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Synthesis and biological evaluation of nitric oxide releasing derivatives of 6-amino-3-*n*-butylphthalide as potential antiplatelet agents

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ABSTRACT

A series of novel nitric oxide releasing derivatives of 6-amino-3-*n*-butylphthalide were designed, synthesized and evaluated as potential antiplatelet agents. Compound **10b** significantly inhibited the adenosine diphosphate (ADP)-induced platelet aggregation in vitro, superior to 6-amino-3-*n*-butylphthalide, 3-*n*-butylphthalide (NBP) and ticlopidine. Meanwhile **10b** released moderate levels of NO, which could be beneficial for improving cardiovascular and cerebral circulation. Furthermore, **10b** had an enhanced aqueous solubility relative to NBP. These findings may provide new insights into the development of novel antiplatelet agents for the treatment of thrombosis-related ischemic stroke.

Stroke is a major cause of adult disability and death worldwide[1] which can be subdivided into two categories, ischemic and hemorrhagic. Ischemic strokes are more prevalent than hemorrhagic ones, making up approximately 87% of all cases, and have been the target of most drug trials.[2] Previous studies have demonstrated that platelet-mediated thrombosis is a major cause of ischemic stroke, and the activation of platelets and the resultant aggregation play a central role in the pathogenesis of ischemic stroke.[3] Thus, platelet adhesion and aggregation have been identified as important targets for the development of antithrombotic drugs against ischemic stroke. Because of the multifactorial nature of ischemic stroke, the traditional approach of single-target drugs could generally only offer limited and transient benefits. Hence, development of new drugs with multiple actions could be of great significance for the treatment of ischemic stroke.

The racemic 3-*n*-butylphthalide (NBP) (Fig. 1), a component extracted from seeds of *Apium graveolens* Linn, was approved by the State Food and Drug Administration (SFDA) of China as a new drug for the treatment of ischemic stroke in 2002. It has already been reported that NBP possesses beneficial effects on stroke through multiple actions,[4–6] such as inhibiting platelet aggregation and thrombosis, improving microcirculation, and decreasing brain infarct volume. Despite these benefits, the clinic application of NBP has been limited due to its poor aqueous solubility, and NBP should be administered by combination with other antiplatelet drugs to improve therapeutic effects. Thus, more potent drugs than NBP for the treatment of ischemic stroke are needed.

In recent years, it has been reported that the introduction of a proper substituent into position 6 of the phenyl ring of NBP could impact its activity, and 6-amino-3-*n*-butylphthalide, that is, 6-amino-NBP (compound **1**, Fig. 1) showed stronger potency than NBP, and could significantly inhibit the platelet aggregation, reduce the area of cerebral infarct, improve mitochondrial function and decrease oxidative damage as well as neuronal apoptosis.[7]

Nitric oxide (NO), a free radical gas, is a multifunctional messenger molecule with diverse physiological roles, such as dilation of blood vessels, immune responses, and potentiation of synaptic transmission.[8,9] Indeed, increasing NO production either from endothelial NOS (eNOS) or NO-donors mimicking eNOS-derived NO has already been applied as a therapeutic approach for thrombotic complications, such as myocardial infarction and ischemic stroke.[10] Recently, we reported several NO-based ring-opening derivatives of NBP (**2a**–**l**, (*S*)- and (*R*)-ZJM-289, Fig. 1) as potent anti-ischemic stroke agents which showed excellent antiplatelet and antithrombotic activities.[11,12]

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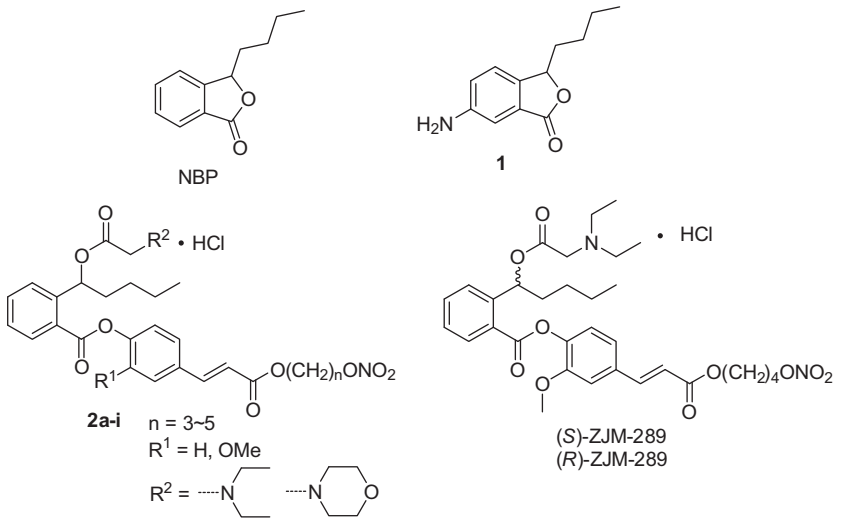


Figure 1. Structrues of NBP, **1**, **2a–i**, (*S*)- and (*R*)-ZJM-289.

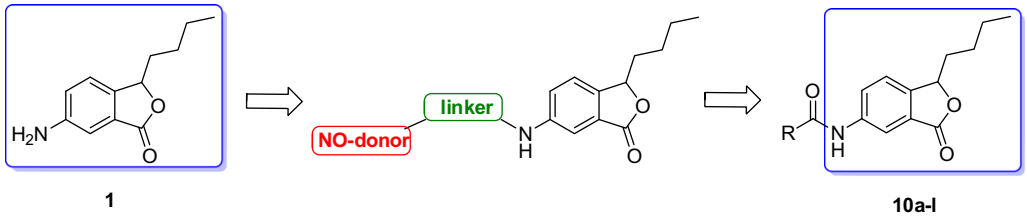
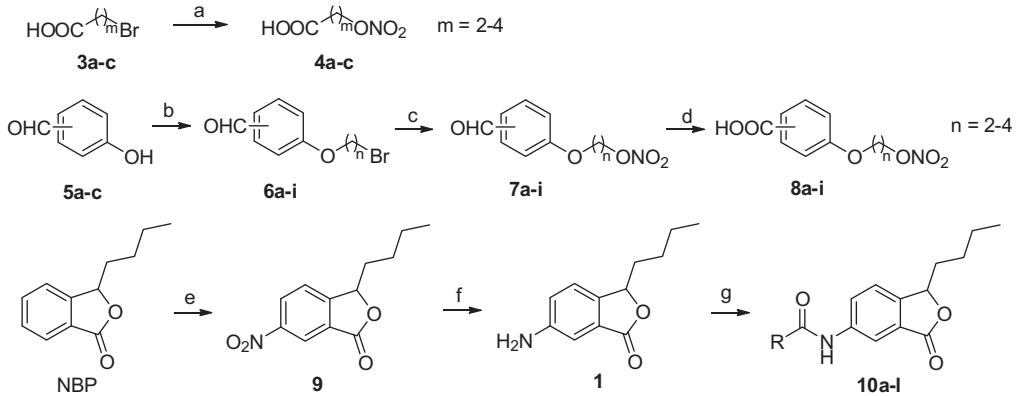


Figure 2. Strategy for the design of NO releasing derivatives of 6-amino-3-*n*-butylphthalide.

In the present study, to obtain new agents with stronger potency, we employed **1** as the lead compound to design and synthesize a novel class of NO-releasing derivatives (**10a–l**, Fig. 2). Their antiplatelet activity in vitro, NO-releasing ability as well as aqueous solubility were evaluated.

The synthesis of target compounds **10a–l** was depicted in Scheme 1. The bromo-substituted alkylcarboxylic acids **3a–c** were converted to the corresponding nitrates **4a–c** using $AgNO_3$ in CH_3CN. Treatment of hydroxybenzaldehyde **5a–c** with $Br(CH_2)_mBr$ yielded bromids **6a–i**, which were treated with $AgNO_3$ leading to nitrats **7a–i**. Subsequent oxidation reactions of **7a–i** using potassium permanganate yielded acids **8a–i**. On the other hand, NBP was nitrated using potassium nitrate in H_2SO_4 to give nitro compound **9**, which was reduced with Fe and NH_4Cl in THF/H_2O to give amino compound **1**. Finally, the target compounds **10a–l** were obtained by acylation of **1** with **4a–c** or **8a–i**, respectively. All of new compounds were purified by column chromatography and characterized by IR, ESI-MS, ^1H NMR, ^{13}C NMR, and HRMS.[13,14]

The in vitro inhibitory effects of the target compounds **10a–l** on adenosine diphosphate (ADP)-induced platelet aggregation in rabbit platelet rich plasma (PRP) were assayed using Born's turbidimetric method.[15] As shown in Table 1, **1** (inhibition rate 18.43%



10a: R = CH_2ONO_2; **10d**: R = *o*-$ArO(CH_2)_2ONO_2$; **10g**: R = *o*-$ArO(CH_2)_3ONO_2$; **10j**: R = *o*-$ArO(CH_2)_4ONO_2$;
10b: R = $(CH_2)_2ONO_2$; **10e**: R = *p*-$ArO(CH_2)_2ONO_2$; **10h**: R = *p*-$ArO(CH_2)_3ONO_2$; **10k**: R = *p*-$ArO(CH_2)_4ONO_2$;
10c: R = $(CH_2)_3ONO_2$; **10f**: R = *m*-$ArO(CH_2)_2ONO_2$; **10i**: R = *m*-$ArO(CH_2)_3ONO_2$; **10l**: R = *m*-$ArO(CH_2)_4ONO_2$

Scheme 1. Synthesis of compounds **10a–l**. Reagents and conditions: (a) $AgNO_3$, CH_3CN, 70 °C, 2 h; (b) $Br(CH_2)_mBr$, K_2CO_3, acetone, reflux, 2–4 h; (c) $AgNO_3$, CH_3CN, 70 °C, 3 h; (d) K_2MnO_4, acetone, reflux, 10 h; (e) KNO_3, H_2SO_4, rt, 12 h; (f) Fe, NH_4Cl, THF/H_2O, 65 °C, 2 h; (g) **4a–c** or **8a–i**, EDCI, DMAP, CH_2Cl_2, rt, 6–8 h.

Table 1
The effects on platelet aggregation induced by ADP in vitro ($n = 6$, $\bar{x} \pm s$)

Compd (0.1 mM)	Inhibition rate (%)	Compd (0.1 mM)	Inhibition rate (%)
Ticlid	26.45 ± 6.38	**10f**	26.58 ± 7.14
NBP	15.72 ± 3.99	**10g**	16.71 ± 6.76
1	18.43 ± 4.22	**10h**	22.78 ± 4.38
10a	22.16 ± 4.36	**10i**	28.76 ± 8.64
10b	30.42 ± 5.49*	**10j**	13.57 ± 3.64
10c	28.56 ± 4.54	**10k**	18.25 ± 5.05
10d	15.20 ± 4.41	**10l**	21.55 ± 4.72
10e	20.04 ± 5.14		

PRP were preincubated with tested compounds (0.1 mm) at 37 °C for 5 min followed by the addition of ADP (10 μM). Data are expressed as mean ± SD of each group ($n = 6$) and analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey test.
* $P < 0.05$ versus NBP.

at 0.1 mM) showed slight stronger inhibitory effect than NBP (15.72%) at the same concentration, which was consistent with the previous report.[7] Furthermore, **10b** (30.42%), **10c** (28.56%), **10f** (26.58%) and **10i** (28.76%) displayed significantly inhibitory effects, which were superior to **1** (18.43%) and two clinically used anti-ischemic stroke drugs, NBP (15.72%) and Ticlid (26.45%).[16] Notably, **10b** was almost twofold more potent than NBP and **1**.

Since **10b** is composed of two moieties, that is, 6-amino-NBP (**1**) and NO donor **4b**, we further investigated their individual contribution to the overall activity against ADP-induced platelet aggregation in vitro. We found that although **1** and **4b** displayed a certain degree of antiplatelet activity (18.43% and 8.32%, respectively), both of them were less effective than **10b** (30.42%, Fig. 3), suggesting that the two moieties may have synergistic effects on inhibiting the ADP-induced platelet aggregation in vitro. In addition, the antiplatelet effect of **10b** was dramatically attenuated by treatment with hemoglobin (He), an endogenous NO scavenger, resulting in reduction of inhibition from 30.42% to 23.05% (Fig. 2). These results strongly demonstrate that NO has an important impact on the antiplatelet effects of **10b**.

To further examine the relationship between NO and antiplatelet aggregation activity, the levels of NO produced by **10a–i** were determined by Griess assay.[17] As shown in Figure 4, the active compounds **10b**, **10i**, **10c** and **10f** released moderate amount of NO (12.88, 7.27, 7.10 and 6.82 μmol/L, respectively). In contrast, the less active compounds **10h**, **10a**, **10e**, **10g** and **10d** under the same conditions released lower levels of NO (3.88, 3.67, 2.21, 2.18 and 1.82 μmol/L, respectively). The resluts indicated that the

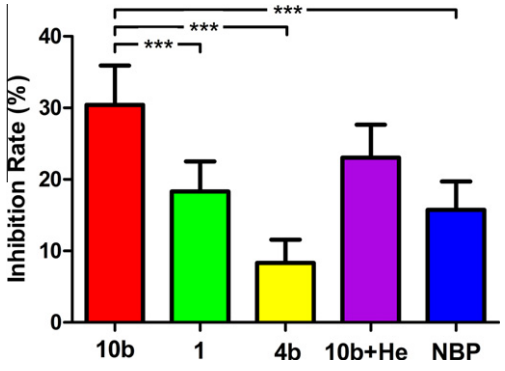


Figure 3. Inhibition of ADP-induced rabbit platelet aggregation by selected compounds in vitro. PRP were pre-incubated with tested compounds (0.1 mM) at 37 °C for 5 min followed by addition of ADP (10 μM) Hemoglobin (20 μM) was added and incubated with the drug-platelet suspension in the indicated group. Data are expressed as mean ± SD ($n = 6$) and analyzed by one-way analysis of variance (ANOVA) followed by post hoc Tukey test. ***$P < 0.001$ versus **10b**.

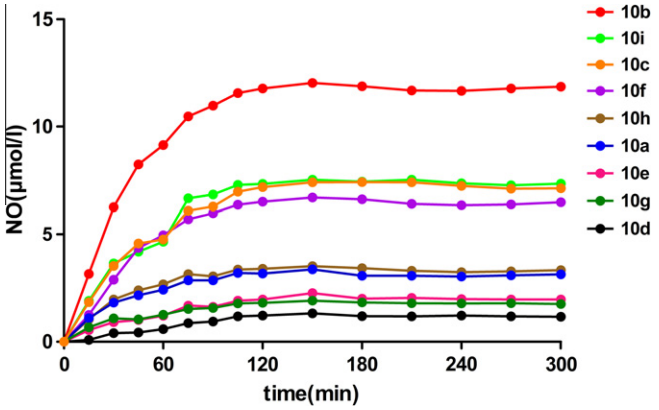


Figure 4. NO-releasing assay of selected compounds in vitro. The levels of NO produced by selected compounds were determined by Griess assay. Individual compounds were incubated for the indicated periods and reacted with Griess reagent, followed by measuring at 540 nm. The levels of NO produced by individual compounds were calculated, according to the standard curve of nitrite. NO data are expressed as mean ± SD (nitrite) in μmol/L at each time point and intra-group variations were less than 10%.

NO-releasing ability of these compounds was positively correlated to their platelet aggregation inhibitory activity ($R^2 = 0.98$, $P < 0.01$, determined by logistic regression analysis).[18]

As the poor aqueous solubility of NBP affects its therapeutic efficacy leading to limited clinical application, we next investigated whether an increase in aqueous solubility of target compounds could be associated with their enhanced activity. Aqueous solubility of compounds **10b**, **10g–i** and NBP with different levels of antiplatelet aggregation activity were tested as described previously.[19] It was observed that the aqueous solubility of **10b** and **10i** with strong antiplatelet aggregation activity were 1.18 and 1.06 mM, respectively, which were higher than that of NBP (0.54 mM) whereas the aqueous solubility of **10g** and **10h** with weak antiplatelet aggregation activity were 0.58 and 0.75 mM, which were similar to that of NBP (Table 2).[20] In addition, the log*P* values of **10b** (2.94) and NBP (3.19) were calculated using the ChemDraw Ultra program, version 12.0, CambridgeSoft company.[21] These results suggest that the appropriate lipid-water partition coefficient of these target compounds might be contributed to their antiplatelet aggregation activity.

Analysis of structure and antiplatelet aggregation activity relationship revealed that the target compounds **10a–l** with different linkers displayed variable antiplatelet aggregation activity. In general, the compounds with straight chain alkanes exhibited higher antiplatelet potency than those with aromatic alkanes. For straight chain alkanes, the compounds with two-carbon alkanes showed best inhibitory activity. For aromatic alkanes, the different position of the substituents showed different activity (*para* > *meta* > *ortho*). Evidentially, **10b** with a two-carbon linker displayed the strongest antiplatelet aggregation activity. These results suggest that varied linkers have different abilities to modulate the structure, stability, metabolism and penetrability of these compounds, which affect

Table 2
The solubility of NBP, **1**, **10b** and **10g–i**[a]

Compd	Solubility (mM)	Compd	Solubility (mM)
NBP	0.54	**10h**	0.75
10b	1.18	**10i**	1.06
10g	0.58		

[a] Data are expressed as mean concentration of individual compounds saturating in 5 ml saline at 25 °C.

the NO production, leading to various bioactivities of the target compounds.

In summary, we designed and synthesized a series of NO-releasing derivatives of 6-amino-NBP and found that some compounds had strong inhibitory effects on ADP-induced platelet aggregation in vitro, among which **10b** was the most potent, superior to NBP, 6-amino-NBP and Ticlid. Further investigation demonstrated that **10b** could produce moderate levels of NO in vitro, which could be beneficial for improving cardiovascular and cerebral circulation. Importantly, since **10b** had an enhanced aqueous solubility and appropriate lipid–water partition coefficient relative to NBP, it may more favorably penetrate the blood–brain barrier (BBB) to exert its action. Altogether, the multifunctional effects of **10b** qualified it as a potential antiplatelet agent for the treatment of thrombosis-related ischemic stroke.

Acknowledgments

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13. General procedure for the synthesis of the target compounds **10a–l**: Compound **1** and one molar amount of EDCI were dissolved in 20 mL of dry CH_2Cl_2 and the mixture was stirred at room temperature for 0.5 h. Then a solution of one equiv molar amount of **4a–c** or **8a–i** in 5 mL of dry CH_2Cl_2 was added, followed by a catalytic amount of DMAP. The solution was left stirring at room temperature for 6–8 h. Then the resulting mixture was washed sequentially with 1 M HCl, water, and brine. The solution was then dried, filtered, and evaporated to dryness. The residue was purified by column chromatography (petroleum ether/EtOAc 5:1 v/v) to obtain the target compounds **10a–l** as white solids.
14. *Analytical data for* ***10b***: Mp: 116–120 °C. MS (ESI): *m*/*z* 345.1 $[M+Na]^+$. IR (cm^{-1}, KBr): ν_{max} 763, 1284, 1400, 1618, 1752, 2925. ^1H NMR (300 Hz, $CDCl_3$): δ 0.90 (t, 3H, CH_3, *J* = 6.9 Hz), 1.26–1.50 (m, 4H, 2 × CH_2CH_3), 1.71–2.07 (m, 2H, $CHCH_2$), 2.92 (t, 2H, $NHCOCH_2$, *J* = 6.1 Hz), 4.87 (t, 2H, ONO_2CH_2, *J* = 6.7 Hz), 5.47–5.51 (m, 1H, $COOCHCH_2$), 7.41 (d, 1H, ArH, *J* = 8.3 Hz), 7.93 (s, 1H, ArH), 8.26 (dd, 1H, *J* = 1.4, 8.3, ArH), 8.70 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.08, 167.45, 145.66, 139.16, 126.57, 126.40, 122.52, 115.96, 82.13, 68.48, 34.48, 29.66, 26.76, 22.36, 13.79. HRMS (ESI): *m*/*z* Calcd for $C_{15}H_{18}N_2O_6$, $[M+Na]^+$ 345.1063; found 345.1067. *Analytical data for* ***10i***: Mp: 103–106 °C. MS (ESI): *m*/*z* 451.1 $[M+Na]^+$. IR (cm^{-1}, KBr): ν_{max} 763, 1280, 1400, 1501, 1752, 2924. ^1H NMR (300 Hz, $CDCl_3$): δ 0.902 (t, 3H, CH_3, *J* = 6.8 Hz), 1.38–1.50 (m, 4H, 2 × CH_2CH_3), 1.70–2.06 (m, 2H, $CHCH_2$), 2.17–2.27 (m, 2H, $ONO_2CH_2CH_2$), 4.16 (t, 2H, ONO_2CH_2, *J* = 6.8 Hz), 4.68 (t, 2H, OCH_2, *J* = 6.3 Hz), 5.45–5.49 (m, 1H, $COOCHCH_2$), 7.06–7.60 (m, 5H, ArH), 8.07 (s, 1H, ArH), 8.32 (dd, 1H, *J* = 1.9, 8.3 Hz, ArH), 8.69 (s, 1H, NH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.70, 165.69, 158.81, 145.62, 139.42, 135.68, 129.88, 126.76, 123.34, 119.72, 118.95, 116.63, 112.95, 81.75, 69.87, 63.88, 34.39, 29.65, 26.94, 26.78, 22.38, 13.80. HRMS (ESI): *m*/*z* Calcd for $C_{22}H_{24}N_2O_7$, $[M+Na]^+$ 451.1481; found 451.1486.
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16. Antiplatelet aggregation effect in vitro: Blood samples were withdrawn from rabbit carotid artery and mixed with 3.8% sodium citrate solution (9:1, v/v), followed by centrifuging at 500 rpm for 10 min at room temperature. After the resulting platelet-rich plasma (PRP) supernatant was collected, the residue was centrifuged at 3000 rpm for another 10 min at room temperature to obtain platelet-poor plasma (PPP). The PRP was adjusted with PPP in order to obtain platelet counts of 400–450 × 10^9 Pl/L. Platelet aggregation was determined by Born's turbidimetric method using a four-channel aggregometer (LG-PABER-I Platelet-Aggregometer, Beijing, China) within 3 h after blood collection. Briefly, PRP (240 μl) was pre-incubated with vehicle, positive control or different concentrations of individual compounds (30 μL) for 5 min at 37 °C, followed by the addition of 10 μM of Adenosine 5′-diphosphate sodium salt (ADP, Sigma–Aldrich, USA) to induce the platelet aggregation. The maximum aggregation rate (MAR) was recorded within 5 min at 37 °C. The inhibition rate of the tested compounds on platelet aggregation was calculated with the following formula: Inhibition rate (%) = (100% − MAR of tested compound/MAR of vehicle).
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18. Nitrate/nitrite measurement in vitro: Brifly, 0.1 mM of each compound in phosphate buffer solution (PBS) was incubated at room temperature for 15–300 min and were sampled every 15 min for 120 min and then every 30 min for the remaining time. The collected samples (50 μl) were mixed with 50 μl of sulfanilamide solution and 50 μL of *N*-[-1-naphthyl-ethylenediamine dihydrochloride], then incubated 5–10 min at room temperature, protected from light. Nitrite concentration was determined by microtiter plate reader at 540 nm from a standard curve (0–100 mmol L^{-1}) derived from $NaNO_2$ (Beyotime Biotechnology). NO data are expressed as mean ± SD (nitrite) in μmol L^{-1}.
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20. Aqueous solubility assays: Individual compounds at ca. 1 mg were dissolved in 10 ml of methanol and the maximum UV absorption of each compound was determined in a UV755B spectrophotometer, eventually diluting the solution (with MeOH) as necessary. A saturated solution of each compound was then prepared by stirring magnetically a small volume of normal saline in the presence of excess compound for 5 h. The saturated solution was filtered with a Millipore 0.45-mm filter to remove solid compound and measured by UV-spectrometry at the wavelength determined. Total solubility was determined by the relationship: $C' = A'CA^{-1}$, where C = the concentration of standard solution (mg mL^{-1}), A = absorbance of standard solution, A' = absorbance of saturated solution, and C' = concentration of saturated solution (mg mL^{-1}).
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