

SERCA-Inhibiting Activity of C-19 Terpenolides from *Thapsia garganica* and Their Possible Biogenesis

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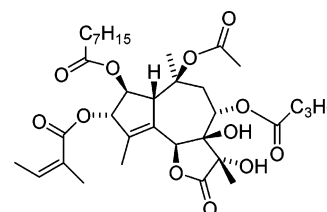
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An investigation of *Thapsia garganica* afforded a series of tetracyclic C-19 dilactones, whose production was dependent on the time and location of the collection. These unusual tetrahomosesquiterpenoids are presumably biosynthesized via a carbon dioxide-triggered electrophilic polyolefin cyclization. Despite the structural differences with thapsigargin, these compounds showed SERCA-inhibiting properties.

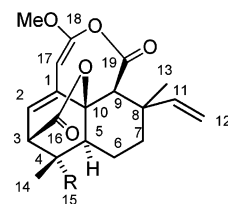
The biomedical relevance of the Mediterranean umbelliferous plant *Thapsia garganica* L. can hardly be overestimated, since the guaianolide thapsigargin (**1**), its major constituent, has become an indispensable tool in cell physiology and in the study of calcium homeostasis.¹ Compound **1** is a powerful and selective (K_i ca. 2 pM) inhibitor of sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPases (SERCA)¹ and holds considerable potential as an anticancer drug.² Thapsigargin is expensive,³ and its concentration in *T. garganica* varies considerably on account of the existence of several chemotypes.⁴ As part of a research program on anticancer natural products, multi-gram amounts of compound **1** were needed. Since *T. garganica* is common in Sardinia, we investigated its potential as a thapsigargin source. Most Sardinian samples of *T. garganica* showed a relatively high content (around 1%) of **1**, but we noticed that two summer (late June) collections of roots also contained an additional more polar compound that was characterized as an unusual C-19 dilactone (**2c**). A further winter collection from the same location afforded three additional analogues (**2a,b,d**) that were absent in the summer collection. While these studies were in progress, a report on the occurrence of C-19 terpenolides, including compounds **2a,b**, in a Moroccan collection of *T. transtagana* appeared,⁵ prompting us to disclose the characterization of analogues **2c,d** as new members of this unusual class of compounds. Despite marked structural differences with thapsigargin, C-19 terpenolides behave as biological analogues of this poly-oxygenated guaianolide, showing potent SERCA-inhibiting properties.

Results and Discussion

The dilactones **2a–d** were obtained as white powders by gravity column chromatography of an acetone extract from the roots of a winter collection of *T. garganica*. Crystallization from ether of various fractions more polar than those containing compound **1** afforded **2a–d** as white powders. Compounds **2a** and **2b** were identified as transtaganolides D and C, respectively.⁵ The NMR spectra of



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R	
2a Me	C-8 α -Methyl, β -Vinyl
2b Me	C-8 β -Methyl, α -Vinyl
2c COOMe	C-8 α -Methyl, β -Vinyl
2d CH ₂ OAc	C-8 α -Methyl, β -Vinyl

the new compounds **2c** and **2d** (see Table 1) showed the peculiar ¹³C resonances of the enol lactone moiety (δ ca. 80 and 155, C-17 and C-18, respectively) and of the vinyl group (δ ca. 140 and 110) of C-19 *Thapsia* terpenolides. The downfield resonance of the vinyl methine proton at around 7 ppm showed that **2c** and **2d** belong to the 8S series, where the vinylic H-11 is deshielded by the combined effect of the *syn*-oriented ethereal lactone oxygen at C-10 and the carbonyl oxygen of the enol lactone moiety. High-resolution MS experiments revealed the elemental composition of **2c,d** as C₂₁H₂₄O₇ and C₂₂H₂₆O₇, respectively, showing 10 degrees of unsaturation in both compounds. The structures of compounds **2c,d** were determined by 2D NMR spectroscopy, and diagnostic HMBC correlations for compound **2c** are shown in Figure 1. The methoxy group gave HMBC correlations to both C-17 and C-18, and the value of ¹J_{C,H} (164 Hz) and the marked difference in chemical shift between these carbons (over 70 ppm!) suggested that they are part of a highly polarized trisubstituted olefin moiety. H-17 correlated with C-2 and C-10, and the HMBC correlations from H-2, H-5, and H-9 to both C-1 and C-10 established the connection between C-2, C-1 and C-10. The COSY correlation between H-2 and H-3 and the HMBC correlations from the 14-methyl to C-3, C-4, C-5, and C-15

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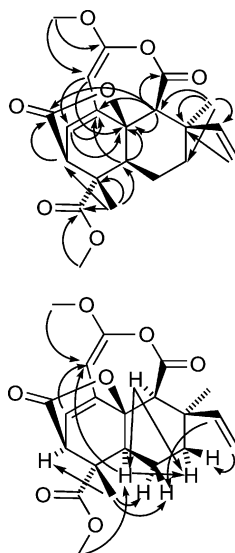
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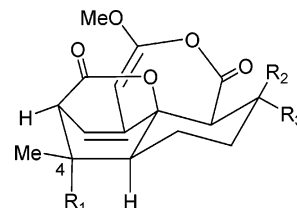
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Table 1. ^1H (500 MHz) and ^{13}C (125 MHz) NMR Data of Compounds **2c** and **2d**

pos.	2c		2d	
	δ_{H} (mult., J in Hz)	δ_{C} (mult.)	δ_{H} (mult., J in Hz)	δ_{C} (mult.)
1		139.3 (s)		138.6 (s)
2	6.08 (dd; 1.2, 6.3)	123.5 (d)	6.06 (dd; 1.1, 6.4)	122.8 (d)
3	3.67 (d; 6.3)	50.0 (d)	3.29 (d; 6.4)	49.7 (d)
4		44.7 (s)		37.0 (s)
5	2.30 (dd; 5.3, 12.6)	44.8 (d)	1.29 (dd; 4.6, 12.9)	44.7 (s)
6 α	1.75 (dddd; 2.8, 12.6, 12.6, 13.8)	20.8 (t)	1.76 (dddd; 2.8, 12.9, 13.0, 13.8)	21.1 (t)
6 β	1.67 (m)		1.54 (dddd; 2.8, 3.9, 4.6, 13.8)	
7 α	1.96 (ddd; 2.8, 3.9, 13.7)	40.2 (t)	1.94 (ddd; 2.8, 3.9, 13.7)	40.2 (t)
7 β	1.52 (ddd; 3.1, 12.6, 13.7)		1.42 (ddd; 2.8, 13.0, 13.7)	
8		38.5 (s)		38.4 (s)
9	3.18 (s)	53.3 (d)	3.15 (s)	53.3 (d)
10		87.0 (s)		86.9 (s)
11	7.01 (dd; 11.2, 17.7)	142.7 (d)	7.00 (dd; 11.1, 17.7)	142.6 (d)
12a	5.16 (dd; 1.0, 11.2)	112.2 (t)	5.17 (dd; 1.0, 11.)	112.3 (t)
12b	5.07 (dd; 1.0, 17.7)		5.06 (dd; 1.0, 17.7)	
13	1.24 (s)	28.6 (q)	1.24 (s)	28.6 (q)
14	1.31 (s)	20.9 (q)	1.12 (s)	19.6 (q)
15a		175.2 (s)	3.74 (d; 10.8)	70.4 (t)
15b			3.70 (d; 10.8)	
16		170.0 (s)		170.7 (s)
17	4.97 (d; 1.2)	79.2 (d)	5.01 (d; 1.1)	79.0 (d)
18		156.8 (s)		156.9 (s)
19		162.3 (s)		162.3 (s)
15-OMe	3.71 (s)	56.4 (q)		56.4 (s)
15-OAc			2.09 (s)	170.8 (s)
18-OMe	3.72 (s)	52.8 (q)	3.74 (s)	20.7 (q)
				56.4 (s)

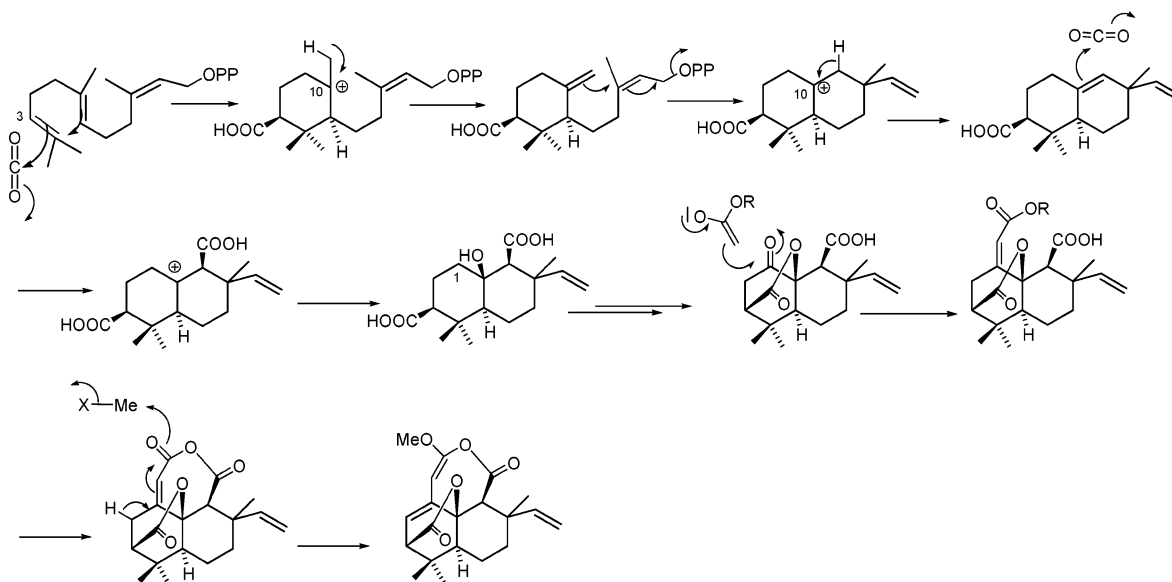
**Figure 1.** Diagnostic HMBC (top) and NOESY (bottom) correlations observed with compound **2c**.

established the first ring and that the methoxycarbonyl group is geminal to C-14. COSY correlations from H-5 to the C-7 methylene and the HMBC correlations of the 13-methyl to C-7, C-8, C-9, and C-11 permitted definition of a decalin system and located the vinyl group at C-8. The two remaining carbons are carbonyls, and the HMBC correlations from H-3 to C-16 and from H-9 to C-19 located them at C-3 and C-9 of the decalin framework. The chemical shifts of these carbons and the elemental composition of **2c** required that both carbonyls were involved in lactone rings. Thus, the complementary ethereal oxygen terminus must be at C-10 and C-18, whose chemical shift requires oxygenation. H-9 gave a weak, but significant, 4J HMBC correlation to C-16, suggesting a 16,10-olide structure, a proposal further confirmed by the analysis of the NOESY data (see *infra*). As indicated in Figure 1, NOESY correlations between H-5, H-7b and H-9 and their coupling

**Figure 2.** Stereostructure of basililolides.

patterns (Table 1) showed that these protons are axial and that the B-ring exists in a chair conformation. This makes the C-19 carbonyl group equatorial and prevents any connection to the C-3 carbonyl group. Similar considerations also rule out a C-3–C-18 connection. The second oxygen bridge therefore has to be between C-18 and C-19 and should be of the enol lactone type. This pattern of oxygen connectivity explained several other features of the NOESY spectra. Thus, the closure of the acylal bridge twists the enol lactone double bond out of the plane of the endocyclic double bond and orients 17-H toward H-5, as demanded by the detection of a NOESY correlation between these protons. The β -axial orientation of the vinyl group was supported by NOESY correlations between the β -oriented protons at C-6 and C-7, while the equatorial α -orientation of the C-8 methyl was supported by NOESY correlations with both H-7 protons as well as with H-9. Ring A is forced by the 10,16-lactone bridge into a boat conformation, and the detection of a diagnostic NOESY correlation between the 4-carboxymethyl and H-5 located the ester group at C-4 on the β -face of the molecule (Figure 2). Compound **2d** differed from **2c** by replacement of the methoxycarbonyl group at C-4 with an acetoxymethyl functionality and showed the same basic pattern of HMBC and NOESY correlations as **2c**, indicating the same relative configuration.

With the structure of the new compounds established, we speculated on their biogenetic origin. We reasoned that the 4,8-geminal substitution pattern of the decalin moiety is typically “terpenoid”. While the acetate moiety at C-1

Scheme 1. Possible Biogenesis of the C-19 Terpenolides from *Thapsia*

might be introduced by a two-carbon homologation, a process well established in the biosynthesis of isoprenoids, the two extra carboxylic groups are strategically located at C-3, the position required for electrophilic triggering of an olefin cyclization, and at C-9, a carbon adjacent to a tertiary hydroxyl. This suggests introduction of the two carboxylic groups by electrophilic addition of carbon dioxide (or an electrophilic carbon dioxide equivalent)⁶ to an olefinic substrate. Thus, carbon dioxide might trigger the cyclization of the ω -double bond of farnesyl pyrophosphate,⁶ affording a monocyclic compound bearing a C-3 carboxylic group (Scheme 1). Loss of a proton from the methyl adjacent to the tertiary cationic center would generate an exocyclic methylene, which might displace in a vinylogous fashion the pyrophosphate group. Proton loss from the 10-carbon then generates an endocyclic olefin, which undergoes electrophilic carboxylation in a Markovnikov fashion to generate a tertiary 10-cation, eventually quenched by water. The two additional carbons might then be inserted, after oxidation of C-1 to the ketone, by addition of an acetate equivalent to C-1, in a process similar to that postulated for the bis-homologation of pregnanes to cardenolides⁷ and of jatrophanes to terracinolides,⁸ but leading to a cyclic anhydride rather than a lactone because of the presence of the carboxylate at C-9. Finally, the enol lactone moiety could originate by methylation-induced vinylogous enolization of the anhydride C-18 carbonyl oxygen. The C-19 dilactones from *Thapsia* should therefore be considered as tetrahomosesquiterpenoids, in accordance with the bias for the production of sesquiterpenoids observed in plants from this genus.⁴ The most unusual step in our biosynthetic proposal for the C-19 terpenolides from *Thapsia* is the electrophilic carboxylation step. This process, which represents a formal nonphotosynthetic carbon dioxide fixation, is unprecedented in terpenoid biosynthesis and therefore worth investigating with feeding experiments as well as a synthetic mimic.⁶

Diversity within the C-19 terpenolides is essentially due to the oxidation of the *gem*-dimethyls at C-4, which can lead to a different pattern of oxygen bridging. We suggest reserving the name transtaganolides for compounds having the 3-carboxy group oxygen bound to C-15, while naming compounds where the 3-carboxylate is oxygen bound to C-10 basilolides, from the name of the Sardinian village

(San Basilio) from which the samples of *T. garganica* containing **2a–d** were obtained.

The occurrence of unusual tetrahomosesquiterpenoids in *T. garganica* further highlights the unique profile of secondary metabolites from this species. While better known for the presence of thapsigargin, plants form the genus *Thapsia* contain, in addition to C-19 tetrahomoterpenoids, also a remarkable portfolio of chemical oddities, including tethered lipids, a class of compounds typical of Archaeobacteria and unprecedented in higher plants,⁹ as well as sesquiterpenoids of the thapsane class, a type of compounds for which no biogenetic scheme has so far been proposed.⁴ The taxonomic value of the accumulation of C-19 terpenolides is highlighted by their occurrence in only two of the samples investigated, while the seasonal variation of their contents suggests a possible, but still elusive, physiological role.

Finally, we evaluated whether **2a–d** share the ability of thapsigargin to inhibit SERCAs. Sea urchin egg homogenates¹⁰ contain IP₃-sensitive stores that use a thapsigargin-sensitive calcium pump.¹¹ Using a standard protocol,¹² incubation with 10 μ M thapsigargin resulted in a Ca²⁺ leak from the endoplasmatic reticulum that peaked in 15–20 min (Figure 3). At the same concentration, basilolides induced a similar calcium leak, with **2c** and **2d** showing a slightly higher efficacy than thapsigargin (Figure 3a). To confirm that this effect was due to emptying of the endoplasmatic reticulum, 1 μ M IP₃ was added to pretreated homogenates, resulting in markedly reduced responses in pretreated compared to control homogenates (Figure 3b). Taken together, these data strongly suggest that basilolides behave as biological analogues of thapsigargin and represent a new type of SERCA inhibitors, well worth further investigation.

Experimental Section

General Experimental Procedures. IR spectra were obtained on a Shimadzu DR 8001 spectrophotometer. ¹H NMR (500 MHz) and ¹³C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in CDCl₃, and the solvent signals (7.26 and 77.0 ppm, respectively) were used

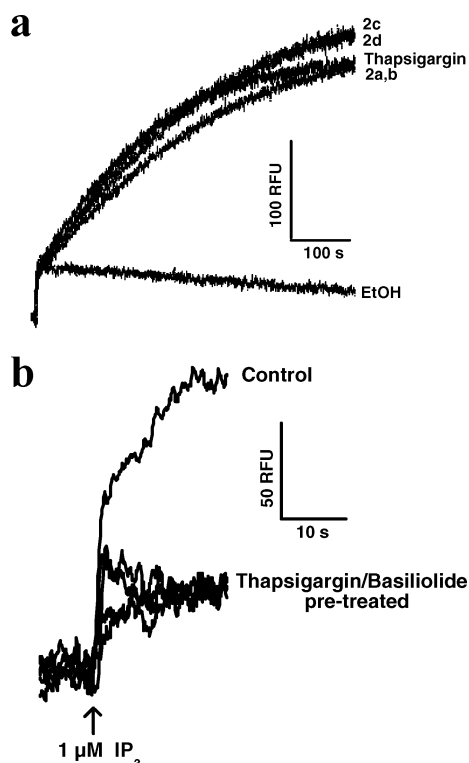


Figure 3. (a) Representative fluorimetric calcium traces showing calcium leak from sea urchin egg calcium stores induced by thapsigargin (**1**) (10 μ M) and by basiliolides (10 μ M). The effect of the vehicle ethanol (EtOH) alone is also shown. Traces are representative of at least 3 independent determinations. (b) Calcium release induced by 1 μ M IP_3 in both TG or basiliolide pretreated and control homogenates. Traces are representative of at least 3 separate determinations.

Table 2. Initial Calcium Leak Rates Induced by Basiliolides Expressed as a Percentage of the Initial Leak Rate Induced by Thapsigargin (81 ± 11.0 RFU/min)^a

compound (10 μ M)	leak rate (% of TG)
1	100 \pm 13.6
2a,b	65.4 \pm 12.6
2c	61.4 \pm 7.7
2d	64.2 \pm 10.2

^a Data are expressed as average \pm SEM of 4 or 5 separate determinations. RFU = relative fluorescence units.

as reference signals. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC, and HMBC experiments were recorded with gradient enhancements using sine-shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{\text{CH}} = 145$ Hz and $^nJ_{\text{CH}} = 10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra (HRESI) were recorded with a Micromass Q-TOF MICRO instrument. Silica gel 60 (70–230 mesh) was used for gravity column chromatography.

Plant Material. *T. garganica* L. was collected in January 2001 near San Basilio (CA, Sardinia) and identified by M.B. A voucher specimen (TG 180105) is deposited at the Dipartimento di Scienze Botaniche, Università di Cagliari.

Isolation of Basiliolides. A sample (452 g) of roots was powdered and extracted with acetone (3×3 L). The pooled extracts were evaporated to give 12.4 g of a brownish syrup, part of which (7.8 g) was fractionated by gravity column chromatography (175 g silica gel, petroleum ether–EtOAc gradient), to give thapsigargin (**1**) (petroleum ether–EtOAc 7:3, 3.9 g, 1.3%), and a mixture of basiliolides (ca. 500 mg), further purified by gravity column chromatography on alumina (petroleum ether–EtOAc, 5:5) to afford, after trituration with ether, a mixture (120 mg, 0.042%, ca. 2:1) of basiliolides A1

and A2 (= transtaganolides D and C, **2a,b**), 80 mg (0.028%) of basiliolide B (**2c**), and 130 mg (0.046%) of basiliolide C (**2d**).

Basiliolide B (2c): white powder (ether); mp 146 $^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{25} -16$ (c 1.0, MeOH); IR (KBr) ν_{max} 1763, 1700, 1623, 1440, 1332, 1110, 960, 908 cm^{-1} ; ^1H and ^{13}C NMR, see Table 1; HREIMS m/z 388.1501 $[\text{M}]^+$ (calcd for $\text{C}_{21}\text{H}_{24}\text{O}_7$, 388.1522).

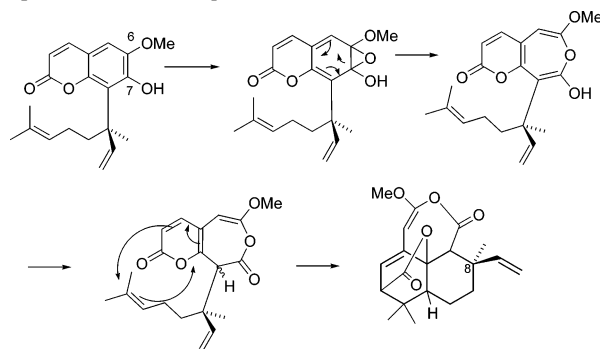
Basiliolide C (2d): white powder (ether); mp 168 $^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{25} -21$ (c 1.1, MeOH); IR (KBr) ν_{max} 1760, 1740, 1670, 1370, 1246, 1187, 1030, 973 cm^{-1} ; ^1H and ^{13}C NMR, see Table 1; HREIMS m/z 402.1665 $[\text{M}]^+$ (calcd for $\text{C}_{22}\text{H}_{26}\text{O}_7$, 402.1679).

Biological Assays. Sea urchin egg homogenates (*Lytechinus pictus*, Marinus, Long Beach, CA) were prepared and used according to a standard protocol.¹² In brief, homogenates were diluted to 1.25% in buffer (250 mM Kgluconate, 250 mM *N*-methylglucamine, 20 mM HEPES, 1 mM MgCl_2 , pH 7.2) containing an ATP regenerating system (1 mM ATP, 10 U/mL creatine phosphokinase, and 10 mM phosphocreatine). Calcium leak from intracellular stores was monitored in real time using Fluo-3 (3 μM) in a Perkin-Elmer LS-50B fluorimeter. Standard solutions of TG and basiliolides were prepared in ethanol.

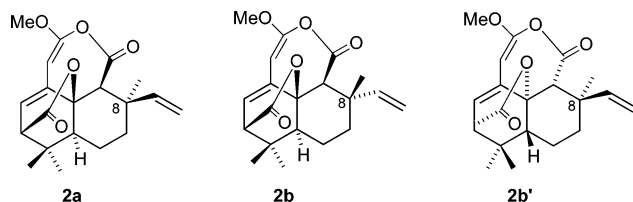
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Note Added in Proof: Following a discussion with Dr. Jasmin Jakupovic (Analyticon GmbH, Potsdam, Germany), an alternative biogenetic proposal for the C-19 terpenolides from *Thapsia* was elaborated. This proposal (Scheme 2) regards these compounds as meroterpenoids, derived from a *C*-prenylated coumarin precursor. Formal epoxidation of the aromatic 6,7-bond, followed by electrocyclic epoxide opening and tautomerization, would generate, in an overall unprecedented example of phenol cleavage, an oxepane Diels–Alder precursor, next evolving into C-19 terpenolides via a transannular cycloaddition.

Scheme 2. Meroterpenoid Biogenetic Derivation of C-19 Terpenolides from *Thapsia*



The occurrence of C-8 epimeric optically active terpenolides fits well with the tetrahomoterpenoid biogenetic derivation, but would be difficult to accommodate in a meroterpenoid derivation. This would require an enzyme tolerant of enantiomeric C-8 (basiliolide numbering) substrates, but capable of kinetically resolving them by induction of the same absolute configuration in all stereocenters formed in the cascade pericyclic process (tandem electrocyclic opening–Diels–Alder cycloaddition). A pseudoenantiomeric (**2a** vs **2b'**) rather than an epimeric (**2a** vs **2b**) relationship would thus fit better into a meroterpenoid derivation. We are actively pursuing this issue by using a combination of spectroscopic and synthetic approaches.



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