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Understanding the Competing Pathways Leading to Hydropyrene and Isoelisabethatriene

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Abstract

Terpene synthases are responsible for the biosynthesis of terpenes, the largest family of natural products. Hydropyrene synthase generates hydropyrene and hydropyrenol as its main products along with two byproducts, isoelisabethatriene A and B. Fascinatingly, a single active site mutation (M75L) diverts the product distribution towards isoelisabethatriene A and B. In the current work, we study the competing pathways leading to these products using quantum chemical calculations in the gas-phase. We show that there is a great thermodynamic preference for hydropyrene and hydropyrenol formation, and hence most likely in the synthesis of the isoelisabethatriene products kinetic control is at play.

Keywords

Diterpenes; quantum mechanics; enzyme mechanism; thermodynamic and kinetic control; terpene synthases

Introduction

Terpenes constitute a ubiquitous class of natural molecules that are synthesized by terpene synthases (TPS). TPS generate a plethora of terpenes employing rich carbocation chemistry from a very limited number of substrates, known as geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), to produce mono-, sesqui-, and diterpenes, respectively. The formation of terpenes relies on an assortment of carbocation steps like cyclization, methyl migrations, rearrangements, proton or hydride transfers, hydroxylations and epoxidations. TPS and downstream functionalizing enzymes, like P450s, together produce more than 80,000 known terpenes and terpenoids. [1-3].

Hydropyrene synthase (HpS) from *Streptomyces clavuligerus* generates a mixture of diterpenes named hydropyrene (HP) (52%) and diterpenoid named hydropyrenol (HPol) (26%) as its main GGPP cyclization products, along with two minor compounds, namely the isoelisabethatrienes (IE) A (13%) and B (9%), respectively. Interestingly, the elisabethatriene diterpene macrocycle and its isoforms can act as biosynthetic precursors of the bioactive compounds erogorgiaene and pseudopterosin, having antibiotic and anti-inflammatory activities, respectively. [4, 5] Unexpectedly, a single active site mutation, M75L, significantly shifts the product distribution and IE A becomes the dominant product (44%) in this enzyme variant. [6]

As suggested by Rinkel et al., both routes (HP and IE routes) proceed from the same substrate (GGPP). However, in the IE pathway a substrate isomerization occurs, shifting the covalent attachment point between the substrate hydrocarbon and the pyrophosphate group (Figure 1.). [7] Presumably, this isomerization is responsible for a slightly different substrate fold inside the active site, hence shifting the product distribution in favor of the IE products rather than the HP products.

Oxidation of IE A and B by lipases results in formation of the advanced Pseudopterosin (P) precursor erogorgiaene and 1R-epoxy-elisabetha-5,14-diene (EED), respectively. [6, 7] Ps, marine amphilectane-type diterpenoids from the gorgonian coral *Antillogorgia elisabethae*, feature superior anti-inflammatory properties which render them innovative target compounds for drug development. [8, 9] Hence, increasing the IE products at the expense of the HP products is an important biotechnology mission for sustainable supply of the latter. In order to modulate the IE and HP enzyme pathways accordingly, it is important to understand the factors determining both synthetic routes.

In the current work, we focus on the mechanistic details of the HP and IE pathways using computational methods in the gas phase. Gas phase studies have been crucial in understanding terpene chemistry.[10-22] This work sheds light on

the thermodynamic and kinetic parameters of the inherent chemistry in these reactions and also points to some understanding of the possible thermodynamic and kinetic control in the enzyme.

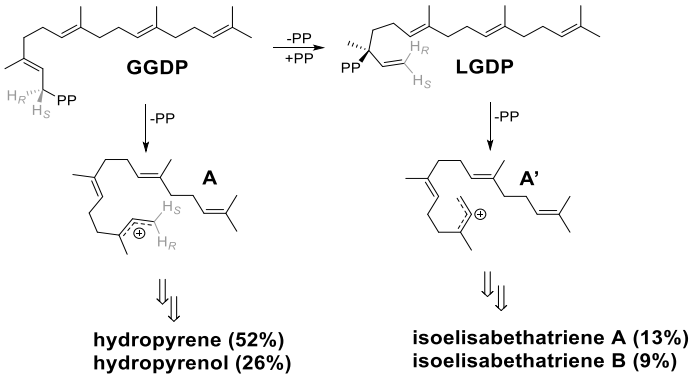


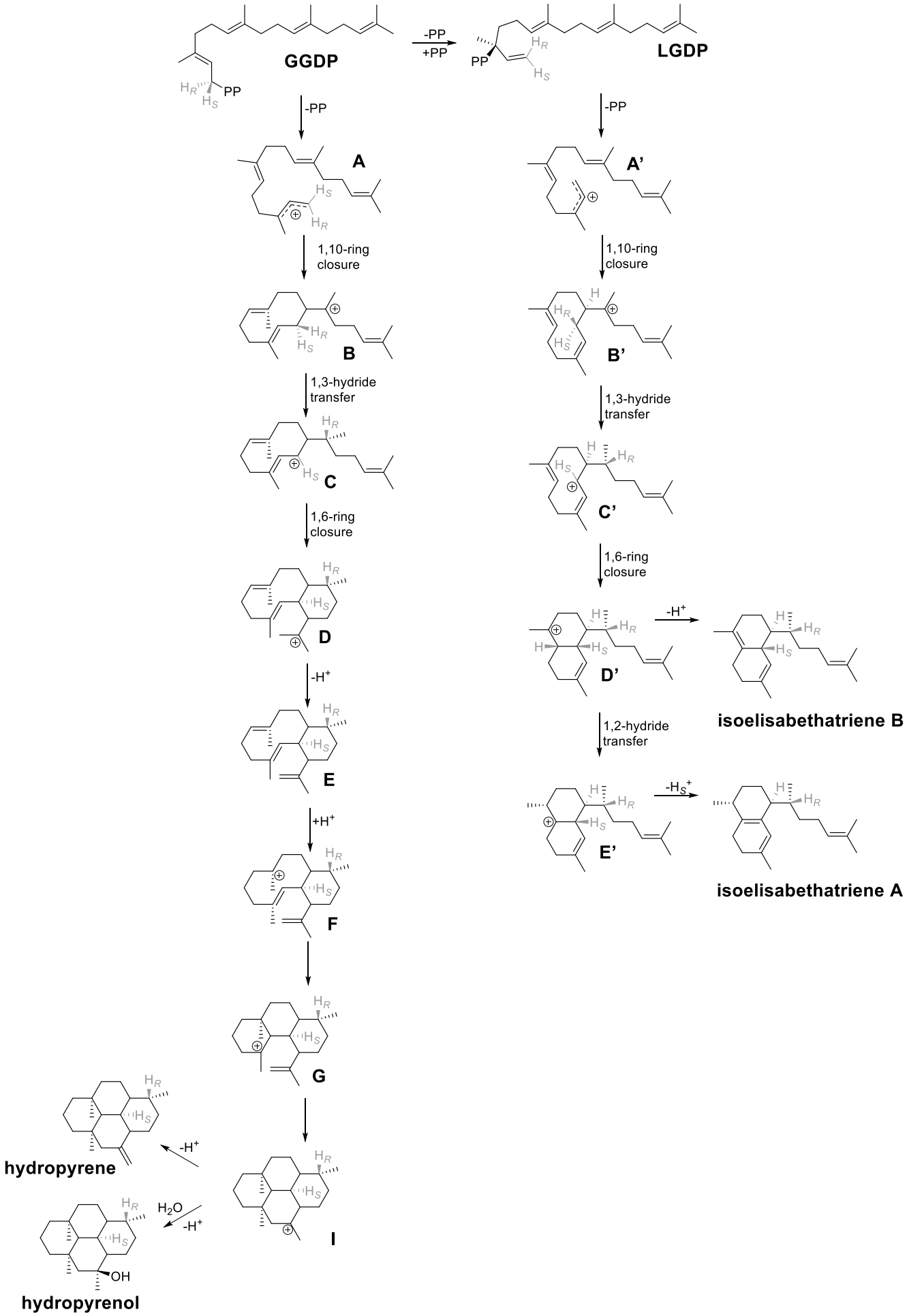
Figure 1. Summary of yields of HP and IE products in hydropyrene synthase.

Results

Reaction Mechanism

To better understand the HP and IE reaction pathways, we performed quantum mechanics (QM) calculations using density functional theory (DFT). We studied the inherent chemistry of the reaction leading to HP and IE using gas-phase calculations. This provided the free energy of distinct carbocation intermediates and transition states along the proposed reaction path leading to products in the gas-phase. The gas phase is a natural choice as a reference environment for terpene synthases.[10-12, 15, 16, 21-26]

The proposed reaction mechanisms yielding HP and IE and are presented in Scheme 1, while the reaction free energy profile is presented in Figure 2. Here, we modeled the transformations **A→I** (HP) and **A→E'** (IE). The gas-phase calculations commenced with geranylgeranyl cation (**A**) in a fully extended form.


GGDP
-PP
+PP
LGDP
-PP
A
1,10-ring closure
B
1,3-hydride transfer
C
1,6-ring closure
D
-H+
E
+H+
F
G
I
-H+
hydropyrene
H2O
-H+
hydropyrenol
-PP
A'
1,10-ring closure
B'
1,3-hydride transfer
C'
1,6-ring closure
D'
-H+
isoelisabethatriene B
1,2-hydride transfer
E'
-Hs+
isoelisabethatriene A

Scheme 1. Proposed mechanism for HP and IE routes.

HP pathway

The HP gas-phase pathway commences with a C1-C10 cyclization, which yields cation **B**, which is more stable than **A** by -11.4 kcal/mol. A subsequent 1,3-hydride transfer results in an allyl cation (**C**), which is -18.5 kcal/mol more stable than **A**. The barrier for the 1,3-hydride transfer is 16.2 kcal/mol for **B**→**C**. Subsequently, the double bond on C14-C15 reacts with the cation on C1 to form intermediate **D**, which is slightly less stable than **C** (-17.3 kcal/mol) In the enzyme environment intermediate **D** deprotonates to form intermediate **E**, while intermediate **E** is re-protonated to form intermediate **F**, which immediately transforms to intermediate **G (**i.e., **F** is not stable**)**. **G** is significantly more stable than **D** (by -16.5 kcal/mol). **G** then transforms to the very stable 4-ring intermediate **I** without any free energy barrier. The overall exergonicity of this process which transforms four π-bonds to σ-bonds, with accompanying gains in intramolecular dispersion interactions, is -62.8 kcal/mol.

IE pathway

As describe above both pathways commence with a C1-C10 cyclization. However, in the IE pathway a preliminary isomerization step occurs via rotation around the C2-C3 bond, transforming from the trans to the cis form. In the enzyme this process occurs with the help of a pyrophosphate group. In the current gas-phase study, we employ cation **A** as our reference point.

The C1-C10 cyclization yields cation **B'**, which are more stable than **A** -15.4 kcal/mol. A subsequent 1,3-hydride transfer results in an allyl cation (**C'**, -31.8 kcal/mol relative to **A**), with a barrier of 5.4 kcal/mol. Cation **C'** collapses into **D'** via a barrierless 1,6-ring closure (ΔG_r of -35.6 kcal/mol relative to **A**). **D'** can deprotonated to yield **IE B** or conversely may undergo a 1,2-hydride transfer, forming carbocation **E'** (ΔG_r of -34.1 kcal/mol relative to **A**) This transformation has a $\Delta G^{\ddagger}$ of 6.6 kcal/mol. **E'** may then deprotonate to form IE A. Overall, the exergonicity for the formation of IE A/B from carbocation **A** is significant, due to the exchange of two π-bonds for σ-bonds, as well as gain in dispersion interactions on folding of the extended geranylgeranyl cation.

DISCUSSION

Although the current calculations were performed in the gas-phase without inclusion of the enzyme environment, we may still generate some hypotheses regarding the enzymatic process. First, considering the similar free energy of IE A and B and the small kinetic barrier separating them, these isomers may exist in equilibrium in the enzyme active site. The relative amount of IE A and B may then be determined by their proximity to an active site base. Second, considering the huge thermodynamic preference for the HP pathway, it is unlikely that a thermodynamic equilibrium exists between this pathway and the IE pathway. Rather, an equilibrium may exist between GGDP and LGDP,

but once cyclization commences, the reactions will proceed until completion along their respective pathways. Hence, the difference in product profile in WT and enzyme variants may be largely due to different folding of the initial substrate. Future *in-enzyme* studies can shed light on the preferred folding of GGDP inside the WT and variant enzymes and potentially the roles specific active site residues play during catalysis.[23, 24, 27]

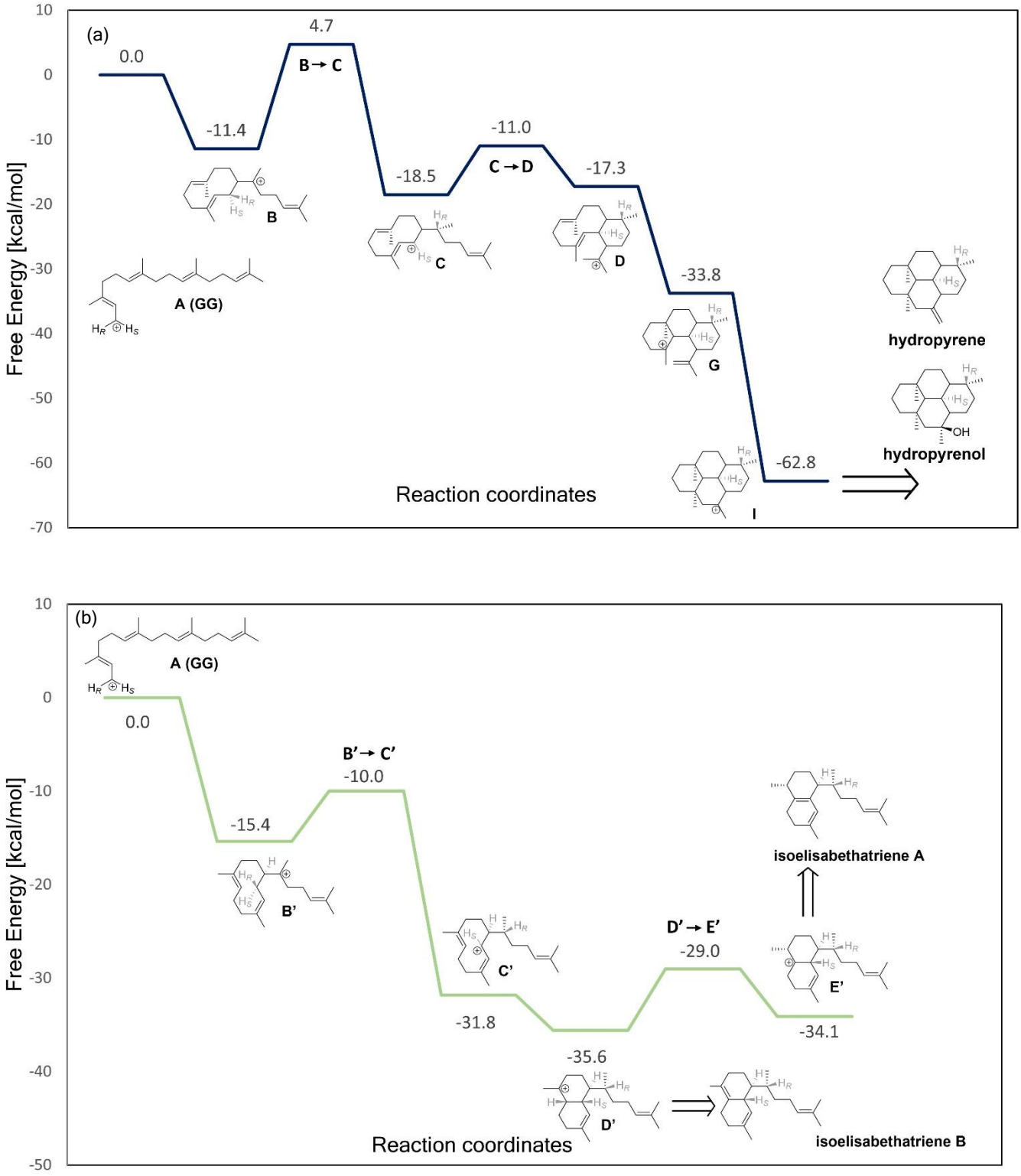


Figure 2. Free energy profile of hydropyrene cation (a), and IE cation (b) formation in the gas phase. The free energy of cation A is set to zero.

Conclusion

In the current work we performed gas-phase quantum chemistry calculations for the competing reaction pathways leading to IE A and B as well as hydropyrene and hydropyrenol. In the former two reactions there is an exchange of two π-bonds for σ-bonds, resulting in a total exergonicity of -34.1 and -35.6 kcal/mol, respectively. In the latter two reactions which replace four π-bonds for σ-bonds and share a common final carbocation, the exergonicity is -62.8 kcal/mol. These values reflect the energetics of exchanging π-bonds for σ-bonds. In spite of the current calculations being performed in the gas-phase, we may still generate some propositions regarding the enzymatic process. First, considering the similar free energy of IE **A** and **B** and the low barrier between them, IE **A** and **B** may exist in equilibrium in the enzyme active site. The proximity to an active site base may then determine the relative amount of IE **A** and **B**. Second, it is unlikely that a thermodynamic equilibrium exists between the HP and IE pathways, due to the significant free energy barriers required for reverse barriers in the enzyme. Rather, an equilibrium may exist between GGDP and LGDP.

Experimental

Dynamics and Monte-Carlo simulations.

We generated conformers using simulated annealing (SA) molecular dynamics followed by SA Monte Carlo simulation using CHARMM. [28] Force field parameters were generated using CGenFF [29] and an in-house code which modifies parameters for cations from existing parameters for neutral molecules. For each intermediate we created 100 conformers which were subsequently clustered (a cluster width of 1.0 Å was used). For each unique conformer we performed QM calculations and then choose the lowest energy conformer as representative of each carbocation intermediate.

Quantum chemistry calculations.

All QM calculations were performed using the Gaussian 16 program [30] using M06-2X/6-31G+(d,p).[31, 32] This combination has been employed previously to TPS reactions.[18, 20, 24, 25, 33-37]

Supporting Information

All coordinate files for intermediates and TS structures are available.

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References

1. Croteau, R. *Chem. Rev.* **1987**, *87*, 929-954.
2. Christianson, D. W. *Chem. Rev.* **2017**, *117* (17), 11570-11648.
3. Cane, D. E. *Chem. Rev.* **1990**, *90*, 1089-1103.
4. Movahhed, S.; Westphal, J.; Kempa, A.; Schumacher, C. E.; Sperlich, J.; Neudörfl, J.-M.; Teusch, N.; Hochgürtel, M.; Schmalz, H.-G. *Chemistry – A European Journal* **2021**, *27* (45), 11574-11579, https://doi.org/10.1002/chem.202101863 .
5. Kohl, A. C.; Kerr, R. G. *Marine Drugs* **2003**, *1* (1).
6. Ringel, M.; Reinbold, M.; Hirte, M.; Haack, M.; Huber, C.; Eisenreich, W.; Masri, M. A.; Schenk, G.; Guddat, L. W.; Loll, B.; Kerr, R.; Garbe, D.; Brück, T. *Green Chemistry* **2020**, *22* (18), 6033-6046, 10.1039/D0GC01697G.
7. Rinkel, J.; Rabe, P.; Chen, X.; Köllner, T. G.; Chen, F.; Dickschat, J. S. *Chemistry – A European Journal* **2017**, *23* (44), 10501-10505.
8. Berrué, F.; McCulloch, M. W. B.; Kerr, R. G. *Biorg. Med. Chem.* **2011**, *19* (22), 6702-6719.
9. Mayer, A. M. S.; Jacobson, P. B.; Fenical, W.; Jacobs, R. S.; Glaser, K. B. *Life Sci.* **1998**, *62* (26), PL401-PL407.
10. Hong, Y. J.; Tantillo, D. J. *Nat. Chem.* **2009**, *1* (5), 384-389.
11. Tantillo, D. J. *WIREs Computational Molecular Science* **2020**, *10* (3), e1453, https://doi.org/10.1002/wcms.1453.
12. Tantillo, D. J. *Angew. Chem. Int. Ed.* **2017**, *56* (34), 10040-10045.
13. Smentek, L.; Hess, B. A. *J. Am. Chem. Soc.* **2010**, *132* (48), 17111-17117.
14. Hess Jr, B. A.; Smentek, L. *Angew. Chem. Int. Ed.* **2013**, *52* (42), 11029-11033, https://doi.org/10.1002/anie.201302886.
15. Tantillo, D. J. *Chem. Soc. Rev.* **2018**, *47* (21), 7845-7850.
16. Tantillo, D. J. *Nat. Prod. Rep.* **2011**, *28* (6), 1035-1053.
17. Dixit, M.; Weitman, M.; Gao, J.; Major, D. T. *ACS Catal.* **2017**, *7*, 812-818.
18. Major, D. T. *ACS Catal.* **2017**, *7* (8), 5461-5465.
19. Weitman, M.; Major, D. T. *J. Am. Chem. Soc.* **2010**, *132* (18), 6349-6360.
20. Ansbacher, T.; Freud, Y.; Major, D. T. *Biochemistry* **2018**, *57* (26), 3773-3779.
21. Hong, Y. J.; Tantillo, D. J. *Journal of the American Chemical Society* **2009**, *131* (23), 7999-8015.
22. Hong, Y. J.; Tantillo, D. J. *J. Am. Chem. Soc.* **2014**, *136* (6), 2450-63.
23. Raz, K.; Levi, S.; Gupta, P. K.; Major, D. T. *Curr. Opin. Biotechnol.* **2020**, *65*, 248-258.
24. Major, D. T.; Freud, Y.; Weitman, M. *Curr. Opin. Chem. Biol.* **2014**, *21*, 25-33.
25. Raz, K.; Driller, R.; Dimos, N.; Ringel, M.; Brück, T.; Loll, B.; Major, D. T. *J. Am. Chem. Soc.* **2020**, *142* (51), 21562-21574.
26. Hong, Y. J.; Tantillo, D. J. *J. Am. Chem. Soc.* **2014**, *136* (6), 2450-2463.
27. Das, S.; Shimshi, M.; Raz, K.; Nitoker Eliaz, N.; Mhashal, A. R.; Ansbacher, T.; Major, D. T. *J. Chem. Theory Comput.* **2019**, *15* (9), 5116-5134.
28. Brooks, B. R.; Bruccoleri, R. E.; Olafson, B. D.; States, D. J.; Swaminathan, S.; Karplus, M. *J. Comput. Chem.* **1983**, *4* (2), 187-217.
29. Vanommeslaeghe, K.; Hatcher, E.; Acharya, C.; Kundu, S.; Zhong, S.; Shim, J.; Darian, E.; Guvench, O.; Lopes, P.; Vorobyov, I.; Mackerell, A. D., Jr. *J. Comput. Chem.* **2010**, *31* (4), 671-690.
30. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Petersson, G.

A.; Nakatsuji, H.; Li, X.; Caricato, M.; Marenich, A. V.; Bloino, J.; Janesko, B. G.; Gomperts, R.; Mennucci, B.; Hratchian, H. P.; Ortiz, J. V.; Izmaylov, A. F.; Sonnenberg, J. L.; Williams; Ding, F.; Lipparini, F.; Egidi, F.; Goings, J.; Peng, B.; Petrone, A.; Henderson, T.; Ranasinghe, D.; Zakrzewski, V. G.; Gao, J.; Rega, N.; Zheng, G.; Liang, W.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Throssell, K.; Montgomery Jr., J. A.; Peralta, J. E.; Ogliaro, F.; Bearpark, M. J.; Heyd, J. J.; Brothers, E. N.; Kudin, K. N.; Staroverov, V. N.; Keith, T. A.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A. P.; Burant, J. C.; Iyengar, S. S.; Tomasi, J.; Cossi, M.; Millam, J. M.; Klene, M.; Adamo, C.; Cammi, R.; Ochterski, J. W.; Martin, R. L.; Morokuma, K.; Farkas, O.; Foresman, J. B.; Fox, D. J. *Gaussian 16 Rev. A.03*, Wallingford, CT, 2016.

31. Zhao, Y.; Truhlar, D. G. *Theor. Chem. Acc.* **2008**, *120* (1-3), 215-241.
32. Nagy, B.; Jensen, F. Basis Sets in Quantum Chemistry. In *Rev. Comput. Chem.*; 2017; pp 93-149.
33. Das, S.; Dixit, M.; Major, D. T. *Biorg. Med. Chem.* **2016**, *24* (20), 4867-4870.
34. Dixit, M.; Weitman, M.; Gao, J. L.; Major, D. T. *ACS Catal.* **2017**, *7* (1), 812-818.
35. Pahima, E.; Zhang, Q.; Tiefenbacher, K.; Major, D. T. *J. Am. Chem. Soc.* **2019**, *141* (15), 6234-6246.
36. Levi, S.; Zhang, Q.; Major, D. T. *ACS Catal.* **2020**, *10* (12), 6843-6853.
37. Zev, S.; Gupta, P. K.; Pahima, E.; Major, D. T. *J. Chem. Theory Comput.* **2022**, *18* (1), 167-178.