

Communication

Synthesis and Pharmacological Evaluation of (+)-Usnic Acid Derivatives as Hypoglycemic Agents

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Abstract: Usnic acid is produced by lichens and exhibits different biological properties, including hypoglycemic ones. However, this effect becomes noticeable only at relatively high doses, something that may lead to some adverse effects. The chemical modification of the molecule is able to enhance its safety profile and its hypoglycemic properties. We synthesized six enamine derivatives of (+)-usnic acid, and two of them are novel. All compounds were evaluated for the hypoglycemic effect after oral introduction in mice with alloxan-induced diabetes mellitus at a dose of 50 mg/kg. The derivative containing a 4-trifluoromethylphenyl fragment showed the most pronounced hypoglycemic effect, which was detected starting from sixth day of the experiment. Also, OGTT was performed in mice without alteration of glucose metabolism (C57BL/6), which showed no hypoglycemic effect after oral introduction of all studied compounds.

Keywords: usnic acid; enamines; hypoglycemic action; diabetes mellitus



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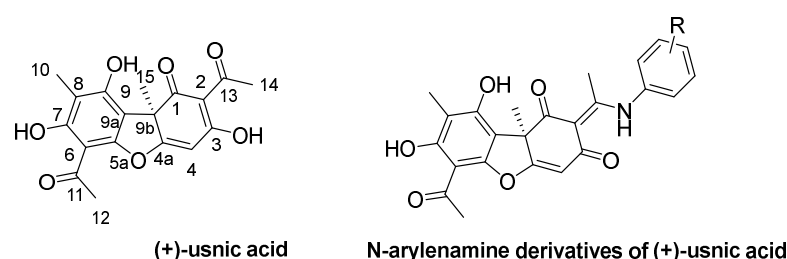
1. Introduction

Lichens are symbiotic organisms composed of fungi and algae. Usnic acid (UA) 1 is one of the most studied and widely distributed metabolites of lichens. There is abundant evidence that UA is capable of inhibiting the growth of a number of microorganisms (bacteria, mycobacteria, viruses, fungi) and that it exhibits some antitumor properties [1,2]. Much rarer are in vivo studies that suggest that UA has analgesic and anti-inflammatory effects [3], acute and chronic anti-inflammatory effects [4], and antiulcer and antioxidant effects in mice and rats [5]. However, the medical use of UA is limited by its low solubility in organic solvents and water, cytotoxicity, and hepatotoxicity, as well as the ability of UA to cause oxidative damage to the liver in high doses [6] and other side effects [7].

There is a single article in the literature demonstrating the anti-diabetic activity of UA [8]. The authors of that paper assumed that anti-inflammatory and antioxidant properties are often very useful for the treatment of many different diseases, including diabetes mellitus. Given that diabetes mellitus is a very complex disease with a potential risk of fatal cardiovascular complications, and that its therapy is still far from optimal, new sugar-lowering drugs of natural or synthetic origin are greatly needed. The aforementioned work showed that UA shows no activity in the OGTT test in healthy animals, although at a dose of 75 mg/kg it has beneficial effects on glucose homeostasis and kidney function in Sprague-Dawley rats with diabetes mellitus induced by streptozocin. Inhibition of SLGT [8] and α -glucosidase [9] has been mentioned as a potential mechanism.

Previous studies by our group have shown that chemical modification of UA leads to a decrease in its toxicity [10,11]. UA enamines are among the most accessible and well-studied UA derivatives. They are formed by the reaction of UA with primary amines. During this reaction, the double bonds in the resulting imine are displaced, which leads to a rearrangement of the β -triketone system and the formation of enamine (Scheme 1) [12]. As

a rule, the configuration of the newly formed double bond is either not indicated, or the E-configuration is assigned by analogy with enamine from [13]. Chemical modification of the β -triketone fragment is one of the factors contributing to the decrease in the toxicity of UA, which leads to the loss of protonophoric properties of UA [14]. Derivatives of this kind have been found to possess many useful properties, including antibacterial, antimycobacterial, antiviral, antimalarial, insecticidal, and enzymatic activities [1,2,15]. N-aryl derivatives have been shown to inhibit tau-protein aggregation and neuroinflammation [16] and can act as inhibitors of DNA polymerase beta [17] and tyrosyl-DNA phosphodiesterase 1 [18]. Most of the described enamines are synthesized from (+)-usnic acid, which is due to the fact that only this enantiomer of usnic acid is commercially available. The protonophoric properties responsible for cytotoxicity do not depend on the configuration of the methyl group. However, the configuration of the chiral center can be crucial for binding to proteins (e.g., enzymes and receptors), that has been repeatedly confirmed for pairs of enantiomeric derivatives [17,19].

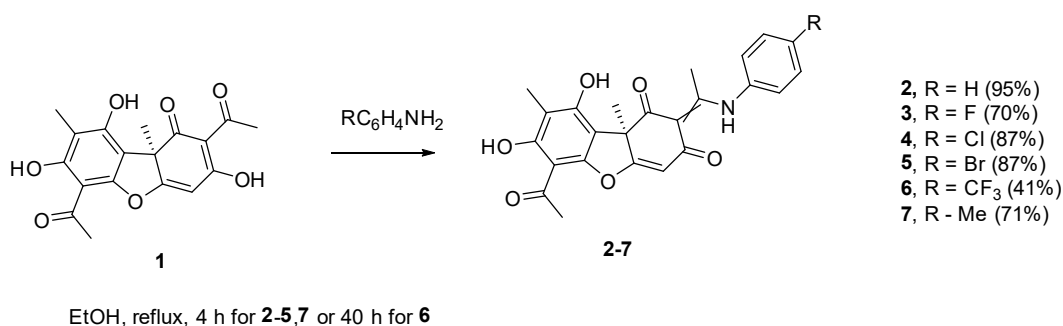


Scheme 1. Structures of (+)-usnic acid (with numbering of atoms) and N-arylenamines of (+)-usnic acid.

In this work, the hypoglycemic action of a number of known and novel enamine derivatives of (+)-usnic acid was investigated using an oral glucose tolerance test (OGTT) and an experimental model of alloxan-induced diabetes mellitus. Using the OGTT, performed after a single oral administration of a test compound in mice without any metabolic disturbances (C57Bl/6), it is possible to detect substances with a very limited set of mechanisms of action, such as those that increase insulin secretion (DPP4 inhibitors, FFAR1 agonists). To evaluate the hypoglycemic effects mediated by other mechanisms of action, other experimental models, such as alloxan- or streptozocin-induced diabetes, should be used [8].

2. Results and Discussion

To study the hypoglycemic activity, enamines 2–7 with different types of substituents in the *para*-position of the aniline fragment were synthesized (Scheme 2); two of them (compounds 6 and 7) were new and not previously described. (For ^1H , ^{13}C NMR, and DFS spectra of compounds, see Figures S1–S14 in Supplementary Materials.) The configuration of the newly formed double bond was not determined, though according to the NMR spectra, the only isomer of enamines was formed.



Scheme 2. Synthesis of compounds 2–7. Compound 2 was synthesized according the procedure described in [17], compound 3—[19], compounds 4, 5—[12].

The reaction proceeds by refluxing the reagents in ethanol. The reaction time for the formation of enamines 2–5 and 7 is 4 h. We found that the reaction of (+)-UA with 4-trifluoromethylaniline proceeds slower, which seems to be due to the strong acceptor action of the CF₃-group and, consequently, the lower nucleophilicity of the amino group. Acceptable reaction conversion was achieved by increasing the excess of aniline and reaction time. Refluxing for 40 h with three equivalents of 4-trifluoromethylaniline resulted in conversion of 70% and after chromatography gave the desired enamine 6 with 41% yield.

All obtained compounds were pre-tested in an experimental model of alloxan-induced diabetes mellitus in CD-1 mice. According to the results of the experiment, compound 6 at a dose of 50 mg/kg was found to most effectively arrest severe hyperglycemia in diabetic mice (Figure 1). It should be noted that its effect on day 6 of the experiment was higher than that of the positive control metformin. Moreover, the data suggest that a course of administration of this compound is necessary to achieve the desired hypoglycemic effect, which is consistent with the presumed mechanism of action of UA, which does not include rapid mechanisms of hypoglycemia, such as increased insulin secretion or insulin sensitization. [8,9]. This is supported by the data we obtained when we performed OGTT in mice without metabolic abnormalities (C57Bl/6). In this experiment, none of the compounds studied at a dose of 50 mg/kg exhibited hypoglycemic activity (Figure 2).

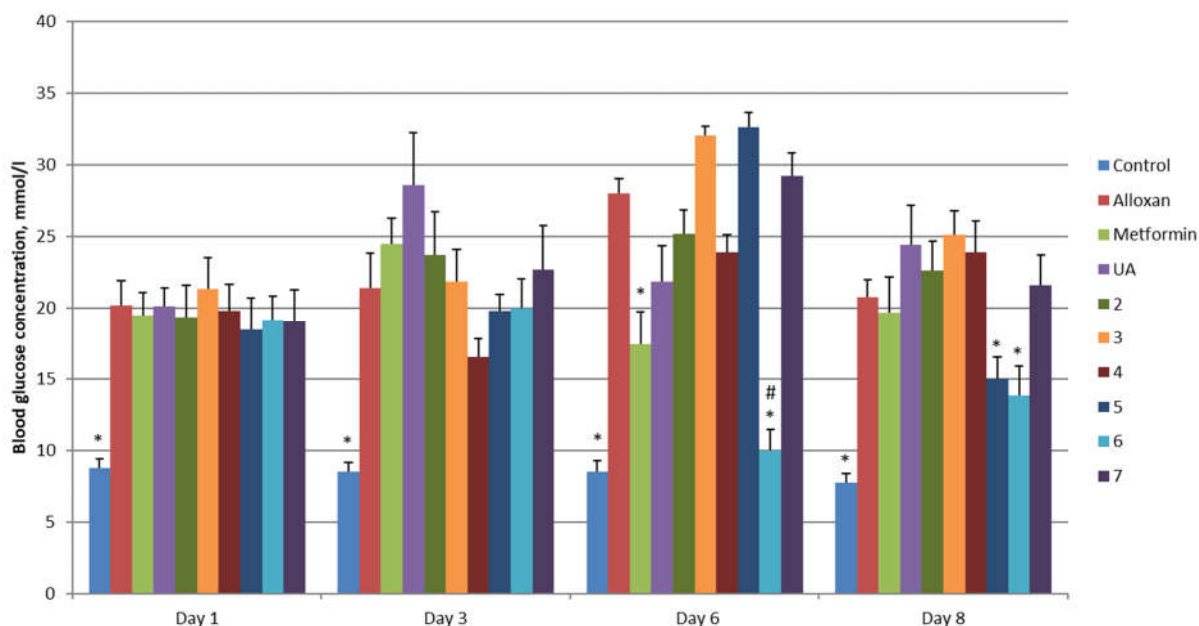


Figure 1. The effect of compounds 2–7 on the blood glucose level of mice with diabetes mellitus induced by alloxan. * $p < 0.05$ compared to the “Alloxan” group, # $p < 0.05$ compared to the “Metformin” group.

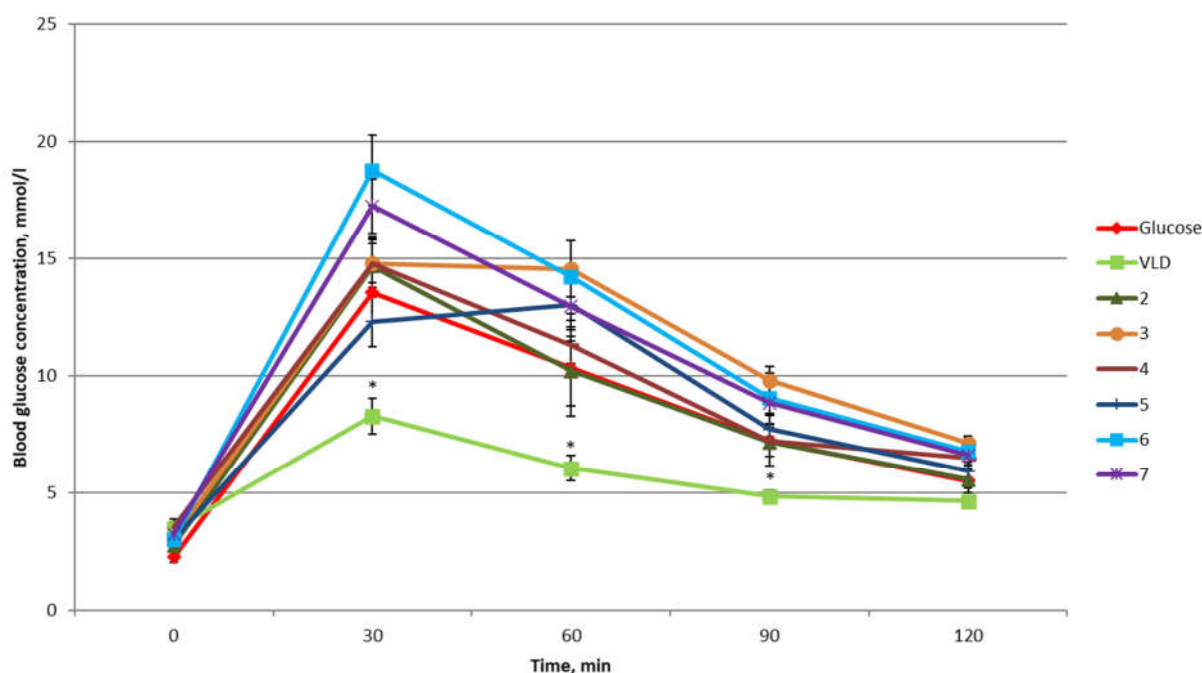


Figure 2. Results of the OGTT. * $p < 0.05$ compared to the «Glucose» group. VLD—vildagliptin.

3. Materials and Methods

3.1. Materials

Synthetic starting materials and reagents were acquired from Acros Organics. (+)-Usnic acid was obtained from Zhejiang Yixin Pharmaceutical Co., Ltd., (Lanxi, China).

3.2. Animals

The study involved male C57BL/6, CD-1 mice weighing 22–25 g that were obtained from the SPF vivarium of the Institute of Cytology and Genetics SB RAS. The animals were housed in polycarbonate cages with ad libitum access to water and feed. In the vivarium room, humidity, temperature, and the 12/12 h light-and-dark cycle were controlled. All manipulations with animals were carried out in strict accordance with the laws of the Russian Federation, the decree of the Ministry of Health of the Russian Federation no. 199n of 4 January 2016, and Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

3.3. Methods

Synthetic starting materials and reagents were purchased from Acros Organics (Belgium). (+)-Usnic acid (CAS 7562-61-0) was purchased from Zhejiang Yixin Pharmaceutical Co., Ltd. (Lanxi, China).

^1H and ^{13}C NMR spectra were recorded in CDCl_3 with solvent resonances (^1H 7.24, ^{13}C 76.90 ppm) as internal standards on a Bruker AV-400 spectrometer (operating frequency 400.13 MHz for ^1H and 100.61 MHz for ^{13}C). ^{19}F NMR spectra were recorded on a Bruker AV 300 spectrometer (282.4 MHz). IR spectra were recorded on a Vector 22 instrument in KBr. Mass spectra (ionizing-electron energy 70 eV) were recorded on a DFS high-resolution mass spectrometer (Thermo Scientific). Melting points were measured on a Kofler block. Specific optical rotation was recorded on a PolAAR 3005 polarimeter (Optical Activity Ltd., Huntingdon, UK) and expressed in $(\text{deg} \times \text{mL})/(\text{g} \times \text{dm})$; solution concentrations, in $\text{g}/(100 \text{ mL})$. Column chromatography was performed on silica gel (Merck, 60–200 mesh). TLC was performed on Sorbfil plates (UV 254). Atomic numbering in compounds is given for the assignment of resonances in NMR spectra according to the rules, which are set for this class of natural products.

3.3.1. Alloxan-Induced Diabetes Mellitus

Diabetes was induced in CD-1 mice. After a 14 h fast, all animals were intraperitoneally injected with freshly prepared physiological alloxan solution at a dose of 200 mg/kg. After the injections, the animals were given water, and 5 h later, pelleted feed; 3 days after the alloxan treatment and after a 4 h fast, the animals were injected again with alloxan solution at the same dose and according to the same scheme as before; 3 h after that, the mice were given access to pelleted feed. Three days after the second alloxan treatment and after a 4 h fast, blood glucose levels were measured in all animals. Mice with blood glucose levels >11.1 mmol/L were considered diabetic. All diabetic animals were divided into groups (7–9 mice per group) with the same average blood glucose level. Subsequently, the studied compounds were administered to the diabetic mice intragastrically in water with a drop of Tween 80 solution at a dose of 50 mg/kg. Animals in the negative control group received only the solvent (water + drop of Tween 80). Metformin at a dose of 250 mg/kg was used as a comparison drug. All substances were administered daily for 7 days. On days 3, 6, and 8 of the experiment after a 4 h fast, blood glucose levels were measured (blood was taken by tail notch) using a ONE TOUCH Select glucometer (LIFESCAN Inc., Milpitas, CA, USA).

3.3.2. OGTT

To assess animal glucose tolerance, an oral glucose tolerance test was performed on C57BL/6 mice, with 6 animals in each group. The compounds tested were dissolved in water with a drop of Tween 80. Vildagliptin at a dose of 10 mg/kg was used as a positive control. All animals received glucose at a dose of 2.5 g/kg through a probe after a 12 h fast. All tested compounds were administered intragastrically at a dose of 50 mg/kg 30 min before glucose administration. Blood glucose levels (collected by tail snip) were measured before the experiment (0 time point) and then every 30 min for 2 h after drug administration. Glucose concentration in the collected blood was assessed using a ONE TOUCH Select glucometer (LIFESCAN Inc., Milpitas, CA, USA).

3.3.3. Synthesis of (+)-Usnic Acid Enamine Derivatives

Compound **2** was synthesized from (+)-UA by the published method [17]. Compound **3** was synthesized from (+)-UA by the published method [19]. Compounds **4** and **5** were synthesized from (+)-UA by the published method [12].

General procedure for the synthesis of compounds **6** and **7**.

(+)-UA (1 mmol) was treated with appropriate amine (3 mmol), dissolved in ethanol (12 mL), refluxed on a water bath for 40 h (reaction with 4-trifluoromethylaniline) or 4 h (reaction with 4-methylaniline), cooled, and treated with distilled water (10 mL) and 1 M HCl up to pH 5. A white precipitate formed and was filtered off, washed with water, and dried in air. The precipitate was separated using column chromatography over silica gel with elution by CHCl₃ with an ethyl acetate gradient from 0 to 20% to afford **6** or **7**.

(2*R*,4*E*)-10-acetyl-4-{1-[(4-trifluoromethylphenyl)amino]ethyliden}-11,13-dihydroxy-2,12-dimethyl-8-oxatricyclo[7.4.0.0^{2,7}]trideca-1(9),6,10,12-tetraen-3,5-dione **6**: light yellow amorphous powder; Yield 41%; m.p. 181–182 °C; [α]_D²⁷ +224 (c 0.15; CHCl₃). ¹H NMR spectrum (CDCl₃, 400 MHz) δ 1.74 (3H, s, H-5), 2.08 (3H, s, H-10), 2.58 (3H, s, H-12), 2.67 (3H, s, H-14), 5.88 (1H, s, H-4), 7.32 and 7.73 (4H, d and d, J = 7.7 Hz, Har), 11.64 (1H, s, H-9), 13.35 (1H, s, H-7), 15.31 (1H, s, NH). ¹³C NMR spectrum (CDCl₃, 100 MHz) δ 7.4 (C-15), 20.6 (C-12), 31.2, 31.7 (C-14, C-10), 57.6 (C-9b), 101.3 (C-6), 102.1 (C-4), 102.9 (C-2), 104.6 (C-4a), 108.2 (C-8), 123.4 (CF₃, q, J = 272.5), 126.0 and 126.8 (2 C-17 and 2 C-18), 130.2 (C-19, q, J = 33.2), 139.2 (C-16), 155.7 (C-5a), 157.9 (C-9), 163.5 (C-7), 173.6 (C-11), 175.2 (C-4a), 191.0 (C-3), 198.9 (C-1), 200.5 (C-13). ¹⁹F NMR spectrum (CDCl₃, 282 MHz) δ 99.12 (CF₃, s); IR (KBr, cm⁻¹): 1064.5, 1135.9, 1292.1, 1322.9, 1371.2, 1461.8, 1544.7, 1629.6, 1697.3, 2927.5, 2979.6, 3079.9. Found: m/z 487.1238 [M]⁺ C₂₅H₂₀F₃O₆N. Calcd: M = 487.1237.

(2*R*,4*E*)-10-acetyl-4-{1-[(4-methylphenyl)amino]ethyliden}-11,13-dihydroxy-2,12-dimethyl-8-oxatricyclo[7.4.0.0^{2,7}]trideca-1(9),6,10,12-tetraen-3,5-dione **7**: light yellow amorphous powder; Yield 71%; m.p. 203–204 °C; [α]_D²⁷ +256 (c 0.20; CHCl₃). ¹H NMR spectrum (CDCl₃, 400 MHz):

δ 1.44 (3H, s, H-15), 2.08 (3H, s, H-10), 2.38 (3H, s, H-CH₃(Ph)), 2.55 (3H, s, H-12), 2.67 (3H, s, H-14), 5.85 (1H, s, H-4), 7.05 and 7.25 (2H and 2H, d and d, $J = 8.22$), 11.84 (1H, s, H-9), 13.35 (1H, s, H-7), 14.91 (1H, s, NH). ¹³C NMR spectrum (CDCl₃, 100 MHz): δ 7.4 (C-15), 20.5 and 21.0 (C-12 and C-CH₃(Ph)), 31.2 and 31.8 (C-14, C-10), 57.2 (C-9b), 101.2 (C-6), 102.3 (C-4), 102.6 (C-2), 104.8 (C-9a), 108.0 (C-8), 125.3 and 130.1 (C-17 and C-18), 133.2 and 138.3 (C-16 and C-19), 155.7 (C-5a), 158.1 (C-9), 163.4 (C-7), 174.0 (C-4a), 174.4 (C-11), 190.5 (C-3), 198.5 (C-1), 200.5 (C-13). IR (KBr, cm⁻¹): 1062.6, 1268.9, 1290.2, 1365.4, 1375.0, 1457.9, 1540.9, 1625.7, 1689.4, 1733.7, 2719.2, 2919.8, 2979.6, 3066.4. Found: m/z 433.1521 [M]⁺ C₂₅H₂₃O₆N. Calcd: $M = 433.1520$.

4. Conclusions

Thus, we have synthesized two novel UA derivatives, and one of them containing a 4-trifluoromethylphenyl fragment exhibited marked hypoglycemic activity on the alloxan-induced experimental model of diabetes in mice at a dose of 50 mg/kg/day. UA itself at the same dose showed no activity. The positive effect of the fluorine atom is known for large numbers of marketed drugs (e.g., fluoxetine and ezetimibe), which may be based on improved metabolic stability, changes in pKa, lipophilicity, molecular conformation, and protein-ligand interactions. [20,21]. So, the reduction in the effective hypoglycemic dose in the case of the UA derivative may help in reducing the risk of side effects known for this natural metabolite.

Supplementary Materials: The following are available online, Figure S1: The ¹H NMR spectrum of compound 2; Figure S2: The ¹H NMR spectrum of compound 3; Figure S3: The ¹H NMR spectrum of compound 4; Figure S4: The ¹H NMR spectrum of compound 5; Figure S5: The ¹H NMR spectrum of compound 6; Figure S6: The ¹³C NMR spectrum of 6; Figure S7: The DFS spectrum of 6; Figure S8: The ¹H NMR spectrum of compound 7; Figure S9: The ¹³C NMR spectrum of 7; Figure S10: The DFS spectrum of 7; Figure S11: The ¹H NMR spectrum of compound 7; Figure S12: The ¹³C NMR spectrum of 7; Figure S13: The IR spectrum of 7; Figure S14: The DFS spectrum of 7.

Author Contributions: Conceptualization, O.A.L. and M.V.K.; methodology, S.A.B. and M.V.K.; investigation, O.A.L. and S.A.B.; writing—original draft preparation, O.A.L., S.A.B. and M.V.K.; writing—review and editing, S.A.B., O.A.L. and M.V.K.; supervision, T.G.T. and N.F.S.; project administration, O.A.L. funding acquisition, O.A.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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