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Higginsianins D and E, Cytotoxic Diterpenoids Produced by *Colletotrichum higginsianum*

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2 ABSTRACT

3 Two new diterpenoids with tetrasubstituted 3-oxodihydrofuran substituents, named higginsianins D
4 (**1**) and E (**2**), were isolated from the mycelium of the fungus *Colletotrichum higginsianum* grown
5 in liquid culture. They were characterized as methyl 2-[6-hydroxy-5,8a-dimethyl-2-methylene-5-(4-
6 methylpent-3-enyl)-decahydronaphthalen-1-ylmethyl]-4,5-dimethyl-3-oxo-2,3-dihydrofuran-2-
7 carboxylate and its 21-epimer by using NMR, HRESIMS, and chemical methods. The relative
8 configurations of higginsianins D and E, which did not afford crystals suitable for X-ray analysis,
9 were determined by NOESY experiments and by comparison with NMR data of higginsianin B.
10 The absolute configuration was established by comparison of experimental and calculated
11 electronic circular dichroism data. The evaluation of **1** and **2** for antiproliferative activity against
12 human A431 cells derived from epidermoid carcinoma and H1299 non-small-cell lung carcinoma
13 cells revealed that **2** exhibited higher cytotoxic activity than **1**, with an IC₅₀ value of 1.0 μM against
14 A431 cells. Remarkably, both **1** and **2** were almost ineffective against immortalized keratinocytes,
15 used as a preneoplastic cell line model.

16 *Colletotrichum* is a fungal genus comprising a large number of endophytic, saprophytic, and
17 plant pathogenic species. The latter are responsible for severe diseases to many crops, such as
18 peaches, apples, pecans, and other hosts (Bernstein et al., 1995) and are considered some of the
19 most harmful species in agriculture (Dean et al., 2012). However, the production of phytotoxic
20 secondary metabolites potentially involved in plant pathogenesis by various *Colletotrichum* species
21 is only partially explored. Among these, the phytotoxic metabolites named colletochlorins and
22 colletorins, grouped in prenyl or diprenyl orsellinaldehyde derivatives, were isolated from
23 *Colletotrichum tabacum* (synonym of *Colletotrichum nicotianae*), causing anthracnose in tobacco
24 (García-Paión et al., 2003), and *Colletotrichum gloeosporioides*, a fungus proposed for biocontrol
25 of *Ambrosia artemisiifolia* (Masi et al., 2018). The genus is also interesting for the capability of the
26 species to produce a wide array of secondary metabolites possessing various biological properties
27 (Kim et al., 2019). During a preliminary screening carried out on 89 strains belonging to many
28 species of the genus *Colletotrichum* and aimed at finding novel bioactive metabolites, a strain of
29 *Colletotrichum higginsianum*, belonging to the *Colletotrichum destructivum* species complex
30 (Damm et al., 2014), was selected because its culture filtrate showed phytotoxic activity, while the
31 EtOAc extract of its mycelium showed promising anticancer activity (Cimmino et al., 2016; Masi et
32 al., 2017a). *C. higginsianum* is the causal agent of anthracnose leaf spot disease of several
33 Brassicaceae crop species. This disease was also recently attributed to *Colletotrichum capsici*,
34 causing anthracnose on bok choy (*Brassica chinensis*) in Malaysia (Mahmodi et al., 2013), or to
35 *Colletotrichum truncatum*, found to cause severe anthracnose of Chinese flowering cabbage
36 (*Brassica parachinensis*) (He et al., 2016).

37 From the culture filtrate of *C. higginsianum* a tetrasubstituted pyran-2-one and a
38 tetrasubstituted dihydrobenzofuran, named colletochlorins E and F, respectively, were isolated
39 together with the known chlorinated 3-diprenylorsellinaldehyde derivative colletochlorin A, 4-
40 chlororcinol, and colletopyrone. Colletochlorin F and 4-chlororcinol showed significant activity on
41 both weedy and parasitic plants, while colletochlorin A and colletopyrone showed modest activity

42 (Masi et al., 2017a). Subsequently, a new tetrasubstituted indolyidenepyranone named
43 colletopyranone and a tetrasubstituted chroman- and a tetrasubstituted isochroman-3,5-diol,
44 named colletochlorins G and H, respectively, were isolated from the culture filtrates of the same
45 fungus. Only the colletopyranone showed modest phytotoxicity (Masi et al, 2017b).

46 The bioguided purification of the EtOAc extract obtained from the mycelium of *C.*
47 *higginsianum* led to the isolation of two new diterpenoids having an α -pyrone moiety located at C-
48 4, named higginsianins A and B (Cimmino et al., 2016). In a preliminary evaluation against a six
49 cancer cell panel together with three semisynthetic derivatives prepared from higginsianin A, both
50 higginsianins A and B showed promising cytostatic rather than cytotoxic activity, while the activity
51 of the derivatives provided the first structure–activity relationship correlations (Cimmino et al.,
52 2016). Further investigations have been carried out on the anticancer activity of higginsianins A and
53 B, and they have been demonstrated to induce cell cycle arrest in the S-phase associated with an
54 increase of γ H2AX positive nuclear foci, indicating the occurrence of DNA lesions (Sangermano et
55 al., 2019).

56 The *CclA* subunit of the COMPASS complex mediates the trimethylation of the lysine unit at
57 position 4 of histone H3 (H3K4). Such epigenetic modification plays a critical role in regulating
58 fungal growth, development, pathogenicity, and secondary metabolism in *C. higginsianum*. It was
59 recently shown that a *C. higginsianum* strain with a deleted version of *CclA* ($\Delta cclA$) exhibited
60 strongly reduced mycelial growth and spore germination as well as attenuated virulence on plants
61 (Dallery et al. 2019a; Dallery et al. 2019b). The secondary metabolite profile of the $\Delta cclA$ mutant
62 was different with respect to that of the wild type, with the presence of other metabolites belonging
63 to the three different families of terpenoids, namely, the colletochlorins, higginsianins, and
64 sclerosporide. From the liquid culture of the mutant $\Delta cclA$ were also isolated the new higginsianin
65 C and 13-*epi*-higginsianin C, sclerosporide, colletorin D, and colletorin D acid (Dallery et al. 2019a;
66 Dallery et al. 2019b).

67 Further investigation of the EtOAc extract obtained from the mycelium of *C. higginsianum*
68 permitted isolation of two new metabolites structurally related to higginsianins, which were named
69 higginsianins D and E, bearing an unusual trisubstituted 2-carboxymethyldihydrofuran-3-one
70 moiety located at C-21. This article reports the isolation and the chemical and biological
71 characterization of higginsianins D and E.

72

73 RESULTS AND DISCUSSION

74 The EtOAc extract of the *C. higginsianum* mycelium was further investigated. The chromatographic
75 purification as reported in the Experimental Section led to the isolation of the known cytotoxic α -
76 pyrone diterpenoids higginsianins A and B (**3** and **4**, Figure 1) (Cimmino et al., 2016; Sangermano
77 et al., 2019) and the new natural products named higginsianins D (**1**) and E (**2**).

78 Preliminary investigation of the ^1H and ^{13}C NMR data of **1** and **2** showed that they share
79 similar structures and are related to **4**, while their HRESIMS spectra showed the same sodium
80 adduct ion from which the molecular formula of $\text{C}_{28}\text{H}_{42}\text{O}_5$ and eight hydrogen deficiencies were
81 deduced. These findings were corroborated by the bands observed in the IR spectra for hydroxy,
82 carbonyl, and olefinic groups (Nakanishi and Solomon, 1977), while the UV spectra showed bands
83 typical of a conjugated carbonyl group (Pretsch et al., 2000). However, a noteworthy difference was
84 observed for the moiety attached to C-4 when **1** and **2** were compared to **4**.

85 The ^1H NMR and COSY spectra of higginsianin D (**1**) (Table 1) showed a broad triplet (J
86 = 7.1 Hz) and two coupled broad singlets, due to the protons of a trisubstituted olefinic and an
87 exocyclic methylene group at δ 5.15 (H-13) and 4.69 and 4.51 (H₂C-19), the broad singlet of a
88 proton of a secondary oxygenated carbon (HC-8) at δ 3.62, and the singlet of a methoxy group at δ
89 3.67. Furthermore, the signals for four vinylic methyl groups at δ 2.26, 1.68, 1.72, and 1.66 (Me-27,
90 Me-26, Me-15, and Me-16), with the latter two coupled with H-13, were observed. Two other
91 singlets, due to two tertiary aliphatic methyl groups (Me-18 and Me-17), resonated at δ 0.96 and
92 0.82. The C-8 proton coupled with the protons of the adjacent methylene group (H₂C-7), resonating

93 as a triplet of doublets ($J = 14.3$ and 2.2 Hz) at δ 2.15 and a multiplet at δ 1.94, being also coupled
94 with the multiplets of the protons of the adjacent methylene group ($\text{H}_2\text{C}-6$) observed at δ 1.60 and
95 0.81. The H-10 multiplet at δ 1.59 coupled with protons of the adjacent methylene group ($\text{H}_2\text{C}-1$)
96 that resonated as two multiplets at δ 1.60 and 1.33. These latter protons ($\text{H}_2\text{C}-1$) also coupled with
97 the protons of the adjacent methylene group ($\text{H}_2\text{C}-2$) present as a triplet of doublets ($J = 13.9$ and
98 5.8 Hz) at δ 2.38 and a doublet of doublets of doublets ($J = 13.9$, 4.4 , and 1.6 Hz) at δ 2.18.
99 Furthermore, the multiplet of an aliphatic methine (HC-4), observed at δ 1.96, coupled with the
100 protons of the adjacent methylene group ($\text{H}_2\text{C}-20$) resonating as a doublet of doublets ($J = 14.6$ and
101 10.8 Hz) and a multiplet at δ 2.53 and 2.12, respectively. Finally, the olefinic H-13 also coupled
102 with the protons of the adjacent methylene group ($\text{H}_2\text{C}-12$) appearing as two multiplets at δ 1.95
103 and 1.61, which also coupled with the protons of the adjacent methylene group ($\text{H}_2\text{C}-11$) observed
104 as a multiplet at δ 1.27. These findings suggested the presence of hexasubstituted decalin and 4-
105 methylpent-3-en-1-yl moieties in **1** similar to those observed in **4**. In fact, the couplings observed in
106 the HSQC spectrum (Berger and Braun, 2004) permitted assignment of the signals to the protonated
107 carbons in the ^{13}C NMR (Table 1) spectrum and in particular those observed at δ 124.9, 110.4, 71.9,
108 52.7, 52.3, 39.9, 39.5, 38.1, 31.0, 25.6, 25.5, 22.7, 22.5, 18.8, 17.8, 14.8, and 5.9 to C-13, C-19, C-
109 8, C-4, OMe, C-10, C-11, C-5, C-2, C-15, C-12, C-1, C-17, C-18, C-16, C-27, and C-26,
110 respectively (Breitmaier and Voelter, 1987).

111 The ^{13}C NMR spectrum also showed signals typical of two carbonyls, two sp^3 and four sp^2
112 quaternary carbons, and an oxygenated tertiary carbon, which were assigned on the basis of
113 couplings observed in the HMBC spectrum (Berger and Braun, 2004) (Table 1). In fact, C-22
114 coupled with Me-26, C-24 with Me-26 and Me-27, C-25 with H-20B and OMe, C-3 with H₂-20 and
115 H-4, C-14 with Me-15 and Me-16, C-23 with Me-26 and Me-27, C-21 with H₂-20, C-9 with Me-17,
116 and C-5 with Me-18. Thus, the signals at δ 198.8, 185.1, 166.1, 147.8, 131.8, 109.5, 89.9, 39.4, and
117 38.1 were assigned to C-22, C-24, C-25, C-3, C-14, C-23, C-21, C-9, and C-5, respectively
118 (Breitmaier and Voelter, 1987). These finding supported the presence of a hexasubstituted decalin

119 moiety as in **4** but also showed the absence of the α -pyrone moiety that is replaced by a
120 tetrasubstituted 3-oxodihydrofuran-2-one moiety carrying a methoxycarbonyl group at C-21. Such a
121 ring structure is extremely rare among natural products. It has been previously found only in some
122 spiroditerpenoids where the dihydrofuran-2-one ring is part of the spiro moiety (Fujimoto et al.,
123 1996; Kwon et al., 2015).

124 Thus, the chemical shifts were assigned to all the protons and corresponding carbons as
125 reported in Table 1, and the structure of **1** was defined as methyl 2-[6-hydroxy-5,8-dimethyl 1-2-
126 methylene-5-(4-methylpent-3-enyl)-decahydronaphthalen-1-ylmethyl]-4,5-dimethyl-3-oxo-2,3-
127 dihydrofuran-2-carboxylate. The structure assigned to **1** was confirmed by the HMBC couplings
128 shown in Table 1 and its HRESIMS data. The latter showed the sodiated adduct and protonated
129 dimers $[2M + Na]^+$ and $[2M + H]^+$, the sodium adduct $[M + Na]^+$, and protonated $[M + H]^+$ ions at
130 m/z 939, 917, 481, and 459.3129, respectively.

131 Higginsianin E (**2**) has the same molecular formula as **1** and showed similar IR, UV, and 1H
132 and ^{13}C NMR spectra. In particular, the 1H NMR spectra of **1** and **2** differed with respect to the
133 shielded ($\Delta\delta$ 0.33) and deshielded ($\Delta\delta$ 0.42) shifts of the C-20 methylene protons. These results
134 suggested that higginsianin E (**2**) is a diastereomer of **1**, in particular, its epimer at C-21.

135 The relative configurations of **1** and **2** were deduced from their NOESY data (Table 2)
136 (Berger and Braun, 2004). In particular, significant crosspeaks were observed between H-4 and Me-
137 18, H-8 and Me-17, and H-7A, Me-17 and Me-18, confirming in both isomers the same relative
138 configuration at C-4, with H-4 oriented equatorial and cis to Me-18.

139 To definitely confirm the unusual structure of the dihydrofuran-3-one moiety, we performed
140 NMR calculations using density functional theory (DFT) with the gaugeindependent atomic orbital
141 (GIAO) method. Because of the pronounced conformational flexibility of **1** and **2**, the structures
142 employed for NMR calculations were cut at the C-11/C-12 bond; that is, the chain attached at C-9
143 was replaced by a methyl group. Moreover, a computational protocol was employed purposely
144 developed for the prediction of ^{13}C NMR chemical shifts of flexible compounds (Hehre et al.,

145 2019). The protocol consists of NMR calculations run at the ω B97X-D/6-31G(d) level with an
146 empirical chemical shift correction; the input structures are generated with a sequence of steps with
147 final ω B97X-V/6-311+G(2df,2p)// ω B97X-D/6-31G(d) energy estimation and geometry
148 optimization. The protocol leads usually to overall rms (root-mean-square) errors below 2 ppm
149 between experimental and calculated ^{13}C chemical shifts (Hehre et al., 2019). In the current case,
150 the protocol produced a set of ^{13}C signals in good agreement with the experimental spectra for both
151 **1** and **2**. Focusing only on the dihydrofuran-3-one moiety the rms error was 1.75 ppm for (21*S*)-**1**
152 and 1.85 ppm for (21*R*)-**2** (see Supporting Information). When the two isomeric structures were
153 switched, the rms errors were, however, highly similar (1.7 and 1.9 ppm, respectively). Thus, NMR
154 calculations confirmed the structures proposed for **1** and **2**, but they were not sufficient to
155 distinguish the configuration at C-21.

156 This latter piece of information, together with the absolute configuration of **1** and **2**, could
157 eventually be obtained from experimental and calculated ECD data. The experimental ECD spectra
158 of **1** and **2** measured in MeCN were almost mirror images over the measured range (Figure 2 vs
159 Figure 3, bottom panels, solid traces), while the corresponding absorption UV spectra were almost
160 superimposable (Figures 2 and Figure 3, top panels). This simple fact itself reinforces the hypothesis
161 that higginsianins D and E have opposite configurations at C-21, which is the center of chirality
162 closest to the main chromophore, namely, the substituted enone included in the dihydrofuran-3-one
163 ring. To simulate the ECD spectra (Pescitelli and Bruhn, 2016; Superchi et al., 2018), low-energy
164 structures found during NMR calculations were reoptimized at the ω B97X-D/6-311+G(d,p)/PCM
165 level and employed as input in time-dependent DFT calculations run at the ω B97X-D/def2-
166 TZVP/PCM level, including in both cases a polarizable continuum solvent model for MeCN.
167 Despite the presence of several low-energy minima, the final Boltzmann-averaged UV and ECD
168 spectra agreed well with the experimental ones (Figures 2 and 3, solid traces vs dotted traces). We
169 rely in particular on the UV and ECD bands centered at 270 nm, due to the enone π - π^* transition,
170 which is red-shifted by the presence of substituents on the enone system, thus obscuring the

171 otherwise diagnostic $n-\pi^*$ transition (Xue et al., 2012; Yang et al., 2012). The absolute
172 configurations of the new compounds may be assigned as (4*R*,5*R*,8*R*,9*S*,10*R*,21*S*) for higginsianin
173 D (**1**) and (4*R*,5*R*,8*R*,9*S*,10*R*,21*R*) for higginsianin E (**2**), respectively.

174 To summarize, two new diterpenoid dihydrofuran-3-ones, named higginsianins D (**1**) and E
175 (**2**), were isolated from the mycelium of the fungus *C. higginsianum* grown in liquid culture. Their
176 structures, including relative and absolute configurations, were fully elucidated using NMR
177 techniques and experimental and calculated ECD. Obviously, their structures resemble those of the
178 diterpenoid α -pyrones higginsianin A and B (**3** and **4**) previously isolated from the same fungus,
179 subglutinols previously isolated from *Fusarium subglutinans* (Lee et al., 1995), higginsianin C and
180 13-*epi*-higginsianin C produced by another strain of *C. higginsianum* (Dallery et al., 2019b), and the
181 diterpenoid BR-050 previously isolated from *Torrubiella luteostrata* (Pittayakhajonwut et al.,
182 2009). Although a tetrasubstituted 3-oxodihydrofuran-2-one bearing a methoxycarbonyl group has
183 not been found in nature, a similar structure has been synthesized (Arimoto et al., 1994).

184 The evaluation of the in vitro cytotoxicity of higginsianins D and E was performed by MTT
185 assays in A431 and H1299 carcinoma cells as well as in HaCaT immortalized keratinocytes, used as
186 a preneoplastic cell line model. The experiments with the new higginsianins were performed in
187 parallel with the previously described higginsianin B as a positive control (Sangermano et al.,
188 2019).

189 After 24 h of treatment, HaCaT cell viability was significantly reduced by higginsianin E,
190 40% at 1 μ M and 37% at 10 μ M, while higginsianin D had no effect. A moderate but significant
191 reduction of cell viability was observed after 48h of incubation with a concentration of 10 μ M
192 higginsianin D or E. Interestingly, however, HaCaT cells fully recover after 72 h of treatment
193 despite the presence of higginsianin E or D (Figure 4). In H1299, a similar effect was observed with
194 higginsianin D at both concentrations, while higginsianin E caused 22% (at 1 μ M) and 26% (at 10
195 μ M) reduction after 72 h of treatment. A431 cell viability, instead, was strongly affected by both
196 higginsianins in a time- and dose-dependent manner, and the IC₅₀ of higginsianin E was 1 μ M after

197 72 h of incubation (Figure 4). It is important to take into consideration that, unlike higginsianin B,
198 higginsianin E showed no toxicity in HaCaT cells, at the same experimental conditions.

199

200 **EXPERIMENTAL SECTION**

201 **General Experimental Procedures.** Optical rotations were measured in a MeOH solution on a
202 Jasco P-1010 digital polarimeter; IR spectra were recorded as a glassy film on a PerkinElmer
203 Spectrum One FT-IR spectrometer, and UV spectra were recorded in MeOH solution on a
204 PerkinElmer Lambda 25 UV/vis spectrophotometer. ECD spectra were recorded with a Jasco J-715
205 spectropolarimeter, on solutions of 3.3 mM in CH₃CN and using a quartz cell with a 0.01 cm path
206 length. ECD measurement parameters were the following: scan speed 100 nm/min; time constant
207 0.5 s; bandwidth 1 nm; 4 accumulations. ¹H and ¹³C NMR spectra were recorded at 400 and 100
208 MHz, respectively, in CDCl₃ on a Bruker spectrometer. The same solvent was used as an internal
209 standard. Carbon multiplicities were determined by DEPT spectra (Berger and Braun, 2004). DEPT,
210 COSY-45, HSQC, HMBC, and NOESY experiments (Berger and Braun, 2004) were performed
211 using Bruker microprograms. HRESI and ESI mass spectra and liquid chromatography (LC)/MS
212 analyses were performed using the LC/MS TOF system Agilent 6230B, HPLC 1260 Infinity. The
213 HPLC separations were performed with a Phenomenex LUNA (C₁₈ (2) 5 μ 150 × 4.6 mm).
214 Analytical and preparative TLC were performed on silica gel plates (Merck, Kieselgel 60, F₂₅₄, 0.25
215 and 0.5 mm, respectively) or on reverse-phase (Whatman, KC18 F₂₅₄, 0.20 mm) plates; the
216 compounds were visualized by exposure to UV light and/or iodine vapors and/or by spraying first
217 with 10% H₂SO₄ in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating
218 at 110 °C for 10 min. CC: silica gel (Merck, Kieselgel 60, 0.063–0.200 mm).

219 **Fungal Strain.** The *C. higginsianum* isolate used in this study is IMI 349063 (CABI Culture
220 Collection), as previously described (Cimmino et al., 2016).

221 **Production, Extraction and Purification of Fungal Metabolites.** The strain of *C.*
 222 *higginsianum* was grown in M1-D as previously reported (Cimmino et al., 2016). The harvested
 223 mycelium was lyophilized (14.5 g from 4.1 L of culture filtrate) and macerated with EtOAc (3 × 1
 224 L) for 24 h at room temperature in the dark. The organic extracts were combined, dried with
 225 anhydrous Na₂SO₄, and evaporated under reduced pressure, yielding a brown oil residue (1.8 g).
 226 This oil was purified by CC eluted with CHCl₃–*i*-PrOH (97:3), yielding 10 groups of homogeneous
 227 fractions. The residue of the fourth fraction (234.5 mg) was purified by CC eluted with *n*-
 228 hexane–acetone (7:3), yielding six groups of homogeneous fractions. The residue (15.8 mg) of the
 229 third fraction of the latter column was purified on TLC eluted with *n*-hexane–EtOAc (7:3),
 230 affording two homogeneous amorphous solids, higginsianin D (**1**, 2.7 mg, R_f 0.36) and higginsianin
 231 E (**2**, 3.7 mg, R_f 0.39). The residue of the sixth fraction (65.1 mg) of the first column was
 232 crystallized using EtOAc–*n*-hexane (1:1), obtaining higginsianin A (**3**, 39.9 mg, R_f 0.80) as white
 233 crystals. The eighth fraction of the first column was obtained as a homogeneous solid and identified
 234 as higginsianin B (**4**, 84.6 mg, R_f 0.50).

235 *Higginsianin D (1), Methyl 2-[6-Hydroxy-5,8a-dimethyl-2-methylene-5-(4-methylpent-3-*
 236 *enyl)decahydronaphthalen-1-ylmethyl]-4,5-dimethyl-3-oxo-2,3-dihydrofuran-2-carboxylate:*
 237 amorphous solid, [α]_D²⁵ –34 (*c* 0.2); IR ν_{max} 3714, 1731, 1706, 1632, 1226 cm⁻¹; UV λ_{max} nm (log ε)
 238 276 (3.6); ¹H and ¹³C NMR see Table 1; HRESIMS (+) *m/z* 939 [2M + Na]⁺, 917 [2M + H]⁺, 481
 239 [M + Na]⁺, 459.3129 [calcd for C₂₈H₄₃O₅ 459.3111, M + H]⁺.

240 *Higginsianin E (2), Methyl 2-[6-Hydroxy-5,8a-dimethyl-2-methylene-5-(4-methylpent-3-*
 241 *enyl)decahydronaphthalen-1-ylmethyl]-4,5-dimethyl-3-oxo-2,3-dihydrofuran-2-carboxylate:*
 242 amorphous solid, [α]_D²⁵ +62 (*c* 0.2); IR ν_{max} 3710, 1748, 1706, 1630, 1205 cm⁻¹; UV λ_{max} nm (log ε)
 243 275 (3.6); ¹H and ¹³C NMR see Table 1; HRESIMS (+) *m/z* 939 [2M + Na]⁺, 917 [2M + H]⁺, 481
 244 [M + Na]⁺, 459.3130 [calcd for C₂₈H₄₃O₅ 459.3111, M + H]⁺.

245 **Cell Culture and Reagents.** HaCaT, spontaneously immortalized keratinocytes from adult skin,
246 were purchased from Service Cell Line and cultured as described ([Amoresano et al., 2010](#)). Human
247 non-small-cell lung carcinoma cells H1299 (CRL-5803) and human epidermoid carcinoma cells
248 A431 (ATCC-CRL1555) were from American Type Culture Collection (ATCC). According to the
249 p53 compendium database (<http://p53.fr/tp53-database/the-tp53-cell-linecompendium>), HaCaT cells
250 contain mutant p53 (H179Y/R282W), H1299 are p53 null, while A431 contain only one p53
251 mutated allele (R273H). All mentioned cell lines were cultured in Dulbecco's modified Eagle's
252 medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified
253 atmosphere of 5% CO₂. All cell lines were routinely tested for mycoplasma contamination and were
254 not infected.

255 **Determination of the IC₅₀ Growth Inhibitory Concentrations In Vitro.** The MTT
256 colorimetric assay was performed as previously described ([Montano et al., 2019](#)). Briefly, 2×10^4
257 cells were seeded on 24-well plates and exposed to increasing concentrations of either 1 or 10 μ M
258 higginsianins B, D, or E for 24, 48, and 72 h. MTT/DMEM without phenol red (0.5 mg/mL) was
259 added to the wells and incubated for 3 h at 37 °C in a humidified atmosphere. The reaction was
260 stopped by the removal of the supernatant, followed by dissolving the formazan product in acidic
261 isopropanol. Optical density was measured with an ELISA reader (Bio-Rad) in a dual-wavelength
262 mode (570 and 630 nm) filter using an iMark microplate reader (Bio-Rad) and calculated as
263 follows: Absorbance (570 nm) – Absorbance (630 nm). Each experiment was performed in
264 quadruplicate, in three independent experiments. The cell viability was calculated as (Absorbance
265 of test sample)/(Absorbance of control).

266 Statistical analyses were carried out using the GraphPad Prism 8 software. Data were represented
267 as the mean \pm standard deviation and analyzed for statistical significance using ordinary one-way
268 analysis of variance (ANOVA) and multiple comparisons. For all tests, $P < 0.5$ was considered to
269 indicate a statistically significant difference.

270 **Computational Methods.** Molecular mechanics, Hartree–Fock (HF), and density functional
271 theory (DFT) calculations were run with Spartan’18 (Wavefunction, Inc., Irvine, CA, 2018), with
272 standard parameters and convergence criteria. DFT and TDDFT calculations were run with
273 Gaussian16 (Frisch et al., 2016) with default grids and convergence criteria. All calculations were
274 run on truncated models of **1** and **2** that were cut at the C-11/C-12 bond, that is, with the chain
275 attached at C-9 replaced by a methyl group.

276 For NMR calculations, the conformers obtained by a conformational search run with the Monte
277 Carlo algorithm using the Merck molecular force field (MMFF) were geometry-optimized at the
278 HF/3-21G level, screened by single-point calculations at the ω B97X-D/6-31G(d) level, and
279 geometry-optimized at the same level. Final energies and populations were estimated at the
280 ω B97X-V/6-311+G(2df,2p) level, according to the procedure described by Hehre et al., 2019. The
281 procedure afforded 20 energy minima for (21*S*)-**1** and 21 minima for (21*R*)-**2** within the final
282 energy threshold (10 kJ/mol at the ω B97X-D/6-31G(d) level). ¹³C NMR chemical shifts were then
283 calculated with the GIAO method at the ω B97X-D/6-31G(d) level. Finally, an empirical correction
284 was applied depending on the number of bonds to the carbon and on the bond lengths (Hehre et al.,
285 2019).

286 For ECD calculations, the sets of low-energy minima found as described above were reoptimized
287 at the ω B97X-D/6-311+G(d,p)/PCM level including the IEF-PCM continuum solvent model for
288 MeCN and rechecked for duplicates and energy threshold. This led to 14 conformers for (21*S*)-**1**
289 and 15 conformers for (21*R*)-**2**, which were used as input structures for TDDFT calculations run at
290 the ω B97X-D/def2-TZVP/PCM level, including 36 excited states (roots) in each case. Other
291 functionals (CAM-B3LYP and B3LYP) were checked for consistency on selected structures.
292 Average ECD spectra were computed by weighting component ECD spectra with Boltzmann
293 factors at 300 K estimated from DFT internal energies. ECD spectra were generated using the
294 program SpecDis (Bruhn et al., 2017), using dipole-length rotational strengths; the difference from
295 dipole-velocity values was negligible in all cases.

296

297 **ASSOCIATED CONTENT**298 **Supporting Information**

299 The Supporting Information is available free of charge at
300 <https://pubs.acs.org/doi/10.1021/acs.jnatprod.9b01161>.

301 Additional spectra of 1 and 2, low-energy DFT structures, and details on NMR calculations
302 (PDF)

303

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329 **Notes**

330 The authors declare no competing financial interest.

331

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407 **Table 1. ^1H and ^{13}C NMR Data of Higginsianins D and E (1 and 2)^{a,b}**

position	1			2		
	$\delta_{\text{C}}^{\text{c}}$	δ_{H} (J in Hz)	HMBC	$\delta_{\text{C}}^{\text{c}}$	δ_{H} (J in Hz)	HMBC
1	22.7 CH ₂	1.60, m 1.33, m	H-10	22.6 CH ₂	1.62, m 1.32, m	H-10
2	31.0 CH ₂	2.38, td (13.9, 5.8) 2.18, ddd (13.9, 4.4, 1.6)	H-4, H-19B	31.5 CH ₂	2.24, m 2.18, m	H-4, H ₂ -19
3	147.8 C		H ₂ -20, H-4	148.5 C		H ₂ -20, H-4
4	52.7 CH	1.96, m	H ₂ -20, Me-18	52.3 CH	1.82, dd (9.9, 2.4)	H ₂ -19, Me-18, H-20B
5	38.1 C		Me-18	38.1 C		Me-18
6	28.1 CH ₂	1.60, m 0.81, m	Me-18	28.4 CH ₂	1.60, m 0.81, m	H-8, H-7A, Me-18
7	21.5 CH ₂	2.15, td (14.3, 2.2) 1.94, m		25.7 CH ₂	1.93, m 1.58, m	
8	71.9 CH	3.62, br s	Me-17	71.9 CH	3.61, br s	Me-17
9	39.4 C		Me-17	39.4 C		Me-17
10	39.9 CH	1.59, m	Me-18, Me-17	39.9 CH	1.59, m	Me-18, Me-17
11	39.5 CH ₂	1.27, m (2H)	H-10, Me-17	39.5 CH ₂	1.27, m (2H)	Me-17
12	25.5 CH ₂	1.95, m 1.61, m		25.8 CH ₂	1.93, m 1.58, m	
13	124.9 CH	5.15, br t (7.1)	Me-15, Me-16	125.0 CH	5.11, br t (7.0)	Me-15, Me-16
14	131.8 C		Me-15, Me-16	131.8 C		Me-15, Me-16
15	25.6 CH ₃	1.72, br s ^d	Me-16	25.4 CH ₃	1.69, br s ^e	Me-16
16	17.8 CH ₃	1.66, br s ^d	Me-15	17.8 CH ₃	1.62, br s ^e	Me-15
17	22.5 CH ₃	0.82, s		22.4 CH ₃	0.81, s	H ₂ -11, H-10
18	18.8 CH ₃	0.96, s	H-1	18.7 CH ₃	0.96, s	H-1
19	110.4 CH ₂	4.69, br s 4.51, br s	H-4	110.6 CH ₂	4.65, br s 4.34, br s	H-4
20	31.4 CH ₂	2.53, dd (14.6, 10.8) 2.12, m	H-4	32.2 CH ₂	2.20, dd (14.2, 9.9) 2.54, dd (14.2, 2.4)	H-4
21	89.9 C		H ₂ -20	90.1 C		H ₂ -20
22	198.8 C		Me-26	198.3 C		Me-26
23	109.5 C		Me-26, Me-27	109.4 C		Me-26, Me-27
24	185.1 C		Me-26, Me-27	184.8 C		Me-26, Me-27
25	166.1 C		H-20B, OMe	166.4 C		H-20B, OMe
26	5.9 CH ₃	1.68, s		5.8 CH ₃	1.65, s	
27	14.8 CH ₃	2.26, s		14.6 CH ₃	2.24, s	
408 OMe	52.3 CH ₃	3.67, s	H-20B	53.0 CH ₃	3.73, s	

409 ^aThe chemical shifts are in δ values (ppm) from TMS. ^b2D ^1H , ^1H (COSY), ^{13}C , ^1H (HSQC) NMR
410 experiments delineated the correlations of all protons and their corresponding carbons.
411 ^cMultiplicities were assigned by the DEPT spectrum. ^{d,e}These signals could be reversed.

412

413 **Table 2. NOESY Data of Higginsianins E and D (1 and 2)**

1		2	
irradiated	observed	irradiated	observed
H-4	Me-18	H-4	Me-18
H-8	Me-17, H-7A	H-8	Me-17, H-7A
Me-17	Me-18	Me-17	Me-18
H-20A	H-20B	H-20A	H-20B

414

415 **Figure Legend**
416

417 **Figure 1.** Structures of higginsianins D and E (**1** and **2**) and higginsianins A and B (**3** and **4**).

418 **Figure 2.** UV–vis absorption (top) and ECD spectra (bottom) of higginsianin D (**1**) measured in
419 acetonitrile (solid lines, 3.3 mM, 0.01 cm cell) compared with spectra calculated for (21*S*)-**1**
420 at the ω B97XD/def2-TZVP/PCM level as a Boltzmann average of 14 conformers at 300 K
421 (dotted lines). Calculated spectra were obtained as sums of Gaussian bands with 0.3 eV
422 exponential half-width, red-shifted by 15 nm, no vertical scaling.

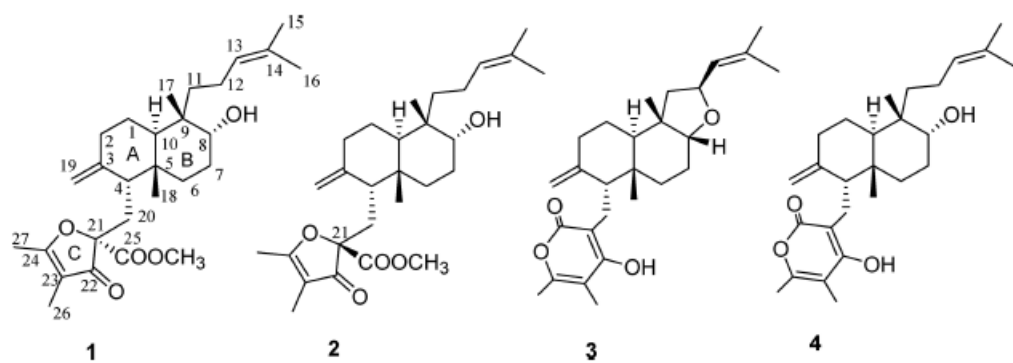
423 **Figure 3.** UV–vis absorption (top) and ECD spectra (bottom) of higginsianin E (**2**) measured in
424 acetonitrile (solid lines, 3.3 mM, 0.01 cm cell) compared with spectra calculated for (21*R*)-**2**
425 at the ω B97XD/def2-TZVP/PCM level as a Boltzmann average of 15 conformers at 300 K
426 (dotted lines). Calculated spectra were obtained as sums of Gaussian bands with 0.3 eV
427 exponential half-width, red-shifted by 15 nm, ECD spectrum scaled by a factor 1.5.

428 **Figure 4.** Effects of higginsianins D, E, and B on HaCaT cell viability. MTT assay of HaCaT cells
429 incubated for 24, 48, and 72 h with higginsianins B, D, and E at 1 or 10 μ M, as indicated.
430 Data are expressed as absorbance and presented as mean \pm SD of three independent
431 experiments, each done in triplicate. Analysis of variance was performed by one-way Anova
432 and multiple comparisons. * $P < 0.5$ when compared with the control.

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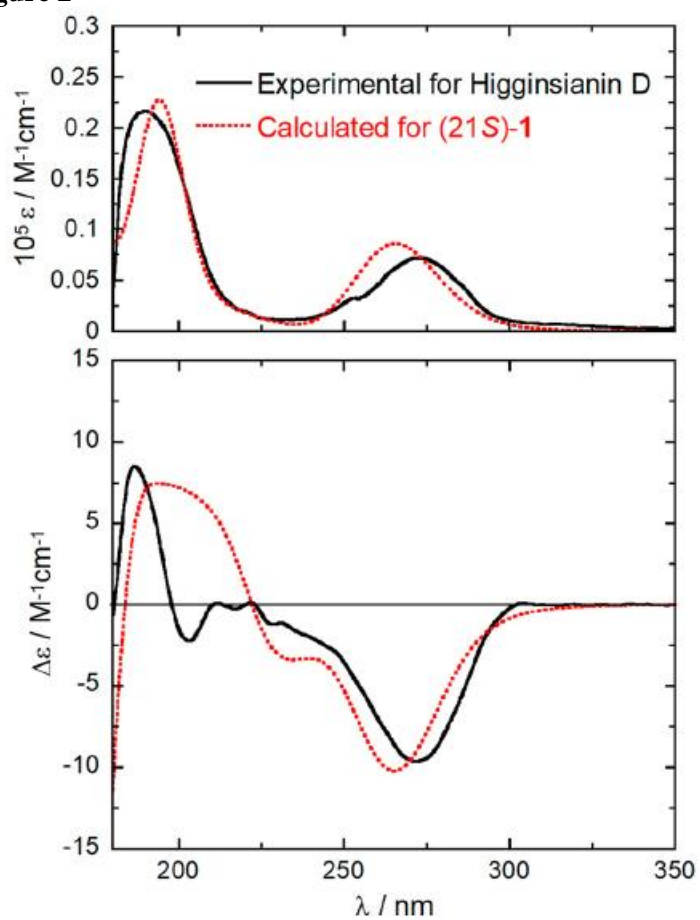
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436 **Figure 1**

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439 **Figure 2**

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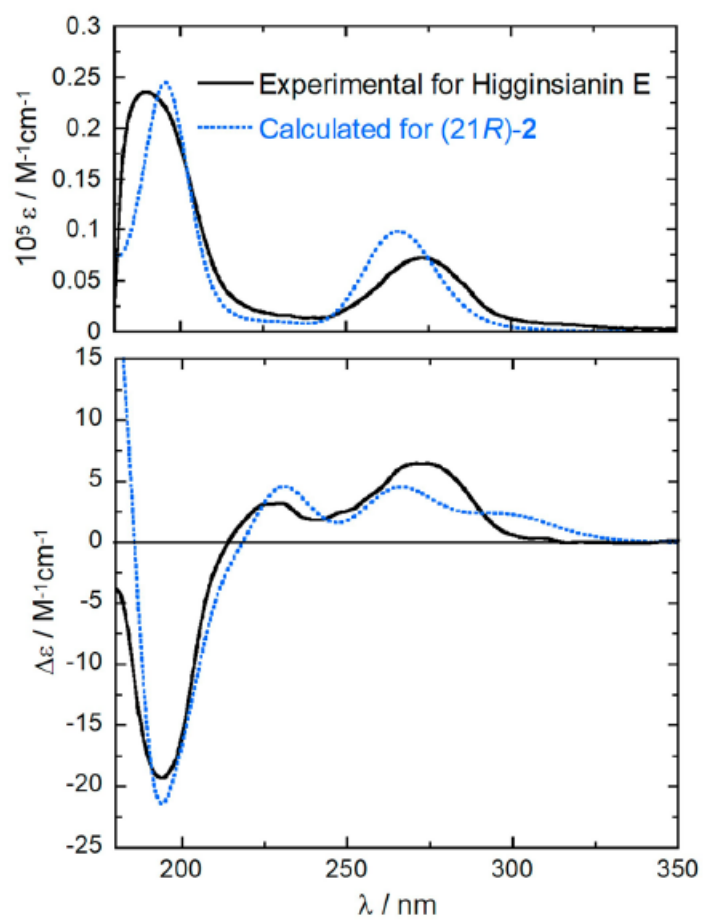
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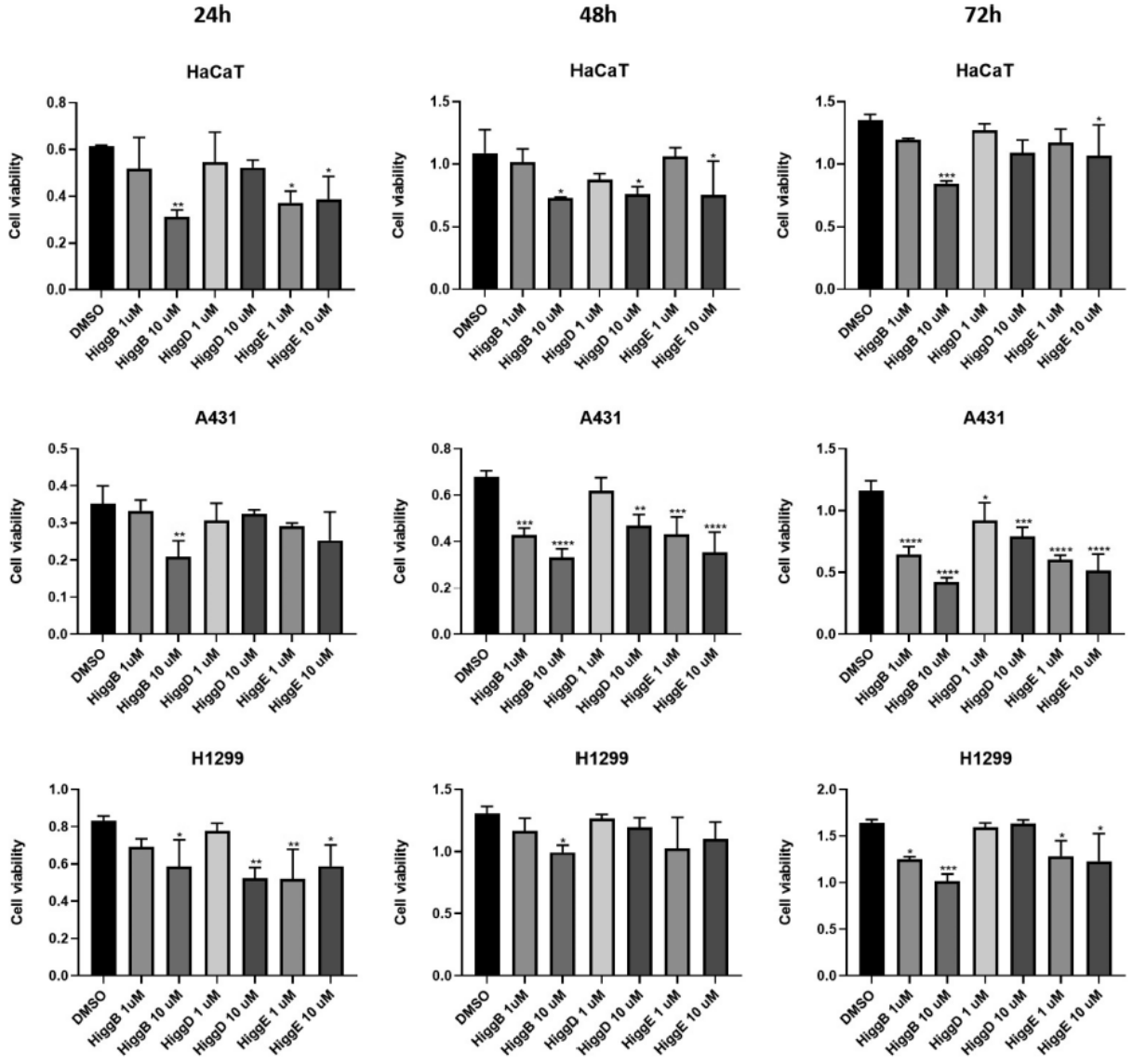
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453 **Figure 3**

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480 **Figure 4**



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