced, including versions with groups that may be considered pharmacophore equivalents for a nitro group, (**4-12**) were less active (Figure 2 A-B). The derivative with a (trifluoromethyl) sulfonyl group substituted for nitro (**8**), inhibits 65.6% separase enzyme at 100 μM concentration, was the most active among those molecules.

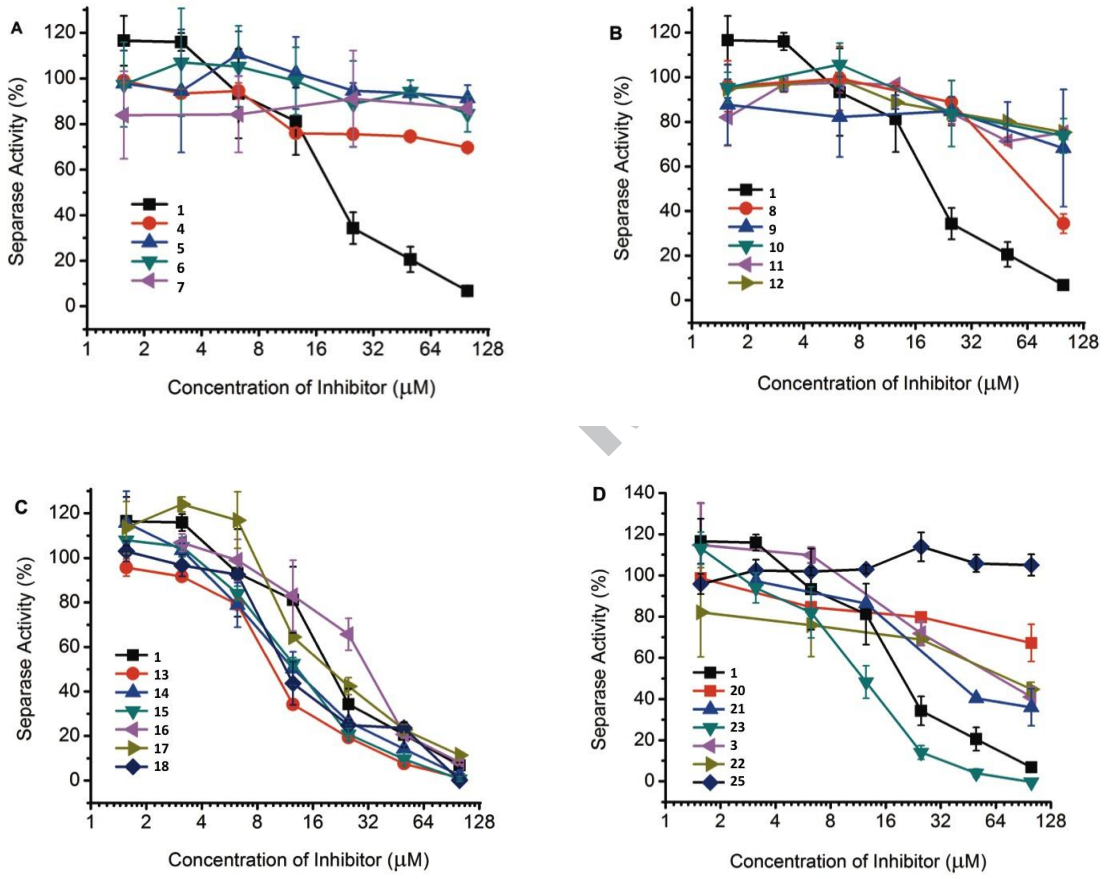

Figure 2. Inhibitory activity of derivatives in separase enzymatic assay. The inhibitory activity of **1** and its derivatives modified at C5 (A-B) and C2 (C) and on the heterocyclic ring (D) was determined using separase activity assay.

As shown in Table 3 the inhibitory activity toward separase seems to increase with the strength of the electron withdrawing group ($-NO_2$ > $-SO_2CF_3$ > -COOH > -F > -Br > $-C_6H_5$ > $-COOCH_3$ > $-NH(C_4H_9)$ > $-CF_3$ > -OEt). Derivatives that have functional groups with an electron-donating characteristics (-OEt, $-NH(C_4H_9)$) in (**6** and **10**, respectively) or weakly-electron-withdrawing effects (-COOH, $-COOCH_3$, $-CF_3$, -F, -Br, and $-C_6H_5$ in **4**, **5**, **7**, **9**, **11** and **12**, respectively) show low inhibitory activity toward separase. Although derivatives with stronger electron-withdrawing groups such as $-SO_2CF_3$ in **8** possesses better biological activity, the separase inhibition observed with these compounds is still lower than that of **1** with the NO_2 group (Table 3).

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Replacing the dimethyl group at the C2 position with different alkyl chains had a moderate effect on the inhibition of separase activity (Figure 2C). Compound **13** which possesses an ethyl group is 1.4 fold more active than the original compound (Figure 2C, Table 3). Modifying the methyl groups to propyl (**14**), butyl (**15**) or spiro-alkane (**17** and **18**) results in a better or equivalent effect on the inhibition of separase activity (Figure 2C, Table 3).

The data suggest that this position could be used to attach a photo-labeling moiety, which would be used to study the mechanism of inhibition of the separase enzyme. With that in mind, we have synthesized **16**, which has a COOH group at the para position of a phenyl group attached to C2 of 2H-bezimidazole dioxide skeleton (Scheme 2). This molecule retained moderate activity compared to the lead compound with an IC_{50} of 29.2 μM (Figure 2C, Table 3).

Table 3. Inhibitory activity of Sepin compounds in *in vitro* separase enzymatic assay shown in Figure 2

Compound (#)	X	R¹	R²	IC_{50} (μM)[d]	Inhibitoryactivity at 100 μM (%)[d]
Sepin-1 (**1**)	$-NO_2$	$-CH_3$	$-CH_3$	17.8±2.3	95.9±7.0
(**4**)	-COOH	$-CH_3$	$-CH_3$	>100	24.4±8.8
(**5**)	$-COOCH_3$	$-CH_3$	$-CH_3$	>100	14.6±7.3
(**6**)	$-OC_2H_5$	$-CH_3$	$-CH_3$	>100	21.2±12.3
(**7**)	$-CF_3$	$-CH_3$	$-CH_3$	>100	8.0±7.0
(**8**)	$-SO_2CF_3$	$-CH_3$	$-CH_3$	50.1±11.4	64.6±3.7
(**9**)	-F	$-CH_3$	$-CH_3$	>100	35.4±9.1
(**10**)	$-NH(C_4H_9)$	$-CH_3$	$-CH_3$	>100	40.9±17.3
(**11**)	-Br	$-CH_3$	$-CH_3$	>100	24.6±8.4
(**12**)	$-C_6H_5$	$-CH_3$	$-CH_3$	>100	39.3±21.4
(**13**)	$-NO_2$	$-CH_3$	$-C_2H_5$	12.7±1.0	99.6±0.7
(**14**)	$-NO_2$	$-C_2H_5$	$-C_3H_7$	14.1±2.9	99.2±1.3
(**15**)	$-NO_2$	$-CH_3$	$-C_4H_9$	14.6±2.1	99.8±0.3
(**16**)	$-NO_2$	$-CH_3$	$-C_6H_4$(*p*-COOH)	29.2±10.8	84.1±6.7
(**17**)	$-NO_2$	$-(CH_2)_4-$		17.1±2.5	96.2±6.6
(**18**)	$-NO_2$	$-(CH_2)_5-$		16.2±2.3	99.5±0.8
(**20**)[a]	-F; $-OC_2H_5$ (*o*)	$-CH_3$	$-CH_3$	>100	25.2±22.4
(**21**)[a]	$-NO_2$; -F (*o*)	$-CH_3$	$-CH_3$	87.2±15.6	60.1±4.6
(**23**)[a]	$-NO_2$; -Br (*m*)	$-CH_3$	$-CH_3$	12.1±2.6	100
(**3**)[b]	$-NO_2$	-	-	>100	0
(**22**)[b]	$-NO_2$	-	-	>100	43.1±13.2
(**25**)[c]	$-NO_2$	-	-	>100	0

[a] The first group is at C5 position, the position for the second group is given in parenthesis and relative to the first group (*o*: ortho; *m*: meta). [b] These molecules are the precursors to Sepin-1. [b,c] See Scheme 3 for chemical structures.
[d] The data were from three independent assays.

Structural modifications of other aromatic positions on the 6-member ring resulted in changes in separase inhibition (Figure 2D, Table 3). The derivatives with fluorine and ethoxy groups at C5 and C6 (**20**) exhibit poorer inhibitory activity. While the analog that has a fluorine atom substituted at C6 (**21**) shows modest activity relative to that of **1**. Interestingly, the derivative with a bromine atom at C7 (**23**) possesses a 1.5 fold lower IC_{50} (12.1 μM) compared to that of the lead structure **1** (Figure 2C, Table 3), and thus far is the derivative with best activity toward separase enzyme identified.

Due to the inherent simplicity of the NMR spectra of these molecules, the molecular structure of a number of derivatives was confirmed by single-crystal X-ray diffraction. To our knowledge, these structures are the first examples of isolated 2*H*-benzimidazole-1,3-dioxides derivatives that have been analyzed

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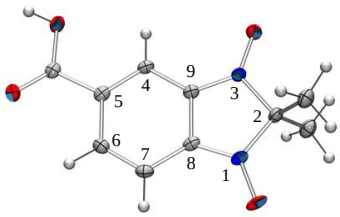
using single-crystal X-ray diffraction. To date, the only available structure of 2*H*-benzimidazole-1,3-dioxide characterized by X-ray diffraction was reported by Keller *et al.* in which the molecule is co-crystallized with $H[AuCl_4]$.[10] For all structures reported in our work, the bond distance of C4-C5 and C6-C7 bonds in the benzene ring are significantly shorter than the C9-C4, C5-C6, C7-C8, and C8-C9 bonds (Table 4). The crystal structures of **4**, **6**, **7**, **9**, **12, 16, 17**, **18**, **20** and **23** are all reported in the supplementary material. Interestingly, although this difference in the bond lengths of C-C bonds of the benzene ring was also observed in other 2*H*-benzimidazole derivatives,[11] there is very little variation for aromatic C-C bonds in the 2*H*-benzimidazole-1,3-dioxide reported by Keller *et al.*, which may be due to the interaction of the molecule with the $[AuCl_4]^-$ anion.[10]

Density-functional theory (DFT) calculations have been performed to explore the distribution of electron density on derivatives, with the hope of gaining insight into the biological activity of the tested compounds (Figure 3). The atomic coordinates from the crystal structures of molecules (when available) were used as starting geometry for structural optimization using B3LYP/6-31G* methodology. Figure 3 shows the electrostatic potential surface mapping of selected derivatives and the Mulliken charges of the atoms on the

Table 4. Selected Bond Lengths



	Bond Length (Å)
C9-C4	1.414(2)
C4-C5	1.364(2)
C5-C6	1.451(2)
C6-C7	1.357(2)
C7-C8	1.417(2)
C8-C9	1.432(4)
N1-O1	1.3048(14)
N2-O2	1.2712(15)
C8-N1	1.3253(19)
C9-N2	1.3326(18)

2*H*-benzimidazole-1,3-dioxide ring skeleton. When comparing the results from calculation and the inhibitory activity toward separase enzyme, a correlation between the charge on the 5-member ring and the activity of analogs was observed. Derivatives that have more positive charge on the 5-member ring, possess better activity as separase inhibitors. This charge distribution is dependent on the substituent group on the 6-member ring, particularly at C5. It was also noted that derivatives with more negative charge on the N-O oxygens appear to possess better activity. While there are certainly a number of structural features responsible for the activity observed with the reported molecules, the local electron density appears to play a role.

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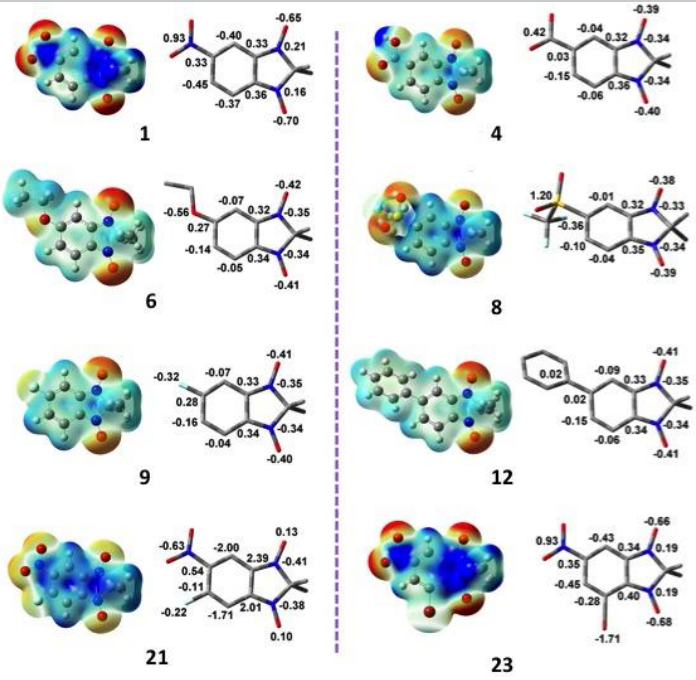

Figure 3. Electrostatic potential surface mapping and Mulliken charges of selected derivatives.

We report the synthesis, structural information and the structure-activity relationship of a novel class of separase inhibitors based on our lead compound **1**. Various analogs of **1** were synthesized by modifying three main positions on the lead molecule. The bioassay data revealed that, despite our efforts to find an alternative, the NO_2 group at C5 is essential for the activity of analogs in the inhibition of separase enzyme. Modification at the C2 has a moderate effect on the biological activity of those compounds, suggesting that derivatives from this group could be used to help identify the actual active site in enzyme by photoaffinity labeling studies, which will assist substantially in the search for better inhibitors. Changing the substituents at other positions on 2*H*-benzimidazole-1,3-dioxide or the skeleton of the molecule modifies the inhibition of separase by those analogs. The derivative with highest activity was found to be the version with bromine-substituted at C7 on skeleton (**23**). DFT calculations illustrate a correlation between the charge on the oxide nitrogen and oxygen atoms and the inhibitory activity toward separase, suggesting that the oxide moieties on molecule's skeleton may be involved in binding to the separase enzyme.

While only modest improvement in the activity of the basic scaffold has been achieved, this paper clearly illustrates that the nitro group at C-5 appears to be critical for activity. These molecules represent an important tool for use in the study of separase activity and the potential role of separase inhibitors.

Supporting Information. Single crystal X-ray crystallographic data and CCDC numbers are reported in the supporting information.

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# Equal Contribution

H.T.D. synthesized all analogs and performed the DFT calculations, structural identification and analysis of them. N.Z. performed all the biological assays. All authors analyzed the data and made decisions on what analogs to synthesized.

ACKNOWLEDGMENT

We thank Minh Nguyen for collecting X-ray diffraction data and solving X-ray structures. The authors gratefully acknowledge support by the Cancer Prevention and Research Institute of Texas Grant # DP150064, and Department of Defence Award W81XWH-15-1-0122 awarded to D. Pati.

Supplementary data

Single crystal X-ray crystallographic data and supplementary data associated with this article can be found online.

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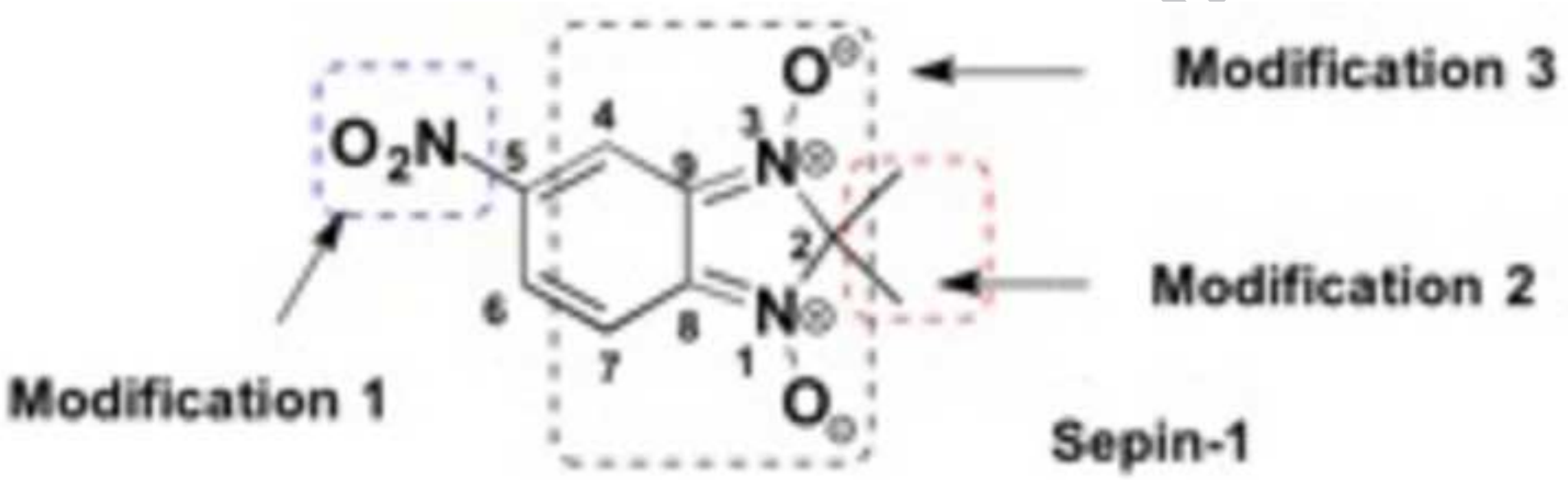

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