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The case for a Diels-Alderase-free, bis-pericyclic, [4+2] dimerization in the biosynthesis of (±)-paracaseolide A

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Abstract

The natural product paracaseolide A is a tetracyclic dilactone containing six adjacent stereocenters. It has an unprecedented skeleton and occupies unique structural space among the >200,000 characterized secondary metabolites. Six different research groups have reported a chemical synthesis of this compound, five of which used a thermal, net Diels–Alder [4+2] cycloaddition and dehydration at 110 °C to access the target by dimerization of a simple butenolide precursor. Here we report that this dimerization proceeds under much milder conditions and with a different stereochemical outcome than previously recognized. This can be rationalized by invoking a bis-pericyclic transition state. Furthermore, we find that spontaneous epimerization, necessary to correct the configuration at one key stereocenter, is viable and that natural paracaseolide A is racemic. Together these facts point to the absence of enzymatic catalysis (i.e., Diels–Alderase activity) in the cycloaddition and strongly suggest that a non-enzyme-mediated dimerization is the actual event by which paracaseolide A is produced in Nature.

Paracaseolide A (**1a**), a secondary metabolite first reported by Guo and coworkers in 2011,[1] was isolated from the stem bark of *Sonneratia paracaseolaris*, a mangrove plant that was collected in Guangdong Province, China. It is reported to inhibit the action of the biologically relevant phosphatases CDC25B and PTP1B, kinases involved in cell cycle regulation (IC_{50} of 6.4 μM)[1,2] and signal transduction in the insulin pathway (IC_{50} of 1.5 μM),[3] respectively. Compound **1a** has a fascinating, skeletally unique structure that quickly captured the attention of synthetic chemists. Our analysis led us to hypothesize that the carbon skeleton in **1a** is assembled in Nature by a spontaneous, non-enzyme-mediated[4] (here, Diels–Alderase-free) dimerization of the simple α-dodecenylbutenolide **3a** as well as a facile epimerization event of the resulting Diels–Alder (DA) adduct. In fact, **3a** was proposed as the possible biosynthetic precursor to **1a** by Guo et al. The relevance of Diels–Alderase enzymatic action (or lack thereof) in biosynthesis[5,6] remains a timely topic.[7,8] Other biosynthetically relevant, spontaneous Diels–Alder dimerizations have been proposed

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Author Contributions

T. W. and T. R. H. conceived and designed the experiments, analyzed the data, and co-wrote the paper. T. W. performed the experiments.

Additional Information

Supplementary information is available in the online version of the paper.

and demonstrated; notable examples include those leading to carpanone[9,10] and the endiandric acids.[11,12]

In the short period of time since the structure of paracaseolide A was revealed,[1] researchers in five different laboratories have reported a total synthesis of **1a** involving the same final key step.[3,13,14,15,16] Namely, dimerization of the butenolide **3a** (Fig. 1) accompanied by dehydration has been repeatedly used to produce **1a**. In every instance **3a** was heated to 110 °C to effect this overall transformation. In every instance the researchers initially rationalized the reaction to occur via an *endo* orientation of the two reacting monomers (see Fig. 1) and via the intermediacy of the *endo* Diels–Alder dimer **2**. (In this manuscript, *endo* and *exo* refer to the relative orientation between the carbonyl group on the dienophilic component and the internal carbons of the 1,3-butadiene derivative in the cycloaddition transition state geometry and products derived therefrom.) This necessitated the supposition of a subsequent epimerization at C5 in the cyclohexenyl ring of **2** in order to account for the final formation of **1a**. It is noteworthy that attempts to interconvert *5-epi*-**1a** (not shown) with **1a** (and vice-versa) by two groups of workers have been unsuccessful.[14,15] These mechanistic and stereochemical dilemmas have recently been explored computationally by the research groups of Ganguly, Khan, and Mehta, leading those workers to conclude that a "stepwise mechanism [leading to *5-epi*-**2** (not shown)] is the likely pathway" and that "a concerted [4+2] cycloaddition and post-DA epimerization" is less likely.[17]

Results and Discussion

As reported here, our studies of this key Diels–Alder reaction reveal considerably different reaction energetics and stereochemical features to those reported by previous investigators. Namely, we show that dimerization of the alkenylbutenolide **3a** [as well as its truncated analog **3b** (Fig. 2)] occurs under much milder conditions than have been previously used. Moreover, the reaction proceeds, surprisingly, through an *exo* approach of the two reactants, as discussed in detail below. (Throughout this document compound structure numbers ending in "**a**" all contain *n*-dodecyl groups that correspond to the substituents present in the natural product; the structure numbers ending in "**b**" all contain a methyl in place of the *n*-dodecyl group and comprise a series of unnatural, truncated analogs.)

Following similar strategies to those used by others,[13,14,15] we synthesized the butenolide monomers **3a** and **3b** by the concise routes shown in Fig. 2, commencing with 4-bromo-2-methylfuran (**4**). The key step is the conversion of the silylated furan **5a**/**b** to the racemic butenolide hemiacylal **3a**/**b** with singlet oxygen, a reaction that proceeds by spontaneous cleavage of an initial endoperoxide to the intermediate *O*-silylated keto acid[18] **6a**/**b** (observable by ^{1}H NMR analysis). Desilylation provides **3a**/**b** in which only the closed hemiacylal (or hydroxybutenolide) form was detected (>99%,^{1}H NMR analysis in CD_3OD). We too observed that heating **3a** converted it to **1a**, whose spectral data were in excellent accord with those reported.[1,3,13,14,15,16,19]

Insights gained using unnatural, truncated analogs (i.e., the “b” series of methyl-bearing compounds)

We used the simpler methyl-bearing analog **3b** to gain useful insights that guided our later experiments with the *n*-dodecyl-containing **3a**. We first verified that it behaved similarly to **3a** when heated to 110 °C as a neat sample under nitrogen. That is, dimer **1b**, a side-chain truncated analog of **1a**, was formed as the dominant product (Fig. 2). More excitingly, we observed that when **3b**, a liquid, was held as a neat sample, a new product appeared under much milder conditions (35 °C) than had been reported for **3a**. The resulting dimer had not yet dehydrated. A crystalline sample of that new material was subjected to single crystal X-ray analysis, which revealed it to be the Diels–Alder adduct **7b** (Fig. 3a). This dimerization occurs with a very high level of regio- and stereoselectivity. Most notably, the relative configuration among carbons 5/5a/7b/7c indicated that the [4+2] cycloaddition had proceeded by a pathway in which the diene had approached the dienophile with an *exo orientation* with respect to the carbonyl group on the dienophile (Fig. 3b).

In an attempt to understand this stereochemical outcome, we performed density functional theory [DFT; SMD/M06-2X/6-311+G(d,p) in 2-butanol] calculations to evaluate the relative energies of transition state structures corresponding to the *exo* vs. the *endo* modes of dimerization of **3b** (Fig. 3b vs. Fig. 3c). Consistent with the experimental observation, the lowest energy transition state (TS) structure for all possible (see Supplementary Information for details) *exo* modes of dimerization is favored by 3.9 kcal·mol^{-1} relative to the overall lowest energy for an *endo* approach of the two reactants. Moreover, the computed free energy of activation for the *exo* dimerization of **3b** to **7b** was 25.2 kcal·mol^{-1}. That this value is in reasonably good agreement with the observed dimerization rate provides validity to the computational methodology. The overall reaction of two molecules of **3b** to the initial bis-acylal-containing dimer, which subsequently ring-opens to the more stable keto acid **7b**, is computed to be exergonic by 14.0 kcal•mol^{-1}, suggesting that the dimerization should not be significantly reversible under the experimental conditions. We also admixed dimer **7b** with the “real” dimeric diacid **7a** (formed in analogous fashion, see Fig. 4a below) and incubated that neat sample at 35 °C for 7 days. Analysis by ESI-MS gave no evidence of any mixed dimer, which presumably would have formed had there been any appreciable reversibility back to **3b** and **3a**.

How can one rationalize this atypical preference for an *exo* over an *endo* approach for a Diels–Alder reaction (i.e., $\mathbf{TS_{exo}}$ rather than $\mathbf{TS_{endo}}$)? It is enlightening to recognize that the geometry of the lowest energy TS ($\mathbf{TS_{exo}}$, Fig. 3b) is C_2-symmetric, as reflected, for example, by the identical distance between carbons C2–Cβ in both pairs of reactants, (cf. y and y′ in Fig. 3b). This is an example of a bis-pericyclic process,[20] passing through a TS in which the two possible modes of cycloaddition, [4+2] vs. [2+4], have fully merged. Subsequent (and degenerate) bifurcation,[21] in which the partial bonding in $\mathbf{TS_{exo}}$ between one or the other of the two C2–Cβ pairs is forfeited, forms the product **7b**. This type of symmetrical TS structure was first invoked to account for Diels–Alder dimerizations of simple 4π-systems like acrolein[22] and cyclopentadiene.[20] More recently, a C_2-symmetric, bis-pericyclic TS assembly has also been used to rationalize the stereochemical outcome of the self-dimerization of a complex orthoquinol in a synthesis of the secondary metabolite

aquaticol.[23] The extra stabilization that attends a bis-pericyclic TS has been nicely explained as its being "admirably suited to take advantage of the maximum accumulation of unsaturated centers".[20]

We also established that the dimer **7b** was a competent precursor to the paracaseolide A analog **1b**. When a neat sample of crystalline **7b** was melted (mp 170–174 °C, with sweating), the resulting cooled substance had cleanly been transformed to **1b**. Moreover, holding crystalline **7b** well below its melting point (110 °C) resulted in its virtually quantitative conversion to crystalline **1b**. It should be noted that the stereochemical outcome of this transformation requires a change in the configuration at C7c, a point that is further discussed below.

The dimer **7b** (Fig. 3a) contains a ring-opened keto acid in its "northern" region in both the solid state (X-ray) as well as in solution (^{1}H NMR). This observation led us to wonder about the role of the analogous ring-opened "tautomer" of the precursor hemiacylal **3b** as a participant in the Diels–Alder reaction itself. The ^{1}H NMR spectrum of **3b** in CD_3OD showed no clear evidence for any of the isomeric keto acid **8b** (Fig. 3d). However, when this solution was treated with slightly over one equivalent of the weak base Et_3N, the NMR spectral data indicated that the Et_3NH^+ salt of the ring-opened carboxylate **9b** was now the dominant species (Fig. 3d).[24]

These observations raised the question of the relevance of a ring-opened keto acid like **8b** or carboxylate like **9b** as a potential reactant in the dimerization involved in the biosynthesis of paracaseolide A (**1a**) itself. In particular, it might be expected that either of **8b** or **9b**, each having an alkene bearing two electron-withdrawing groups, would be a more reactive dienophile than the butenolide **3b**. Previous workers have not considered the Diels–Alder reactivity of an open-chain tautomer like **8b** or **9b**. We used the TBS ester **6b**, the direct product of singlet oxygen oxidation of furan **5b** (Fig. 2), to probe this possibility. Interestingly, a neat sample of this acyclic analog of **3b** dimerized to give, highly selectively, **10b** with a rate about two times faster than that of **3b** (Fig. 3e). The relative configuration of **10b** was securely established by its conversion to **7b** upon removal of both TBS esters. The structure of **10b** indicated that the dimerization of **6b** had also occurred through an approach involving an *exo* orientation of the two reacting molecules.

Extrapolation to the natural set of compounds (i.e., the "a" series)

We then examined the ambient temperature behavior of the 'parent' hydroxybutenolide **3a**. By extrapolation, the fact that **3b** dimerized exclusively to the *exo* adduct **7b** implied that **7a** should be formed in the dimerization of **3a** that ultimately gives rise to (±)-paracaseolide A (**1a**). Recall that, in contrast, it is the *endo* adduct **2** (Fig. 1) that has been previously invoked by those who have delineated a structure for the initial Diels–Alder dimer.[13,14,15] Indeed, when held as a highly concentrated sample at ambient temperature, **3a** was observed to slowly but cleanly dimerize to the *exo* adduct **7a** (Fig. 4a, below). This transformation is *much* faster (ca. 2% conversion per day at 21 °C) than what is implied by every previous experiment, all of which were carried out at 110 °C for 12–34 hours. Presumably these more forcing conditions were used because only the formation of the final dehydrated product **1a**

was being monitored. The relative configuration of **7a** was confidently assigned in view of its many analogous ^{1}H NMR spectroscopic features vis-à-vis those of its lower homolog **7b**. In turn, this revised stereochemical outcome now requires adjustment of the configuration at C7c in **7a** in order to ultimately arrive at the proper relative configuration present in **1a**. This contrasts with previous presumptions that epimerization at C5 was necessary, a consequence of the incorrect assumption that the structure of the initial DA adduct was the *endo* intermediate **2**.

We have also examined the potential of an open-chain analog of the hemiacylal **3a** to undergo spontaneous [4+2] cycloaddition. A neat sample of the methyl ester **11** (obtained by treatment of **3a** with MeI/K_2CO_3/acetone) also slowly dimerized at room temperature to give the *exo* adduct **10a** in a highly diastereoselective process (≤3% of any other single compound was observed). The rate of this reaction was slightly faster than that of the dimerization of **3a** itself. The structure of **10a** was assigned on the basis of analogy, NOE (nuclear Overhauser enhancement) analysis, and ^{1}H NMR spectroscopic comparisons with **10b**. Because the methyl ester in **11** (as well as the TBS ester in the analogous open-chain analog **6b**, Fig. 3e) represents a significant perturbation of the structure of the ring-open keto acid **8a** (or its carboxylate **9a**), we believe that drawing a definitive conclusion about the relative reactivities of the open vs. closed isomers of the acid **8a** (or its carboxylate **9a**, which might well be the species most relevant to the natural event enroute to **1a** in the mangrove) vs. the hemiacylal **3a** is unwarranted.

Facile exchange of the C7c-hydrogen atom—a path to spontaneous epimerization

But how might epimerization at C7c in **7a** (or **7b**) occur? Quite serendipitously, we made a remarkable observation, first using the truncated analog **7b–*h***. When this compound was held in methanol-d_4 at room temperature, the C7c-proton *spontaneously exchanged*, quite cleanly, with a deuterium atom from the solvent to give **7b–*d*** ($t_{1/2}$ 3.2 days at room temperature, Fig. 5a; also page S74 in Supplementary Information)! One rationale for this exchange is by way of intramolecular formation of the zwitterion **12** (Fig. 5b) followed by internal removal of the allylic C7c-proton by the proximal carboxylate to produce the enol **13**. Intervention of a hydroxyl group, specifically the MeO–D bond here, as a proton shuttle to aid this process can also be envisioned. This interconversion of **7** with **13** constitutes an intramolecular, self-organocatalyzed, keto-enol tautomerization. Rapid solvent exchange of RCOOH to RCOOD in **13** followed by the microscopic reverse would return the observed mono-deuterated analog **7b–*d***. Importantly, this facile process provides access to an intermediate having a planar, sp^2-hydribized C7c carbon atom, protonation of which from the β-face would effect the requisite C7c-epimerization leading to the relative configuration mandatory for final dehydration to **1**.

The analogous adduct **7a–*h*** (Fig. 4a) underwent even more facile H-D exchange at C7c to give **7a–*d*** when dissolved in CD_3OD ($t_{1/2}$ ca. 4 h at room temperature). The same experiment using the dimethyl ester **10a** (Fig. 4b) showed that this substrate had a much slower rate of deuteration at C7c (ca. 35% after 10 days), clearly suggesting a catalytic role for the carboxylic acid in **7a–*h*** (cf. **12** and **13** in Fig. 5b). To judge if that process was unimolecular or bimolecular in nature, we prepared a CD_3OD solution containing both **7a–*h***

and **10a**. After 12 hours, ESI-MS analysis indicated that the former had undergone exchange to the extent of ca. 75% whereas exchange in **10a** was not detected. This argues against a bimolecular process in which a carboxylic acid in one molecule promotes the enolization and exchange of H7c alpha to the acetyl group in a second molecule of **7a–*h*** or **10a**.

Is paracaseolide A produced in the mangrove as a racemate?

Finally, we return to a key issue that is highly germane to the question of whether the spontaneous dimerization of diene **3a** is the true biosynthetic event involved in the biosynthesis of paracaseolide A (**1a**). If this reaction is not catalyzed by an enzyme, one would expect the isolated natural sample of **1a** to be racemic. The natural substance was reported to have a specific rotation of $[\alpha]^{24}_{D} = +0.9$.[1] In our view, particularly in light of minor resonances in the ^{1}H NMR spectrum that indicate the presence of contaminants in that sample, the small value of this rotation is within experimental error of zero. To interrogate this point further, we resolved a sample of our synthetic (±)-**1a** on a Chiralcel® OD HPLC column (Fig. 5c). (Our attempts to obtain a small amount of the natural sample of paracaseolide A to analyze by this method were unsuccessful.) Essentially equal amounts (4.5 and 4.4 mg) of each enantiomer were recovered following semi-preparative-scale separation. The rotation values measured for the faster and the slower eluting enantiomers were +92° and −86°, respectively. This evidence strongly suggests that the natural material, like the synthetic, is racemic. This, in turn, is further support of our hypothesis that a Diels–Alderase is neither required nor responsible for the biosynthesis of paracaseolide A (**1a**).

Conclusion

Immediately upon contemplating the structure of paracaseolide A and in parallel with our interpretation of the fact that the natural material showed a near-zero value for its specific rotation,[1] we hypothesized that its biosynthesis proceeded by *spontaneous* dimerization of the hydroxybutenolide **3a**. To test this required that we examine the chemical reactivity of **3a** to learn if it is sufficiently innately active to produce an adduct like **2** or **7a**. Our conviction in that hypothesis led us to undertake the experiments reported here, through which we have established: (i) that the DA dimerization of **3a** (or the isomeric ring-opened keto acid **8a**) is sufficiently energetically accessible to be biosynthetically relevant, (ii) that this DA dimerization is *exo* selective, unlike what previous workers have presumed, (iii) using DFT computational studies, that this stereoselectivity can be explained by a bis-pericyclic pathway involving a C_2-symmetric TS geometry, (iv) that facile H/D exchange of the C7c-hydrogen atom in **7a–*h*** shows that the obligate planarization of C7c is a feasible process and enables a pathway for the required epimerization at C7c in **7a–*h***, and (v) that the natural sample of paracaseolide A is, in all likelihood, racemic—a fact incompatible with its formation under the action of a Diels–Alderase in *Sonneratia paracaseolaris*. Collectively, these observations lend substantial support to the idea that a spontaneous DA dimerization of the butenolide **3a** is the key biosynthetic event leading to paracaseolide A—conceptualization that, until now, has been obscured by biased, but incorrect, assumptions that the dimerization required high temperature and occurred with either *endo* selectivity or by a stepwise mechanism.

Methods

HPLC separation of the antipodes of 1a and specific rotation measurements

A racemic (and synthetic) sample of paracaseolide A (**1a**, 14 mg) was dissolved in a mixture of CH_2Cl_2 (50 μL) and hexanes (100 μL). A Chiralcel® OD column (4.5 mm d × 250 mm l) was pre-equilibrated with 9:1 hexanes:2-propanol. The enantiomers of the racemic paracaseolide A were separated (4 injections) to give, in the order of elution, 4.5 mg of the (+)-enantiomer and 4.4 mg of the (−)-enantiomer. Each was then dissolved in HPLC grade $CHCl_3$ (1.5 mL, ≥99.8%, amylene stabilized, no ethanol present) and transferred to a 1 mL polarimeter cell. For each enantiomer, 5 measurements (10 scans in each measurement) were made and averaged to give a specific rotation value of +92±2 for the first enantiomer and −86±1 for the second enantiomer. Measurements were made on a Rudolph Research Analytical (Autopol III) polarimeter.

All new synthetic compounds have been fully characterized; see Supplementary Information for preparation procedures, line listings of spectroscopic data, and copies of ^{1}H and ^{13}C NMR spectra.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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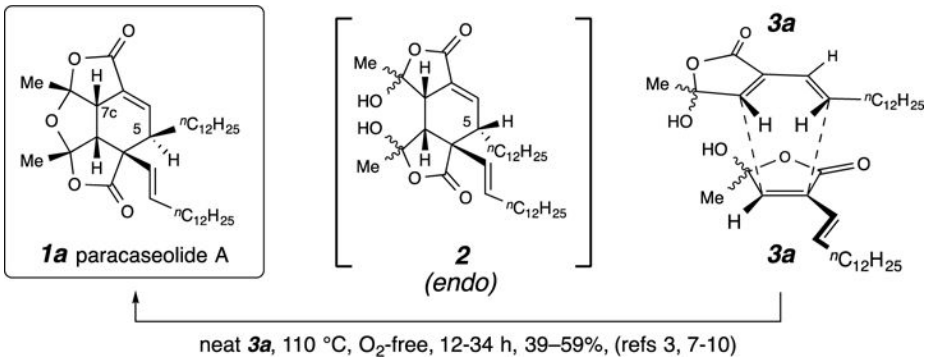

Figure 1. Structure and previous syntheses of paracaseolide A

In several previous studies (refs [3, 7–10]) concomitant thermal dimerization and dehydration of the monomeric alkenylbutenolide **3a** was used as the key step. This cycloaddition has been presumed to proceed through the intermediacy of the *endo*-Diels–Alder adduct **2**.

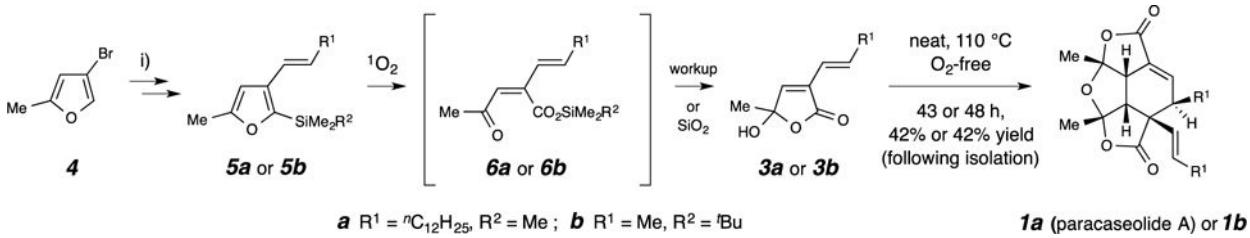

Figure 2. Strategy and methods for the synthesis of paracaseolide A and a truncated (methyl-containing) analogue

Paracaseolide A (**1a)** was prepared from **3a**[3,13,14,15,16] and the truncated analogue **1b** from **3b** by heating the neat compound in an oxygen-free-atmosphere. The monomers **3a** and **3b** were prepared by a two-step or four-step sequence, respectively, from 4-bromo-2-methylfuran.

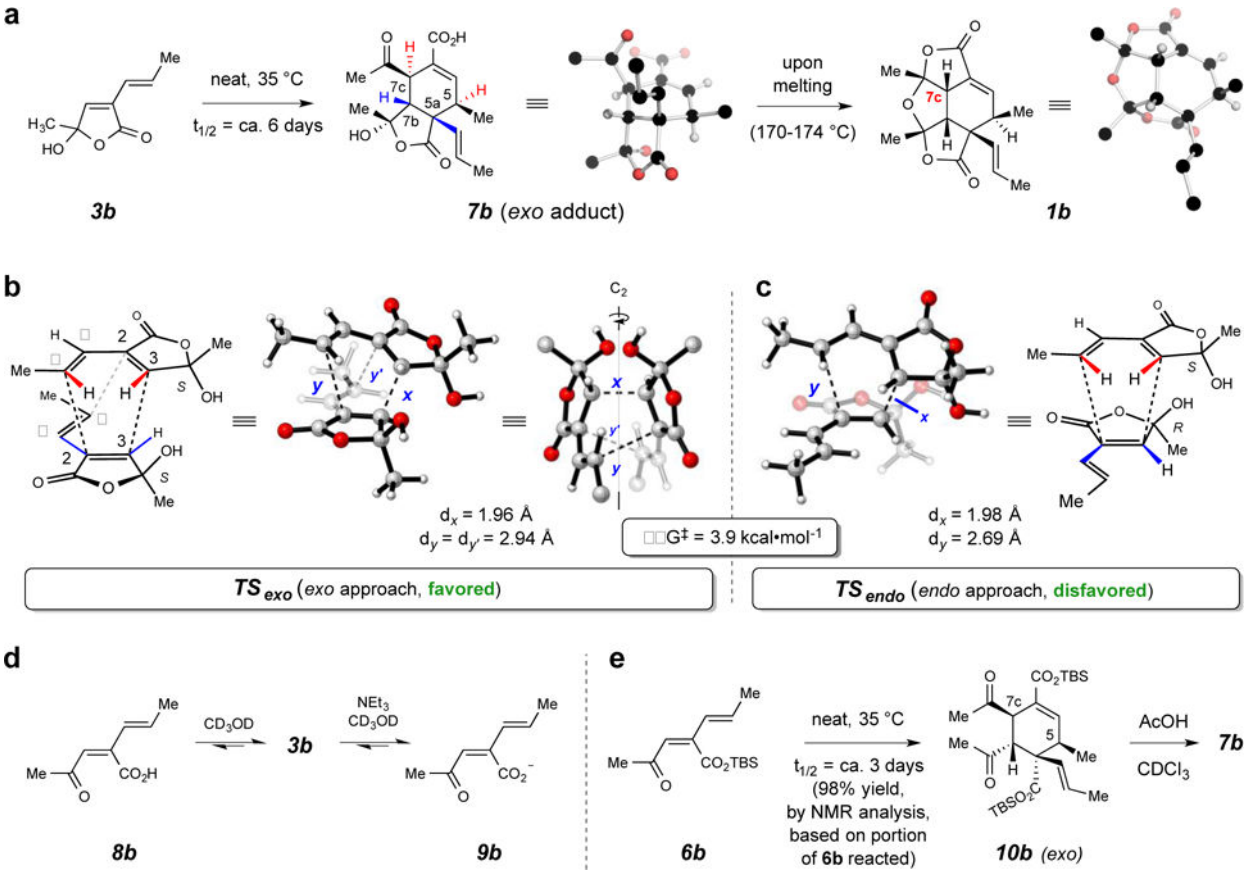

Figure 3. The stereochemical outcome of spontaneous butenolide dimerization

a, Facile dimerization of **3b** cleanly produces the *exo* adduct **7b. b**/**c**, *Exo* vs. *endo* modes of approach in the dimerization of alkenylbutenolide **3b**; the computed TS structures [DFT, SMD/M06-2X/6-311+G(d,p) in 2-butanol] favor the C_2-symmetric $\mathbf{TS_{exo}}$ over $\mathbf{TS_{endo}}$. **d**, Neutral keto acid **8b** prefers the closed hemiacylal **3b**, but weak base promotes conversion of **3b** to the ring-opened carboxylate **9b. e**, *tert*-Butyldimethylsilyl (TBS) ester **6b** readily dimerizes to **10b** [97% yield, as judged by ^{1}H NMR analysis based on the portion of remaining, unreacted **6b** (ca. 10%); see pp S66–S69 Supplementary Information], the *exo* configuration of which was established by its conversion to the diacid **7b**.

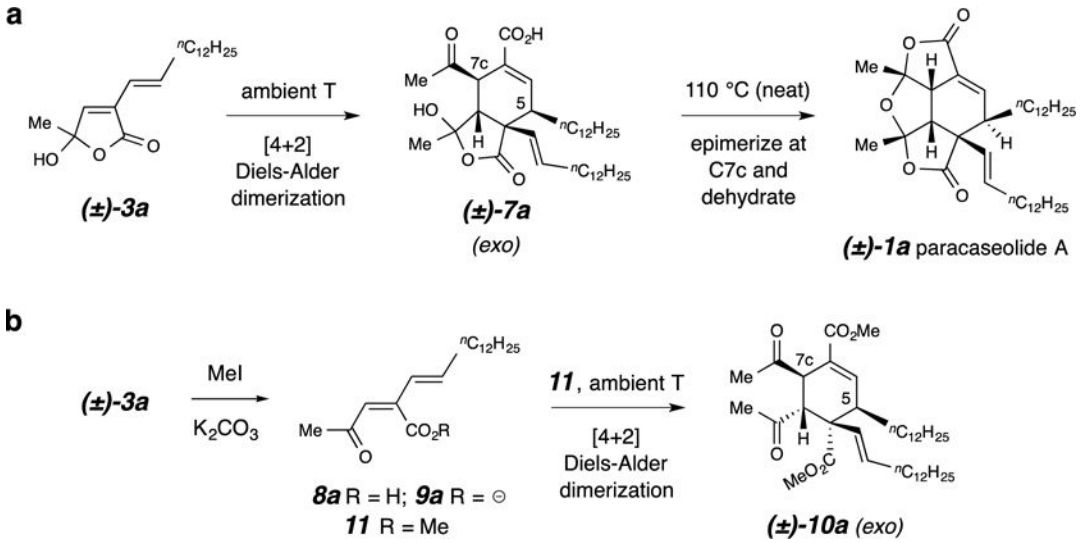

Figure 4. Conversion of the *exo*-Diels-Alder product to the natural product

a, Ambient temperature dimerization of butenolide **3a** produces a racemate of the *exo* adduct **7a**; thermal dehydration requires additional heating. **b**, The acyclic methyl ester derivative of **3a**, the ketoester **11**, also dimerizes in a highly diastereoselective fashion at ambient temperature to produce the *exo* adduct **10a**.

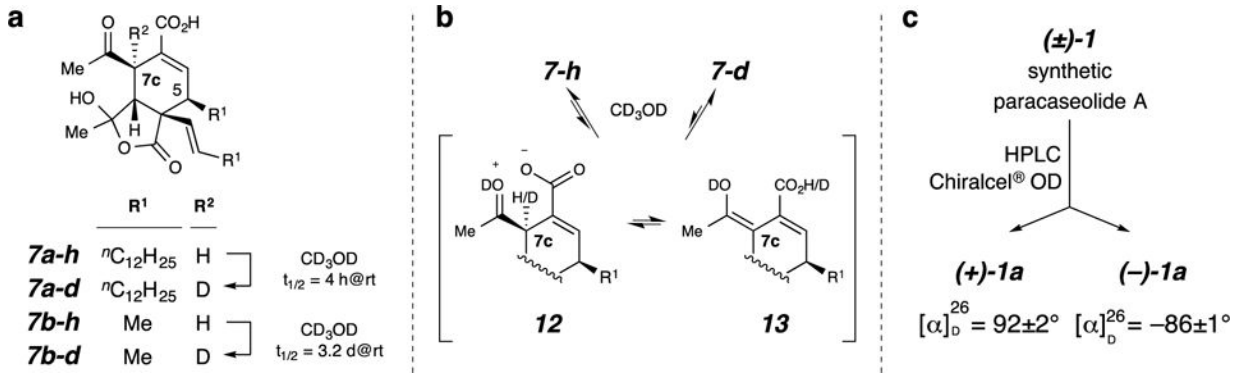

Figure 5. Epimerization of the Diels-Alder adducts may occur spontaneously

a, **b**, Mild, spontaneous H/D exchange at C7c in dimers **7a** and **7b** indicates that epimerization of the configuration of C7c is facile, perhaps via intermediates like **12** and **13.** **c**, Each enantiomer of **1a** has a specific optical rotation value that is much larger than that reported for the natural sample of paracaseolide A (**1a**), suggesting that the natural substance is racemic in nature.