Antibacterial Diterpenes from *Calceolaria pinifolia*

Girma M. Woldemichael,[†] Gerald Wächter,[†] Maya P. Singh,[‡] William M. Maiese,[‡] and Barbara N. Timmermann*,[†]

Department of Pharmacology and Toxicology, Division of Medicinal and Natural Products Chemistry, College of Pharmacy, University of Arizona, Tucson, Arizona 85721-0207, and Wyeth Research, Natural Products Microbiology, Pearl River, New York 10965

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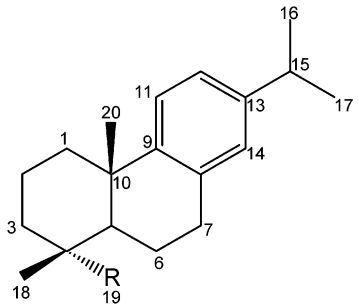
Two new isopimaranes, 19-methylmalonyloxy-*ent*-isopimara-8(9),15-diene (**5**) and 19-malonyloxy-*ent*-isopimara-8(9),15-diene (**6**), were isolated using bioassay-guided fractionation of the CH_2Cl_2−MeOH (1:1) extract of the aerial part of *Calceolaria pinifolia* along with eight other diterpenes (**1**−**4**, **7**−**10**) and two triterpenes (**11**, **12**). All compounds were assayed against *Staphylococcus aureus* (SA), methicillin-resistant *S. aureus* (MRSA), *Bacillus subtilis* (BS), and *Escherichia coli* (EC). 4-Epi-dehydroabietinol (**2**) and *ent*-isopimara-9(11),15-diene-19-ol (**8**) were found to be active against MRSA with MIC values of 8 and 2 μg/mL, respectively. Mechanistic studies of **8** in BS suggested rapid and nonspecific inhibition of uptake and incorporation of radiolabeled precursors into DNA, RNA, and protein consistent with membrane-damaging effects in bacteria. Compound **8** did not afford protection against an acute infection with SA in mice.

Plants of the genus *Calceolaria* (Scrophulariaceae) are found distributed throughout Central and South America and New Zealand.[1] *Calceolaria pinifolia*, a wild shrub, was collected in a semiarid and mountainous region of North Western Argentina, where it is locally used as an astringent. Traditionally, various species belonging to the genus *Calceolaria* are used as bactericidal agents, stomachics, and sweeteners.[2] As part of the International Cooperative Biodiversity Group (ICBG) program "Bioactive Agents from Dryland Biodiversity of Latin America", *C. pinifolia* was included in screening for activity against various pathogens. An initial bioassay of the dichloromethane−methanol extract of the plant showed antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecium* including methicillin- and vancomycin-resistant strains, respectively, of these bacteria. On the basis of this important activity, the plant was selected for further work. Many species of the genus *Calceolaria* are known for their content of diterpenes, and chemical investigations undertaken on other *Calceolaria* species have so far led to the isolation of 55 new diterpenes belonging to six skeletal types. We report here the characterization of constituents of the bioactive fraction, which yielded two novel pimarane-type diterpenes along with several known pimarane and abietane diterpenes from *C. pinifolia*.[3−5] This, to our knowledge, is the first report describing the chemical investigation of *C. pinifolia* and the antibacterial activity of its diterpenes.

Results and Discussion

Upon discovery that the CH_2Cl_2−MeOH (1:1) extract of *C. pinifolia* exhibited activity against *B. subtilis*, *S. aureus*, and *E. faecium* with MIC values of 8, 16, and 16 μg/mL, respectively, bioassay-guided fractionation of the extract was undertaken as described in the Experimental Section. Extensive chromatographic purification of components of several of the bioactive fractions afforded two new diterpenes, **5** and **6**, as well as several known compounds, **1**−**4** and **7**−**12**.



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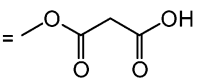
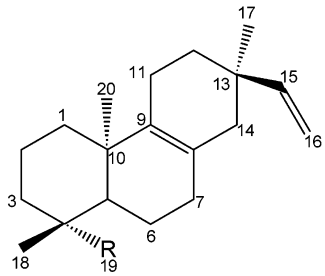
3 R = 



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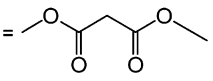
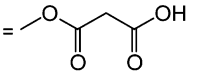
5 R = 

6 R = 

The IR spectrum of **5** indicated the presence of hydroxyl and ester carbonyl groups through characteristic absorptions. Its molecular formula was established as $C_{24}H_{36}O_4$ using HRFABMS. Analysis of its ^{13}C and DEPT spectra revealed that it possesses four methyls, 10 methylenes, two methines, and seven quaternary carbons. Quaternary

* To whom correspondence should be addressed. 1703 E. Mabel St., Tucson, AZ 85721-0207. Tel: (520) 626-2481. Fax: (520) 626-2515. E-mail: btimmer@pharmacy.arizona.edu.
[†] University of Arizona.
[‡] Wyeth Research.

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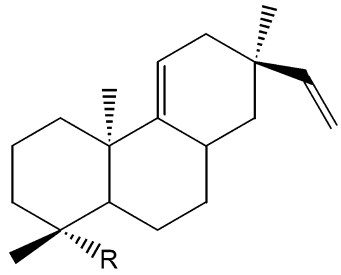

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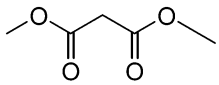
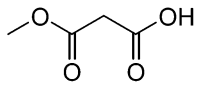
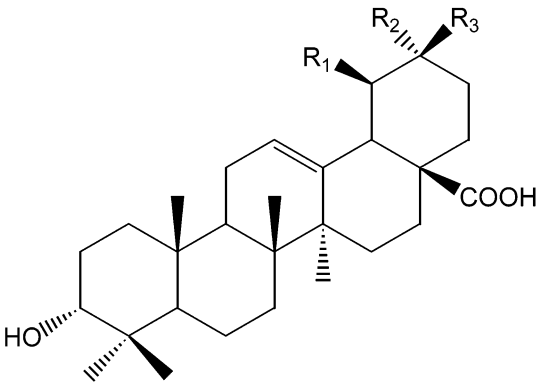
9 R = 

10 R = 



11 R_1 = CH_3 R_2 = CH_3 R_3 = H

12 R_1 = H R_2 = CH_3 R_3 = CH_3

carbon signals observed in the ^{13}C spectrum at δ 124.4 (C-8) and 136.6 (C-9) confirmed the presence of a tetrasubstituted double bond, while those at δ 146.1 (C-15) and 110.7 (C-16) in the ^{13}C and at δ 5.72 (H-15) and 4.82 (H-16a) and 4.87 (H-16b) in the ^1H spectrum made the presence of a vinyl group readily apparent (Table 1). Also identifiable were oxymethylene signals at δ 4.34 (d, J = 10.9 Hz, H-19a) and 3.96 (d, J = 10.9 Hz, H-19b) and signals for three methyl groups at δ 0.95 (s, 6H, H-17, H-18) and 0.94 (s, 3H, H-20) in the ^1H spectrum. Comparison of the spectral data with those of **4** showed that **5** also possessed the same isopimara-8(9),15-diene skeleton. However, a significant downfield shift for C-19 compared with that in **4** indicated substitution at this position. Also consistent with these observations was a methyl malonate substitution at this position, as evidenced by the signals at δ 166.7, 41.5, 167.0, and 52.4 in the ^{13}C spectrum together with those at δ 3.37 (s, 2H) and 3.73 (s, 3H) in the ^1H spectrum. Additional confirmation for this was obtained by the observation of a cross-peak between δ 166.7 and H-19a and H-19b oxymethylene signals in the HMBC spectrum. Confirmation for the α-axial orientation at C-19 was provided by ROESY and selective 1D NOE experiments (Figure 1). Lack of a NOE cross-peak between H-5 and H-20 in the ROESY spectrum showed that the two protons were trans-connected. On the other hand, irradiation of the signals for the two oxymethylene protons at C-19 produced NOE effects only at δ 0.94 (methyl protons at

Table 1. ^{13}C and ^1H NMR Spectral Data for **5** and **6**[a]

	5		**6**	
position	^{13}C	^1H (J in Hz)	^{13}C	^1H (J in Hz)
1	36.4	1.74 dd (8.7, 1.4)	36.3	1.74 ov
		1.01 dd (8.7, 4.1)		1.01 ov
2	18.5	1.53 dt (14.2, 1.4)	18.5	1.51 m
		1.45 ov		1.44 ov
3	36.0	1.67 dd (14.2, 1.4)	35.9	1.67
		1.00 ov		1.00 ov
4	37.1		37.1	
5	52.6	1.27 dd (12.8, 1.7)	52.5	1.27 dd (13.0, 1.5)
6	19.1	1.76 ov	19.0	1.76 ov
		1.44 ov		1.44 ov
7	32.7	1.90 ov (2H)	32.7	1.89 ov (2H)
8	124.4		124.5	
9	136.6		136.5	
10	37.4		37.3	
11	21.2	1.87 ov (2H)	21.2	1.87 ov
12	34.9	1.48 ov	34.9	1.47 ov
		1.29 t (7.8)		1.28 t (8.0)
13	35.1		35.0	
14	41.9	1.79 br s	41.8	1.77 br s
		1.71 br s		1.69 d (2.5)
15	146.1	5.72 dd (17.4, 10.5)	146.1	5.72 dd (17.5, 10.5)
16	110.7	4.82 dd (17.4, 1.4)	110.7	4.82 dd (17.5, 1.5)
		4.87 dd (10.5, 1.4)		4.87 dd (10.5, 1.5)
17	28.0	0.95 s	28.0	0.95 s
18	27.0	0.95 s	27.0	0.96 s
19	68.5	4.34 d (10.9)	68.9	4.38 d (11.0)
		3.96 d (10.9)		4.00 d (11.0)
20	19.9	0.94 s	19.9	0.94 s
$COCH_2CO_2-CH_3$/H	166.7		167.4	
$COCH_2CO_2-CH_3$/H	41.5	3.37 s (2H)	40.6	3.43 s (2H)
$COCH_2CO_2-CH_3$/H	167.0		170.7	
$COCH_2CO_2-CH_3$	52.4	3.73 s		

[a] ov = overlapped.

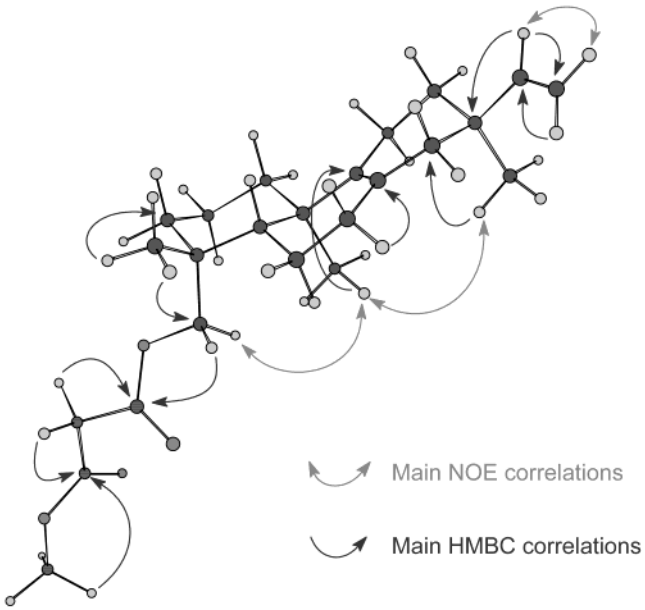


Figure 1. Significant NOE and HMBC correlations of **5**.

C-20) and with each other, confirming that they were both on the same side of rings A and B as the C-20 α-methyl protons. A β-equatorial orientation for the vinyl group at C-13 was determined on the basis of the characteristic ^{13}C

Table 2. Antimicrobial Activity of Compounds **1**−**12** (MIC values in μg/mL)

compound	*Bacillus subtilis* 327	*Staphylococcus aureus* 375	*Staphylococcus aureus* 310[a]	*Escherichia coli* imp389
1	32	16	32	128
2	8	4	4	>128
3	>128	>128	>128	>128
4	32	32	64	>128
5	>128	>128	>128	>128
6	4	8	16	32
7	32	16	32	>128
8	4	2	2	>128
9	>128	>128	>128	>128
10	16	8	32	>128
11	64	>128	>128	>128
12	64	>128	>128	>128
penicillin G[b]	0.03	0.03	128	1

[a] Methicillin-resistant. [b] Control.

chemical shifts for C-15, C-16, and C-17 and multiplicities observed for H-15, H-16, and H-17.[6] These facts, thus, enabled the characterization of **5** as 19-methylmalonyloxy-*ent*-isopimara-8(9),15-diene.

On the other hand, **6**, whose molecular formula was determined as $C_{23}H_{34}O_4$ through HRFABMS, was also identified as an isopimara-8(9),15-diene through characteristic chemical shifts at δ 124.5 (C-8), 136.5 (C-9), 146.1 (C-15), and 110.7 (C-16) in the ^{13}C and at δ 5.72 (H-15), 4.82 (H-16a), 4.87 (H-16b), 0.95 (s, 3H, H-17), 0.96 (s, 3H, H-18), and 0.94 (s, 3H, H-20) in the ^{1}H NMR spectra. The ^{1}H and ^{13}C NMR data suggested a structure similar to compound **5** except for a mass difference of −14 mmu and absence of a methyl group in the malonate moiety at C-19 in the NMR spectra. In addition, the IR spectrum through obvious changes in the carbonyl stretches indicated the presence of both free and ester carbonyl groups. These confirmed that **6** possessed a malonic acid substitution at C-19 instead of a methyl malonate moiety as in **5**. An upfield shift of the methylene carbon and the downfield shift for both carbonyl carbons in the malonate group also confirmed the same. Analysis of its ROESY spectrum and comparison of chemical shifts and coupling constants in the ^{1}H NMR spectrum of **6** with that of **5** established an identical stereochemistry at C-13 and C-4 in **6**. The structure of **6** was, therefore, determined to be 19-malonyloxy-*ent*-isopimara-8(9),15-diene.

Compounds **1**−**12** were tested for their antimicrobial activity against *B. subtilis*, methicillin-susceptible (MSSA) and -resistant *S. aureus*, and *E. coli* imp. Compounds **1**, **2**, **4**, **6**, **8**, and **10** were active at concentrations less than 128 μg/mL against all three Gram-positive organisms including MRSA, but none with the exception of compound **6** were active against the *E. coli* imp strain. Compounds **2** and **8**, however, were found to be the most potent of the tested compounds against the Gram-positive microbes with MIC values between 2 and 8 μg/mL (Table 2). The two triterpenes **11** and **12**, in contrast, exhibited little or no antibacterial effect. Compounds **5** and **9**, methyl esters of **6** and **10**, respectively, showed a significant drop in activity compared to the free acids, indicating that a free carboxylic group in the malonate moiety at C-19 plays a role in the activity of these compounds. Also, among the diterpenes, it appears that an oxymethylene group at C-19 might also contribute toward antimicrobial activity, since it was observed that replacement of this with a carboxylic group as in **1** and **7**, when compared with **2** and **8**, respectively, shows moderation of activity.

To gain insight into the mechanism of action of the active compounds, the most active compound, compound **8**, was tested for its effects on the uptake of radiolabeled precursors ([^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]amino acids) into drug-treated cells and on their incorporation into DNA, RNA, and protein, respectively, of a logarithmic-phase culture of *B. subtilis*. Compound **8** rapidly and nonspecifically inhibited both uptake and incorporation of radiolabeled thymidine, uridine, and amino acids at its MIC or higher concentrations (Table 3). This rapid and nonspecific inhibition of uptake of macromolecular precursors into the drug-treated cells suggested a strong membrane-damaging effect on bacterial cells, but the precise mode of action was not elucidated. Control drugs used, on the other hand, affected the anticipated macromolecular processes. Polymyxin B, a known membrane-active antibiotic, significantly prevented the uptake and incorporation of all three precursors.

Since compound **8** appeared to affect the integrity of bacterial membrane function, its effect on human red blood cells was evaluated. Compound **8** was not found to be hemolytic at the MIC range of 1−2 μg/mL, but did show significant hemolytic activity at 32−128 μg/mL, suggesting some selectivity for the bacterial membrane.

The in vivo results of compound **8** are summarized in Table 4. A single subcutaneous dose of the compound did not afford protection against *S. aureus* infection in a murine model.

Experimental Section

General Experimental Conditions. Optical rotation and IR (as a film on a diamond cell) were measured on a Jasco P-1020 digital polarimeter and a Thermo Nicolet Avatar 360 FT-IR spectrometer, respectively. A JEOL HX110A mass spectrometer was used in recording HRFAB mass spectra. NMR spectra (^{1}H, selective 1D NOE, ^{13}C, DEPT-135, DEPT-90, HSQC, HMBC, DQF-COSY, ROESY) were recorded using either a Bruker DRX-500 or DRX-600 spectrometer in $CDCl_3$. Chemical shifts were expressed in ppm (δ) using partially deuterated solvent chemical shifts at δ 77.0 (^{13}C) and 7.24 (^{1}H) as reference for ^{13}C and ^{1}H NMR signals, respectively. The mixing times used in recording ROESY and selective 1D NOE spectra were 200 and 600 ms, respectively. Purification of fractions was carried out using both open and flash column chromatography with silica gel (63−200 and 32−63 μm, respectively, SAI) and/or 0.5 mm silica gel 60 (Merck) PTLC

Table 3. Effect of **8** on Incorporation of Radiolabeled Precursors into Macromolecules in *B. subtilis*[a]

	conc	% ^{3}H-thymidine		% ^{3}H-uridine		% ^{3}H-amino acid	
compound	(μg/mL)	uptake	incorporation	uptake	incorporation	uptake	incorporation
8	8	1	1	0	0	1	2
	4	1	1	0	0	1	2
	2	2	2	1	1	1	4
	1	13	20	1	2	2	36
ciprofloxacin	0.25	73	61	100	94	104	94
rifampin	0.25	94	108	143	29	90	25
chloramphenicol	8	74	80	141	166	83	10
polymyxin B	8	22	20	2	2	37	16

[a] Data presented as % of untreated control.

Table 4. Activity of **8** in *S. aureus* Infection Model (in mg/kg)

	8		vancomycin	
route	ED_{50}	LD_{50}/ED_{50}	ED_{50}	LD_{50}/ED_{50}
SSC[a]	>64	ND[b]	1.00	>1024

[a] Single subcutaneous dose. [b] Not determined.

Table 5. Effect of **8** on Human Red Blood Cells

	percentage lysis	
conc (μg/mL)	**8**	amphotericin B
128	88.08	NT[a]
64	80.15	NT
32	71.7	NT
16	14.79	117.84
8	3.32	96.87
4	0.30	74.63
2	0.45	27.54
1	0.08	9.70
0.5	−0.3	0.00
untreated	0.00	0.00

[a] Not tested.

plates. Compounds were finally isolated with a Varian ProStar semiprep HPLC system equipped with 9012 pump and a 9065 PDA detector.

Plant Material. The plant material was collected in January 1997, 46 km west of Puesto de Gendarmeria in the province of San Juan, Argentina (30°21′ S; 69°40′ W), by Renée H. Fortunato. A voucher specimen (RHF 5540) has been deposited at the Instituto Nacional de Tecnologia Agropecuaria (INTA), Buenos Aires, Argentina. Intellectual Property Rights Agreements for plant collections and collaborative research have been fully executed between the University of Arizona and INTA.

Extraction and Isolation. Initial extraction of 200 g of aerial parts of the plant with CH_2Cl_2−MeOH, 1:1, followed by silica gel CC using 5−50% acetone in hexanes yielded 36 fractions. Repeated CC of fraction 12 using 2.25−3% acetone in hexanes and repeated HPLC purification using a Reliasil C-18 column (10 × 250 mm, 10 μm, 5.4 mL/min, Column Engineering) with 80% acetonitrile in water gave **6** and **10** (t_R = 13.6 min, through peak shaving). A further 800 g of powder of the aerial part of the plant was again extracted with 10 L of CH_2Cl_2−MeOH, 1:1, to yield 200 g of extract. The extract was then fractionated using a 1−3% gradient of Me_2CO in hexanes collecting 400 mL fractions on a flash column. Fractions 10−18 showed antibacterial activity and were thus combined and further fractionated using open CC eluting with CH_2Cl_2, collecting 200 mL fractions. The combined fractions 13−39 of the CH_2Cl_2 wash yielded compounds **1**−**5** and **7**−**9** on HPLC purification (t_R = 18.5, 20.3, 30.2, 31.4, 39.2, 34.4, 36.3, and 41.3 min, respectively) using a Reliasil C-18 column (10 × 250 mm, 10 μ, 5.4 mL/min, Column Engineering) and a MeOH−0.15% formic acid in H_2O, 80:20 to 100:0 in 45 min, gradient. The combined fractions 40−59, on the other hand, yielded a mixture of **11** and **12** after PTLC (CH_2Cl_2−MeOH, 98:2), which were then separated using HPLC on a Lichrosphere RP-18 column (10 × 250 mm, 10μ, 5.2 mL/min, Column Engineering) using a MeOH−0.15% formic acid in H_2O gradient, 75:20 to 100:0 in 12 min to 100:0 in 5 min (t_R = 11.5 and 11.7 min, respectively). Isolated compounds were identified after analysis of their NMR and FAB spectral data.

In Vitro Antimicrobial Activity Testing. The in vitro antibacterial activities were determined by the agar diffusion or microbroth dilution method as previously described.[7] The minimum inhibitory concentrations (MICs) were determined by the microbroth dilution method as recommended by the National Committee for Clinical Laboratory Standards.[8] Mueller-Hinton II broth was used for nonfastidious aerobic bacteria, and the medium was supplemented with 5% lysed horse blood for the testing of streptococci. Microtiter plates containing 2.5 μL per well of 2-fold serial dilutions of each antimicrobial agent were inoculated with 100 μL of bacterial suspension to yield the appropriate density ((1−5) × 10^5 CFU/mL), as previously described.[9] The plates were incubated for 18 h at 35 °C in ambient air. The MIC was defined as the lowest concentration of a compound that completely inhibited the growth of the organism as determined by the unaided eye.

A modified version of the agar diffusion method was used to evaluate the activities of the crude extract, fractions, and pure compounds against selected bacterial isolates. Assay plates (12 × 12 in. Sumilon) were prepared by pouring 125 mL of agar medium (tempered at 50 °C) inoculated with an overnight broth culture of the test organisms (adjusted to a final inoculum density of approximately 10^6 cells per mL). The medium was allowed to solidify, and 144 wells (5 mm diameter) were bored into the agar layer using an automated plate-welling machine. Volumrd of 10−20 μL of antibiotic solutions diluted in a suitable solvent were dispensed into wells, and the plates were incubated at 37 °C for 18 h. The zones of growth inhibition were measured using a hand-held digital caliper. Fractions showing zones of inhibition of 8 mm or greater were selected for further purification. The bacterial strains used are laboratory cultures maintained in the Wyeth research collection.

Investigation of Inhibition of Macromolecular Synthesis. Mechanistic studies using *B. subtilis* were undertaken through measurement of incorporation of appropriate radiolabeled precursors into trichloroacetic acid (TCA)-precipitable material.[7] An overnight culture was diluted 1:500 in fresh modified minimal medium (50 mL medium/250 mL Erlenmeyer flask) and incubated at 37 °C and 200 rpm to an A_{600} of 0.20. Aliquots of 100 μL were dispensed into microtiter wells containing 5 μL of antibiotic, and plates were incubated for 2−14 min at 37 °C with vigorous agitation. Cells were pulse-labeled for 5 min by adding one of the following radiolabeled precursors at the indicated final concentrations: [^{3}H]thymidine (Tdr) (1 μCi/mL with 0.05 μg/mL of unlabeled Tdr/mL), [^{3}H]-uridine (Udr) (1 μCi/mL with 0.12 μg/mL of unlabeled Tdr/mL), or [^{3}H]amino acid (AA) (10 μCi/mL). To determine specific incorporation into DNA, RNA, and protein, 100 μL of chilled (4 °C) TCA (10%) supplemented with 0.5 mg of unlabeled precursors per mL was added to each well, and the plate was immediately refrigerated for 1 h. The precipitate was collected on a glass fiber filter (Wallac filtermat B, Wallac 1205−404) using a Skatron 96-well cell harvester (Model 11050) programmed for a 3 s prewet with chilled DI water, a 12 s wash with 5% chilled TCA, and a 5 s drying cycle. Filter mats were dried for 7 min at high power in a microwave oven (Quasar, 700 W), solid scintillant (Meltilex B, Pharmacia 1205−402) was applied, and the isotope that was retained on the filter was quantitated in an LKB Betaplate scintillation counter (Wallac 1205). The levels of incorporation of [^{3}H]Tdr, [^{3}H]Udr, and [^{3}H]AA were expressed as a percentage of that of the untreated control.

Effect on Red Blood Cells. RBCs from 1 mL of freshly pooled human blood were washed four times with normal saline and resuspended in 1 mL of RBC buffer (10 mM Na-phosphate + 150 mM NaCl, pH 7.4). Twenty five microliters (25 μL) of the RBC suspension was added to the microfuge tube containing 1 mL of drug solution prepared in duplicate in RBC buffer to a final concentration in the range 1−128 μg/mL. After 2 h of treatment, the tube was centrifuged and the A_{540} of the supernatant was measured. For 100% lysis, 25 μL of RBC suspension was added into 1 mL of water.

In Vivo Activity Testing. The in vivo activity was assessed in female mice, strain CD-1 (from Charles River Laboratories, NY), weighing 20 ± 2 g each, infected intraperitoneally with sufficient bacterial cells suspended in broth or about 5% mucin to kill 95−100% of the untreated mice within 48 h. Antibiotic was administered subcutaneously in single doses 30 min after infection. Seven-day survival ratios from 3 or 4 separate tests each with 2 dose levels and 5 animals per dose level were pooled for the determination of the median effective dose (ED_{50}) by probit analysis.[7]

Methyl 19-malonyloxy-*ent*-isopimara-8(9),15-diene (5): white powder; $[\alpha]^{25}_D$ +41.3° ($CHCl_3$, *c* 0.8); HRFAB$^+$ 389.2630, FAB$^-$ 387.9328, $C_{24}H_{36}O_4$; IR ν_{max} (cm^{-1}) 2928, 1753, 1737,

1340, 1152. 1021; ^{13}C NMR (125 MHz, $CDCl_3$) and ^1H NMR (600 MHz), see Table 1.

19-Malonyloxy-*ent*-isopimara-8(9),15-diene (6): white crystal; $[\alpha]^{25}_D$ +90.4° ($CHCl_3$, *c* 0.54); HRFAB$^+$ 375.2546, $C_{23}H_{34}O_4$; IR ν_{max} (cm^{-1}) 3241, 2930, 2845, 1753, 1699, 1302, 1146; ^{13}C NMR (125 MHz, $CDCl_3$) and ^1H NMR (500 MHz), see Table 1.

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Supporting Information Available: Spectroscopic data for compounds **1**−**4** and **7**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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