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Structural Reassignment of *rel*-(3′*Z*,3*R*,6*R*,7*R*,3a′*R*,6′*R*)-3,8-Dihydrodiligustilide and the Activity of Diligustilide and 3,8-Dihydro- and 3,8,7′,7a′-Tetrahydrodiligustilides as Progestins

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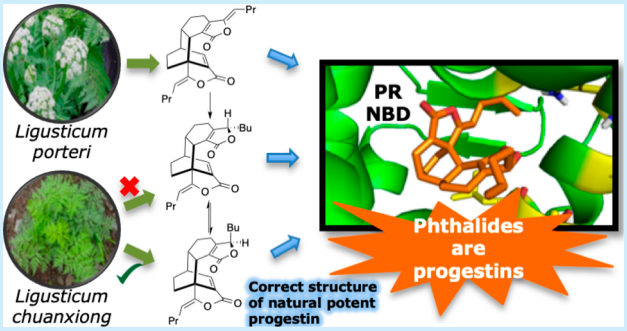
Supporting Information

ABSTRACT: Several phthalides were semisynthesized, including a 3,8-dihydrodiligustilide with progesterone-like activity, previously isolated from *Ligusticum chuanxiong*, the structure of which was earlier assigned to a semisynthetic product with nonidentical spectroscopic constants. The structure of this natural phthalide was reassigned with a proposal of its absolute configuration. Phthalides acted as progestins in cell viability assays, immunofluorescence microscopy, and docking analysis. Therefore, the structures for natural and semisynthetic phthalides with potential use in hormone-related therapies were reassigned.



Progestins are substances that act as progesterone receptor (PR) agonists and can induce the natural activity of progesterone.[1] These compounds are used in hormone replacement therapy[2−4] and contraception[5,6] and for the treatment of infertility, although there are risks associated with these uses, such as an increased breast cancer incidence, depending upon the progestin.[4,7] Therefore, identifying new progestins, with fewer side effects, that could be used for therapeutic purposes is warranted.

Phthalides are a group of compounds isolated from several natural sources, such as plants, fungi, and lichens,[8] and are secondary metabolites of some plants belonging to the Apiaceae family.[9] The latter are widely used in traditional medicines in Asia and North America.[8−10] For example, *Ligusticum porteri* is employed in folk medicine in Northern Mexico and Southern USA for headaches, colds, stomach disorders, diabetes, etc.[11,12] The bioactivity of phthalides correlates in several cases with the traditional uses of the plants; e.g., compound **1** has antinociceptive activity,[13] phthalides **1**, **2**, *rac*-**3**, and *rac*-**4** have sedative effects,[14] and *rac*-**3** is a gastroprotector (see Figure 1).[15]

Chuanxiong rhizome (*L. chuanxiong*) is recommended for menstrual disorders in traditional Chinese medicine, and this use correlates with the isolation of progestins *rac*-**5** and, specifically, **6**, which activate the PR according to a gene reporter assay.[16,17] However, structure **6** had been previously assigned to one of the hydrogenation products of *rac*-**3**.[18] Therefore, two sets of different physical data were allocated to the same structure, and one was incorrectly assigned. Analysis of the spectroscopic data allowed us to hypothesize that we were observing epimers at C3 of 3,8-dihydrodiligustilide.

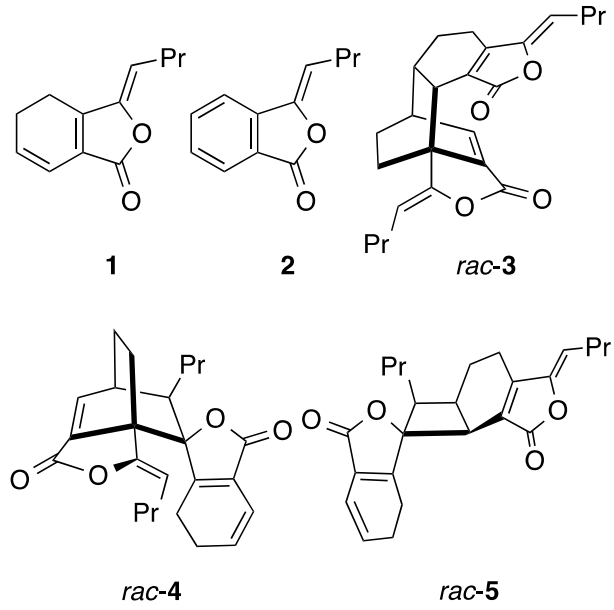


Figure 1. Phthalides from Apiaceae plants.

Herein, we report the semisynthesis of both C3 epimers of 3,8-dihydrodiligustilide from diligustilide (*rac*-**3**), the structural correction and absolute configuration of the bioactive Diels−Alder adduct of *L. chuanxiong* (reported as **6**), as well as the

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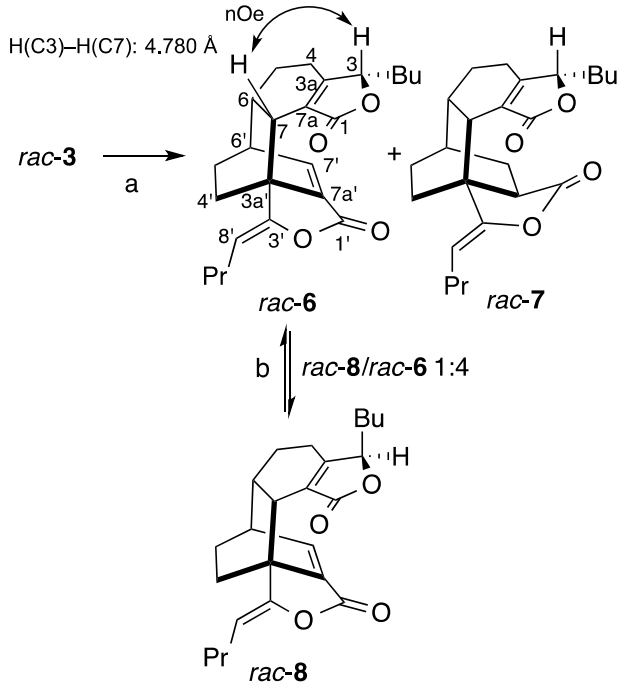
evaluation of these compounds as progestins. Theoretical calculations were also performed to explore the interaction between phthalides and PR.

In the first stage, the hydrogenation of the natural product *rac*-**3** (reisolated from *L. porteri*) was achieved, yielding 3,8-dihydro- and 3,8,7′,7a′-tetrahydro-derivatives, *rac*-**6** and *rac*-**7**, respectively (Scheme 1), which were characterized through

Scheme 1. Derivatization of Diligustilide (*rac*-3)[a]



[a]Reagents: (a) H_2/Pd–C (*rac*-**6**: 66%, *rac*-7: 12%); (b) DBU/THF (MW or reflux, *rac*-**8**: 18%).

spectroscopic methods. ^{1}H and ^{13}C NMR assignments of the obtained compounds (Table 1) were coincident with the semisynthetic products obtained previously in our group.[18] The orientation of the hydrogen attached to C3 of *rac*-**6** was determined by the NOE existing between this hydrogen (δ_H 4.56) and the one bonded to C7 (δ_H 3.18), indicating that both atoms should be oriented toward the same side of the molecule. This observation was confirmed by X-ray crystallography, which showed that the butyl substituent pointed to the inner side of the molecule, and the calculated distance between hydrogens bound to C3 and C7 is 4.780 Å. The structure of the tetrahydroderivative (*rac*-7) was also confirmed by X-ray crystallography (see the Supporting Information), being consistent with the former observation. Consequently, the metabolite from *L. chuanxiong* could not be **6**.

A substantial difference in the spectroscopic constants was the ^{1}H NMR chemical shift of the hydrogen bound to C3, which is δ_H 4.56 for the semisynthetic product (*rac*-**6**)[18] and δ_H 4.80 for the natural product isolated from *L. chuanxiong*.[16] We envisaged the possibility of epimerizing *rac*-**6** to confirm that the natural product corresponded to *rac*-**8**. This task was attempted by reacting *rac*-**6** with DBU in refluxing THF for 24 h and for 30 min upon microwave irradiation. By increasing the temperature, or the reaction length, a retro-Diels–Alder reaction was observed. As a result, a separable 1:4 mixture of *rac*-**8** and *rac*-**6** was obtained (^{1}H NMR control). The spectroscopic data of compound *rac*-**8** were consistent with those reported for the *L. chuanxiong* metabolite (minor corrections in ^{13}C NMR chemical shifts were performed for C4 and C8, Table 1). Therefore, the correct structure for this potent natural progestin is one of the enantiomers of *rac*-**8**.

The fact that the progestogenic 3,8-dihydrodiligustilide isolated from *L. chuanxiong* was found as an enantiomerically pure compound could be explained through a Diels–Alder reaction between (*Z*)-ligustilide (**1**) as a diene and one enantiomer of 3,8-dihydroligustilide as a dienophile. Thus, the absolute configuration of this substance could be deduced as follows: considering the characterization of the racemic epimers at C3 of 3,8-dihydrodiligustilides and that only the 3*S*-enantiomer of 3,8-dihydroligustilide has been characterized as a natural product (senkyunolide A, **9**), then the biosynthetic Diels–Alder reaction for producing the enantiomerically pure adduct (the metabolite of *L. chuanxiong*)[16] should proceed via a face-differentiated *supra,supra* approach between the C3a′*si*,C6′*si* face of diene **1** and the C6*si*,C7*re* face of **9** (*endo-β* approach of the dienophile to the diene) to produce (3′*Z*,3*S*,6*R*,7*R*,3a′*R*,6′*R*)-3,8-dihydrodiligustilide (*ent*-**8** in Scheme 2).

Thus, *ent*-**8** (not **6**) is the structure of the phthalide isolated from *L. chuanxiong*. We then verified its activity (and that of the other phthalides) as progestin.

The T47D cancer cell line viability was determined through MTT assays. Given that it is a measure of the mitochondrial metabolic activity, the assay provides information on cell proliferation. The results showed that *rac*-**6**, *rac*-7, and *rac*-**8** increased cell proliferation after 24 h of treatment, depending on the concentration (see Figure 2). Of note, *rac*-**6** and *rac*-7 had a similar effect to that of progesterone (P4). In contrast, the effect of *rac*-**3** was not significantly different than the control. The observed effect could be attributed to the interaction of phthalides with PR because this observation is consistent with the fact that progestins up-regulate the proliferation of T47D cells.[19−22]

The translocation of PR from the cytoplasm to the nucleus, due to the nuclear translocation signal depending on the hormone, and its retention there, may be a consequence of exposure to progestins, which induces PR phosphorylation, particularly at residue Ser 294.[23,24] It was observed through immunofluorescence microscopy (IMF) that after 20 min of treatment, phosphorylated PR (phospho^{294}-PR) is distributed mainly throughout the cytoplasm for the control. The same occurs for *rac*-**3** and *rac*-**8** (shown in the Supporting Information). On the other hand, phospho^{294}-PR was in perinuclear or nuclear localization after treatment with compounds *rac*-**6** and *rac*-7 (see Figure 3). This result is evidence that this phthalide acts as a progestin, i.e., it is an agonist of PR, capable of inducing its phosphorylation, which in turn allows for the translocation of this nuclear receptor from the cytoplasm to the nucleus after 20 min, where it remains and activates signaling pathways concerning PR function.

Furthermore, molecular docking of tested phthalides (both enantiomers of *rac*-**3**, *rac*-**6** and *rac*-**8**) on PR supported the agonist activity of these compounds toward this protein. Docking calculations between the studied phthalides and PR (using three different crystallized structures of this protein, 4APU,[25] 3ZR7,[26] and 1ZUC[27]), performed in both AutoDock 4.2[28] and AutoDock VINA,[29] showed that all of the tested phthalides interact with the protein near the ligand binding

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Org. Lett. XXXX, XXX, XXX−XXX

Table 1. ^{1}H NMR (700 MHz) and ^{13}C NMR (125 MHz) Data for *rac*-6, *rac*-7, and *rac*-8 ($CDCl_3$)

	rac-6		*rac*-7		*rac*-8	
position	δ_C	δ_H, multiplicity (*J*, Hz)	δ_C	δ_H, multiplicity (*J*, Hz)	δ_C	δ_H, multiplicity (*J*, Hz)
1	171.9		171.7		172.2	
3	82.5	4.56, ddd (7.5, 4.0, 2.0)	83.3	4.55, dt (9.5, 3.0)	81.5	4.80, dd (7.5, 3.5)
3a	168.2		166.2		168.2	
4	22.4	2.08, dddd (17.5, 12.5, 5.0, 2.0); 1.99, dd (17.5, 4.5)	22.7	2.59, dddd (18.5, 12.5, 6.0, 2.5); 2.23, dd (18.5, 6.0)	21.7	2.07−1.97, m
5	28.8	1.54, dddd (13.5, 12.0, 7.0, 5.0); 1.90, dddd (13.5, 4.5, 1.0, 0.5)	27.5	1.92−1.88, m; 1.70−1.51, m	29.4	1.91, ddt (14.0, 4.5, 1.5); 1.50−1.44, m
6	38.3	2.54, t (8.0)	33.0	2.21−2.17, m	38.4	2.52, dd (8.0)
7	41.1	3.18, d (9.0)	38.0	2.86, d (10.0)	41.1	3.20, d (9.0)
7a	127.1		126.2		127.8	
8	32.2	1.68, dtd (8.0, 4.5, 1.9); 1.45−1.35, m	31.6	1.70−1.51, m	31.2	1.43−1.37, m; 1.36−1.22, m
9	26.5	1.35−1.25, m	28.3	1.70−1.5, m; 1.47−1.41, m	26.5	1.17, dddd (11.0, 4.5, 3.5, 2.0); 2.07−1.97, m
10	22.3	1.25−1.35, m	22.3	1.41−1.32, m	22.5	1.43−1.37, m; 1.36−1.22, m
11	13.7	0.90, t (7.0)	13.9	0.92, t (7.5)	14.0	0.85, t, (7.0)
1′	165.0		175.3		165.3	
3′	150.6		153.1		150.6	
3a′	47.4		43.8		47.6	
4′	30.9	2.03, ddd (12.5, 10.0, 5.0); 1.45−1.35, m	29.8	1.95, ddd (13.0, 9.5, 7.5); 1.85, ddd (13.0, 11.0, 1.5)	31.3	2.07−1.97, m; 1.81, dddd (14.0, 10.5, 4.5, 3.5)
5′	25.7	1.86, dq (9.8, 3.1); 1.35−1.25, m	27.3	1.83−1.78, m; 1.70−1.51	25.7	1.91, ddt (13.5, 4.5, 1.5); 1.87, ddt (13.5, 10.0, 3.5)
6′	41.6	2.97, dq (5.5, 2.5)	33.6	1.83−1.78, m	41.8	2.98, dq (6.5, 2.5)
7′	141.9	7.33, d (6.5)	23.0	1.83−1.78, m	142.2	7.33, d (6.5)
7a′	134.5		41.0	2.47 t (10.0)	134.6	
8′	108.6	4.98, t (7.5); 1.45−1.35, m	105.7	4.70, t (7.5)	108.9	4.98, dd (8.0, 7.0)
9′	27.5	2.18 m q (7.5)	27.1	2.13, q (7.5)	27.6	2.20−2.10, m
10′	22.3	1.51−1.45, m; 1.45−1.35, m	22.3	1.52−1.47, m; 1.41−1.32, m	22.3	1.51−1.4, m; 1.43−1.37, m
11′	14.0	0.92, t (7.5)	13.9	0.91, t (7.5)	14.1	0.91, t (7.5)

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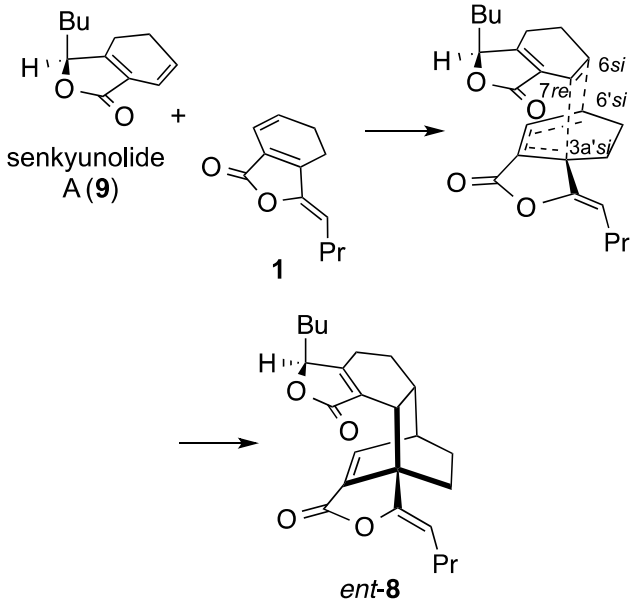
Org. Lett. XXXX, XXX, XXX−XXX

Scheme 2. Regio- and Stereo-Differentiated Biosynthetic Diels–Alder Reaction (1 + 9) for Producing *ent*-8



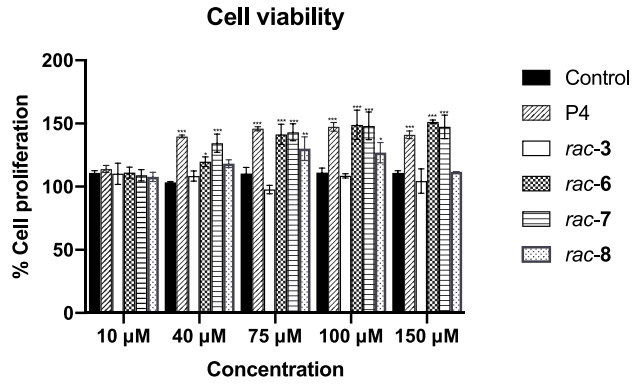


Figure 2. T47D cell proliferation assays with tested compounds at 24 h. Proliferation increased upon treatment with *rac*-**6**, *rac*-7, and progesterone at 40, 75, 100, and 150 μM. Results are presented as the means ± SD (n = 3); ***p < 0.001, **p < 0.002, *p < 0.033 compared with the control.

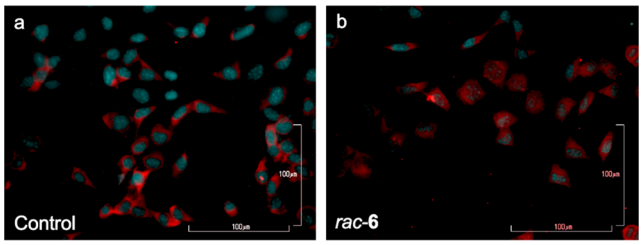


Figure 3. Immunofluorescence microscopy showing subcellular localization of PR in T47D cells labeled with phospho^{294} PR (red) and nucleus stained in blue with 4′,6-diamidino-2-phenylindole (DAPI) for: (a) untreated cells, with PR located in the cytoplasm, and (b) cells after treatment with test *rac*-**6**, pPR is notably located inside the nucleus. Scale bars: 100 μm.

domain (LBD), similar to progesterone and other agonists.[25,30] The respective docking scores are shown in Table 2. On the basis of these results, all proven natural and semisynthetic phthalides could be PR agonists.

Table 2. Calculated Affinity Energies for Interaction between Phthalides and Different Crystallized Structures of PR

	−ΔG (kcal/mol)					
	4APU		3ZR7		1ZUC	
compd	AD4	VINA	AD4	VINA	AD4	VINA
3	10.44	4.9	11.24	6.9	10.56	4.6
6	10.99	4.7	10.76	6.2	10.05	4.7
7	10.61	5.1	10.98	5.8	9.88	3.9
8	11.10	4.8	11.33	6.5	11.67	7.4
ent-**3**	10.71	4.9	10.96	6.5	9.81	5.0
ent-**6**	10.97	4.7	10.88	6.2	10.43	3.7
ent-7	10.79	4.6	10.87	6.7	9.46	3.7
ent-**8**	11.04	4.7	10.77	7.1	10.45	4.0
P4	11.14	5.3	22.48	7.6	24.27	10

As mentioned above, these compounds were located near the LBD, consistent with the experimental results of the activity of phthalides as progestins. Of note, the calculated affinity energies were similar among the tested compounds. For example, in the case of structure 4APU using AD4, the energies for all of the compounds are in the range of −10.44 and −11.14 kcal/mol, like P4. With VINA, a similar behavior was observed, and the range was between −4.7 and −5.3 kcal/mol, very close to, and similar to, P4. In particular, we found that compound **6** showed an interaction between the sulfur (from residue Met-756) and carbonyl oxygen from the ligand, as shown in Figure 4 (S–O attractive interactions, though

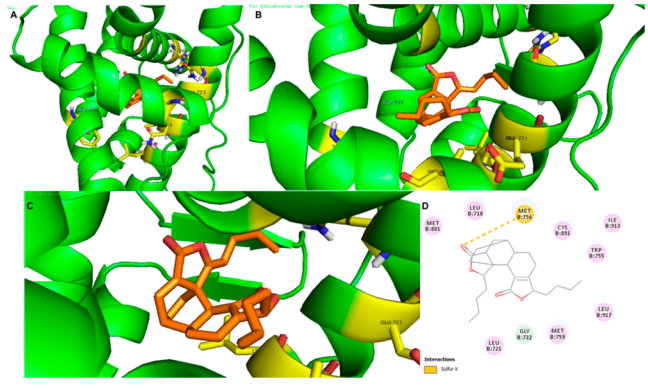


Figure 4. (A–C) Docking position of **6** with PR. (D) 2D interaction diagram between **6** and PR.

counterintuitive, are common).[31] The correct interaction of ligands with this residue is relevant to increase agonistic activity, which correlates with the in vitro results for *rac*-**6**.[26]

Hence, we demonstrated that, despite the misassignment of the structure of the compound from *L. chuanxiong* (*ent*-**8**), it actually acts as a progestin, as do other hydrodiligustilides. Furthermore, this class of compounds could be a family of phytoprogestins useful in therapy, different from the natural flavonoids displaying this activity.[32,33] The impact of any side effects of these phthalides and its optimization remain unstudied.

In summary, we prepared two bioactive compounds, correcting the originally reported structure for the natural

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Org. Lett. XXXX, XXX, XXX–XXX

progestin isolated from *L. chuanxiong* and proposing its absolute configuration (formula (3′*Z*,3*S*,6*R*,7*R*,3a′*R*,6′*R*)-**8**). These compounds increased cell viability in hormonodependent T47D cancer cells and led to the phosphorylation of PR and its translocation into the nucleus, according to experimental and computational studies. These results are evidence of the progestin-like activity of dimeric phthalides, such as dihydro- and tetrahydrodiligustilides, which may be considered as possible candidates for hormone replacement therapies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b02762.

Experimental details, ^{1}H and ^{13}C NMR spectra, and X-ray crystallography details (PDF)

Accession Codes

CCDC 1921193−1921194 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interest.

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Org. Lett. XXXX, XXX, XXX−XXX