Labdanes and Isopimaranes from *Platycladus orientalis* and Their Effects on Erythrocyte Membrane and on *Plasmodium falciparum* Growth in the Erythrocyte Host Cells

Javad Asili,[†,‡] Maja Lambert,[†] Hanne L. Ziegler,[†] Dan Stærk,[†] Majid Sairafianpour,[†] Matthias Witt,[§] Gholamreza Asghari,[‡] Ismaiel S. Ibrahimi,[⊥] and Jerzy W. Jaroszewski*[,†]

Department of Medicinal Chemistry, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen, Denmark, Department of Pharmacognosy, Faculty of Pharmacy, Isfahan University of Medical Sciences, Hezar Jerib Avenue, P.O. Box 81745-319, Isfahan, Islamic Republic of Iran, Bruker Daltonik GmbH, Fahrenheitstrasse 4, D-28359 Bremen, Germany, and Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Engehab Avenue, P.O. Box 14155-6451, Tehran, Islamic Republic of Iran

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Six labdanes (**1**−**6**) and four isopimaranes (**7**−**10**), including three new natural products (**7**, **9**, and **10**), were isolated from *Platycladus orientalis*, and their structures determined using 1D and 2D NMR methods, ion-cyclotron resonance HRMS, and optical rotation data. Relative configurations of all chiral centers in the isopimaranes were determined using NOESY experiments at 600 and 800 MHz. Specific optical rotation data were used to correlate absolute configurations. Compounds **1**−**9** and aframodial (**11**) were tested for their in vitro antiplasmodial activity and for their ability to induce changes of erythrocyte shape in order to obtain data about possible correlation between the two effects. All compounds tested exhibited weak (IC_{50} > 25 μM) in vitro antiplasmodial effects against *Plasmodium falciparum* strain 3D7. At the same time, the compounds caused echinocytic or stomatocytic changes of the erythrocyte membrane curvature, indicative of their incorporation into the lipid bilayer, in the concentration region where the antiplasmodial activity was observed. The antiplasmodial effect of these compounds thus appears to be an indirect effect on the erythrocyte host cell. Weak or moderate antiplasmodial activity observed with many other apolar natural products, in particular those with amphiphilic structures, is also likely to be an indirect effect.

A major proportion of commercially available therapeutic agents against malaria are based on secondary metabolites from plants, and screening of plant extracts and isolates for antiplasmodial activity continues to attract great interest.[1−11] Selection of active extracts for bioactivity-guided fractionation using malaria parasite (*Plasmodium*) cultures poses a major challenge, since many crude extracts exhibit moderate to high activities, but it is not known a priori which extracts contain small amounts of highly potent *Plasmodium* inhibitors and which contain large amounts of weak inhibitors, exhibiting additive and possibly synergistic effects. Because of these difficulties, bioactivity-guided fractionations frequently result in isolation of inhibitors with IC_{50} values in the 10−50 μM range,[6,7] i.e., compounds that are several orders of magnitude less active than commercial antimalarial drugs. Moreover, since the in vitro antiplasmodial assay is carried out with *Plasmodium* parasites multiplicating inside erythrocytes, and growth of the parasites relies on the integrity of the erythrocyte host cells, the assay is prone to artifacts resulting from indirect effects of the compounds on the erythrocytes. We have recently demonstrated that various natural products and synthetic amphiphiles that incorporate into erythrocyte lipid membrane, which is manifested as stomatocytic or echinocytic changes of the erythrocyte cell curvature, cause inhibition of *P. falciparum* growth that correlates well with the extent of erythrocyte membrane alterations.[12−15] In such cases, the growth inhibition is considered to be an indirect effect, rather than a toxic effect on the parasites themselves. The erythrocyte membrane-modifying effects paralleling the inhibition of *P. falciparum* growth have been observed with a number of triterpenoids[12,13,15] and with an abietane-type diterpene, dehydroabietinol.[14]

In the present work, in vitro antiplasmodial and erythrocyte membrane-modifying effects of a series of diterpenes belonging to the labdane and isopimarane types are compared. Most of the diterpenes, including three compounds described as natural products for the first time, have been isolated from *Platycladus orientalis* (L.) Franco (Cupressaceae), a well-known source of terpenoids.[16−29]

Results and Discussion

Leaves or branches of *P. orientalis* were extracted with EtOAc, and the extracts fractionated by silica gel chromatography followed by normal-phase or reversed-phase preparative HPLC. This resulted in isolation of the diterpenoids **1**−**10**. Molecular formulas of all compounds were determined by ion-cyclotron resonance mass spectrometry with relative differences (ΔM) between calculated and experimental molecular masses (as MH^+ or MNa^+ ions) less than 0.7 ppm (see Experimental Section). ^1H and ^{13}C NMR (1D and 2D) and optical rotation measurements, and comparison with literature data, allowed identification of the compounds **1**−**6** as the labdanes pinusolide (**1**),[26,30] pinusolidic acid (**2**),[23−25,27,31] isocupressic acid (**3**),[24,32] lambertianic acid (**4**),[33,34] and the isomeric lactols **5**[27,31,35] and **6**.[27,33] The configuration of the olefinic bond between C-13 and C-14 in **3** was confirmed[24,32] by a NOESY spectrum. Compounds **5** and **6** are approximately 1:1 mixtures of C-16 and C-15 epimers, respectively, expected to be exchange-

* To whom correspondence should be addressed. Tel: (45) 35306372. Fax: (45) 35306040. E-mail: jj@dfuni.dk.
† The Danish University of Pharmaceutical Sciences.
‡ Isfahan University of Medical Sciences.
§ Bruker Daltonik.
⊥ Tehran University of Medical Sciences.

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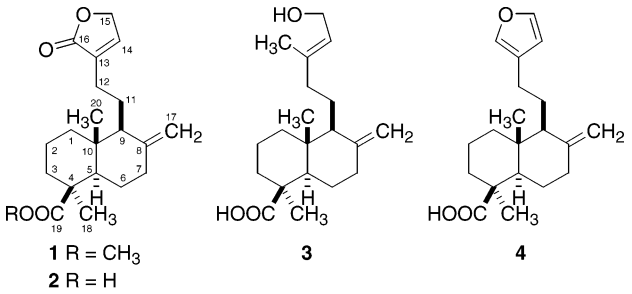
able via an acyclic form.[36] Interestingly, the epimers differed in chemical shifts not only of carbon atoms close to the epimeric centers but also of many carbon atoms in the bicyclo[4.4.0]decane system, suggesting a close proximity of the latter and the lactol ring, possibly involving a hydrogen bond to the C-19 carboxylic group. While H-16 signals of the epimeric mixture **5** appeared as two singlets with chemical shift difference of 0.03 ppm, the corresponding H-15 signals of the epimeric mixture **6** were not separated (600 MHz, $CDCl_3$). In **5**, the geminal hydrogens attached to C-17 gave two broadened singlets at δ 4.50 and 4.89, similarly as in **1**−**4**, whereas in the epimeric mixture **6** the olefinic hydrogen appearing at a higher field gave two broadened singlets separated by 0.01 ppm. Because **5** and **6** are relatively poorly characterized in the literature,[27,31,33,35] their ^{1}H and ^{13}C NMR data are reported in the Experimental Section.



1 R = CH_3
2 R = H

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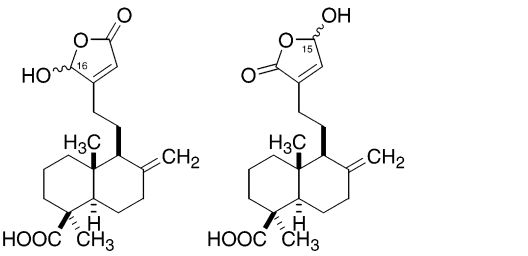

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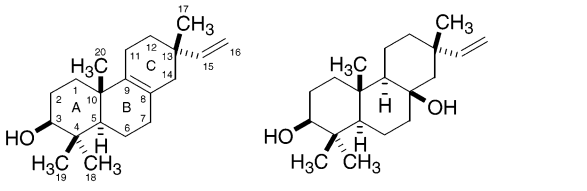

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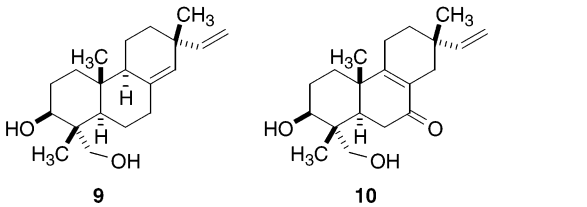

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10

The remaining compounds were identified as isopimaranes **7**−**10**. All compounds contained a double bond between C-15 and C-16, as shown by a characteristic ABX spin system of the vinyl group. In each case, the hydrogen and carbon resonances of the vinyl group showed HMBC correlations to those of a methyl group, identifying the methyl group attached to C-13. Moreover, NOESY correlations from H-15 to H-17 were observed in each case. Each of the compounds **7**−**10** contained an equatorial hydroxy group attached to C-3, as shown by the presence of a large diaxial splitting of the H-3 methine resonance ($^3J_{2ax,3ax}$ about 11.5 Hz). The H-3 methine resonance of **7** and **8** showed HMBC correlations to the methyl carbons C-18 and C-19. NOEs between the 1,3-diaxially oriented methyl groups H-19 and H-20 provided the basis for the distinction of the resonances of the geminal methyl groups attached to C-4 and at the same time established the relative configuration of C-10. In compounds **9** and **10**, one of the geminal methyl groups was replaced with a hydroxymethyl group. The methylene hydrogens gave a characteristic AB pattern ($^2J_{AB}$ = 10.4 Hz) and showed HMBC correlations to C-3. The hydroxymethyl group present in **9** and **10** is equatorial because the NOEs between the 1,3-diaxial methyl groups H-19 and H-20 were still observed.

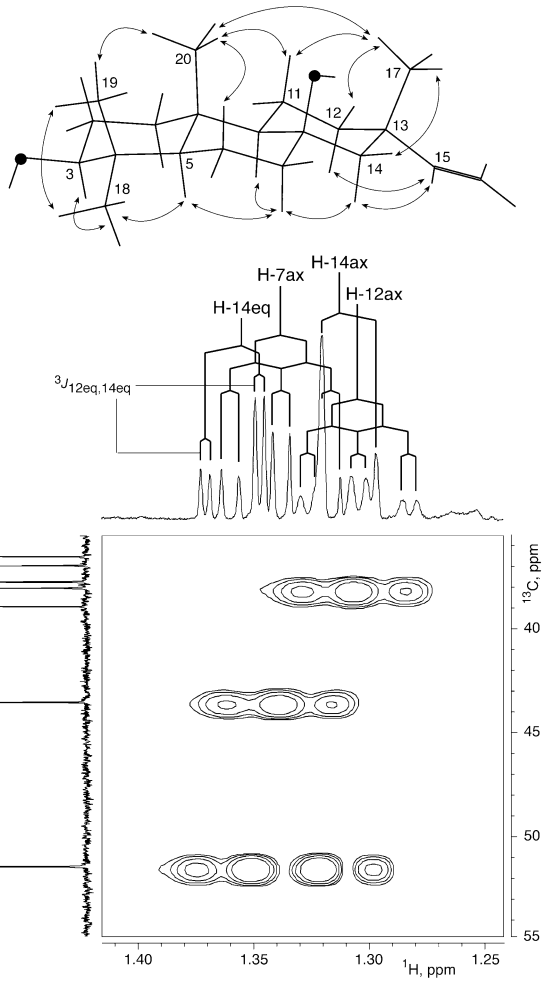

Figure 1. (Top) Selected diagnostic NOESY correlations (600 MHz, mixing time 600 ms, $CDCl_3$) for **8** (molecular model optimized using the MM2 force field). (Bottom) Region of a gHSQC spectrum of **8** (600 MHz, $CDCl_3$) illustrating stereospecific assignment of the diastereotopic hydrogens H-14.

The relative orientation of the substituents at C-13 in **8** was proved by NOEs between H-17 and H-20 and between H-17 and H-11ax, H-12eq, and H-14eq (Figure 1). Although the diastereotopic hydrogens attached to C-12 and C-14 appear in crowded regions of the spectra, their regio- and stereoselective assignment necessary for interpretation of the NOESY spectrum was possible using HSQC correlations combined with the observation of four-bond coupling between the equatorial hydrogens ($^4J_{12eq,14eq}$ = 2.3 Hz) along a planar zigzag path (Figure 1). The same splitting was observed in C_6D_6 at 600 and 800 MHz, proving that it represents a genuine *J*-coupling and not a non-first-order (strong coupling) effect. The relative configuration of **9** was determined similarly. In the NOESY spectra of **7** and **10**, the two compounds containing a double bond between C-8

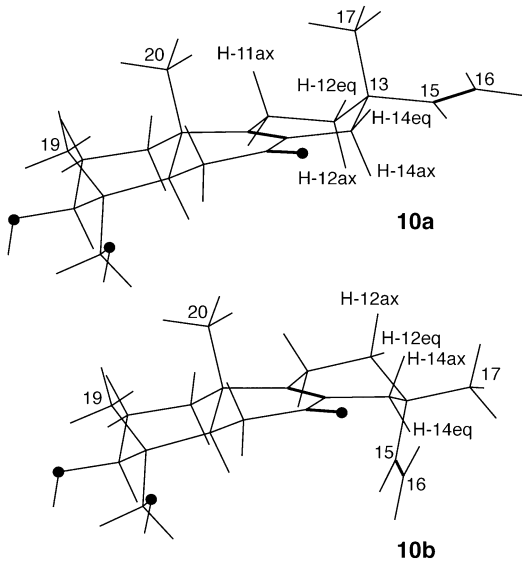

Figure 2. Two low-energy conformations of **10** optimized using the MM2 force field.

and C-9, no correlations between H-17 and H-20 were observed. In contrast to the conformationally fixed **8**, compounds **7** and **10** are expected to have two conformations representing two half-chair conformers of the cyclohexene ring C (conformations **10a** and **10b**, Figure 2). Conformation **10a** implies an NOE between H-17 and H-20; these methyl groups are oriented similarly as in **8** and **9**, where the corresponding NOEs are indeed observed. On the other hand, no NOE is expected between H-17 and H-20 in the conformer **10b**. According to MM2 calculations[37,38] the conformers **10a** and **10b** are essentially isoenergetic, with a steric energy difference less than 0.2 kcal/mol. Lack of correlation between H-17 and H-20 in the NOESY spectra of **10** suggests that the dominating conformation is in fact **10b**. This is confirmed by the presence of NOEs from H-15 to H-12eq and H-14eq and lack of NOEs from H-15 to H-12ax and H-14ax, expected for **10a**. Similar arguments were used for the determination of the relative configuration of C-13 in **7**. This completes the determination of relative configurations of C-3, C-4, C-10, and C-13 in the whole series. Fully assigned ^{1}H and ^{13}C NMR data for **7**−**10** are collated in Tables 1 and 2, and a summary of 2D NMR correlations is included as Supporting Information.

A compound with the structure **7** has not yet been reported as a natural product, but was previously obtained as a racemate by acid-catalyzed cyclization of derivatives of geranylgeraniol[39,40] and also as a dextrorotatory material formed in a series of chemical transformations from virescenols A and B.[41] The optical rotation found in the present work ($[\alpha]_D$ +105°) is practically identical with that of the synthetic, dextrorotatory compound[41] ($[\alpha]_D$ +92°), proving the absolute configuration of **7**.

A compound formulated as the antipode of **8**, i.e., *ent*-**8**, was reported recently.[17] The reported rotation of this compound ($[\alpha]_D$ +3°) is indeed approximately opposite to that found for **8** in the present work ($[\alpha]_D$ −9°). Thus, the present report is the first on the occurrence of the levorotatory enantiomer **8**. Recently another stereoisomer of **8** belonging to the *ent*-pimarane series was isolated;[42] as expected, its ^{13}C NMR spectrum differs from that of **8** (Table 2) for the carbon atoms in the vicinity of C-13.[42]

Both enantiomers of compound **9** have been described,[43,44] and the NMR and optical rotation data of the levorotatory **9** isolated in the present work correspond well to those

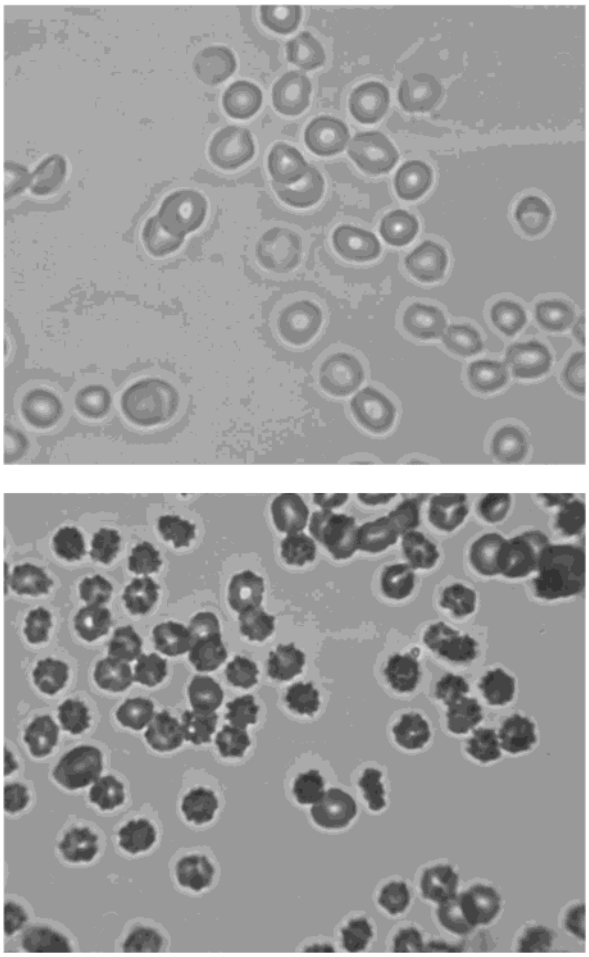

Figure 3. Phase-contrast light microscope photographs of erythrocytes (magnification 700 times). (Top) Control erythrocytes incubated for 48 h in the parasite growth medium; the erythrocytes have discocytic shape. (Bottom) Erythrocytes incubated in the medium containing 25 μg/mL (82 μM) of compound **9**, resulting in formation of echinocytes.

reported.[43,44] Compound **10** is new. Its C-4 epimer was recently isolated from a marine source.[45] The ^{13}C NMR spectrum of the latter[45] shows expected differences from that of **10** (Table 2) for the carbon atoms close to C-4. The absolute configuration of **10** is assumed to correspond to that of **1**−**9** on biosynthetic grounds. Thus, the present results agree with previous findings that *P. orientalis* and the Cupressaceae in general typically produce isopimarane-type diterpenes rather than pimaranes, *ent*-pimaranes, or *ent*-isopimaranes.[20,23−27,43,46]

The labdanes **1**−**6** and the isopimaranes **7**−**9** were tested for their ability to inhibit growth of *P. falciparum* parasites in vitro as well as for their eryhthrocyte membrane-modifying effects in order to obtain data about possible correlation between the two effects (the isolated amount of **10** was insufficient for the tests). The labdane **11**, previously found to be a weak antiplasmodial agent,[47] was included in the study. The results are summarized in

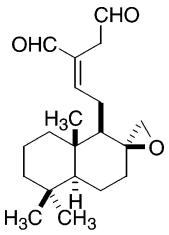

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Table 1. ^{1}H NMR Spectra (600 MHz) of Isopimaranes **7**−**10** ($CDCl_3$)[a]

hydrogen	**7**	**8**[b]	**9**	**10**
H-1	ax: 1.15 (td, $J_{1ax,1eq}$ = $J_{1ax,2ax}$ = 13.2, $J_{1ax,2eq}$ = 3.8)	ax: 0.99 (td, $J_{1ax,1eq}$ = $J_{1ax,2ax}$ = 13.4, $J_{1ax,2eq}$ = 3.7) (0.69)	ax: 1.17 (td, $J_{1ax,1eq}$ = $J_{1ax,2ax}$ = 13.2, $J_{1ax,2eq}$ = 3.5)	ax: 1.34 (td, $J_{1ax,1eq}$ = $J_{1ax,2ax}$ = 13.2, $J_{1ax,2eq}$ = 3.6)
	eq: 1.75 (dt, $J_{1eq,1ax}$ = 13.2, $J_{1eq,2ax}$ = $J_{1eq,2eq}$ = 3.8)	eq: 1.76 (dt, $J_{1eq,1ax}$ = 13.4, $J_{1eq,2eq}$ = $J_{1eq,2ax}$ = 3.7) (1.53)	eq: 1.75 (dt, $J_{1eq,1ax}$ = 13.2, $J_{1eq,2ax}$ = $J_{1eq,2eq}$ = 3.5)	eq: 1.87 (dt, $J_{1eq,1ax}$ = 13.2, $J_{1eq,2ax}$ = $J_{1eq,2eq}$ = 3.6)
H-2	ax: 1.59 (tdd, $J_{2ax,2eq}$ = $J_{2ax,1ax}$ = 13.2, $J_{2ax,3ax}$ = 11.6, $J_{2ax,1eq}$ = 3.8)	1.64 (m, 2H) (1.45)	ax: 1.60 (m)	ax: 1.72 (tdd, $J_{2ax,1ax}$ = $J_{2ax,2eq}$ = 13.2, $J_{2ax,3ax}$ = 11.6, $J_{2ax,1eq}$ = 3.6)
	eq: 1.67 (m)		eq: 1.65 (m)	eq: 1.76 (m)
H-3	3.24 (d, br, $J_{3ax,2ax}$ = 11.6)	3.21 (dd, $J_{3ax,2ax}$ = 11.4, $J_{3ax,2eq}$ = 5.2) (3.03)	3.67 (dd, $J_{3ax,2ax}$ = 11.3, $J_{3ax,2eq}$ = 4.5)	3.72 (dd, $J_{3ax,2ax}$ = 11.6, $J_{3ax,2eq}$ = 4.6)
H-5	1.10 (dd, $J_{5ax,6ax}$ = 12.6, $J_{5ax,6eq}$ = 2.1)	0.83 (dd, $J_{5ax,6ax}$ = 12.7, $J_{5ax,6eq}$ = 3.1) (0.55)	1.15 (m)	1.82 (dd, $J_{5ax,6ax}$ = 14.2, $J_{5ax,6eq}$ = 3.8)
H-6	ax: 1.49 (m)	ax: 1.61 (m) (1.49)	1.42 (m, 2H)	ax: 2.45 (dd, $J_{6ax,6eq}$ = 17.7, $J_{6ax,5ax}$ = 14.2)
	eq: 1.68 (m)	eq: 1.52 (ddt, $J_{6eq,6ax}$ = 13.7, $J_{6eq,7ax}$ = 4.4, $J_{6eq,7eq}$ = $J_{6eq,5ax}$ = 3.1) (1.33)		eq: 2.33 (dd, $J_{6eq,6ax}$ = 17.7, $J_{6eq,5ax}$ = 3.8)
H-7	1.94 (m, 2H)	ax: 1.34 (td, $J_{7ax,7eq}$ = $J_{7ax,6ax}$ = 13.3, $J_{7ax,6eq}$ = 4.4) (1.01)	ax: 2.03 (m)	
		eq: 1.67 (dt, $J_{7eq,7ax}$ = 13.3, $J_{7eq,6eq}$ = $J_{7eq,6ax}$ = 3.1) (1.29)	eq: 2.25 (dt, $J_{7eq,7ax}$ = 14.0, $J_{7eq,6ax}$ = $J_{7eq,6eq}$ = 3.5)	
H-9		0.82 (dd, $J_{9ax,11ax}$ = 13.2, $J_{9ax,11eq}$ = 3.4) (0.44)	1.70 (td, $J_{9ax,11ax}$ = $J_{9ax,11eq}$ = 7.4, $J_{9ax,14}$ = 1.8)	
H-11	1.89 (m, 2H)	ax: 1.65 (m) (1.68)	ax: 1.49 (m)	2.19 (m, 2H)
		eq: 1.46 (dq, $J_{11eq,11ax}$ = 3.3, $J_{11eq,12eq}$ = $J_{11eq,12ax}$ = $J_{11eq,9ax}$ = 3.4) (1.31)	eq: 1.59 (m)	
H-12	ax: 1.31 (m)	ax: 1.30 (td, $J_{12ax,12eq}$ = $J_{12ax,11ax}$ = 12.8, $J_{12ax,11eq}$ = 3.4) (1.26)	ax: 1.35 (td, $J_{12ax,12eq}$ = $J_{12ax,11ax}$ = 12.6, $J_{12ax,11eq}$ = 3.7)	ax: 1.33 (m)
	eq: 1.49 (m)	eq: 1.56 (dtd, $J_{12eq,12ax}$ = 12.8, $J_{12eq,11eq}$ = $J_{12eq,11ax}$ = 3.4, $J_{12eq,14eq}$ = 2.3) (1.56)	eq: 1.46 (m)	eq: 1.60 (dtd, $J_{12eq,12ax}$ = 13.1, $J_{12eq,11ax}$ = $J_{12eq,11eq}$ = 4.7, $J_{12eq,14eq}$ = 2.2)
H-14	ax: 1.73 (d, $J_{14ax,14eq}$ = 17.2)	ax: 1.31 (d, $J_{14ax,14eq}$ = 14.1) (1.15)	5.23 (q, $J_{14,7ax}$ = $J_{14,9ax}$ = $J_{14,12eq}$ = 1.8)	ax: 2.03 (dt, $J_{14ax,14eq}$ = 17.6, $J_{14ax,11ax}$ = $J_{14ax,11eq}$ = 2.2)
	eq: 1.81 (d, $J_{14eq,14ax}$ = 17.2)	eq: 1.36 (dd, $J_{14eq,14ax}$ = 14.1, $J_{14eq,12eq}$ = 2.3) (1.09)		eq: 2.33 (dq, $J_{14eq,14ax}$ = 17.6, $J_{14eq,11ax}$ = $J_{14eq,11eq}$ = $J_{14eq,12eq}$ = 2.2)
H-15	5.73 (dd, $J_{15,16Z}$ = 17.5, $J_{15,16E}$ = 10.8)	5.72 (dd, $J_{15,16Z}$ = 17.4, $J_{15,16E}$ = 10.7) (5.77)	5.76 (dd, $J_{15,16Z}$ = 17.5, $J_{15,16E}$ = 10.4)	5.67 (dd, $J_{15,16Z}$ = 16.8, $J_{15,16E}$ = 10.9)
H-16	*Z*: 4.84 (dd, $J_{16Z,15}$ = 17.5, $J_{16Z,16E}$ = 1.6)	*Z*: 4.87 (dd, $J_{16Z,15}$ = 17.4, $J_{16Z,16E}$ = 1.3) (4.97)	*Z*: 4.90 (dd, $J_{16Z,15}$ = 17.5, $J_{16Z,16E}$ = 1.4)	*Z*: 4.84 (dd, $J_{16Z,15}$ = 16.8, $J_{16Z,16E}$ = 1.2)
	E: 4.89 (dd, $J_{16E,15}$ = 10.8, $J_{16E,16Z}$ = 1.6)	*E*: 4.81 (dd, $J_{16E,15}$ = 10.7, $J_{16E,16Z}$ = 1.3) (4.93)	*E*: 4.88 (dd, $J_{16E,15}$ = 10.4, $J_{16E,16Z}$ = 1.4)	*E*: 4.93 (dd, $J_{16E,15}$ = 10.9, $J_{16E,16Z}$ = 1.2)
H-17	0.97 (s)	1.22 (s) (1.30)	1.04 (s)	1.01 (s)
H-18	1.00 (s)	0.99 (s) (1.02)	A: 3.43 (d, $J_{18A,18B}$ = 10.4)	A: 3.42 (d, $J_{18A,18B}$ = 10.4)
			B: 3.70 (d, $J_{18B,18A}$ = 10.4)	B: 3.68 (d, $J_{18B,18A}$ = 10.4)
H-19	0.81 (s)	0.81 (s) (0.83)	0.93 (s)	0.98 (s)
H-20	0.96 (s)	0.99 (s) (1.00)	0.85 (s)	1.14 (s)
OH	1.31 (br s)	1.45 (br s, 2H) (0.75)	2.42 (s, 2H)	2.06 (br s) and 2.63 (br s)

[a] Multiplicity of signals is given in parentheses: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; coupling constants (apparent splittings) are reported as numerical values in Hz; eq and ax designate equatorial and axial hydrogen atom, respectively, and A and B designate magnetically nonequivalent geminal hydrogens; the assignments are based on homo- and heteronuclear 2D correlations (see Supporting Information). [b] Chemical shifts in C_6D_6 (800 MHz) are given in parentheses.

Tables 3 and 4. The IC_{50} values for the antiplasmodial effect are generally above 50 μM except for **7**, which shows an IC_{50} about 25 μM (Table 3). The values obtained are very similar to the few IC_{50} values available for isopimaranes[47,49] and labdanes.[47]

The antiplasmodial activity of **1**−**9** and **11** was paralleled by changes of erythrocyte membrane shape, as assessed by light microscopy after 19 and 48 h of incubation. The results for the incubation time of 48 h, corresponding to the duration of the antiplasmodial assay, are shown in Table 4 (the changes observed after 19 h were similar but usually less pronounced). An example of erythrocyte shape modifications is shown in Figure 3. The deviations from the normal erythrocyte shape (discocyte) are described using nomenclature according to Bessis.[50] All isopimaranes caused formation of echinocytic forms, suggesting their incorporation into the outer leaflet of the erythrocyte membrane.[51−53] The labdanes **1**−**4** and **11** caused formation of stomatocytes, indicative of incorporation into the inner membrane leaflet.[51−53] The extent of membrane modifications increased with concentration (Table 4) and correlated well with the concentrations at which *P. falciparum* growth inhibition was observed (Table 3). Compound **7**, showing the lowest IC_{50} value, caused progressive transformation

Table 2. ^{13}C NMR Spectra (100 MHz) of Isopimaranes **7**−**10** ($CDCl_3$)

carbon	7	8	9	10
C-1	34.73	37.75	36.96	33.50
C-2	27.77	27.19	27.23	26.71
C-3	78.97	79.03	77.15	75.17
C-4	38.86	38.94	42.21	41.90
C-5	51.05	55.51	48.66	43.54
C-6	18.63	17.59	22.48	34.94
C-7	32.66	43.56	35.59	198.99
C-8	124.63	72.35	136.31	129.06
C-9	136.41	56.79	50.32	164.93
C-10	37.35	36.97	38.01	39.18
C-11	21.18	17.16	18.80	23.09
C-12	34.94	38.05	34.49	33.59
C-13	35.12	36.55	37.43	34.38
C-14	41.84	51.47	129.09	33.38
C-15	146.20	151.54	148.91	145.07
C-16	110.74	108.64	110.16	111.74
C-17	27.96	24.26	25.98	28.03
C-18	28.03	28.21	72.16	70.44
C-19	15.52	15.48	11.49	11.21
C-20	19.42	15.66	15.51	18.31

Table 3. In Vitro Antiplasmodial Activity of Labdanes and Isopimaranes[a]

compound	IC_{50}, μg/mL	IC_{50}, μM
1	18.5 ± 1.6	53.4 ± 4.6
2	54.5 ± 1.4	163.9 ± 4.2
3	33.5 ± 1.7	104.5 ± 5.3
4	41.2 ± 4.0	130.2 ± 12.6
5	29.3 ± 0.8	84.1 ± 2.3
6	50.6 ± 3.0	145.2 ± 8.6
7	7.1 ± 0.6	24.6 ± 2.1
8	24.8 ± 2.1	80.9 ± 6.9
9	28.4 ± 1.4	93.3 ± 4.6
11	24.3 ± 0.6	76.3 ± 1.9
chloroquine[b]	0.0264 ± 0.0025	0.0512 ± 0.0048

[a] IC_{50} values expressing concentration necessary to inhibit growth of *P. falciparum* strain 3D7 by 50%. [b] As chloroquine diphosphate.

of discocytes toward echinocytes starting at the lowest concentrations. This compound differs from the remaining compounds in the series in that it contains only a single oxygen function and can hence be attributed the most pronounced amphiphilic character. Also compound **9**, in which the polar groups are located at one end of the molecule, induced echinocytosis at low concentrations and caused pronounced cell lysis at 100 μg/mL (Table 4). Compounds **3**, **5**, **6**, and **8**, containing two hydroxy groups located at the opposite ends of the molecule, exhibited less pronounced membrane effects. Similarly as in the betulinic acid series,[15] the methyl ester **1** was more potent than the corresponding carboxylic acid **2**. Control experiments demonstrated that the assay conditions by themselves do not affect erythrocytes to any appreciable degree.

In conclusion, the antiplasmodial effect of labdanes and isopimaranes studied in this work was associated with a pronounced membrane-modifying effect on erythrocytes, and both effects were observed in the same concentration region. Although the two effects may in principle be independent, it must be pointed out that the erythrocyte membrane plays an important role in parasite vacuolization and schizont growth.[54−58] It may therefore be expected that changes in chemical composition of the erythrocyte membrane, manifested as deviations from the discocyte form, are likely to affect parasite proliferation. In such cases, the observed in vitro inhibition of growth of *Plasmodium* parasites is indirect and cannot be regarded as a genuine antiplasmodial effect. We believe that many literature reports on antiplasmodial activities of natural products represent in fact the indirect effects on erythrocyte host cells, especially for natural products with amphiphilic character. An example is the recently reported[47] in vitro antiplasmodial activity of a series of labdanes, including **11**. Saponins, which cause lysis of erythrocytes,[59] belong to an extreme case of membrane-active chemicals and have predictably an antiplasmodial activity in vitro.[60,61] We believe, therefore, that antiplasmodial assays using erythrocyte cultures should always be supplemented with microscopical investigations in order to disclose possible erythrocyte membrane-mediated effects. This is especially important with crude extracts, commonly containing membrane-active terpenoids such as those described previously[12−15] and in the present work.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Perkin-Elmer 241 polarimeter. NMR spectra were recorded on a Bruker Avance 600, Bruker Avance 400, or Varian Unity Inova spectrometer (proton frequency 600.13, 400.13, or 799.75 MHz, respectively) at 25 °C or on a 300 MHz Varian Mercury Plus spectrometer at ambient temperature, using $CDCl_3$ as solvent and TMS as internal standard. NOESY spectra were obtained with mixing times of 600 or 700 ms. gHMBC and gHSQC spectra were optimized for $^nJ_{C,H}$ = 7.7 Hz and $^1J_{C,H}$ = 145 Hz, respectively. High-resolution mass measurements for exact mass determination were carried out using a Bruker APEX-Q III 7 T ion-cyclotron resonance mass spectrometer equipped with electrospray ionization (ESI) ion source (Combi source) and operating in positive ion mode. The spectra were externally calibrated with a collision-induced dissociation spectrum of luteinizing hormone releasing hormone. Samples were dissolved in methanol and diluted with spray solution, 0.1% HCOOH in MeOH−H_2O (1:1). Column chromatography was performed on Matrex silica gel 60A (particle size 37−70 μm). VLC separations were performed on Merck silica gel 60H for TLC, particle size <45 μm (90%). TLC separations were performed on Merck precoated silica gel 60 F_{254} plates. Normal-phase preparative HPLC separation were carried out on a 25 × 1.6 cm Lichrosorb Si60 column, 7 μm, eluted isocratically with heptane−THF or heptane−EtOH mixtures (see below) at flow rates of 10 mL/min, with UV absorbance detection at 220 nm. Reversed-

Table 4. Erythrocyte Shape Changes Caused by Incubation with Compounds **1**−**9** and **11**[a]

	compound									
concentration, μg/mL	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**11**
100	SS1***	S2*	S2*	S2**	E1-D	E1-D	E3	E1	SE2****	SS2***
50	S3-S2	E1-D	D	S1-D	E1-D	D	E3	D	SE1-E3	SS1
25	S2-D	D	D	D	D	D	E2	D	E3	S2-S1
12.5	D	D	D	D	D	D	E1	D	E1	D
0[b]	D	D	D	D	D	D	D	D	D	D

[a] Dominating erythrocyte shapes (designated according to Bessis[50]) observed after 48 h of incubation; D, discocyte; E1, E2, E3, echinocyte types 1, 2, and 3; SE1, SE2, spheroechinocyte types 1 and 2; S1, S2, S3, stomatocyte types 1, 2, and 3; SS1, SS2, spherostomatocyte types 1 and 2; the number of stars (*) designates the degree of erythrocyte lysis observed in the culture. [b] Control; identical control cultures were obtained after incubation with or without 0.5% of DMSO in the medium.

phase preparative HPLC separations were carried out on a 25 × 1.6 cm Lichrosorb RP18 column, 5 μm, eluted isocratically with H_2O−MeCN mixtures at 6 mL/min, with UV absorbance detection at 220 nm.

Plant Material. Leaves and branches of *P. orientalis* (L.) Franco (Cupressaceae), syn. *Biota orientalis* (L.) Endl., syn. *Thuja orientalis* L., were collected from wild trees in the Soorkesh Valley, Gollestan, Iran. A voucher specimen (accession number 72894) was deposited in the herbarium TARI (Botanical Department, Research Institute of Forests and Rangelands, Tehran, Iran).

Extraction and Isolation. Dry, powdered leaves of *P. orientalis* were defatted with light petroleum and extracted with 4 × 1.5 L of EtOAc. The combined extracts were evaporated, the residue dissolved in MeOH−H_2O (9:1), and the solution extracted repeatedly with light petroleum. The methanolic solution was evaporated to dryness, and the residue was suspended in MeOH, coated on silica gel, and applied on top of a 75 × 4 cm silica gel column. The column was eluted with light petroleum−EtOAc 8:2 (6 L) and then with light petroleum−EtOAc 7:3 (4 L), collecting 25 mL fractions. The fractions were pooled according to TLC profiles and evaporated, and repeatedly purified by normal-phase preparative HPLC. This yielded 31 mg of **1**, 10 mg of **2**, and 6 mg of **8** (purified by HPLC with heptane−THF 9:1), 3 mg of **4** (purified with heptane−THF, 94:6), 4 mg of **7** (heptane−THF, 4:1), and 20 mg of **5**, 6 mg of **6**, and 6 mg of **9** (heptane−EtOH, 88:12).

Dry, powdered branches of *P. orientalis* were extracted with 4 × 1.5 L of EtOAc and the extract (29.4 g) fractionated on an 8 × 10 cm VLC column eluted with light petroleum (discarded) and then with 3 L of EtOAc, pooling fractions according to their TLC profiles. Repeated VLC using toluene−EtOAc mixtures, followed by repeated purification using preparative reversed-phase HPLC afforded 12 mg of **3** (purified using H_2O−MeCN, 9:1) and 2 mg of **10** (purified using H_2O−MeCN, 4:1).

Pinusolide (1): colorless oil; $[\alpha]^{25}_D$ +47° (*c* 3.0, $CHCl_3$), lit.[26] $[\alpha]^{23}_D$ +58.5° (*c* 0.1, $CHCl_3$); HR ESI FT MS *m/z* 347.22159 (74%) and 369.20351 (100%), $C_{21}H_{31}O_4^+$ and $C_{21}H_{30}O_4Na^+$ require 347.21159 (Δ*M* 0.29 ppm) and 369.20351 (Δ*M* 0.33 ppm), respectively; ^{1}H and ^{13}C NMR data as reported.[26]

Pinusolidic acid (2): colorless oil; $[\alpha]^{25}_D$ +53° (*c* 0.36, $CHCl_3$), lit.[25] $[\alpha]^{23}_D$ +54.5° (*c* 0.1, $CHCl_3$); HR ESI FT MS *m/z* 333.20608 (28%) and 355.18796 (100%), $C_{20}H_{29}O_4^+$ and $C_{20}H_{28}O_4Na^+$ require 333.20604 (Δ*M* 0.12 ppm) and 355.18798 (Δ*M* 0.06 ppm), respectively; ^{1}H and ^{13}C NMR data as reported.[23,24,27,31]

Isocupressic acid (3): colorless oil; $[\alpha]^{25}_D$ +43° (*c* 0.27, $CHCl_3$), lit.[32] $[\alpha]^{25}_D$ +42° (*c* 2.5, $CHCl_3$); HR ESI FT MS *m/z* 343.22457 (100%,), $C_{20}H_{32}O_3Na^+$ requires 343.22437 (Δ*M* 0.58 ppm); ^{1}H and ^{13}C NMR data as reported.[24,32]

Lambertianic acid (4): colorless oil; $[\alpha]^{25}_D$ +53° (*c* 0.26, $CHCl_3$), lit.[33] $[\alpha]^{25}_D$ +55° (*c* 0.69, EtOH); HR ESI FT MS *m/z* 317.21120 (100%) and 339.19308 (33%), $C_{20}H_{29}O_3^+$ and $C_{20}H_{28}O_3Na^+$ require 317.21112 (Δ*M* 0.25 ppm) and 339.19307 (Δ*M* 0.03 ppm), respectively; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 12.84, 19.89, 23.59, 24.28, 26.07, 28.98, 37.98, 38.69, 39.05, 40.38, 44.13, 55.25, 56.23, 106.51, 110.96, 125.45, 138.74, 142.68, 147.83, 182.55; ^{1}H NMR data as reported.[34]

16-Oxo-8(17),13-labdadiene-15,19-dioic acid, lactol form (5): colorless oil; $[\alpha]^{25}_D$ +38° (*c* 1.03, $CHCl_3$), no lit. value available; HR ESI FT MS *m/z* 349.20108 (14%) and 371.18291 (100%), $C_{20}H_{29}O_5^+$ and $C_{20}H_{28}O_5Na^+$ require 349.20095 (Δ*M* 0.37 ppm) and 371.18290 (Δ*M* 0.03 ppm), respectively; ^{1}H HMR (600 MHz, chemical shifts from HSQC correlation) δ H-1ax 1.11, H-1eq 1.84, H-2ax 1.89, H-2eq 1.53, H-3ax 1.05, H-3eq 2.17, H-5 1.33, H-6ax 1.87, H-6eq 1.99, H-7ax 1.89, H-7eq 2.42, H-9 1.65, H-11A 1.64, H-11B 1.78/1.82, H-12A 2.18/2.33, H-12B 2.52/2.64, H-14 5.85/5.86, H-16 5.97/6,00, H-17A 4.50, H-17B 4.90, H-18 1.26, H-20 0.63; ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 12.75/12.78 (C-20), 19.86 (C-2), 21.00/21.12 (C-11), 26.00 (C-6), 26.76/26.84 (C-12), 28.97 (C-18), 37.85 (C-3), 38.57 (C-7), 39.18 (C-1), 40.55/40.63 (C-10), 44.18 (C-4), 55.49/55.71 (C-9), 56.15/56.20 (C-5), 98.86/99.37 (C-16), 106.73/106.85 (C-17), 117.09/117.31 (C-14), 147.23 (C-8), 170.29 (C-13), 171.64 (C-15), 183.35 (C-19).

15-Oxo-8(17),13-labdadiene-16,19-dioic acid, lactol form [15ξ-hydroxypinusolidic acid] (6): colorless oil; $[\alpha]^{25}_D$ +30° (*c* 0.65, $CHCl_3$), lit.[27] $[\alpha]^{25}_D$ +30.5° (*c* 1.0, $CHCl_3$); HR ESI FT MS *m/z* 349.20104 (33%) and 371.18292 (100%), $C_{20}H_{29}O_5^+$ and $C_{20}H_{28}O_5Na^+$ require 349.20095 (Δ*M* 0.26 ppm) and 371.18290 (Δ*M* 0.03 ppm), respectively; ^{1}H HMR (600 MHz, chemical shifts from HSQC correlation) δ H-1ax 1.08, H-1eq 1.83, H-2ax 1.84, H-2eq 1.52, H-3ax 1.06, H-3eq 2.16, H-5 1.34, H-6ax 1.87, H-6eq 1.99, H-7ax 1.89, H-7eq 2.43, H-9 1.62, H-11A 1.60, H-11B 1.77, H-12A 2.10, H-12B 2.45, H-14 6.84, H-15 6.09, H-17A 4.56/4.57, H-17B 4.89, H-18 1.24, H-20 0.60; ^{13}C NMR (150.9 MHz, $CDCl_3$) δ 12.78 (C-20), 19.86 (C-2), 21.73/21.81 (C-11), 24.51/24.55 (C-12), 26.03 (C-6), 28.97 (C-18), 37.93 (C-3), 38.60 (C-7), 39.18 (C-1), 40.52 (C-10), 44.17 (C-4), 55.77 (C-9), 56.23 (C-5), 96.50/96.51 (C-15), 106.83/106.88 (C-17), 138.94/138.96 (C-13), 142.82/142.85 (C-14), 147.27/147.33 (C-8), 171.40 (C-16), 182.90 (C-19).

8(9),15-Isopimaradien-3β-ol (7): colorless oil; $[\alpha]^{25}_D$ +105° (*c* 0.36, $CHCl_3$), lit.[41] $[\alpha]^{25}_D$ +92° (*c* 0.82, $CHCl_3$); HR ESI FT MS *m/z* 311.23463 (100%), $C_{20}H_{32}ONa^+$ requires 311.23454 (Δ*M* 0.29 ppm); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

15-Isopimaren-3β,8β-diol (8): colorless oil; $[\alpha]^{25}_D$ −9° (*c* 0.36, $CHCl_3$), lit.[17] $[\alpha]^{25}_D$ +3° (*c* 1.0, $CHCl_3$) for *ent*-**8**; HR ESI FT MS *m/z* 329.24533 (100%), $C_{20}H_{34}O_2Na^+$ requires 329.24510 (Δ*M* 0.70 ppm); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

8(14),15-Isopimaradien-3β,19-diol (9): colorless oil; $[\alpha]^{25}_D$ −23° (*c* 1.18, $CHCl_3$), lit.[43] $[\alpha]^{23}_D$ −17° ($CHCl_3$); HR ESI FT MS *m/z* 327.22947 (100%,), $C_{20}H_{32}O_2Na^+$ requires 327.22945 (Δ*M* 0.06 ppm); ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

3β,19-Dihydroxy-8(9),15-isopimaradien-7-one (10): colorless oil; $[\alpha]^{25}_D$ +52° (*c* 0.06, $CHCl_3$); HR ESI FT MS *m/z* 319.22686 (100%) and 341.20887 (56%), $C_{20}H_{31}O_3^+$ and $C_{20}H_{30}O_3Na^+$ require 319.22677 (Δ*M* 0.28 ppm) and 341.20872 (Δ*M* 0.44 ppm), respectively; ^{1}H and ^{13}C NMR data, see Tables 1 and 2.

Assay for Antiplasmodial Activity. The assay was carried out essentially as previously described[12,62] with asynchronous cultures of chloroquine-sensitive *P. falciparum* strain 3D7 (initial parasitemia 1.5%), adding test compounds **1**−**9** and **11**[47] dissolved in DMSO solutions (to give a final DMSO concentration in the growth medium up to 0.5%) and using incorporation of [^{3}H]phenylalanine as the index of growth. Inhibition curves were obtained with eight different concentrations of the test compounds, with each concentration used in triplicate. Chloroquine diphosphate was employed as a positive reference.

Assay for Erythrocyte Membrane Shape Modifications. Nonparasitized erythrocytes were incubated in 96-well microtiter plates in medium containing test compounds **1**−**9** and **11**[47] as in the assay for antiplasmodial activity,[12,62] but omitting addition of the radioactive tracer. Control wells contained 0% or 0.5% of DMSO but no test compounds; no difference between these two controls was observed. After 19 h (approximately in the middle of the incubation period) and after 48 h (end of incubation), 20 μL samples were spread on microscope slides, allowed to dry, fixed with methanol, and stained with Giemsa for phase-contrast light microscopy.

Molecular Mechanics Calculations. Implementation of the MM2 force field[37,38] in Chem3D Pro software from CambridgeSoft Corporation, Cambridge, MA (ver. 5.0, 1999), was used to calculate molecular models.

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Supporting Information Available: 2D NMR connectivities (600 MHz COSY, NOESY, HMBC) for **7**−**10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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