Biosynthesis

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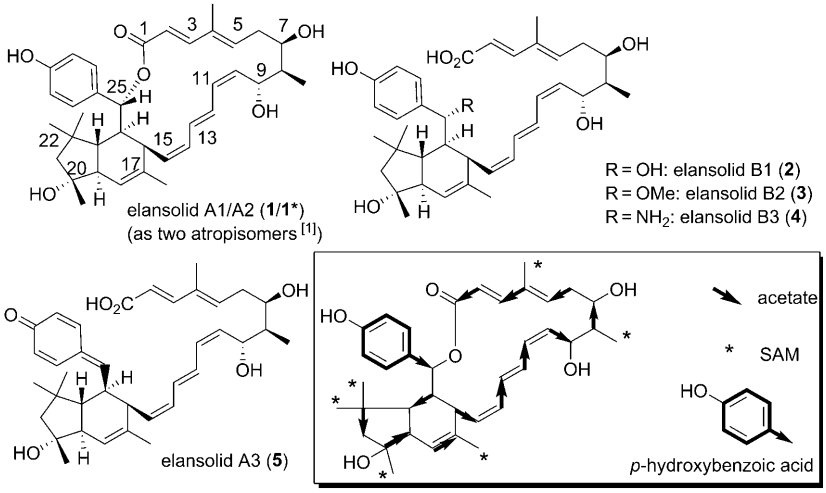
Molecular Basis of Elansolid Biosynthesis: Evidence for an Unprecedented Quinone Methide Initiated Intramolecular Diels–Alder Cycloaddition/Macrolactonization**

Richard Dehn, Yohei Katsuyama, Arne Weber, Klaus Gerth, Rolf Jansen, Heinrich Steinmetz, Gerhard Höfle, Rolf Müller, and Andreas Kirschning**

Elansolids A1/A2 (**1**)[1] and B1–B3 (**2**–**4**) and the structurally unusual and highly reactive elansolid A3 (**5**)[2] are new metabolites from the gliding bacterium *Chitinophaga sancti* (formerly *Flexibacter spec.*; Scheme 1). While elansolid A2 (**1***) shows antibiotic activity against Gram-positive bacteria in the range of 0.2 to 64 $\mu g\,mL^{-1}$ and cytotoxicity against L929 mouse fibroblast cells with an IC_{50} value of 12 $\mu g\,mL^{-1}$, the atropisomer elansolid A1 (**1**) is significantly less active.[2,3] The elansolids feature a bicyclo[4.3.0]nonane core which in the case of elansolids A1/A2 is part of a 19-membered macrolactone. Elansolid B1 is the corresponding seco acid of elansolids A1/A2, while the elansolids B2 and B3 are workup artifacts that result from nucleophilic addition of methanol and NH_3, respectively, to the quinone methide moiety in elansolid A3 (Scheme 1). Noteworthy, the unique bicyclo[4.3.0]nonane core may arise from an intramolecular Diels–Alder cycloaddition (IMDA).[4]



Scheme 1. Elansolids A1/A2 (**1**/**1***), A3 (**5**), and B1–B3 (**2**–**4**) as well as results from feeding studies with ^{13}C-labeled precursors. SAM: *S*-adenosylmethionine.

Herein we describe the identification of the elansolid biosynthetic gene cluster for elansolid biosynthesis plus details on the unique aspects of elansolid biosynthesis and focus particularly on the proposed IMDA cycloaddition and macrolactonization. Conceptually we approach this topic from two directions: a) identification and detailed analysis of the biosynthetic polyketide synthase machinery including feeding studies and b) synthesis of model precursors and synthetic studies on the IMDA cycloaddition.

Initial feeding studies with *Chitinophaga sancti* employing isotopically labeled precursors revealed that the elansolids are polyketide-derived metabolites (Scheme 1) exhibiting a chorismate-derived *p*-hydroxybenzoic acid starter unit (see Table S3 and Figure S9 in the Supporting Information).

We next intended to gain molecular insight into elansolid biosynthesis by identifying the biosynthetic gene locus in *Chitinophaga sancti*. As this genus has not been described to harbor polyketide synthase (PKS) gene clusters, a cosmid gene library of our producer strain was probed for the presence of PKSs eventually enabling sequencing of the elansolid biosynthetic gene locus (Figure S1A, Table S1; Genbank accession number: HQ680975). In the course of our study it became apparent that *Chitinophaga pinensis* DSM2588 available from public strain collections had been

[*] Dr. R. Dehn,[+] A. Weber, Prof. Dr. A. Kirschning
Institut für Organische Chemie und Biomolekulares Wirkstoffzentrum (BMWZ), Leibniz Universität Hannover
Schneiderberg 1B, 30167 Hannover (Germany)
Fax: (+49) 511-762-3011
E-mail: andreas.kirschning@oci.uni-hannover.de
Dr. Y. Katsuyama,[+] Prof. Dr. R. Müller
Helmholtz-Institut für Pharmazeutische Forschung Saarland
Helmholtz Zentrum für Infektionsforschung
and Universität des Saarlandes
Pharmazeutische Biotechnologie, Campus Gebäude C23
Postfach 151150, 66041 Saarbrücken (Germany)
Fax: (+49) 681-302-70201
E-mail: rom@helmholtz-hzi.de
Dr. K. Gerth, Dr. R. Jansen, H. Steinmetz, Prof. Dr. R. Müller
Mikrobielle Wirkstoffe, Helmholtz Zentrum für Infektionsforschung
Inhoffenstrasse 7, 38124 Braunschweig (Germany)
Prof. Dr. G. Höfle
Helmholtz Zentrum für Infektionsforschung
Braunschweig (Germany)
[+] These authors contributed equally to this work.

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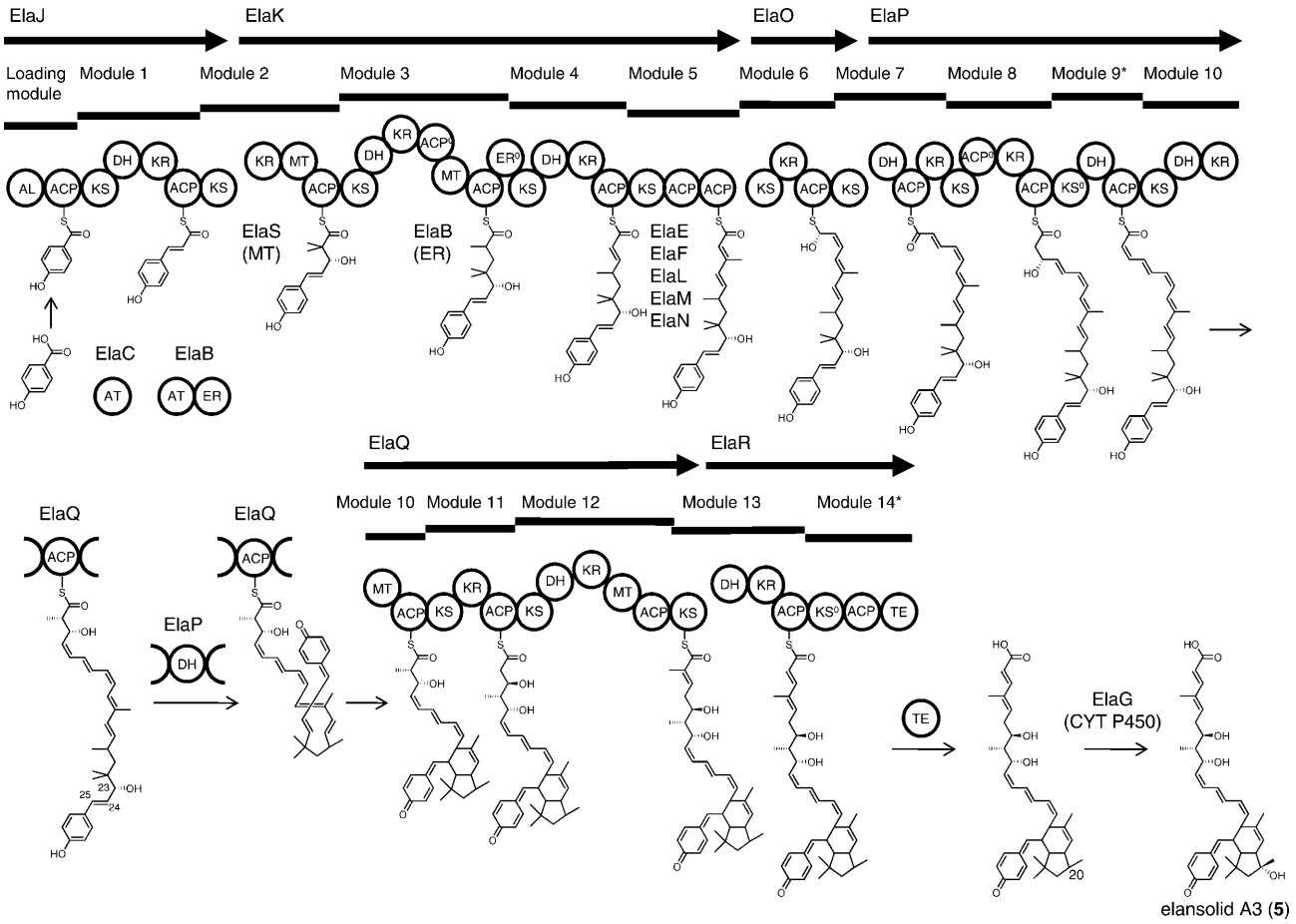
genome-sequenced and contained a highly similar set of genes (84.6% identity in nucleotide sequence with the cluster from *C. sancti*; Table S2). We next found this strain to produce elansolids, albeit at much lower titer (data not shown). The biosynthetic machinery identified turned out to represent a *trans*-AT PKS including all functionalities for β branching (C32) and methylation (Scheme 2).[5,6] Detailed analysis of the gene locus allowed us to delineate a biosynthetic scheme (Scheme 2 and Figure S1B). The identified megasynthetase incorporates many features characteristic of *trans*-AT systems, for example, unusual domain organization and modules split across two subunits.[5] The assembly line consists of six AT-less PKS subunits (J,K,O,P,Q,R) interacting with two *trans*-AT functions encoded by *elaB* and *elaC*.

Based on the deduced domain architecture of the PKS as well as phylogenetic analysis of the KS domains for their predicted substrate specificity (Scheme 2 and Figure S2),[7] we propose the biosynthetic model outlined in Scheme 2. Feeding experiments plus the presence of a putative chorismate lyase (ElaI) strongly indicate that *p*-hydroxybenzoic acid serves as the starter unit. This starter is activated and attached to the ACP of the loading module of ElaJ and subsequently further processed in 12 consecutive elongation steps (although this hypothesis does not correlate with the predicted substrate specificity of the KS in module 1 as shown in Figure S2). Within the 14 identified elongation modules, the KS domains of modules 9 and 14 do not contain the highly conserved HGTGT motif essential for decarboxylative elongation (Figure S3),[5] and are therefore assumed to be inactive. Except for the extra ACP domains in modules 3 and 8, which lack the essential serine residue (Figure S7), and the ElaK-ER domain, which shows some mutations in the conserved region (Figure S8), all other domains of the elansolid assembly line are proposed to be functional during polyketide assembly. After the second elongation step, geminal methyl groups are presumably introduced by the MT domain in module 2 and the *trans*-acting MT ElaS. However, we cannot exclude the possibility that both methylations are catalyzed by the internal MT domain, making ElaS superfluous for elansolid biosynthesis, and it is also possible that ElaS acts at a later stage of the biosynthesis.

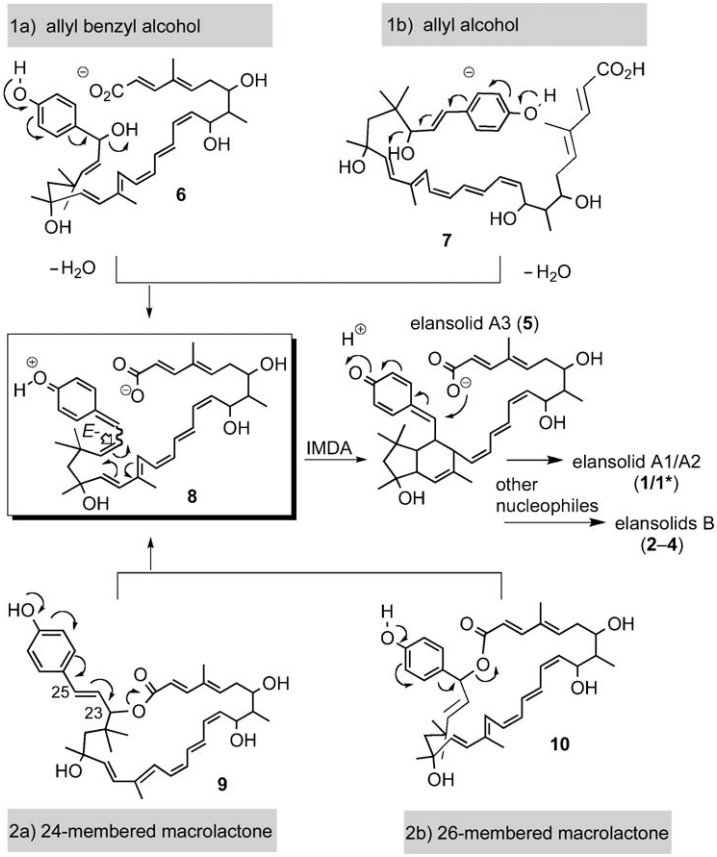


Scheme 2. Proposed biosynthetic pathway of elansolid A3 (**5**) deduced from the elansolid biosynthetic gene cluster. The timing of the IMDA reaction cannot be deduced with certainty and thus dehydration centered at C23 plus subsequent IMDA reaction might occur at a different stage (see Scheme 4). Modules 9 and 14 do not insert extender units and are marked with an asterisk. Additionally, TE-catalyzed lactonization (via C23–OH) might take place prior to the IMDA reaction resulting in the formation of the hypothetical intermediate **9** (see below). AL: AMP ligase, ACP: acyl carrier protein, KS: ketosynthase, DH: dehydratase, KR: ketoreductase, MT: methyltransferase, AT: acyltransferase, ER: enoylreductase, TE: thioesterase; inactive domains are labeled with °.

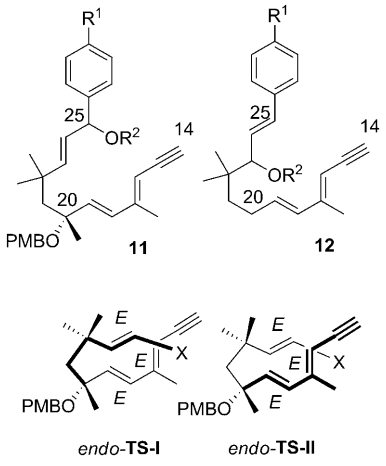
The C-terminal ER domain in the downstream module (module 3) is a typical example of unusual domain arrangements in *trans*-AT PKS.[5] However, ER sequence analysis indicates its similarity to non-functional ER domains from the bryostatin pathway.[5] This may imply that this domain is inactive and thus the double-bond reduction is carried out by ElaB. In analogy to other polyketide biosynthetic pathways[6] a set of five proteins (E,F,L,M,N) is encoded in the elansolid biosynthetic gene cluster catalyzing the β alkylation at C17. This modification is carried out during chain assembly while the intermediate is bound to module 5, which might explain the presence of tandem ACP domains. Modules 6/7 and 8/9 represent dehydrating bimodules often found in *trans*-AT PKS.[5]

The configurations of the double bonds generated during elansolid biosynthesis as well as the configurations at C7 and C9 correlate well with those predicted[8,9] for the respective KR domains (Figure S5 and S6). Especially the predicted double-bond geometries (C16–C19) as shown in Scheme 2 are crucial for the subsequent IMDA reaction (see below and Scheme 3). Interestingly, module 10 harbors an extra DH domain which apparently does not catalyze the dehydration of the respective β-hydroxy group. We speculate that this domain instead catalyzes the dehydration of the OH group at C23 (elansolid numbering), resulting in the quinone methide moiety (Scheme 2 and Scheme 4).

Notably, the SAM-dependent formation of the geminal methyl groups at C22, most likely on the nascent β-keto intermediate, excludes a subsequent



Scheme 4. Biosynthetic considerations for IMDA cycloaddition starting from open-chain (**6** and **7**; cases 1a, 1b) and macrocyclic precursors (**9** and **10**; cases 2a, 2b); for clarity, the configurations of the stereogenic centers are not represented.



Scheme 3. all-*E* IMDA precursor **11**, its positional isomer **12**, and the *endo* transition states **TS-I** and **TS-II**.[12]

standard PKS dehydration reaction. The quinone methide intermediate may subsequently undergo IMDA cycloaddition to form the bicyclo[4.3.0]nonane core (see below). However, it cannot be excluded that PKS processing continues with the linear-chain polyketide (see Scheme 4). In the former case, the quinone methide cycloadduct would be further extended and subsequently hydrolyzed by the TE domain at the final module to yield deoxyelansolid A3. As the final step of elansolid A3 biosynthesis, ElaG, a cytochrome (CYT) P450 monooxygenase, is supposed to catalyze the oxidation at C20. An alternative scenario would be the further extension of the linear chain without an IMDA reaction; this would result in product **9** which would be eventually formed by TE-catalyzed lactonization. As this macrolactone would also be prone to IMDA cycloaddition (Scheme 1), both routes implicate the presence of highly reactive quinone methide intermediates in several consecutive biotransformations.

In brief, the analysis of the PKS provides a logical scenario for the biosynthesis of deoxyelansolid A3, which is thus the presumed final product of the assembly line. The proposed unusual dehydratase reaction setting up the IMDA reaction is most intriguing and warrants further analysis. We cannot exclude that this dehydration is carried out by another dehydratase present in the cluster and the order of the reactions may differ from that in Scheme 2 including the IMDA reaction which might occur on a "shorter" intermediate coupled to ElaO or ElaP.

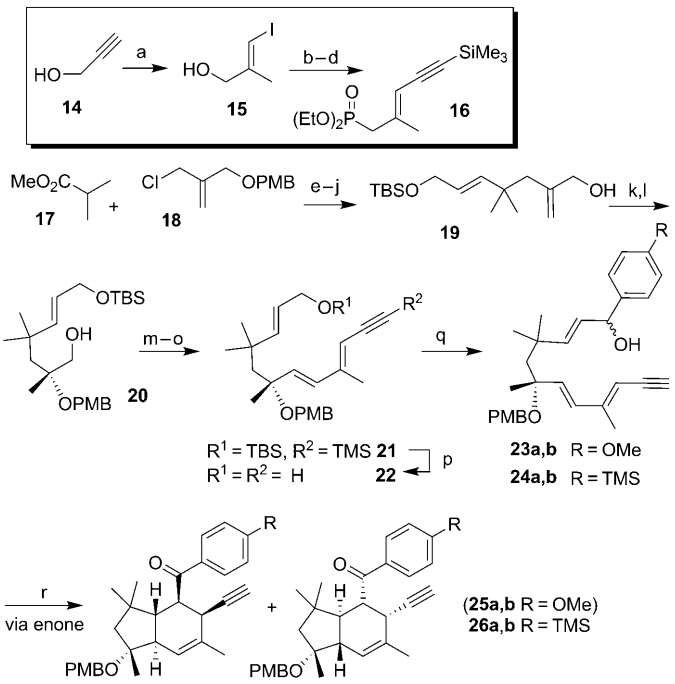
The information obtained from the gene cluster analysis prompted us to analyze the role of quinone methide moieties and the unprecedented IMDA cycloaddition in greater detail and relate them to macrolactonization. If the quinone

methide moiety is not formed by dehydration during PKS assembly and the IMDA cycloaddition takes place at a later stage of polyketide assembly, open-chain seco acid **6** (allyl benzyl alcohol) and—with respect to the gene analysis more likely—allyl alcohol **7** can be envisaged as possible open-chain precursors (cases 1a and 1b in Scheme 4). At this stage the vinylogous quinone methide **8**, the key intermediate of this biosynthetic sequence, is generated which would directly undergo IMDA cycloaddition to give tetrahydroindane quinone methide **5** (elansolid A3). In a tandem fashion, this highly reactive species would cyclize by a Michael-type attack of the carboxylate onto the quinone methide moiety. As a result elansolid A **1** is formed. If water, methanol, or ammonia serve as nucleophiles, elansolids B1–B3 (**2**–**4**) are generated, instead.

In principle it cannot be excluded that IMDA substrate **8** originates from either the 24-membered macolactone **9** or alternatively the 26-membered macolactone **10** after lactone activation (cases 2a and 2b; Scheme 1). Intermediate **9** is clearly favored over **10** by the proposed biosynthetic outlined above. This scenario would require a PKS-associated cyclizing thioesterase (TE) instead of the hydrolyzing TE as shown in Scheme 2. Bioinformatic analysis currently does not allow a distinction between TEs that preferentially hydrolyze or lactonize. Importantly, in both cases 1 and 2 presented in Scheme 4 the Diels–Alder cycloaddition generates elansolid A3 (**5**) which would then directly cyclize through a Michael-type addition of the carboxylate onto the quinone methide moiety and result in elansolid A (**1**).

Based on the feeding experiments and the principal considerations mentioned above we consequently addressed the question of the IMDA cycloaddition[10] by preparing the synthetic model compound all-*E* triene **11** (resembling IMDA precursors **6** and **10**) and the simplified regioisomer **12** (resembling precursors **7** and **9**). It must be noted that model substrate **11** was designed first to be closely related to the natural system in order to study all factors of diastereocontrol during IMDA cycloaddition in more detail.[11] The *E* configuration of all olefinic double bonds would result in the correct relative configuration at C16, C19, C23, and C24[12] via the two possible *endo* transition states **TS-I** and **TS-II**. The stereocenter at position 20 has to exert diastereofacial control.[13]

The synthesis of allyl alcohol **22** commenced with allyl chloride **18**[14] which was transformed into allyl alcohol **19** by a standard sequence that included α-allylation of **17**, reduction/oxidation followed by Horner–Wadsworth–Emmons (HWE) olefination with phosphonate **13**, ester reduction, O-silylation of the intermediate allyl alcohol, and finally PMB removal (Scheme 5). The methylidene moiety in **19** was epoxidized under Sharpless conditions with good enantiomeric excess and the epoxy alcohol was then reductively ring-opened to yield a 1,2-diol.[15] Formation of the cyclic PMB acetal and cleavage under reductive conditions gave alcohol **20**. Oxidation furnished an aldehyde which was fused with phosphonate **16** by a HWE olefination, and the resulting alkyne **21** was finally desilylated to afford allyl alcohol **22**. Phosphonate **16**[16] was prepared from vinyl iodide **15** (from **14**[17]) which was first elaborated by a Sonogashira–Hagihara alkynylation followed by Appel bromination and Michaelis–Arbusov reaction.



Scheme 5. Synthesis of allyl alcohol **22** and IMDA studies. Reagents and conditions: a) 1. $[Cp_2ZrCl_2]$, $AlMe_3$, CH_2Cl_2, RT, 15 h, 2. I_2, 50%; b) Me_3SiCCH, $[Pd(PPh_3)_4]$, CuI, pyrrolidine, RT, 2 h, 80%; c) CBr_4, PPh_3, CH_2Cl_2, RT, 30 min; d) $P(OEt)_3$, microwave irradiation, 100 °C, 30 min, 70% for two steps; e) 1. DIPA, *n*BuLi, THF, −78 °C to 0 °C, then addition of **17**, −78 °C→−40 °C, 2. addition of **18**, TBAI, −40 °C to RT, 82%; f) Dibal-H, THF, −78 °C→RT, 99%; g) PCC, CH_2Cl_2, RT; h) NaH, $(EtO)_2P(O)CH_2CO_2Et$ (**13**), THF, 50 °C, then addition of aldehyde (from g), 80 °C, 91% for two steps; i) 1. Dibal-H, THF, −78 °C→RT, 2. TBSCl, imidazole, DMAP, CH_2Cl_2, RT, 99% for two steps; j) DDQ, CH_2Cl_2, pH 7 phosphate buffer, RT, 93%; k) $Ti(OiPr)_4$, D-(−)-DET, *t*BuOOH, CH_2Cl_2, −25 °C, 93%, 95% *ee* (determined by ^1H NMR spectroscopy after formation of the *S* Mosher ester[15]); l) $LiAlH_4$, THF, 0 °C to RT, 86%; m) 1. 4-MeO-C_6H_4-$CH(OMe)_2$, PPTS, CH_2Cl_2, RT, 2. Dibal-H, toluene, −78 °C→RT, 89% for two steps; n) DMP, $NaHCO_3$, CH_2Cl_2, 0 °C, 86%; o) NaHMDS, **16**, THF, −78 °C, then addition of aldehyde, −78 °C→RT, 84%, all-*E*/other isomers = 10:1; p) TBAF, THF, 0 °C to RT, 99%; q) 1. TPAP, NMO, CH_2Cl_2, −30 °C; 2. RC_6H_4–MgBr, THF, TMEDA, −78 °C to −50 °C (R = OMe, 75%; R = TMS, 81% for two steps); r) **24a**,**b**, TPAP, NMO, MS 4 Å, CH_2Cl_2, −30 °C (67%; *de* = 5:1). Compounds **25** were not obtained. Abbreviations: Cp = cyclopentadienyl, DIPA = ethyldiisopropylamine, TBAI = tetra-*n*-butylammonium iodide, Dibal-H = diisobutylaluminum hydride, PCC = pyridinium chlorochromate, TBS = *tert*-butyldimethylsilyl, DMAP = 4-dimethylaminopyridine, DDQ = dichlorodicyanoquinone, DET = diethyl tartrate, PPTS = pyridinium *p*-toluenesulfonate, DMP = Dess–Martin periodinane, NaHMDS = sodium hexamethyldisilazide, TBAF = tetra-*n*-butylammonium fluoride, TPAP = tetra-*n*-propylammonium perruthenate, NMO = *N*-methylmorpholine *N*-oxide.

Using the Ley–Griffith method[18] we next oxidized allyl alcohol **22** at −30 °C to the corresponding aldehyde which was treated directly with 4-methoxyphenylmagnesium bromide or alternatively with 4-trimethylsilylphenylmagnesium bromide[19] to afford allyl benzyl alcohols **23a**,**b** and **24a**,**b**, respectively, as mixture of diastereomers (ca. 1:1 for both examples; Scheme 5). These alcohols served as model quinone methide precursors (see allyl benzyl alcohol **6**, Scheme 4). In no case were Brønstedt or Lewis acids able to

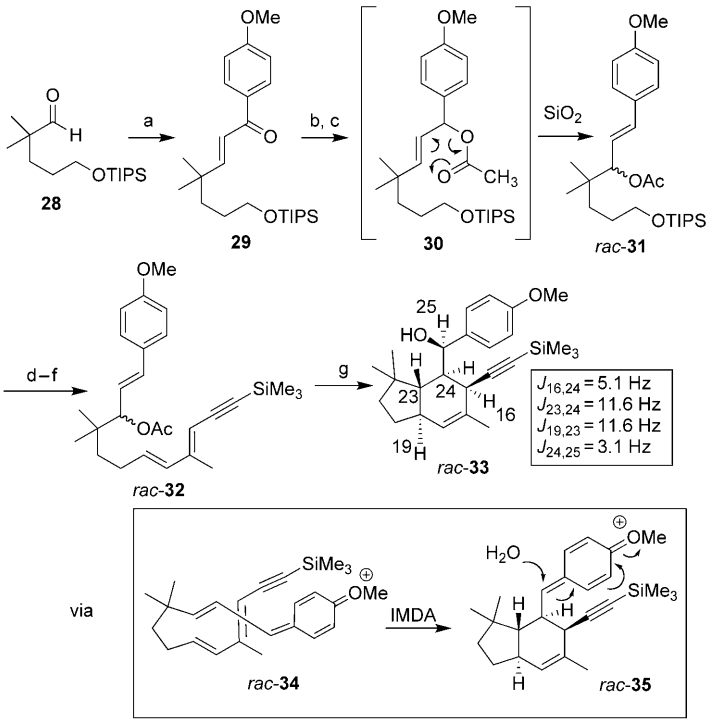
force the formation of the desired IMDA products.[20] Also all attempts to provide the allyl benzyl alcohols **23a**,**b** or **24a**,**b** with a better leaving group (acetate, tosylate) failed. Only when trienes **24** were oxidized to the corresponding enones did spontaneous formation of IMDA products **26** occur with good diastereoselectivity via the preferred **TS-I**.[21] The corresponding 4-methoxyphenyl substituted alcohols **23** did not furnish the desired IMDA products **25**; complete decomposition was observed.

The configuration of the Diels–Alder products **26** was unambiguously determined by analysis of the H,H coupling constants (*J*) and by conducting NOE experiments; the data were compared with the corresponding data collected for authentic elansolids.[1,23] The absolute configurations at C16, C19, C23, and C24[13] were identical to those in the elansolids.

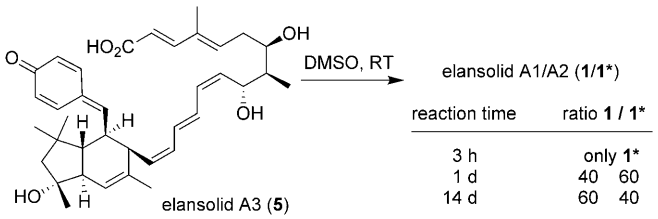
Next, we prepared the model substrate **12** with reversed positioning of the hydroxy group and the double bond at C23–C25 (elansolid numbering). Activation should also lead to the key quinone methide intermediate (resembling **10**, Scheme 4). Thus, aldehyde **28**[23] was condensed with *p*-methoxyacetophenone to yield enone **29** as a single *E* isomer (Scheme 6). Luche reduction followed by acylation furnished allyl acetate **30**. Forced by the electron-donating properties of the *para* methoxy group, **30** spontaneously underwent a [3,3]-sigmatropic rearrangement, which was promoted under the slightly acidic purification conditions during silica gel column chromatography, to furnish the styrene derivative **31**. After deprotection and oxidation, the resulting aldehyde was coupled with phosphonate **16**. The resulting triene **32** was treated with trifluoroacetic acid in wet dioxane to smoothly provide the IMDA product *rac*-**33** as a single diastereomer. The polar aprotic solvent was chosen based on the assumption that it would stabilize the quinone methide cation **34**. As a result of the IMDA reaction the second quinone methide cation **35** is formed which is trapped by water originating from the wet solvent.[24] Analysis of the relevant ^{1}H NMR coupling constants (*J*) revealed that the cycloaddition proceeded through an *endo* transition state yielding the correct relative configuration as present in elansolid A (**1**).[22] Interestingly, nucleophilic attack of water onto intermediate **35** proceeds *syn* relative to the alkynyl substituent at C16 resulting the same relative configuration at C25 as that found in the elansolids. This is indicated by the small vicinal coupling between H24 and H25 which is also observed in authentic samples of the elansolids.[1,22]

The synthetic model studies along with the gene cluster analysis strongly underline our hypothesis, that either an allyl alcohol similar to **7** (see Scheme 4) or, from our point of view less likely, the corresponding lactone **9** serve as biosynthetic substrates for the IMDA cycloaddition, whereas **6** or the 26-membered lactone **10** can almost certainly be excluded as precursors.[25] In fact, the conditions found for activation of **32** closely resemble the biological situation.

Finally, we gained strong support for the proposed unique lactonization based on the nucleophilic addition of the carboxylate onto a quinone methide species when elansolid A3 (**5**) was kept at room temperature in DMSO (Scheme 7).[2] After one day elansolids A1 (**1**) and A2 (**1***) were detected in a 2:3 ratio and after another 13 days this ratio had changed to 3:2.



Scheme 6. Synthesis of triene *rac*-**32** through a [3,3]-sigmatropic rearrangement of allyl acetate *rac*-**31** and Brønstedt acid promoted IMDA cycloaddition to provide the bicyclo[4.3.0]nonane core *rac*-**33**. Reagents: a) *p*-methoxyacetophenone, NaOMe, MeOH, Δ, 59%; b) $NaBH_4$, $CeCl_3$, MeOH, 0°C, 99%; c) AcCl, NEt_3, CH_2Cl_2, RT, 50%; d) TBAF, THF, RT, 64%; e) DMP, CH_2Cl_2, RT, 30%; f) NaHMDS, **16**, THF, −78°C, 62% (all-*E*); g) TFA, wet dioxane, RT, 15 h (55%). TFA = trifluoroacetic acid.



Scheme 7. Direct conversion of elansolid A3 to elansolids A1/A2.

In conclusion, we have conducted a detailed study on the PKS-based biosynthesis of the elansolids and have shed light on unique and unprecedented aspects, namely the setup of the IMDA reaction by an unusual dehydration mechanism, the IMDA reaction itself, and the macrolactonization. In order to achieve such a detailed insight we utilized different approaches and strategies such as feedings studies, analysis of the responsible biosynthetic gene cluster, and the chemical syntehsis of complex model substrates.

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[11] Additionally, the presence of the alkyne group paves the way for a biomimetic total synthesis approach.
[12] Throughout the text atom numbering refers to the numbering of in the natural product elansolid.
[13] To the best of our knowledge no examples of IMDA cycloadditions with a tertiary alcohol in allylic position to the diene have been described so far. In simpler cases, the stereoinduction of this center varies from moderate to excellent, see: a) M. P. Edwards, S. V. Ley, S. G. Lister, P. D. Palmer, D. J. Williams, *J. Org. Chem.* **1984**, *49*, 3503 – 3516; b) W. R. Roush, *J. Am. Chem. Soc.* **1980**, *102*, 1390 – 1404.
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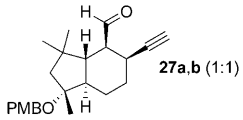
[18] We had to choose an oxidation method that works well at temperatures as low as −30 °C because the intermediate aldehyde spontaneously underwent an IMDA reaction at 0 °C to yield tetrahydroindanes **27a**,**b** as diastereomers (1:1) resulting from both *endo* transition states **TS-I** and **TS-II**.



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[20] Instead, only deprotection of the PMB ether was observed.
[21] This high propensity to cyclization can be explained by 1) a low-energy LUMO of the dienophile, 2) a high-energy HOMO of the diene on account of conjugation to the alkyne, and 3) a double Thorpe–Ingold effect caused by the *gem*-dimethyl group and the tertiary alcohol. However, the gene analysis shown in Scheme 2 almost excludes the possibility of an IMDA precursor bearing a keto group at C25.
[22] See the Supporting Information for details on the analytical assignments.
[23] T. Lampe, H. M. R. Hoffmann, *Tetrahedron Lett.* **1996**, *37*, 7695 – 7698.
[24] Noteworthy, when the weaker acid AcOH in dioxane was employed instead, no IMDA products could be detected.
[25] While this manuscript was under review, Piel and co-workers disclosed a biosynthetic proposal for the elansolids, suggesting a rearranged allyl benzyl alcohol (similar to **6**) as the direct substrate for the IMDA reaction. From our perspective, the electronic requirements for the IMDA cycloaddition are fulfilled only when the quinone methide moiety is involved as the key biosynthesis intermediate: R. Teta, M. Gurgui, E. J. N. Helfrich, S. Künne, A. Schneider, G. Van Echten-Deckert, A. Mangoni, J. Piel, *ChemBioChem* **2010**, *11*, 2506 – 2512.

