Synthesis and Search for Potential Biosynthetic Precursors for Simple Polycyclic Ethers

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Abstract: The biosynthetic processes in which polycyclic ethers are synthesized have long been of interest in the natural product community. Herein, two potential biosynthetic precursors to 6-membered rings were designed, synthesized, and subsequently incubated with extracts from the dinoflagellate Protoceratium reticulatum, in hopes of discovering a pathway to 6-membered rings. However, though no evidence of the desired polycyclic ring formation was observed, progress has been made towards understanding the interactions between the extract and the biosynthetic precursors tested to date.

Introduction
The biological activity, complex structure, and intriguing biosynthetic mechanisms of natural compounds have been of great fascination to biologists and chemists alike. Furthermore, the question of how such complex compounds are assembled by the dinoflagellates that produce them has been an active area of investigation. A hypothesis proposed by Nakanishi over two decades ago attempted to address this question by suggesting that enzymes can polyepoxidize trans-polyene precursors and then further catalyze a cascade-type endo-tet cyclization to yield complex polycyclic ether backbones as shown in figure 1. In hopes of confirming the aforementioned hypothesis, previous work in our group involved the synthesis and incubation of 4 different potential biosynthetic precursors as depicted in figure 2. The study involved incubation of the precursors with extracts from the dinoflagellate Protoceratium reticulatum, an organism known for its production of complex polycyclic ether structures. Though our studies suggest no evidence supporting the existence of such an enzyme, interesting results, most notably the detection of a 16 mass adduct have been attained. Therefore, in an effort to expand on the group’s previous work, two new potential biosynthetic precursors were designed, synthesized, and subject to incubation with Protoceratium reticulatum extract (Figure 3). In doing so, we were able to grasp a better understanding of how
certain biosynthetic precursors react when incubated with *Protoceratium reticulatum* extract.

Figure 3
Proposed biosynthetic precursors

![Proposed biosynthetic precursors](image)

**Synthetic Methods**

The above biosynthetic precursors were synthesized as derivatives of the previously studied precursors. With some intermediate compounds already at hand, precursor 1 was successfully synthesized in 5 steps from the terminal alcohol derivative as depicted in Figure 4. Conversion from a terminal alcohol to an amide is a relatively straightforward process, but the last deprotection step proved difficult. In the first attempt, optimal conditions for purification by silica gel column chromatography were not used and as a result, the epoxide precursor 1 spontaneously cyclized into the undesired 5-membered polycyclic ring. The cyclization was confirmed by both $^1$H NMR and 2D cosy NMR analysis. The second attempt at deprotection using more optimal purification conditions (see Detailed Experimental Section) proved to be a success and the desired precursor 1 was isolated and characterized by ESI mass spec. and $^1$H NMR. The synthesis of precursor 2 involved slightly more complex reactions, but its synthesis was eventually achieved from building block compounds already at hand (Figure 5). The initial step involved a Suzuki-Miyaura coupling reaction between a bromide and iodide fragment. Conventionally, Suzuki-Miyaura coupling reactions are preformed with a halogen and boronate starting material, but the procedure used here required that the boronate be formed *in situ* before adding in the second fragment. The following selective deprotection of a terminal triethylsilyl (TES) group went smoothly, however, some obstacles were encountered in the subsequent epoxidation reaction. The first attempt resulted in almost no formation of the desired epoxide as indicated by $^1$H NMR analysis. As a result, the recovered starting material was resubjected to epoxidation conditions. This time, thin-layer chromatography (TLC) analysis indicated formation of the desired product and the product was eventually isolated and characterized by $^1$H NMR. The final step in the synthesis of precursor 2 involved a fairly difficult deprotection step, mainly because of the compound’s tendency to spontaneously cyclize into the 5-membered ring. This tendency to form the smaller
heterocycle likely arises from a spiro transition state. The desired precursor 2, however, was eventually isolated and characterized by $^1$H NMR.

Figure 5
Synthetic scheme for precursor 2

![Synthetic scheme for precursor 2]

**Incubation Procedures**

With both precursors 1 and 2 at hand, the design of procedures for incubation experiments with *Protoceratium reticulatum* extracts began (Figure 6). Under the hypothesis that dinoflagellates use enzymes to catalyze the formation 6-membered polycyclic ethers, extracts from *Protoceratium reticulatum* were cultured and prepared for incubation using a specially formulated buffer used in previous incubation experiments. The shells of the dinoflagellate cells were also ultrasonicated to ensure complete release of its contents before the precursor substrates were added. Controls consisting of only the extract and only the substrate for both precursors 1 and 2 were prepared. Both experimental and control trials were then incubated for 3 hours at 37 °C before the addition of methanol. The samples were then centrifuged twice at 4 °C for 30 minutes before the supernatant was collected for further analysis.

Figure 6
Scheme of incubation experiments

<table>
<thead>
<tr>
<th>Dinoflagellate <em>Protoceratium reticulatum</em> culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>(200 mL, 1.6 x 10⁴ cells/mL)</td>
</tr>
<tr>
<td>1600 x g, 4 °C, 4 minutes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chilled 50 mM HEPES buffer</td>
</tr>
<tr>
<td>(100 mM NaCl, pH 7.4, 20 mL)</td>
</tr>
<tr>
<td>1600 x g, 4 °C, 4 minutes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Suspension (extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation buffer* (2 mL)</td>
</tr>
<tr>
<td>Homogenized by ultrasonication</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate (ca. 30 nmol)</td>
</tr>
<tr>
<td>37°C for 3 hours</td>
</tr>
<tr>
<td>MeOH (0.5 mL)</td>
</tr>
<tr>
<td>19000 x g, 4 °C, 30 minutes</td>
</tr>
</tbody>
</table>

**Analysis and Results**

The collected supernatant was then prepared for injection into a Liquid Chromatograph-Mass Spectrometer (LC-MS). The concept behind such an instrument lies in the inherent molecular mass and polarity of the compounds subject to testing. Upon injection, the compounds are run through a column that that over time separates them based on difference in polarity. Because different compounds elute at different times, we are able to detect whether or not a new compound is formed post-incubation by comparing it to a standard of the initial
starting material. The mass spectrometer also provides valuable information because in addition to knowing the retention time of a given compound, the molecular weight of the corresponding compound is also given. Upon injection of precursors 1 and 2, there was unfortunately no indication of the desired 6-membered ring. There was mostly a large presence of what seemed to be the undesired 5-membered ring when compared to results of previous experiments. Its formation was confirmed upon verifying matching retention times, suggesting that the existence of a pathway to 6-membered polycyclic ring formation with the tested precursors is slim (see Detailed Experimental Section for figures). With this being said, however, an interesting result was obtained. In past work in the group, an aldehyde analogue of precursor 2 was incubated with the same Protoceratium reticulatum extracts and though there was no evidence of the desired 6-membered ring, there was presence of an unidentified 16 mass adduct. Subsequent analysis suggested that the aldehyde precursor underwent oxidation to form either the carboxylic acid or underwent addition of a second alcohol elsewhere in the molecule. Therefore, precursors 1 and 2 were also designed in order to learn more about potential oxidation pathways that occur during the incubation process. In precursor 1, the terminal alcohol was protected with an MPM group with the thought of intentionally blocking any potential for oxidation at the terminus and so if a +16 mass adduct were to be detected, then it could be concluded that oxidation does not occur at the terminus. Precursor 2 was also designed in order to give a better understanding as to where oxidation takes place. One terminus was left completely unprotected with a primary alcohol to allow for any potential oxidation. According to previous results from the aldehyde analogue, it was hypothesized that oxidation of the primary alcohol should take place. However, the results obtained indicated otherwise. Therefore, it was very surprising when it was determined that the -2, +14, or +16 mass adducts (all potential adducts if the compound were to be oxidized) were not detected after incubation (Figure 7). These results raise new questions, mainly; do the enzymes in Protoceratium reticulatum selectively oxidize only aldehydes and not alcohols? The results of this study seem to suggest that is the case.

**Table of results**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>5-exo</th>
<th>6-endo</th>
<th>-2, +14, +16 mass adducts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor 1</td>
<td>100</td>
<td>0</td>
<td>Not detected</td>
</tr>
<tr>
<td>Precursor 2</td>
<td>100</td>
<td>0</td>
<td>Not detected</td>
</tr>
</tbody>
</table>

**Conclusions and Future Work**

In summary, the aim of this work was to expand on previous studies regarding the search for potential biosynthetic precursors to 6-membered polycyclic ethers. In short, the design and synthesis of precursors 1 and 2 and subsequent incubation experiments were successfully achieved. Though there was no evidence of enzymes catalyzing a pathway to 6-membered rings, interesting results were obtained regarding oxidation processes, mainly that Protoceratium reticulatum extracts seem to only oxidize aldehydes and not alcohols. Future work to confirm this phenomenon include intentionally oxidizing precursor 2 to an aldehyde and running it together with precursor 2. In this way, we can verify this phenomenon of selective oxidation. As a whole, there are still many questions to be answered in this field of research. Moreover, since this research is still in its infancy, different biosynthetic analogues, different dinoflagellate extracts, and different incubation conditions must be
carefully varied and tested in order to increase our understanding of nature’s pathway to 6-membered polycyclic ethers.

References
4. R. V. Snyder et al., Phytochemistry 2005, 66, 1767

Acknowledgements
Though 5 weeks in the lab is an incredibly short amount of time to make any significant advances in any field of research, I feel that I have made some great progress and have many people to thank for that. I must first and foremost thank Professor Tachibana for giving me the opportunity to experience something I could only dream about just a few months ago. I have had a strong passion to study chemistry and Japanese during my early college years and to have finally had the opportunity to do both simultaneously is something I will be forever grateful for. To be able to communicate about topics in chemistry in Japanese has really been a tremendous learning experience for me. I must also thank Professor Satake, who was always readily available when it came to discussions. His kindness and his support throughout the weeks has been immensely motivating and I feel extremely lucky to have had a person like him to have discussions with. I must also thank Atsushi Mizukami and Yuki Takimoto for all their help when in came to setting up certain experiments and using instruments. I have realized that research environments can differ substantially from place to place, but they really helped me adapt to research life in their lab. I must also thank everyone else in the lab who have collectively helped me out with all the little things that come with starting research in a new place. I can confidently state that I have made friendships in this lab that will last a lifetime. I must also thank Sachiko Soeda for her support and hard work in making this program a great success. Her hard work is very admirable and the research achieved in such a short amount of time by the students is proof of that. I must also thank Friends of Todai Inc. especially Professor Hisashi Kobayashi and Professor Masaaki Yamada for providing the funding for me to comfortably stay and work in Japan. I am extremely happy to have been presented with what is truly a once in a lifetime opportunity. I also want to thank my advisor, Professor Moore and the former post-doc I worked with, who can now deservedly be called Professor Gross, for giving me the opportunity to grow so much during my undergraduate research career. I owe a huge part of my interest in chemical research to them. Lastly, I must thank my family for their unconditional support. Without them, I would feel nowhere near as accomplished as I feel now. I will always be appreciative of all that they have done and will continue to do for me.
**Detailed Experimental Section:**

Iodination:
Using a 20 mL roundbottomed flask, 43.5 mg (0.10 mmol) of compound 1, 26.2 mg (0.38 mmol) of imidazole, 50.2 mg (0.19 mmol) of PPh$_3$, and 63.4 mg (0.25 mmol) of I$_2$ were dissolved in approximately 1.5 mL of THF. The reaction was wrapped in aluminum foil and left to stir at room temperature under argon atmosphere for one hour. Analysis of the crude reaction mixture by TLC using 20% EtoAc/Hex solution indicated the presence of the desired product. The reaction was then quenched with Na$_2$SO$_4$ (aq.), extracted three times with Et$_2$O, washed once with brine, and dried over MgSO$_4$ before being filtered by vacuum filtration. The solvent was removed *in vacuo* and the crude product was purified using silica gel column chromatography loading with 3% EtoAc/Hex as the starting eluent before switching to 7% EtoAc/Hex. The solvent was removed *in vacuo* to yield 42.9 mg (79%) of the target compound 2 as a yellowish liquid. The product was then redissolved in benzene before being stored in the freezer.  

Azidation:
Using a 20 mL roundbottomed flask, 42.9 mg (0.08 mmol) of compound 2 was dissolved in approximately 1 mL DMF and stirred on ice before adding 54.7 mg (0.84 mmol) of NaN$_3$. The reaction was wrapped in aluminum foil and left to stir at room temperature under argon atmosphere for two hours before being quenched with water. The reaction mixture was then extracted with Et$_2$O three times, washed once with brine, and dried over Na$_2$SO$_4$ before being filtered by vacuum filtration. The solvent was removed *in vacuo* to yield 33.3 mg (92%) of the target compound 3 as a yellowish liquid. The product was then redissolved in benzene before being stored in the freezer. Note: since analysis by TLC of the crude reaction mixture was inconclusive as to whether the desired reaction took place, a crude mass spec. was taken, verifying the formation of the desired product.
Azide reduction:
Day 1: Using a 20 mL roundbottomed flask, 33.3 mg (0.074 mmol) of compound 3 was dissolved in 1 mL of THF. The solution was left to stir on ice before 27.0 mg (0.10 mmol) of PPh₃ was added. The ice bath was removed after 10 minutes and the reaction was wrapped in aluminum foil and left to stir at room temperature under argon atmosphere overnight.

Day 2: Approximately 0.15 mL of water was added drop wise by syringe directly into the reaction mixture. The reaction flask was then fitted with a reflux condenser, placed into an oil bath, and left to stir at 40 °C overnight.

Day 3: The reaction was then quenched with NaHCO₃ (aq.) solution before being extracted with Et₂O three times, washed with brine once, and dried over Na₂SO₄. The reaction was then filtered by vacuum filtration and the excess solvent was removed in vacuo leaving behind the crude state of compound 4 in liquid form.

Amine acylation:
Day 1: Using a 50 mL roundbottomed flask, 0.741 mmol of compound 4 was dissolved 2 mL of pyridine before 1 ml of Ac₂O was added. The reaction flask was capped and left to stir for 6 hours before a TLC was taken. Analysis by TLC using an anisaldehyde stain suggested that reaction did not go to completion. The reaction was then left to stir overnight.

Day 2: Analysis by TLC indicated almost no change in the product distribution so the reaction was stopped and diluted with toluene. The toluene was evaporated and the process was repeated once more to help ensure the removal of pyridine and Ac₂O. The crude material was then purified by silica gel column chromatography eluting with 80% EtoAc/Hex to yield two different fractions of different purity of the desired compound 5, with the first fraction containing 8.2 mg and the second fraction containing 19.3 mg (79%).
TES deprotection:
Day 1: Using a 20 mL roundbottomed flask, 8.2 mg (0.018 mmol) of compound 5 was dissolved in THF before adding 0.053 mL (0.053 mmol) of TBAF at 0 °C. The reaction was then left to stir overnight at 0 °C under argon atmosphere.

Day 2: The reaction was then raised from the cold bath and placed directly onto a silica gel column loaded with 99:1 EtoAc/Et$_3$N. However, the product did not seem to elute in this solvent system. The polarity of the eluent was then increased by switching to a 95:4:1 EtoAc/MeOH/Et$_3$N solvent system. A product was finally attained in 4.7 mg, but NMR analysis indicated a loss of the epoxy group, suggesting that the compound went through the undesired spontaneous cyclization to the 5-membered ring.

TES deprotection:
Day 1: Because of the undesired result in the previous reaction, the deprotection was reattempted using some of the remaining compound 5. Day 1: Using a 20 mL roundbottomed flask, 11.1 mg (0.024 mmol) of compound 5 was dissolved in THF before adding 0.05 mL (0.05 mmol) of TBAF at 0 °C. The reaction was then left to stir overnight at 0 °C under argon atmosphere.

Day 2: The reaction was then raised from the cold bath and placed directly onto a silica gel column this time using 93:5:2 EtoAc/MeOH/Et$_3$N as the starting eluent to yield 6.6 mg (78%) of the desired compound 6. (Note: triphenylphosphine oxide was identified in the NMR, but should have no effect on the subsequent incubation experiments.)
Suzuki-Miyaura coupling:
Day 1: Using a 50 mL two-neck roundbottomed flask, 102 mg (0.20 mmol) of the iodide fragment was dissolved in 2.0 mL of Et₂O under argon atmosphere. The mixture was then stirred at -78 °C before 0.4 mL of B-OMe 9-BBN was added. After 10 minutes of stirring, 0.5 mL of t-BuLi was added and after another 10 minutes, 1.7 mL of THF was added. The reaction was then raised from the cold bath for 10 minutes before 0.7 mL of 3M Cs₂CO₃ (aq.) was added to the reaction mixture. At this time, 99.1 mg (0.23 mmol) of the bromide fragment dissolved in 1.5 mL of DMF was added via cannula before 13.0 mg of Pd(PPh₃)₄ was added. The reaction was then fitted with a reflux condenser and left to stir overnight at 45 °C under argon atmosphere.

Day 2: The reaction mixture was quenched with NH₄Cl (aq.) and Et₂O before being extracted twice with Et₂O and washed with brine. The solution was then dried over Na₂SO₄, filtered, and purified by silica gel column chromatography starting with 1% EtoAc/Hex as an eluent and moving on to 4% EtoAc/Hex to yield 81.6 mg (56%) of the desired compound 7.

Primary TES deprotection:
Using a 30 mL roundbottomed flask, 81.5 mg (0.11 mmol) of compound 7 was dissolved in THF and left to stir on ice before adding 0.5 mL (0.02 mmol) of 0.05 M CSA(+) solution drop wise. The reaction was monitored by TLC and determined to have reach completion after 10 minutes of stirring. The reaction was stopped, quenched with NaHCO₃ (aq.), extracted twice with Et₂O, washed with brine, dried over Na₂SO₄, and filtered. The crude mixture was then purified by silica gel column chromatography eluting with 10% EtoAc/Hex before switching to 20% EtoAc to yield 49.2 mg (73%) of the desired compound 8.
Sharpless epoxidation:

Day 1: Using a two-neck 20 mL roundbottomed flask, 49.2 mg (0.08 mmol) of compound 8 was dissolved in ~1.5 mL of DCM before adding 56 mg of MS4A. The reaction was then lowered into a -30 °C bath before 0.004 mL (.024 mmol) of (+) –DET and 0.006 mL (0.021 mmol) of Ti(OiPr)₄ was added. After stirring for 25 minutes, 0.073 mL (0.395) of TBHP was added and the reaction was left to stir overnight under argon atmosphere.

Day 2: The reaction was stopped and filtered through celite to remove the MS4A before being quenched with Na₂SO₃ (aq.), extracted with EtoAc 3 times, washed with brine, and dried over NaSO₄. The reaction was then filtered and purified by silica gel column chromatography eluting with 12% EtoAc/Hex. Unfortunately, upon isolation of what was believed to be the desired product, NMR analysis indicated the recovery of what was mostly starting material (31 mg).

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**TBDPSO & TES deprotection:**
Day 1: In a 10 mL roundbottomed flask, 9.2 mg (0.014 mmol) of compound 9 was dissolved in 0.5 mL of THF and lowered into a 0 °C bath before adding 0.04 mL of (0.04 mmol) of TBAF. The reaction was left to stir at 0 °C overnight under argon atmosphere.

Day 2: The reaction was stopped and directly loaded onto a silica gel column eluting with 5:2:93 MeOH/Et₃N/EtoAc to yield 1.6 mg (46%) of the desired compound 10 in decent purity.
**LC-MS Figures**

**SIC: m/z 374**

![LC-MS Figures](image)

**Protoceratium reticulatum extract**
- Nothing found in control experiment

**Starting precursor 1**
- Control showing precursor 1 as majority

**Incubation without extract**
- Shows spontaneous conversion to 5-exo over time

**Incubation with extract**
- Shows conversion to 5-exo upon incubation with extract

x-axis: retention time (min.)  y-axis: intensity
SIC: m/z 307

**2** Absence of epoxide peak indicates complete conversion

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**Proteros motocum reticulatum extract**
- Nothing found in control experiment

**Starting precursor 2**
- Control showing presence of precursor 2

**Incubation without extract**
- Shows spontaneous conversion to 5-exo over time

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- Shows conversion to 5-exo upon incubation with extract

x-axis: retention time (min.) y-axis: intensity
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Sharpless epoxidation:
Day 1: It was decided that the recovered starting material would be resubjected to epoxidation conditions. In a two-neck 20 mL roundbottomed flask, 31 mg (0.050 mmol) of compound 8 was dissolved in ~0.5 mL of DCM before adding 40 mg of MS4A. The reaction was then lowered into a -20 °C bath before 0.004 mL (.024 mmol) of (+)–DET and 0.006 mL (0.021 mmol) of Ti(OiPr)₄ was added. After stirring for 25 minutes, 0.073 mL (0.395) of TBHP was added and the reaction was left to stir overnight under argon atmosphere.

Day 2: The reaction was stopped and filtered through celite to remove the MS4A before being quenched with Na₂SO₃ (aq.), extracted with EtoAc 3 times, washed with brine, and dried over NaSO₄. The reaction was then filtered and purified by silica gel column chromatography eluting with 12% EtoAc/Hex. This time 14 mg (44%) of a different spot was isolated. NMR analysis confirmed it to be the desired compound 9.
TBDPSO & TES deprotection:
Day 1: In a 10 mL roundbottomed flask, 9.2 mg (0.014 mmol) of compound 9 was dissolved in
0.5 mL of THF and lowered into a 0 °C bath before adding 0.04 mL of (0.04 mmol) of TBAF.
The reaction was left to stir at 0 °C overnight under argon atmosphere.

Day 2: The reaction was stopped and directly loaded onto a silica gel column eluting with 5:2:93
MeOH/Et₃N/EtoAc to yield 1.6 mg (40%) of the desired compound 10 in decent purity.
LC-MS Figures

SIC: m/z 374

**Protoceratium reticulatum extract**
- Nothing found in control experiment

**Starting precursor 1**
- Control showing precursor 1 as majority

**Incubation without extract**
- Shows spontaneous conversion to 5-exo over time

**Incubation with extract**
- Shows conversion to 5-exo upon incubation with extract

x-axis: retention time (min.) y-axis: intensity
SIC: m/z 307

Protoporphyra reticulatum extract
- Nothing found in control experiment

Starting precursor 2
- Control showing presence of precursor 2

Incubation without extract
- Shows spontaneous conversion to 5-exo over time

Incubation with extract
- Shows conversion to 5-exo upon incubation with extract

x-axis: retention time (min.) y-axis: intensity