Three New Constituents From the Parasitic Plant *Balanophora laxiflora*

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Abstract

Three new constituents (**1-3**) were isolated from the whole plant of *Balanophora laxiflora*. Their chemical structures were elucidated by extensive analysis of high-resolution electrospray ionization mass spectrometry and nuclear magnetic resonance spectroscopy. Absolute configurations of new compounds were determined by circular dichroism spectra and modified Mosher's method. The isolated compounds weakly inhibited on both NO production and COX-2 mRNA expression in RAW264.7 macrophages.

Balanophora laxiflora (Balanophoraceae) is a parasitic plant and usually parasitizes at the roots of evergreen trees. It is widely distributed in the tropical or subtropical forests of Vietnam, Laos, Cambodia, and China.[1,2] The whole plant of *B. laxiflora* is used in the folk medicinal remedies for antidote, anti-inflammation, improvement of blood circulation, and as a tonic drug.[2,3] Literature survey indicates that *B. laxiflora* and other *Balanophora* species are rich sources of phenolic compounds such as lignans, chalcones, hydrolysable tannins, and acyl glucose of phenolic acids.[2-6] In the present study, the whole plant of *B. laxiflora* was subjected to chemical investigation and led to the isolation of three new compounds including an iridoid (**1**), a rare natural occurring 1-hydroxy-1,3-diarylpropan-2-one glucoside (**2**), and an aryltetralin lignan glucoside (**3**). The anti-inflammatory effects of those compounds were evaluated by inhibition of NO production and reducing COX-2 mRNA expression in RAW264.7 cells.

Compound **1** was obtained as a white amorphous powder. Its molecular formula was determined to be $C_{10}H_{14}O_3$ by a quasi-molecular ion peak at m/z 183.1016 $[M+H]^+$ (calcd. for $C_{10}H_{15}O_3$, 183.1021) in the high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) and in conjunction with the ^{13}C-NMR data. The infrared (IR) spectrum of **1** showed stretching vibration bands at 3427 and 1711 cm^{-1}, suggesting the presence of hydroxyl and carbonyl functional groups, respectively. The ^{1}H-NMR spectrum of **1** contained signals of an exo-olefinic methylene (δ_H 5.09 and 5.19), an oxygenated methylene (δ_H 4.71 and 4.81), an oxygenated methine (δ_H 3.71), and a doublet methyl group (δ_H 1.20). The ^{13}C-NMR spectrum of **1** revealed of 10 carbon signals, which were further classified by heteronuclear single quantum coherence spectra as two nonprotonated carbons, four methines, three methylenes, and one methyl carbon. A deshielded carbon signal at δ_C 176.4 was assigned to a carbonyl group. Two olefinic carbon signals at δ_C 113.9 and 143.7 were demonstrated for the presence of a C-C double bond. In the ^{1}H-^{1}H COSY spectra, a 5-membered ring structure was established by the correlations of neighboring protons including H-5 (δ_H 3.17)/ H_2-6 (δ_H 1.52, 2.36)/ H-7 (δ_H 3.71)/ H-8 (δ_H 2.13)/ H-9 (δ_H 2.74)/ H-5. On the other hand, a COSY correlation between H-8 and methyl group (H_3-10, δ_H 1.20) indicated the attachment of methyl group at C-8 (Figure S1, Supplementary data). The HMBC correlations between

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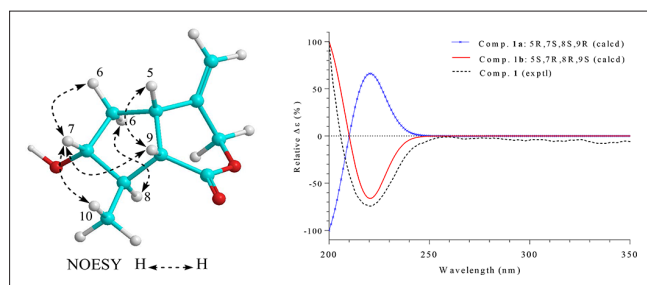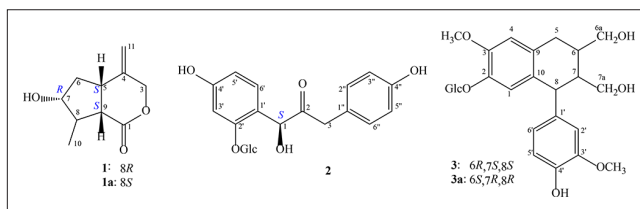Email: phankiem@yahoo.com

Table 1. ^{1}H and ^{13}C-NMR Data for Compounds **1** and **1a**.

No.	$^a\delta_C$	$^a\delta_H$ (mult., J in Hz)	$^b\delta_C$
		1	**1a**
1	176.4	-	171.8
3	71.9	4.71 (d, 11.5)	70.5
		4.81 (d, 11.5)	
4	143.7	-	141.8
5	38.6	3.17 (ddd, 8.0, 9.0, 10.0)	39.3
6	40.4	1.52 (ddd, 10.0, 10.0, 12.5)	40.2
		2.36 (ddd, 5.5, 8.0, 12.5)	
7	78.7	3.71 (ddd, 5.5, 8.5, 10.0)	74.7
8	46.9	2.13 (m)	46.5
9	46.7	2.74 (dd, 9.0, 11.0)	43.9
10	17.5	1.20 (d, 6.5)	10.7
11	113.9	5.09 (s)	113.2
		5.19 (s)	

^{a}Recorded in CD$_3$OD.
^{b}Recorded in CDCl$_3$ and previously reported in Sim et al.[7]

Figure 2. Chemical structures of compounds **1**, **2**, and **3**.

H$_3$-10 (δ_H 1.20) and C-9 (δ_C 46.7)/ C-8 (δ_C 46.9)/ C-7 (δ_C 78.7), and chemical shift of methine C-7 (δ_H 3.71, δ_C 78.7) suggested the presence of hydroxy group at C-7. Moreover, HMBC correlations between H-8 (δ_H 2.13)/ H-9 (δ_H 2.74) and deshielded carbon (δ_C 176.4) indicated that C-1 was a carbonyl carbon (Figure S1, Supplementary data). Location of exocyclic double bond at C-4/C-11 was confirmed by HMBC correlations between H$_2$-11 (δ_H 5.09, 5.19) and C-5 (δ_C 38.6)/ C-4 (δ_C 143.7)/ C-3 (δ_C 71.9). Finally, signal of oxygenated methylene C-3, HMBC correlation between H$_2$-3 (δ_H 4.71, 4.81) and carbonyl carbon C-1 (δ_C 176.4) indicated δ-lactone linkage between C-3 and C-1. Thus, the planar structure of **1** was established and similarly with a previously synthesized compound (**1a**).[7] However, significant difference in their NMR data (Table 1) suggested that they are stereoisomers and expected difference at chiral centers C-7 and/or C-8. Relative configuration of **1** was examined by nuclear Overhauser effect spectroscopy (NOESY) analysis. A NOESY correlation between H-5 (δ_H 3.17) and

Figure 1. Important nuclear Overhauser effect spectroscopy correlations, experimental CD, and theoretical calculated CD spectra of possible enantiomers (**1b** and **1c**) of compound **1**.

H-9 (δ_H 2.74) indicated a *cis*-fused 5/6-bicyclic system (Figure 1). Therefore, H-5 and H-9 were proposed to be β-orientation as notably reported for the iridoids.[8-11] The NOESY correlations between H-7 (δ_H 3.71) and H-9 (δ_H 2.74)/ H$_3$-10 (δ_H 1.20)/ H$_\beta$-6 (δ_H 2.36), H$_\alpha$-6 (δ_H 1.52) and H-8 (δ_H 2.13) suggested for the β-orientation of C-10 and α-orientation of OH-7. Experimental and Time dependent density functional theory calculated circular dichroism (CD) spectra of **1** observed negative Cotton effect at 220 nm, indicating absolute configurations of 5S,7R,8R,9S (Figure 1). Consequently, compound **1** was determined to be a new iridoid and named as balanolaxin (Figure 2).

Compound **2** was isolated as a pale-yellow amorphous powder. A quasi-molecular ion peak at *m/z* 459.1274 [M+Na]$^+$ in the HR-ESI-MS and ^{13}C-NMR data indicated molecular formula of **2** to be C$_{21}$H$_{24}$O$_{10}$ (calcd. for C$_{21}$H$_{24}$O$_{10}$Na, 459.1267). The ^{1}H-NMR spectrum of **2** displayed characteristic signals for an ABX-coupled aromatic proton system [δ_H 6.50 (1H, br d, J = 7.8 Hz), 6.75 (1H, br s), 7.07 (1H, br d, J = 7.8 Hz)] and an AA′BB′-coupled aromatic proton system [δ_H 6.66 (2H, d, J = 7.8 Hz), 6.88 (2H, d, J = 7.8 Hz)], indicating the presence of a 1,2,4-trisubstituted and a 1,4-disubstituted benzene ring, respectively. The ^{13}C-NMR and heteronuclear single quantum coherence spectra of **2** contained signals for 21 carbons including 6 nonprotonated carbons, 13 methines, and 2 methylenes. Of these, 6 carbinol signals at δ_C 103.5, 74.9, 78.2, 71.7, 77.9, and 62.8 were typically recognized for a glucopyranosyl group. Twelve aromatic carbons (δ_C 104.9–160.6) were assigned for 2 benzene rings. Remaining 3 carbons at δ_C 210, 76.6, and 44.9 belonged to a ketone, an oxygenated methine, and an sp^3 methylene group, respectively. The HMBC correlations between H-1 (δ_H 5.32) and C-2 (δ_C 210.0)/ C-1′ (δ_C 120.6)/ C-2′ (δ_C 157.9)/ C-6′ (δ_C 132.1), H$_2$-3 (δ_H 3.61, 3.56) and C-2/C-1″ (δ_C 126.4)/ C-2″ (δ_C 131.7)/ C-6″ (δ_C 131.7) indicated a 1,3-diaryl propan-2-one structure, specifically, a *para*-disubstituted benzene ring at C-3 and 1,2,4-trisubstituted benzene ring at C-1 (Figure S1, Supplementary data). Additional hydroxy group at C-1 was confirmed by its oxygenated methine signals δ_H 5.32/ δ_C 76.6. The large coupling constant of H-6′/H-5′ (J = 7.8 Hz, *ortho*-coupled protons), and HMBC correlations between H-6′ (δ_H 7.07) and C-2′ (δ_C 157.9)/ C-4′ (δ_C 160.6) supported for the assignment of 2 sp^2 oxygenated carbons C-4′ and C-6′. Another sp^2 oxygenated

carbon C-4″ was deduced by HMBC correlation between H-2″ (δ_H 6.88)/ H-6″ (δ_H 6.88) and C-4″ (δ_C 157.2). The HMBC correlations between H-1 (δ_H 5.32)/ H-6′ (δ_H 7.07)/ anomeric proton H-1‴ (δ_H 4.87) and C-2′ (δ_C 157.9) indicated glucopyranosyl group at C-2′. Acid hydrolysis of **2** gave sugar and aglycone portions. The presence of D-glucose in the sugar portions was confirmed by high-performance liquid chromatography (HPLC) analysis of its thiocarbamoylthiazolidine derivative in comparison with those of authentic samples.[12] Aglycone portion was reacted with *S* and *R*-MTPA-Cl reagents to give *R*-MTPA and *S*-MTPA esters, respectively.[13] The difference in the ^{1}H-NMR data between *S*-MTPA and *R*-MTPA esters indicated the *S*-configuration of C-1 (Figure S2, Supplementary data). Additionally, the *S*-configuration of C-1 was also agreed with a negative Cotton effect at 246 nm ($\Delta\varepsilon$: −4.14) and a positive Cotton effect at 299 nm ($\Delta\varepsilon$: +7.81) in the CD spectrum of **2** as described in the literature.[14] Consequently, structure of compound **2** was established and named as balanophoroside A (Figure 2).

Compound **3** was isolated as a pale-yellow amorphous powder. Its molecular formula was deduced as $C_{26}H_{34}O_{11}$ by a quasi-molecular ion peak at *m/z* 545.1998 in the HR-ESI-MS (calcd. for $C_{26}H_{34}O_{11}Na$, 545.1999). The ^{1}H-NMR spectrum of **3** contained signals for one AX-coupled aromatic proton [δ_H 6.82 and 6.74 (each 1H, s)], one ABX-coupled aromatic proton [δ_H 6.71 (1H, d, *J* = 2.0 Hz), 6.67 (1H, d, *J* = 8.0 Hz), 6.46 (1H, dd, *J* = 2.0, 8.0 Hz)], one anomeric proton [δ_H 4.68 (1H, d, *J* = 7.5 Hz)], and two methoxy groups [δ_H 3.87 and 3.78 (each 3H, s)]. The ^{13}C-NMR spectrum of **3** contained signals for 26 carbons. Of these, 6 carbinol signals at δ_C 102.8, 74.8, 77.8, 71.2, 78.1, and 62.4 were typically recognized for a glucopyranosyl group. Two carbon signals at δ_C 56.8 and 56.4 were assigned for 2 methoxy groups. Remaining 18 carbon signals including 12 aromatic carbons (δ_C 113.4−149.3), 2 hydroxymethylene carbons (δ_C 65.5 and 63.4), 3 methines (δ_C 35.4, 44.6, and 46.7), and 1 methylene (δ_C 33.1) suggested for an aryltetralin lignan backbone, containing 2 hydroxymethylene groups (C-6a and C-7a). The HMBC correlations between H-1 (δ_H 6.74) and C-8 (δ_C 46.7)/ C-3 (δ_C 149.3), methoxy protons (δ_H 3.87), and C-3 indicated location of a methoxy group at C-3. Meanwhile, HMBC correlations between H-4 (δ_H 6.82) and C-5 (δ_C 33.1)/ C-2 (δ_C 146.3), anomeric proton (δ_H 4.68) and C-2 indicated an *O*-glucopyranosyl group at C-2 (Figure S1, Supplementary data). In the 1,3,4-trisubstituted benzene ring, HMBC correlations between H-2′ (δ_H 6.71)/ H-6′ (δ_H 6.46) and C-4′ (δ_C 145.9), H-5′ (δ_H 6.67)/methoxy protons (δ_H 3.78) and C-3′ (δ_C 148.3) suggested for the presence of a hydroxy group at C-4′, a methoxy group at C-3′, respectively. The presence of D-glucose in compound **3** was also confirmed by acid hydrolysis and HPLC analysis of thiocarbamoyl-thiazolidine derivatives (Supplementary data). On the other hand, the close similarity NMR spectral data between **3** and sargentodoside A (**3a**)[15] (Table 2) suggested that the aglycone moiety of **3** and **3a** are a

Table 2. ^{1}H and ^{13}C NMR Data for Compounds **3** and **3a** in CD_3OD.

No.	3 δ_C	3 δ_H (mult., *J* in Hz)	3a $^a\delta_C$
I	118.8	6.74 (s)	120.2
2	146.3	-	146.3
3	149.3	-	149.8
4	113.4	6.82 (s)	113.6
5	33.1	3.01 (dd, 4.5, 17.5) 2.70 (dd, 9.0, 17.5)	33.1
6	35.4	2.08 (m)	35.7
6a	65.5	3..60 (dd, 4.0, 11.0) 3.63 (br d, 11.0)	65.6
7	44.6	2.08 (m)	44.7
7a	63.4	3.54 (dd, 5.0, 11.0) 3.41 (dd, 4.5, 11.0)	62.2
8	46.7	4.29 (br s)	46.8
9	131.8	-	132.3
10	133.4	-	133.5
I′	135.7	-	135.9
2′	115.3	6.71 (d, 2.0)	115.5
3′	148.3	-	148.5
4′	145.9	-	146.1
5′	115.5	6.67 (d, 8.0)	115.7
6′	124.0	6.46 (dd, 2.0, 8.0)	124.2
3-OCH₃	56.8	3.87 (s)	56.6
3′-OCH₃	56.4	3.78 (s)	56.9
2-OGlc			
I″	102.8	4.68 (d, 7.5)	103.5
2″	74.8	3.42 (m)	75.1
3″	77.8	3.37 (m)	78.0
4″	71.2	3.37 (m)	71.1
5″	78.1	3.22 (m)	78.0
6″	62.4	3.76 (br d, 12.0) 3.65 (dd, 5.5, 12.0)	63.6

[a]Data were previously reported in Zeng et al.[15]

pair of enantiomers. Because of containing an aryltetralin chromophore, absolute configurations at chiral carbons C-6, C-7, and C-8 of compound **3** were elucidated by CD analysis, which has been reviewed in many reports.[16] The CD spectrum of **3** observed Cotton effects ($\Delta\varepsilon$: +9.46 (238 nm), +3.32 (275 nm), −1.72 (292 nm)) which were in opposite trend with **3a** ([θ]: −50018 (237 nm), −21053 (274 nm), +5901 (291 nm) for 6*S*,7*R*,8*R* configurations),[15] indicating 6*R*,7*S*,8*S* configurations. Thus, compound **3** was determined to be a new aryltetralin lignan glycoside and named as balanophoroside B (Figure 2).

The anti-inflammatory activity of compounds **1-3** was evaluated by inhibition of the NO production[17] and reducing
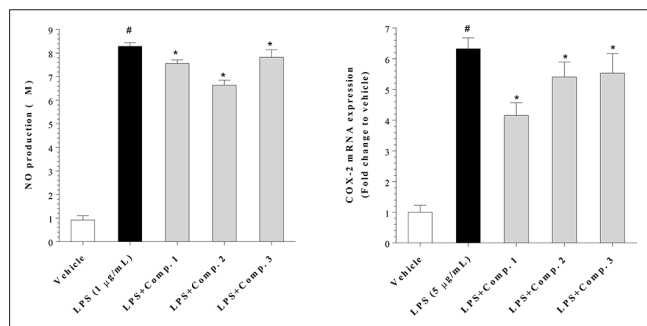
Figure 3. Effect of compounds **1-3** (10 μM) on the NO production and COX-2 expression in LPS-stimulated RAW264.7 cells, $P < 0.05$ vs [#]vehicle or [*]LPS groups.

COX-2 mRNA expression[18] in lipopolysaccharide-stimulated RAW264.7 cells. As shown in Figure 3, the compounds showed weak inhibition in both NO production and COX-2 mRNA expression in the activated RAW264.7 cells at a tested concentration of 10 μM. Their inhibitory values were in the range from 9.91% to 35.61%. Among the three compounds, compound **2** displayed the best NO inhibition (22.39%); meanwhile, compound **1** inhibited higher in the COX-2 mRNA expression (35.61%). At a concentration of 10 μM, all of the compounds (**1-3**) did not show significant cytotoxic effect against RAW264.7 cells by MTT assay.

Experimental

General

Optical rotation was measured on a Jasco P2000 polarimeter. Circular dichroism spectra were acquired on a Chirascan spectrometer. NMR spectra were recorded on Bruker 500 MHz or 600 MHz spectrometer using Tetramethylsilane as an internal standard. High-resolution electrospray ionization mass spectrometry was acquired on an Agilent 6530 Accurate Mass Q-TOF LC/MS system.

Plant Material

The whole plants of *B. laxiflora* Hemsley were collected at Sapa, Lao Cai province, Vietnam in November 2015 and taxonomically determined by botanist Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST. A voucher specimen (NCCT-P01) was deposited at the Institute of Marine Biochemistry, VAST.

Extraction and Isolation

The dried powdered whole plant of *B. laxiflora* (4.0 kg) was extracted with MeOH in ultrasonic bath for 3 times (each 6 L, 30 minutes, at 40°C). After removal of solvent in vacuo, 300 g of crude extract was suspended in 2 L distilled water and separated in turn with dichloromethane and ethyl acetate. Ethyl acetate soluble fraction (110 g) was fractionated on a silica gel column, eluting with dichloromethane/methanol (40/1, 20/1, 10/1, 5/1, 3/1, 1/1, v/v, each 1 L) to give 6 fractions E1-E6. Fraction E3 (9.0 g) was repeatedly chromatographed on a silica gel column, eluting with dichloromethane/acetone (5/1, v/v) to yield 5 fractions E3A-E3E. Fraction E3C was first purified on a reversephase (RP-18) CC, eluting with methanol/water (3/1, v/v) and then futher purified by preparative TLC (dichloromethane/ methanol, 7/1, v/v) to give compound **1** (4 mg). Fraction E5 was subjected on a silica gel column chromatography and eluted with acetone/dichloromethane/water (2/1/0.1, v/v/v) to give 4 fractions E5A-E5D. Fraction E5B was chromatographed using silica gel column and eluent of dichloromethane/ methanol/water (6/1/0.1, v/v/v) to give 3 fractions E5B1-E5B3. Fraction E5B1 was purified on an RP-18 column, eluting with acetone/water (1/2, v/v) to give compound **2** (14 mg). Compound **3** (8 mg) was isolated from fraction E5B3 using RP-18 column and acetone/water (2/5, v/v) as eluent.

Balanolaxin (1)

White amorphous powder.
$[\alpha]_D^{25}$: +19.3 (*c* 0.1, MeOH).
CD (MeOD) $\Delta\varepsilon$ (*λ* nm): −3.31 (220).
IR (KBr): 3427, 2974, 2921, 1711, 1615, 1482, 1393 cm⁻¹.
¹H-NMR (500 MHz, CD₃OD) and ¹³C-NMR (125 MHz, CD₃OD) (Table 1).
HR-ESI-MS: *m/z* 183.1016 [M+H]⁺ (calcd. for $C_{10}H_{15}O_3$, 183.1021).

Balanophroside A (2)

Pale-yellow amorphous powder.
$[\alpha]_D^{25}$: +26.7 (*c* 0.1, MeOH).
CD (MeOD) $\Delta\varepsilon$ (*λ* nm): −4.14 (246), +7.81 (299).
¹H-NMR (600 MHz, CD₃OD) and ¹³C-NMR (150 MHz, CD₃OD) (Table 3).
HR-ESI-MS: *m/z* 459.1274 [M+Na]⁺ (calcd. for $C_{21}H_{24}O_{10}Na$, 459.1267).

Balanophroside B (3)

Pale-yellow amorphous powder.
$[\alpha]_D^{25}$: +41.8 (*c* 0.1, MeOH).
CD (MeOD) $\Delta\varepsilon$ (*λ* nm): −9.38 (206), +3.36 (216), +9.46 (2.38), +3.32 (2.75), −1.71 (292).
¹H-NMR (500 MHz, CD₃OD) and ¹³C-NMR (125 MHz, CD₃OD) (Table 2).
HR-ESI-MS: *m/z* 545.1998 [M+ Na]⁺ (calcd. for $C_{26}H_{34}O_{11}Na$, 545.1999).

Table 3. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Data for Compound **2** in CD$_3$OD.

No.	δ_C	δ_H (mult., J in Hz)	No.	δ_C	δ_H (mult., J in Hz)
1	76.6	5.32 (s)	3″	116.1	6.66 (d, 7.8)
2	210.0	-	4″	157.2	-
3	44.9	3.61 (d, 16.2)	5″	116.1	6.66 (d, 7.8)
		3.56 (d, 16.2)			
1′	120.6	-	6″	131.7	6.88 (d, 7.8)
2′	157.9	-	2′-OGlc		
3′	104.9	6.75 (br s)	1‴	103.5	4.87*
4′	160.6	-	2‴	74.9	3.45 (m)
5′	111.0	6.50 (br d, 7.8)	3‴	78.2	3.46 (m)
6′	132.1	7.07 (br d, 7.8)	4‴	71.7	3.39 (m)
1″	126.4	-	5‴	77.9	3.43 (m)
2″	131.7	6.88 (d, 7.8)	6‴	62.8	3.72 (dd, 5.4, 12.0)
					3.92 (br d, 12.0)

^{a}Overlapped signals, assignment were done by heteronuclear single quantum coherence and HMBC experiments.

Theoretical Calculation of CD Spectra for Compound 1

Conformational searches were performed by Spartan 14 program. Conformations were optimized and subjected to TDDFT calculation using Gaussian 09 program. The calculated CD spectra were combined on the Boltzmann distribution of each stable conformer using SpecDis v1.64 software. Two possible enantiomers of **1** (**1b**: 5*R*,7*S*,8*S*,9*R* and **1c**: 5*S*,7*R*,8*R*,9*S*) were submitted to conformational searches at ground state with semi-empirical AM1 set. The initial stable conformers with Boltzmann distributions over 1.0% (8 conformers) were optimized by DFT calculations at the B3LYP/6-31G(d,p) basic set and polarizable continuum model (PCM) calculation of the solvent methanol. The optimized conformers were then subjected to TDDFT calculations at the B3LYP/6-31G(d,p) level in the presence of methanol as a PCM. The CD spectra at 30 excited states for each conformer were collected and summed to obtain theoretical CD spectra of each enantiomer. Half-band was taken as $\zeta = 0.3$ eV. After UV corrections, the calculated spectra of enantiomers were shifted by 3 nm.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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