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Design and synthesis of novel hybrid sydnonimine and prodrug useful for glaucomatous optic neuropathy

Suchismita Acharya [a,*], Preston Rogers [a], Raghu R. Krishnamoorthy [b,*], Dorota L. Stankowska [b], H. V. Rasika Dias [a,*], Thomas Yorio [c]

[a] Department of Chemistry and Biochemistry, The University of Texas at Arlington, Arlington, TX 76019, USA
[b] North Texas Eye Research Institute (NTERI), University of North Texas Health Science Center, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107, USA
[c] Department of Pharmacology, University of North Texas Health Science Center, 3500 Camp Bowie Boulevard, Fort Worth, TX 76107, USA

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ABSTRACT

Synthesis and bioactivity of novel dual acting nitric oxide releasing and reactive oxygen scavenging hybrid compound **SA-2** is described. The hybrid molecule **SA-2** significantly increased the superoxide dismutase enzyme level and protected the photoreceptor cells from H_2O_2 induced oxidative stress. Synthesis of ocular esterase sensitive aceloxy alkyl carbamate prodrug **SA-4** with improved aqueous half-life is achieved to aid topical ocular formulation. This class of hybrid molecule and prodrug may have dual potential of improved IOP lowering and neuroprotection in glaucomatous optic neuropathy.

Elevated intraocular pressure (IOP) associated axonal degeneration in the optic nerve and progressive loss of retinal ganglion cells (RGCs) leading to visual field loss are characteristic features of both primary open angle and angle closure glaucoma. Some studies have also reported swelling and loss of photoreceptor cells in human as well as experimental models of glaucoma.[1,2] Clinically, the only method of slowing glaucomatous loss of vision is to reduce IOP, but lowering IOP is partially effective, and doesn't address RGCs' underlying susceptibility to degeneration.[3] Recent studies have implicated the role of nitric oxide (NO) directly in the regulation of IOP. In addition, the acute and massive release of NO into the rabbit eye does not induce inflammation or other growth toxic effects on the ocular tissues.[4,5] The nitric oxide system could potentially be targeted to enhance the aqueous outflow by relaxing the normal trabecular meshwork (NTM)[6] or reducing aqueous humor secretion possibly inhibiting Na+, K-ATPase in NPE cells.[7] A beta-blocker compound containing nitrate ester NO donor moiety (nipradilol, Fig. 1) has been shown to be neurotrophic in cat retina and increased the number of regenerating retinal ganglion cells.[8–11] Nitric oxide/cyclic guanosinemonophosphate (NO–cGMP) signaling also contributes to the development of neural precursors derived from human embryonic stem cells and enhances the differentiation of precursors to functional neurons for peripheral nerve regeneration.[12] These actions of nitric oxide lend themselves to the possible development of NO donors for the treatment of glaucoma. The current limitations for the implementation of this intervention based on NO donor therapy are that: IOP elevation induces oxidative stress in RGCs through decreased activity of several enzymes comprising the antioxidant defense system, including superoxide dismutase (SOD), glutathione peroxidase, catalase, thioredoxin (Trx1, Trx2) and has been implicated in RGC death.[13,14] Oxidative stress and mitochondrial dysfunction generate superoxide radicals which may decrease the NO bioavailability by forming toxic peroxynitrite anion ($ONOO^{\cdot-}$). Hence, a balance between superoxide and NO bioavailability is necessary to maintain the IOP and protect the RGC as well as photoreceptor cells. As reported recently by us,[15] a hybrid molecule 4-nitro TEMPOL-H (Fig. 1) containing both NO donor ($-ONO_2$) and a redox anti-oxidant (nitroxide) functional group demonstrated superior IOP lowering activity (20%, 6 h, 150 μg) in lasered monkey eyes as compared to only glyceryl trinitrate (GTN, 13%, 6 h, 500 μg) which contained three $-ONO_2$ groups.

* Corresponding authors.

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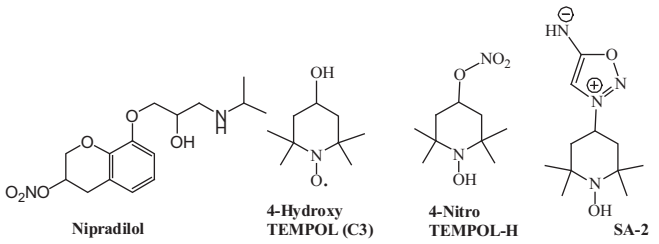


Figure 1. Hybrid NO donor compounds.

Literature report by Anggard and Haj-Yehia[16] supported our observation that the presence of nitroxide may be responsible for enhanced NO bioavailability via scavenging the surrounding ROS and/or up regulating the SOD enzyme. The 4-nitro TEMPOL compound and reported analogs have shown better NO mediated biological actions and act as superior antioxidants. However, organic nitrates are poor nitric oxide donors in ocular tissues and are not efficacious in lowering IOP in a therapeutic manner. In contrast, 3-(4-morpholino)-sydnonimine hydrochloride (linsidomine, **SIN-1**, Fig. 2), a mesoionic class of heterocyclic (1,2,3-oxadiazole amine) NO donor is reported to effectively lower intra ocular pressure (IOP) in rabbit eyes.[4] However the short duration of action (3 h post dose) of **SIN-I** suggested that there is need to design robust compounds with improved in vivo efficacy.

To continue our innovation in discovering small molecules as anti-glaucoma targets,[17–19] we disclose here for the first time the design and synthesis of a novel dual acting nitric oxide donor-antioxidant hybrid compound, **SA-2**. The goal is to provide the additive activity to protect RGC and photoreceptor cells against oxidative stress and enhance the duration of IOP lowering via improved NO availability.

In a simple aerobic aqueous solution, 1,2,3-oxadiazole amine containing NO donor **SIN-1** undergoes base-catalyzed hydrolysis spontaneously to release one mole of NO and one mole of superoxide radical (Fig. 3). Therefore, to improve the drug shelf life, **SIN-1** is used as a carbamate[20] (molsidomine) or amide[21] (ciclosidomine) prodrug (Fig. 2) for treatment of angina pectoris. Both the compounds are hydrolyzed in liver to release **SIN-1**.[22] It is apparent that the stoichiometric production of superoxide ($O_2^{\cdot-}$) generated from **SIN-1A** may combine with NO to form the undesired toxic peroxynitrite ($ONOO^{\cdot-}$) resulting in reduced NO availability.

Our goal was to incorporate a radical scavenger functionality in sydnonimine core structure to enhance the NO availability via scavenging both the superoxide by-product produced during NO release as well as the pathological ROS during IOP mediated oxidative stress. For this purpose, we choose piperidine nitroxide to be the appropriate radical scavenger. Piperidine nitroxide stable radicals as in TEMPOL (compound **C3**, Fig. 1) demonstrated effective antioxidant activity in various biological systems ranging from molecular, cellular and laboratory animal level, and act as catalytic SOD mimics. Besides the catalytic SOD mimetic activity of

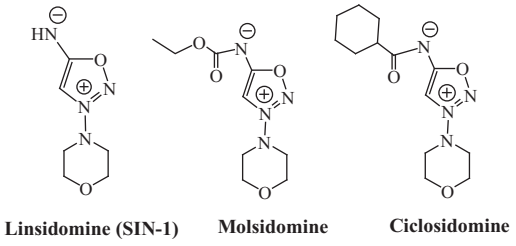


Figure 2. Sydnonimine NO donor.

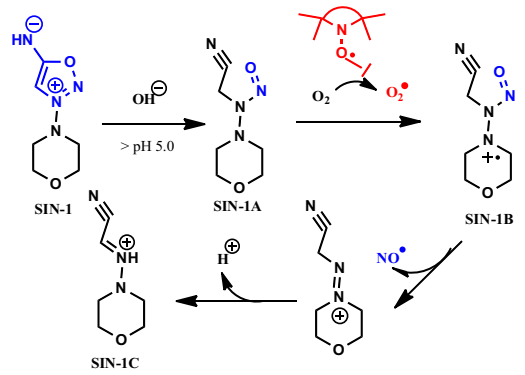


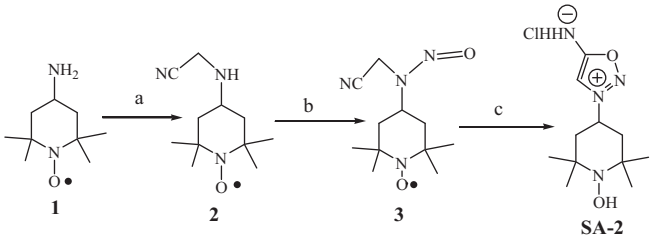
Figure 3. NO release from **SIN-1** (1,2,3-oxadiazole amine) at biological pH.

nitroxides, they can oxidize reduced transition metals that potentiate damage by producing reactive oxygen-derived species (ROS), detoxify hypervalent metals such as the ferryl-heme species, facilitate heme-mediated catalytic removal of H_2O_2, trap carbon-centered radicals, and terminate radical chain reactions. Through competing with NO for $O_2^{\cdot-}$ the nitroxides might also indirectly lower the production of peroxynitrite and elevate NO.[23,24] Compounds such as OT-440, OT-551 containing such nitroxide and *N*-hydroxyl amine functionality have been shown to protect RPE cells as well as RGC against oxidative stress in animal models.[25] The nitroxide radical and *N*-hydroxyl amine are interchangeable inside the cell to render same antioxidant activity.[26,27]

The 4-amino TEMPOL-H sydnonimine **SA-2** was prepared from 4-amino TEMPO (**1**, Scheme 1) following the literature procedure.[28] 4-Amino TEMPO was treated with formaldehyde bisulfite in H_2O followed by heating with KCN at 50 °C to produce the nitrile intermediate **2** which was nitrosylated at 0 °C using $NaNO_2$/concd HCl. The product formation was confirmed from LC/MS. The nitrosylated intermediate **3** is unstable and immediately converted to the 1,2,3-oxadiazole amine hydrochloride salt **SA-2** on treatment with excess methanolic HCl.

Compounds **SIN-1**, **SA-2** and 4-nitro TEMPOL-H (compound **C3**, Fig. 1) were analyzed for total nitrate production in reference to the reported Greiss assay protocol.[29] Sodium nitrite solution (1 mM) in water was used as reference standard. Both **SIN-1** and hybrid compound **SA-2** released nitric oxide similarly as shown in Figure 4.

We believe that the target tissue/cell for NO mediated IOP lowering effect is at the trabecular meshwork cells, the site that appears to be affected during open angle glaucoma where increase in IOP and other pathological stress contribute to glaucoma, and neural cell death. Cell viability study has been done to demonstrate the lack of overt toxicity of this class of molecules in NTM-5 (trabecular meshwork cell line) and 661 W photoreceptor cells. Here we have demonstrated that (Figs. 5 and 6), treatment with **SA-2** did not compromise the viability of neither the NTM5 cells nor



Scheme 1. Reagents and conditions: (a) HCHO, KCN, H_2O, 50 °C, 6 h, 93%, (b) $NaNO_2$, HCl, H_2O, 0 °C, 1 h, 88%, (c) 1 M HCl in MeOH, 12 h, 23 °C, 80%.

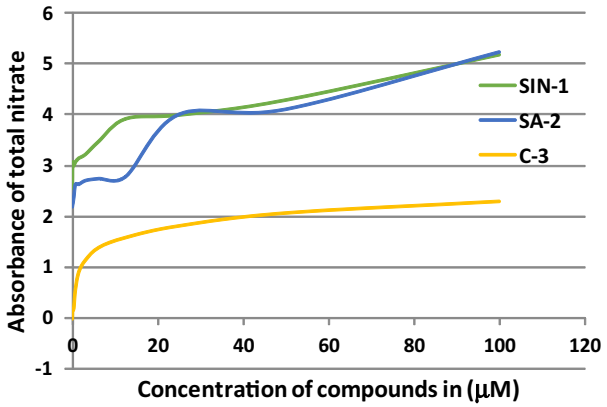


Figure 4. Nitric oxide release from **SIN-1** and **SA-2**.

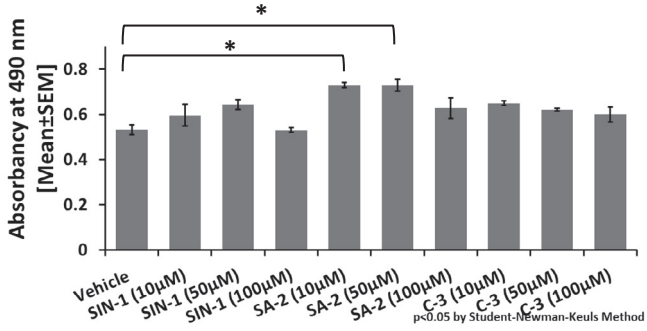


Figure 5. Treatment with **SA-2** did not compromise the viability of 661 W photoreceptor cells.

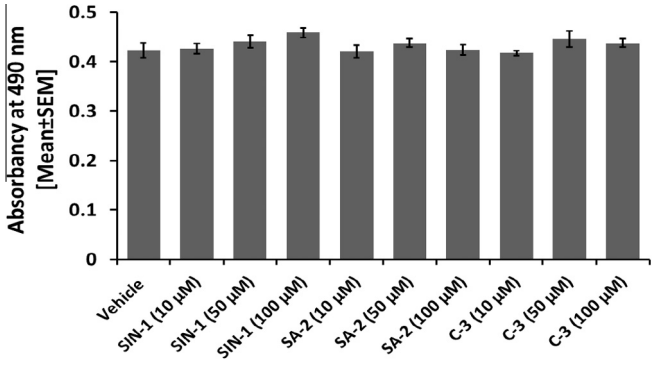


Figure 6. **SA-2** didn't decrease viability of trabecular meshwork NTM-5 cells.

the 661 W photoreceptor cells even as high as 100 μM concentration.

Approximately 3000 normal trabecular meshwork cells (NTM5) or transformed photoreceptor (661 W) cells were seeded in a 96 well plate and treated with different concentrations (10, 50 or 100 μM) of **SIN-1**, **SA-2** or C-3 for 24 h. Vehicle is DMEM/DMSO (1 mL/10 μL) treated cells. A cell survival (MTT) assay was carried out using a kit from Promega which measures the reduction of a tetrazolium substrate to a formazan by NADH/NADPH dehydrogenase secreted by living cells. Formation of the formazan is measured by its absorbance at 490 nm, which is indicative of living cells. Experiments were performed with eight biological replicates.

In addition, as shown in Figure 5, treatment with **SA-2** at two doses (10 μM and 50 μM) produced a modest, yet statistically significant (p <0.05, by Student-Newman Keuls test) increase in number of 661 W cells as compared to the vehicle (DMEM + DMSO) where **SIN-1** and C-3 didn't show such an effect. One of the possible explanations could be the availability of right concentration of nitric oxide from **SA-2** at these two doses to induce a neurogenic effect.[12] Though **SIN-1** is capable of producing NO at the same rate and concentration as **SA-2**, the superoxide byproduct formed during NO release (Fig. 3) may decrease its bioavailability by forming peroxynitrite radicals. In case of hybrid compound **SA-2**, the antioxidant functionality may effectively scavenge the superoxide and maintain the NO level desired for the above observed activity. More mechanistic investigation is underway.

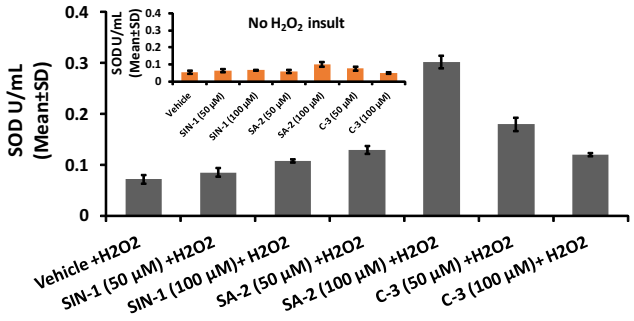


Figure 7. Hybrid compound **SA-2** upregulated SOD in 661 W neuronal cell line.

Deficiency of SOD enzyme causes RGC and photoreceptor cell death as observed in optic nerve head crush insult as well as in conditions of ischemic reperfusion injury.[25,30,31] To our utmost expectation, the hybrid compound **SA-2** upregulated SOD enzymes (Fig. 7) and moderately protects 661 W photoreceptor cells against H_2O_2 induced oxidative insult (Fig. 8) at two doses. Treatment with **SIN-1** (NO donor) in combination with H_2O_2 did not appreciably increase the SOD enzyme level in 661 W cells. However, **SA-2** which is both an NO donor and SOD-mimetic, produced a significant increase in SOD enzyme level at both 50 and 100 μM concentration when treated in combination with H_2O_2, compared to the vehicle alone. Mechanistically, nitroxide SOD mimetics dismutase the peroxy (·OOH) as well as superoxide ($O_2^{\cdot-}$) radicals. Nitric oxide also acts as a powerful antioxidant preserving mitochondrial and cellular integrity during oxidative stress via neutralizing the iron load (Fe^{2+}) in Fenton reaction and inhibit the formation of hydroxyl (·OH) radicals generated from superoxides and H_2O_2.[32] This synergistic effect of nitroxide and nitric oxide may be responsible for improved upregulation of SOD enzyme as observed in case of **SA-2** at 100 μM concentration. We saw a negative dose response in case of C3 (4-hydroxy TEMPOL-H) which implies hybrid molecules are better SOD mimetics than only antioxidants. Detailed dose response and mechanistic investigations are under progress. For measuring the SOD activity the Cayman's Superoxide Dismutase Assay Kit was used according to manufacturer's instructions. Vehicle is DMEM plus DMSO treated cells. The kit utilizes a tetrazolium salt for the detection of superoxide radicals formed by xanthine oxidase and hypoxanthine. SOD assay without H_2O_2 was performed twice with similar results.

In addition, in the MTT assay, treatment of 661 W cells with 400 μM H_2O_2 produced nearly 50% decrease in cell viability (Fig. 8). When cells were treated with H_2O_2 in combination with 50–100 μM of **SA-2**, a modest but significant increase in cell viability was observed at both doses as compared to cells treated with H_2O_2. Again, a dose response study is required to understand the correlation between SOD upregulation, nitric oxide bioavailability and cytoprotection. The MTT assay was performed once with eight biological replicates.

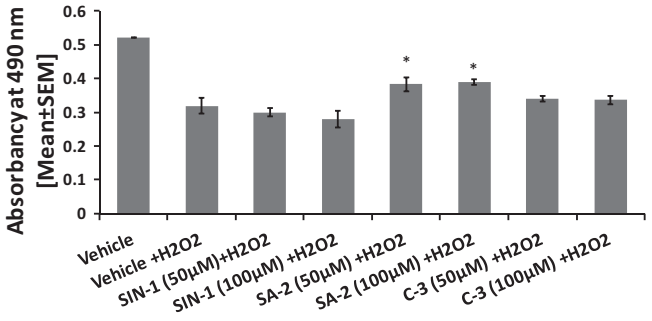


Figure 8. Hybrid compound **SA-2** is protective to 661 W cells against oxidative stress.

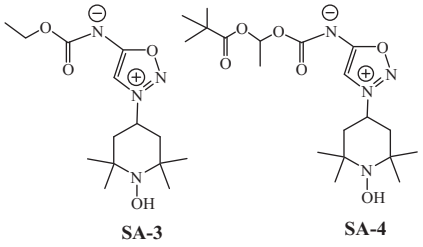
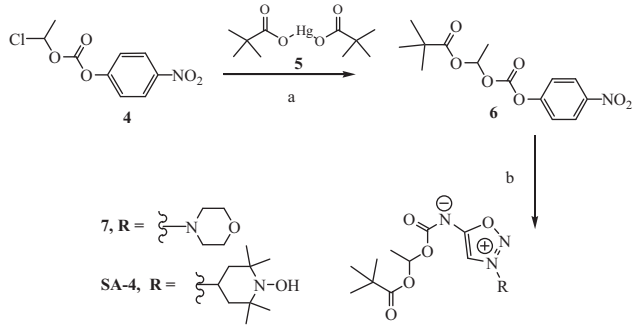


Figure 9. Carbamate prodrugs of hybrid compound **SA-2**.



Scheme 2. Reagents and conditions: (a) Pivalic acid, 50 °C, 12 h, 65%, (b) **SIN-1** or **SA-2**, pyridine, HOBt, 23 °C, 12 h, 35–38%.

Table 1
Half-life of sydnonimine and their AAC pro-drugs in PBS

Compound	$t½$ (PBS $pH_{7.4}$) at 25 °C	$t½$ (PBS $pH_{6.5}$) at 25 °C
SIN-1	<1 d[#]	<1 d[#]
7	9 m[*]	18 m[*]
SA-2	<1 d[#]	<1 d[#]
SA-4	9.1 m[*]	15.3 m[*]

An accelerated aqueous stability was performed where the compounds in 0.5 M of phosphate buffer ($pH_{6.5–7.4}$) were stressed at 45 °C. The half-life was calculated using Arrhenius equation from the % compound remained at different time points as analyzed by LC/MS.

[#] d = days.

[*] m = months.

As proof of concept hybrid compound **SA-2** containing both NO donor and antioxidant functionality demonstrated protective and possible neurogenic potential. Design, synthesis of more efficacious analogs and the in vitro study in both RGC and photoreceptor cells are underway.

Though **SIN-1** effectively lower IOP in rabbit eyes, due to its short half-life in aqueous solution ($t_{1/2}$ = <1 d, pH = 6.5–7.4), it is not suitable for topical ocular formulation. Similar to molsidomine, we have also synthesized the ethyl carbamate prodrug **SA-3**[37] of the parent amine **SA-2** (Fig. 9). Although the carbamate prodrug of **SIN-1** (molsidomine) is chemically stable ($t_{1/2}$ = 92 y, pH = 7.4), no IOP lowering activity is observed[33] indicating lack of enzymatic hydrolysis of ethyl carbamate in ocular tissue to the parent amine **SIN-1**.

Aceloxy alkyl carbamates (AAC) have been reported to have the potential to be used as an ocular prodrug[34] with the ability of getting enzymatically hydrolyzed to the parent drug by corneal esterase. To circumvent this issue and to design an ocular enzyme specific prodrug, we have prepared pivaloxy methyl carbamate prodrugs of **SIN-1** (compound **7**) and studied the aqueous stability in PBS (pH = 6.5–7.4). Synthesis of AAC prodrugs compound **7** and **SA-4** was achieved by reacting **SIN-1** or **SA-2** with pivaloxy anhydride[35] (**6**, Scheme 2) in presence of HOBt (Hydroxybenzotriazole) and pyridine.[36] Compound **7** and **SA-4** were found to have improved aq stability at pH 6.5 ($t_{1/2}$ = 18 months & 15.3 months, respectively) suitable for topical ocular formulation (Table 1). Detail enzymatic hydrolysis of compounds **SA-4** and **7** in ocular tissue is currently been pursued.

In conclusion, we have designed and synthesized a novel hybrid compound **SA-2** which can release nitric oxide similar to a well-known NO donor **SIN-1** and anticipate to lower IOP in an animal model. Compound **SA-2** increased the number of photoreceptor cells (possibly through enhanced viability) in a statistically significant manner, protected them moderately against H_2O_2 induced oxidative stress and rendered significant increases in SOD enzyme activity. This is a very important finding with the projection that **SA-2** and its analogs may be protective to RGC and photoreceptor cells in a glaucoma animal model. Further structure optimization to produce a more efficacious hybrid molecule is in progress.

To further improve the usefulness of this 1,2,3-oxadiazole amine (sydnonimine) class of nitric oxide donor for topical ocular formulation, we have synthesized the ocular esterase sensitive prodrugs such as compound **7** and **SA-4**. This class of carbamate prodrug is expected to be hydrolyzed in ocular tissue by non-selective esterase enzymes to the parent amines **SIN-1** or **SA-2**, respectively, responsible for the release of nitric oxide and lower intra ocular pressure. The stability and hydrophobicity of the prodrug may contribute to the reduced side effects associated with ocular surface phenomena like hyperemia or irritation which might have been caused by the nitric oxide generated from parent amine spontaneously in the surface of the eye before penetration into the intraocular tissues. The design, synthesis of efficacious analogs of **SA-2** as well as evaluation of **SA-2** and prodrugs in glaucoma animal model is ongoing.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.12.030. These data include MOL files and InChiKeys of the most important compounds described in this article.

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28. (a) Masuda, K.; Imashiro, Y.; Kaneko, T. *Chem. Pharm. Bull.* **1970**, *18*, 128; (b) Synthesis of compound **SA-2**: To 4-amino tempamine **1** (1 g, 5.83 mmol) in 20 mL of H_2O, formaldehyde bisulphite salt (0.9 g, 6.42 mmol was added and stirred at 23 °C for 2 h. Then KCN (0.42 g, 6.42 mmol) was added to it and the reaction mixture was heated at 50 °C for 4 h. Extracted with EtOAc, washed with H_2O and dried to get compound **2** as a red liquid (93%). LC/MS (M^+ = 210). To nitrile **2** (1.2 g, 5.71 mmol) in 20 mL H_2O at 0 °C was added $NaNO_2$ (0.43 g, 6.28 mmol) and concd HCl (0.2 mL, 6.28 mmol). The reaction mixture was stirred at 0 °C for 1 h. Light orange solid formed was filtered, washed with water and dried to afford compound **3** (88%). LC/MS (M^+ = 239). The solid was taken in 6 mL of $CHCl_3$/MeOH (1:3) and 5 mL of 1 M HCl in MeOH was added to it. The suspension was stirred at 23 °C for 12 h to obtain a clear solution. Solvent was evaporated, the light yellow solid was recrystallized from ethanol as **SA-2** HCl salt. (80%). ^{1}H NMR (imine form, DMSO-d_6, 500 MHz): δ 1.29 (s, 6H,), 1.48 (s, 6H), 2.29 (m, 2H), 2.57 (m, 2H), 3.62 (br s, 2H), 4.93 (m, 1H), 9.65 (br s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 20.21, 27.73, 32.57, 54.45, 67.71, 114.34. LC/MS (M^+ = 240). Anal. Calcd for $C_{11}H_{20}N_4O_2$.1.0 HCl: C, 47.74; H, 7.65; N, 20.24. Found: C, 46.83; H, 7.70; N, 19.87.
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36. General procedure for synthesis of compound **7** and **SA-4**: Compound **6** (2.4 mmol) and catalytic amount of HOBt were added to **SIN-1** or **SA-2** (1.20 mmol) in pyridine (5 mL) and the resulting mixture was stirred at room temperature for 12 h. Pyridine was evaporated under vacuum and the crude mixture was purified by flash column chromatography using 5% methanol and ethyl acetate as eluent. The products **7** and **SA-4** were obtained as white solids in 35–38% yield. Compound **7** (35%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.18 (s, 9H), 1.50–1.51 (d, 3H, J = 4 Hz), 2.18 (s, 1H), 3.51–3.53 (m, 4H), 3.95–3.97 (m, 4H), 6.86–6.90 (q, 1H, J = 8 Hz), 7.76 (s, 1H); APCI LC/MS = 343 (M^+); Compound **SA-4** (38%); ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.0–1.80 (m, 28 H), 4.94 (m, 1H), 6.75–6.85 (br s, 1H), 8.20 (s, 1H). APCI-LC/MS = 412 (M^+).
37. Compound **SA-3**; ^{1}H NMR (imine form, 500 MHz, $CDCl_3$): δ 1.15 (s, 6H), 1.27 (t, 3H, J = 8.1 Hz), 1.34 (s, 6H), 1.96 (m, 2H), 2.15 (m, 2H), 4.19 (q, 2H, J = 8.1 Hz), 4.26 (s, 2H), 5.02 (m, 1H). LC/MS = 312 (M^+).

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