SUPPLEMENTARY MATERIAL

α-Costic acid, a plant sesquiterpene with acaricidal activity against *Varroa destructor* parasitizing the honey bee

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ABSTRACT: The organic extract of the aerial parts of *Dittrichia viscosa*, a perennial native plant of the Mediterranean basin, showed a significant acaricidal activity against *Varroa destructor*, the parasite mite of *Apis mellifera*, commonly called honey bee. Among the metabolites isolated from the organic extract of this Asteraceae, α-costic acid showed to be one of the compounds responsible for the toxic activity exhibited by the crude plant extract on this parasite mite species. In addition to the toxic effect a clear acaricidal response has been recorded when the parasitic mite was exposed to 1 mg/mL concentration of α-costic acid while no effects have been showed on honey bees using the same compound at the same concentration. This finding suggests a potential use of α-costic acid to control *Varroa* mites. The possibility to reliably achieve absolute configuration of α-costic acid by DFT computational analysis of chiroptical spectra has been also demonstrated.

KEYWORDS: *Dittrichia viscosa*; sesquiterpenes; α-costic acid; *Varroa destructor*; acaricidal activity; absolute configuration; chiroptical spectroscopy

Experimental

1. General experimental procedures

Optical rotations were measured in $CHCl_3$ on a Jasco P-1010 digital polarimeter (Jasco, Tokyo, Japan). IR spectra were recorded as deposit glass film on a Thermo Nicolet 5700 FT-IR spectrometer (Madison, WI, USA). UV spectra were measured in MeCN and MeOH on a Jasco V-530 spectrophotometer (Jasco, Tokyo, Japan). The ECD and UV-vis spectra were recorded on a Jasco J-815 spectropolarimeter in the 180-350 nm range in acetonitrile at a concentration $c = 2.58\times10^{-3}$ M and in a cell with path-length of 0.5 mm. ORD curve was measured on a Jasco Dip-370 digital polarimeter at concentration of 0.375 g/100 mL in $CHCl_3$. ^{1}H- and ^{13}C-NMR spectra were recorded at 400 and 100 MHz, respectively, in $CDCl_3$ on Bruker (Karlsruhe, Germany) spectrometer unless otherwise noted. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectrum. DEPT, COSY-45, HSQC and HMBC experiments were performed using Bruker microprograms. HRESIMS (High Resolution ElectroSpray Ionization Mass Spectroscopy) and ESIMS (ElectroSpray Ionization Mass Spectroscopy), spectra were recorded on an Agilent 6120 Quadrupole LC/MS instrument (Agilent Technologies, Milan, Italy). Analytical and preparative TLC (Thin Layer Chromatography) were performed on silica gel (Kieselgel 60, F254, 0.25 and 0.5 mm respectively) plates. The spots were visualized by exposure to UV radiation (253 nm) or by spraying first with 10% H_2SO_4 in MeOH

and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed using silica gel (Merck, Kieselgel 60, 0.06–0.200 mm).

2. Plant material

Dittrichia viscosa was collected in Apulia region of southern Italy by Dr. Maurizio Vurro and Dr. Nicola Montemurro. A sample of the plant is available at the Istituto di Scienze delle Produzioni Alimentari, Via Amendola 122/O, 70125 Bari, Italy.

3. Extraction and purification of **D. viscosa** *metabolites*

The aerial parts of *D. viscosa* were finely minced in a blender. The resulting material (500 g) was extracted in H_2O-MeOH (1:1, v:v) and the methanolic aqueous phase was extracted with CH_2Cl_2 as recently reported (Andolfi et al. 2013). The combined CH_2Cl_2 phases were dried (Na_2SO_4) and evaporated under reduced pressure. The residue (7.65 g) was purified first by silica gel column eluted with $CHCl_3$-*iso*-PrOH (95:5, v:v) and nine groups of homogeneous fractions were obtained. The second and third fractions, contained the main metabolite. The residues of these two fractions were combined (1 g) and purified by a RP-18 column at medium pressure, using H_2O-MeCN (1:1, v:v) as solvent system and giving three fractions. The two lesser polar fractions, both obtained as homogeneous yellow oil, contained the main metabolite identified as below reported as α-costic acid (**1**, 568 mg, 1.14 g/kg) and another oily homogeneous metabolite, which was identified as inuloxin A (**2**, 131 mg, 262.5 mg/kg) (Andolfi et al. 2013). The residue of more polar fractions from the first column were further purified by a combination of CC and TLC on silica gel eluted with $CHCl_3$-*iso*-PrOH (95:5, v:v) and *n*-hexane-EtOAc (55:45, v:v) yielding a oily compound identified as inuloxin C (**3**, 40 mg, 80 mg/kg) (Andolfi et al. 2013).

4. α-costic acid (1)

Compound **1**: $[\alpha]^{25}_D$ + 15 (*c* 0.5, $CHCl_3$); IR ν_{max} 3403, 2906, 1687, 1618, 1278 cm^{-1}; UV λ_{max} nm (log ε) 225 (3.38) [lit. Shatacher and Kashman 1970: $[\alpha]^{25}_D$ + 10 (*c* 0.8, $CHCl_3$); IR ν_{max} 2900, 2830, 1690, 1615, 1435, 1365, 968 cm^{-1}; lit. Chen et al., 2001: $[\alpha]^{25}_D$ + 8 (*c* 0.24, $CHCl_3$); IR ν_{max} 2916, 2848, 1696, 1621, 1436, 1278 cm^{-1}]; ^1H and ^{13}C NMR: see Table S1; ESIMS (+) *m/z*: 235 $[M + H]^+$, 189 $[M - COOH]^+$.

5. Methyl ester of α-costic acid (4)

An ethereal solution of CH_2N_2 was added to a solution of **1** (15.0 mg) in MeOH (1 mL) to obtain a persistent yellow color. The reaction was carried out at room temperature under stirring and was stopped after 1 h by evaporation under an N_2 stream. The crude residue (15.5 mg) was purified by preparative TLC, using $CHCl_3$ as eluent, to give 10.0 mg of the α-costic methyl ester (**4**) as yellowish oil. **4** had: $[\alpha]^{25}_D$ +42.3 (*c* 0.1, $CHCl_3$) [lit. Zaki et al. 2015: $[\alpha]^{20}_D$ +26.8 (*c* 1.0, CH_2Cl_2)]; ^{1}H NMR: see Table S1; HR ESIMS, *m*/*z* 249.1844 $[M + H]^+$ (calc. for $C_{16}H_{25}O_2$ 249.1855).

6. Computational section

Preliminary conformational analysis was performed by Spartan02 package employing MMFF94s molecular mechanics (MM) force field with Monte Carlo searching and assuming the (*5S, 7R, 10R*) absolute configuration for **4**. All possible conformers were searched, considering the degrees of freedom of the system within a range of 30 kcal/mol and retaining only the structures in an energy range of 10 kcal/mol with respect to the most stable one. The minimum energy conformers found by MM were further fully optimized by Gaussian09 package (Frisch et al. 2009) using the Density Functional Theory (DFT) at the DFT/M06/6-311+(2d,2p) level either in gas phase or in MeCN and $CHCl_3$ by IEFPCM solvation model (Tomasi et al. 2005). All conformers are real minima, no imaginary vibrational frequencies have been found and the free energy values have been calculated and used to get the Boltzmann population of conformers at 298.15 K. Optical rotations were computed by using the DFT/B3LYP/aug-cc-pVDZ level on the DFT optimized geometries. The latter were also employed as input geometries for calculation of UV and ECD spectra at the Time Dependent DFT (TDDFT) TDDFT/CAM-B3LYP/aug-cc-pVDZ level and taking into account the lowest 30 states. UV, ECD, and ORD were computed both in gas phase and by IEFPCM implicit solvation model in MeCN and $CHCl_3$. TDDFT calculations employing the long-range corrected CAM-B3LYP functional (Yanai et al. 2004) provided good reproduction of Cotton effects observed in the theoretical ECD spectra (Evidente et al. 2011). The theoretical ORD, UV, and ECD spectra were obtained as average over the conformers Boltzmann populations. The ECD spectra were obtained from calculated excitation energies and rotational strengths, as a sum of Gaussian functions centered at the wavelength of each transition, with a parameter σ (width of the band at ½ height) of 0.3 eV using SpecDis v1.60 program (Bruhn et al. 2013).

7.* V. destructor *mites collection

Varroa mites have been collected from non-treated bee hives, in an organic farm near to Rome: 42°02'07.8"N; 012°19'41.8"E. One honeycomb has been removed and brought to BBCA facilities. Some of the cells have been open removing the cap of the cover and searching for bee pupal stages infested with the mite. For uncapping the combs and the following *Varroa* mite collection, small entomological soft forceps and a small wet brush have been used. Then the mites were transferred into small Petri dishes (14 cm of diameter) together with some bee pupae as food substrate.

8. Bioassays on Varroa *mites*

The experiment was carried out under laboratory condition exposing 10 mites per extract/compound, replicated 3 or 4 times. The full extract of *D. viscosa* was assayed only at the concentration of 3 mg/mL. The five compounds were first assayed at the concentration of 1 mg/mL, and those showing some active response at this concentration have also been assayed at lower concentrations (0.5 and 0.25 mg/mL, respectively). In each replication 200 microliter aliquots of a stock solution, dissolving the compounds in DMSO (final concentration 2%) and physiological solution of sodium chloride (0.9%), were placed on surface of filter paper in a Petri dish (diameter of 3.5 cm). A solution at 2% of DMSO was used as a negative control. In each Petri dish, 10 mites were placed on the surface of the filter paper favoring direct contact with the solution. The dishes were sealed with parafilm and transferred in incubators at 25°C. After 24 h of treatment, mortality was evaluated by removing the Petri dish cover and touching each *Varroa* with a small brush: those mites that didn't move were transferred into a Petri containing a dry filter paper disk on the bottom to confirm their physiological status. After 1 hour, those *Varroa* mites that were not able to move along a distance equal to or greater than the length of their body after being touched with a brush, were considered as death (Milani 1995).

9. Bioassay on bees

The test on bees was carried out in the first week of April 2019, using bees from the same colony and the same collection site of *Varroa* mites, and fed with a solution of water and sugar for 24 hours before carrying out the experiments. Only the α-costic acid was tested because it was the most active on *Varroa* at the concentration of 1 mg/mL. In each replication filter papers treated with 2 mL of the solution prepared dissolving the compound in DMSO (final concentration 2%) and water, were placed inside glass jar of 500 mL. A solution at 2% of DMSO was used as a negative control. Groups of ten bees were introduced inside glass jar and covered with muslin. Individuals placed in

these jars were therefore exposed to topical, vapor, and potentially oral applications of the treatments. A solution of water and sugar (50%) was supplied for each glass jar inside a petri dish cover of 3.5 cm diameter, and supplied *ad libitum*. Three replicates for each experimental unit were made. The glass jars were kept in an incubator at 29 °C and 70% RH. The mortality was evaluated every 24 hours for three days. The validity of the experiment can be confirmed because the mortality of the negative control was < 10% (OECD, 1998; CEB, 2011), while the mortality of the α-costic acid test was 10%.

10. Statistical analysis

Statistical analyses were performed by using PASW Statistics 17, Release Version 17.0.2 (SPSS Inc., 2008). As regards mites mortality, a generalized linear model was used in order to evaluate multiple factors in the case of data showing a non-normal distribution (Madsen and Thyregod, 2010; Marshall and Sinclair, 2009). Data were analysed within and between treatments by a generalized linear model having a binary error distribution, according to a Bernoulli distribution corresponding to 2 possible events, dead or survived (Hazewinkel 2001) and a logit link function was applied in SPSS PASW. The effects of the compound and concentration were considered in the analysis.

Table S1. ^{1}H, ^{13}C NMR and HMBC data of the α-costic acid (**1**) and ^{1}H NMR data of its methyl ester (**4**).[a,b]

1				**4**
Position	δC[c] c	δH (*J* in Hz)	HMBC	δH (*J* in Hz)
1	37.8 t	1.36 (2H) m	H-6B	1.36 (2H) m
2	23.4 t	2.09 m	H_2-3	2.09 m
		1.96 m		1.96 m
3	121.1 d	5.32 br s	Me-4	5.32 br s
4	134.8 s		H_2-2, Me-14	
5	46.8 d	2.02 m	H_2-2, Me-4, H-6A, H_2-9, Me-10	2.01 m
6	40.1 t	1.27 q (13.1)	H-13B	1.16 q (12.2)
		1.46 dt (13.1, 3.7)		1.47 dt (12.2, 3.7)
7	40.0 d	2.42 br t (13.1, 12,6, 3.7)	H_2-13, H-5, H_2-8, H-6A	2.52 br t (12.6, 12.2, 3.7)
8	29.7 t	1.68 br d (13.0)	H-7, H-9A, H_2-6	1.65 br d (13.0)
		1.56 ddd (13.0, 12.6, 3.7)		1.54 ddd (13.0, 12.6, 3.7)
9	27.4 t	1.86 br d (12.6)	H.5, H.8A, Me-10	1.86 br d (12.6)
		1.28 td (12.6, 2.7)		1.26 td (12.6, 3.7)
10	32.3 s		H-9A, H-8B, H-2B	
11	145.1 s		H-13A, H-7	
12	171.9 s		H_2-13	
13	125.4 t	6.32 s	H-7	6.14 s
		5.68 s		5.57 s
Me-14	15.7 q	0.84 s	H-6B, H-5, H-2B	0.82 s
Me-13	21,1 q	1.62 s		1.60 s
OMe				3.76 s

[a]The chemical shift are in δ-values (ppm) from TMS; [b]2D ^{1}H, ^{1}H (COSY) ^{13}C, ^{1}H (HSQC) NMR experiments delineated the correlations of all the protons and the corresponding carbons; [c]Multiplicities were assigned by DEPT spectrum.

Table S2. Conformers Boltzmann distribution of (5*S*,7*R*,10*R*)-**4**.

	DFT/M06/6-311+(2d,2p)					
	Gas Phase		IEFPCM/CH_3CN		IEFPCM/$CHCl_3$	
Conformers	ΔG (Kcal/mol)	% Pop	ΔG (Kcal/mol)	% Pop	ΔG (Kcal/mol)	% Pop
1	0.000	13.2	0.00	6.0	0.000	4.7
2	-0.432	27.5	-0.84	25.0	-0.867	20.2
3	-0.545	33.2	-1.16	42.6	-1.472	55.8
4	-0.043	14.3	-0.39	11.7	-0.388	9.0
5	0.278	8.3	-0.40	11.8	-0.324	8.0
6	0.793	3.5	0.43	2.9	0.417	2.3

Table S3. Experimental and calculated Optical Rotations (ORs) for **4**.

Wavelength (nm)	Experimental ORs[a] of (+)-**4**	Calculated ORs[b] for (5*S*,7*R*,10*R*)-**4**	
	$CHCl_3$	Gas phase	$CHCl_3$
589	24.5	4.2	66.2
546	29.2	4.8	78.6
435	52.1	5.9	134.1
405	62.1	5.7	160.2

[a]Optical rotations recorded in $CHCl_3$ (*c* 0.056 g/100 mL). [b]Optical rotations obtained as Boltzmann average at TDDFT/B3LYP/aug-cc-pVDZ level in gas phase and, by IEFPCM, in $CHCl_3$.

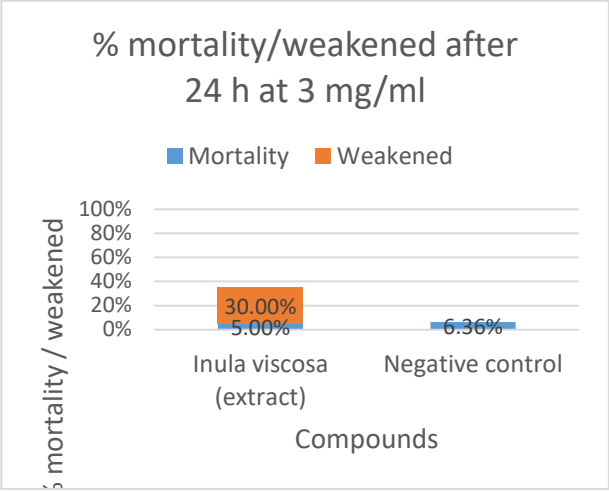

Figure S1. Graph showing the percentage of mortality (blue) and weakened (red) mites after 24 h treatment with the *D. viscosa* extract tested at 3 mg/mL compared to the negative control.

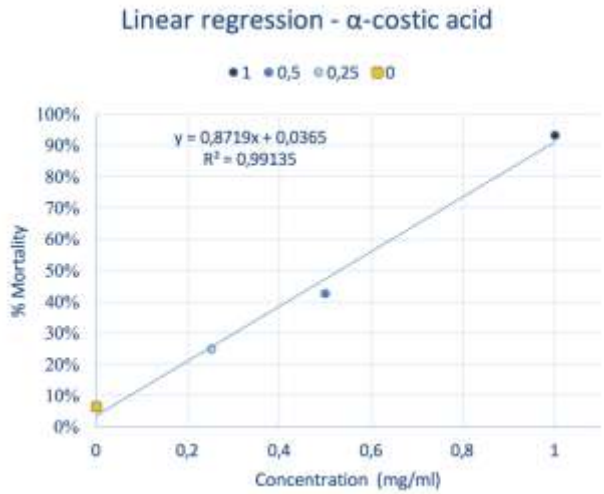

Figure S2. Linear regression referred to α-costic acid (**1**) tested at tested at three different concentrations. The square yellow represents the value of negative control.

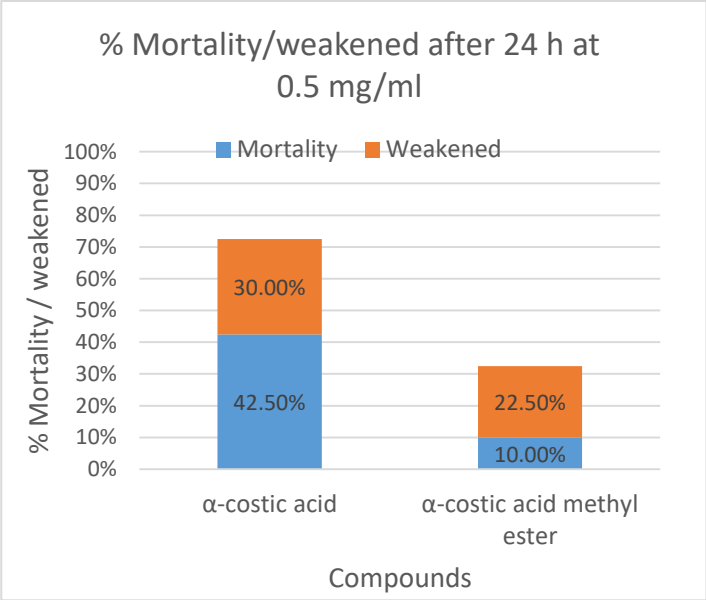

Figure S3. Combination of mortality and weakeness effect after 24 h at the concentration of 0.5 mg/mL.

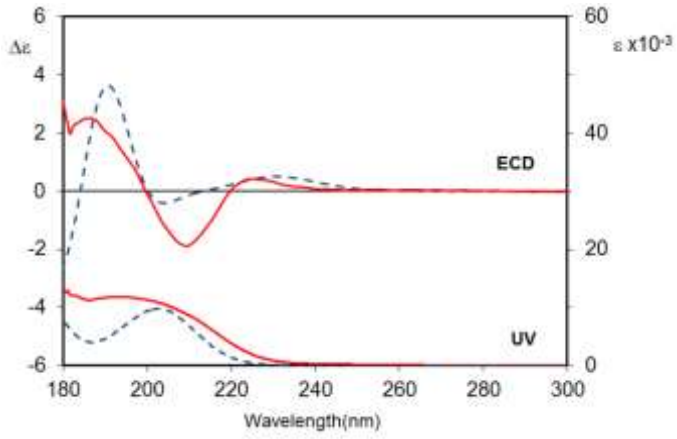

Figure S4. Experimental UV and ECD spectra of (+)-**4** (solid red line, MeCN) and calculated UV and ECD spectra for (5*S*,7*R*,10*R*)-**4** (dashed blue line, TDDFT/CAM-B3LYP/aug-cc-pVDZ/IEFPCM/CH_3CN// DFT/M06/6-311+(2d,2p)/IEFPCM/CH_3CN). Theoretical spectra are blue-shifted by 5.0 nm.

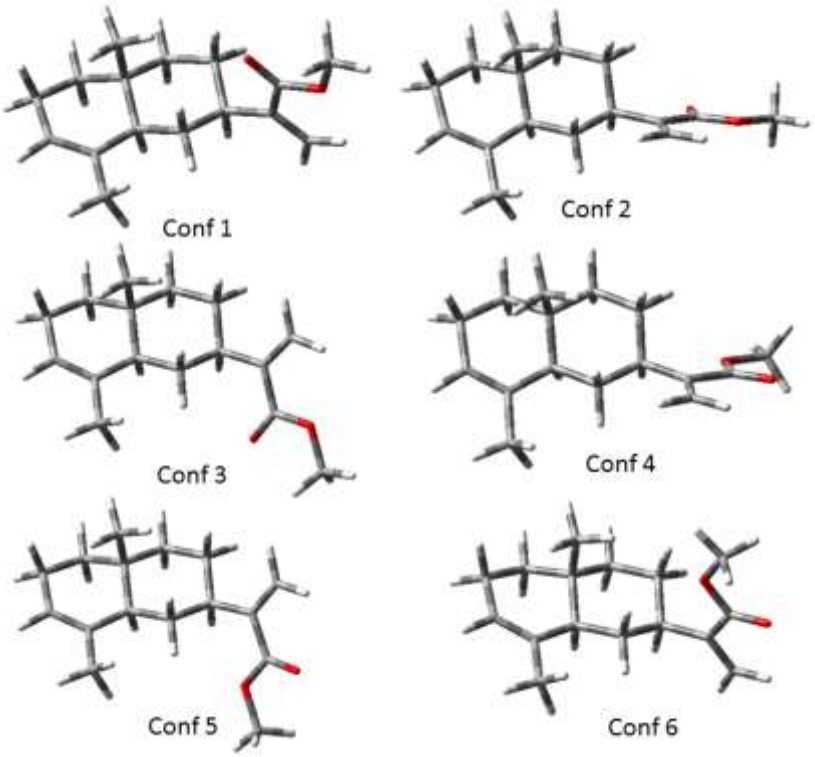

Figure S5. Most stable geometry optimized structures of (5*S*,7*R*,10*R*)-**4**. See Table S1 for relative energies and populations.

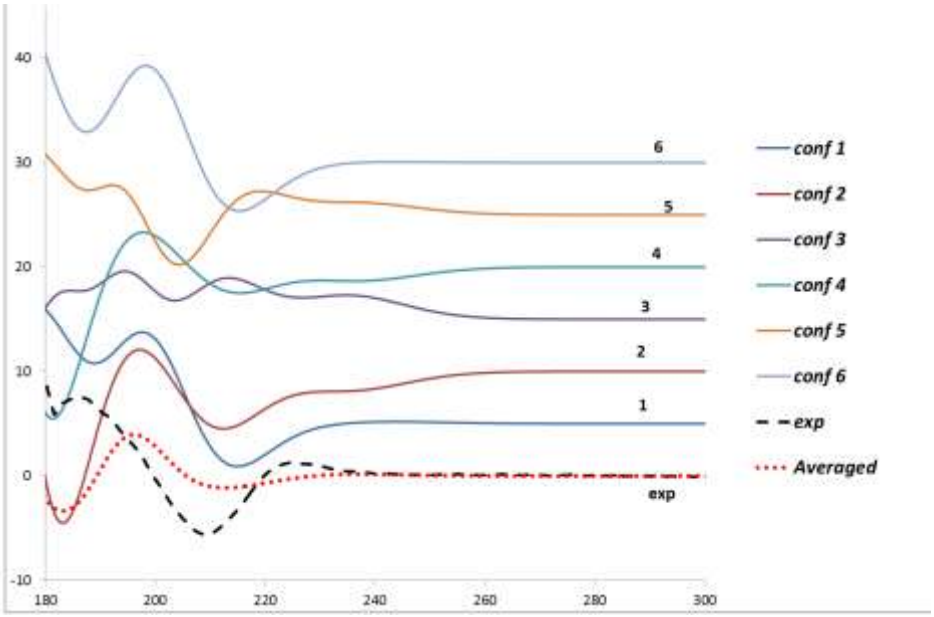

Figure S6. Experimental UV and ECD spectra of (+)-**4** (dashed black line, acetonitrile) and calculated UV and ECD spectra for single conformers of (5*S*,7*R*,10*R*)-**4** (TDDFT/CAM-B3LYP/aug-cc-pVDZ/IEFPCM/CH_3CN//DFT/M06/6-311+(2d,2p)/gas phase).

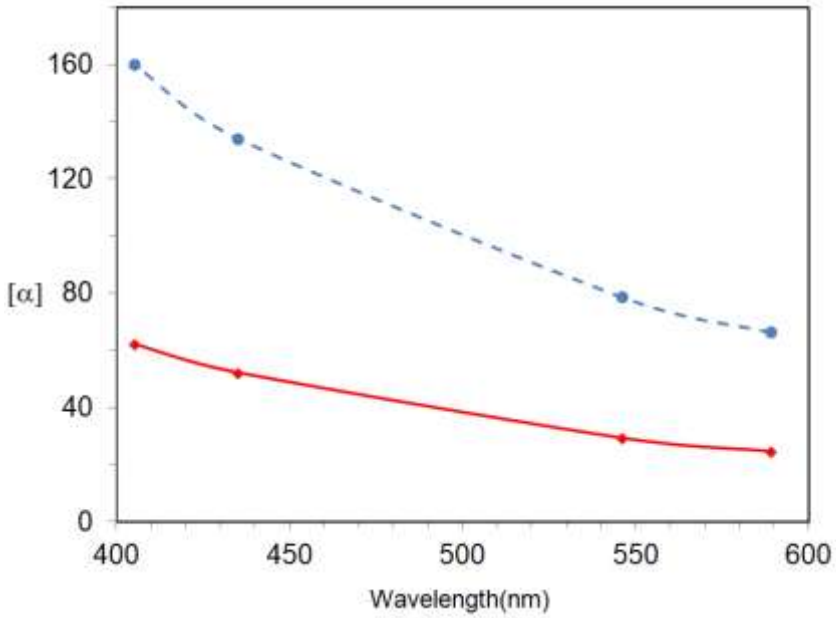

Figure S7. Experimental ORD of (+)-**4** (solid red line, $CHCl_3$) and calculated ORD for (5*S*,7*R*,10*R*)-**3** (dashed blue line, DFT/B3LYP/aug-cc-pVDZ/ IEFPCM/$CHCl_3$//DFT/M06/6-311+(2d,2p)/IEFPCM/$CHCl_3$).

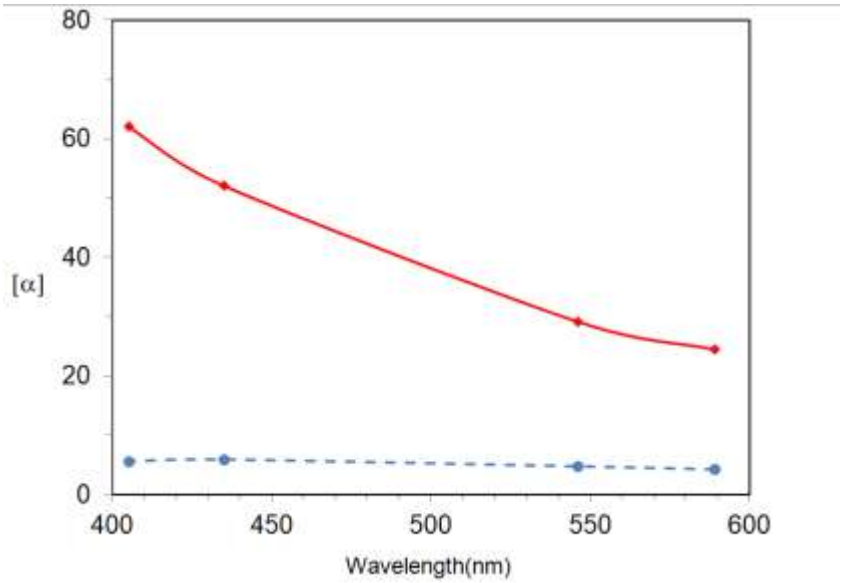

Figure S8. Experimental ORD of (+)-**4** (solid red line, $CHCl_3$) and calculated ORD for (5*S*,7*R*,10*R*)-**3** (dashed blue line, DFT/B3LYP/aug-cc-pVDZ/IEFPCM/$CHCl_3$//DFT/M06/6-311+(2d,2p)/gas phase).

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