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ABSTRACT

Two natural products, spiroxin A (isolated from a marine-derived fungal strain LL-37H248) and angelmarin (isolated from the Japanese medicinal plant *Angelica pubescens*), have exhibited promising antitumor and antibacterial activities. Recent efforts in our laboratory have identified a precursor to spiroxin A, (S)-34, and a coumarin-based angelmarin derivative 50 as lead pharmacophores against human pancreatic adenocarcinoma cell line PANC-1. Both compounds induced PANC-1 cell death exclusively under nutrient-deprived conditions. The research presented herein includes an ongoing structure-activity relationship study based on (S)-34 and an investigation of the correlation between cell culture medium components and the cytotoxicity of 50. For the former, current results showed no substantial effects of the absolute stereochemistry or the protecting group center of (S)-34 on its preferential cytotoxicity, suggesting that hydrophobicity may play an important role in the observed antitumor activity. In addition, the activity of compound 50 was enhanced in the absence of a combination of serum and glucose, which indicates a possible mechanism of action of 50 related to the inhibition of autophagy in PANC-1 cells.
INTRODUCTION

Natural Products in Medicinal Chemistry

For millennia, nature has provided mankind with the necessities to survive and thrive, including our largest medicine cabinet. Living organisms have been a source of medicinal products used for a myriad of diseases. The earliest record of using natural sources to treat illnesses can be dated back to around 2600 BC, documenting approximately 1000 plant species in Mesopotamia.\(^1\) Until the advent of modern pharmaceuticals, the treatment of diseases largely relied on plant concoctions and plant extracts. For example, willow bark extract was used to reduce fever and relieve pain in 400 BC. However, the active ingredient of willow bark was not identified until the 19\(^{th}\) century, when salicylic acid (1, Figure 1), an early example of a physiologically active natural product, was discovered to be responsible for the medicinal properties of the extract. Even in modern times, natural products, which are isolated from living organisms, have played an essential role in medicinal chemistry. In the last 30 years, 64\% of new drugs originated from natural products. For anticancer treatment in particular, about 80\% of therapies were natural products per se or resembled natural products, highlighting their importance in contemporary drug discovery and development.\(^2\)

\[
\begin{align*}
\text{Salicylic Acid (1)} & \quad \text{Aspirin (2)} \\
\end{align*}
\]

**Figure 1.** Structures of salicylic acid and aspirin

However, reliance on solely natural sources poses problems for large-scale drug production due to either the scarcity of some living systems or the low isolation yields of natural
products from their natural sources. Thanks to the development of modern synthetic techniques, organic chemists are able to synthesize natural products in their laboratories. In fact, syntheses of natural products often yield analogs that offer improved medicinal properties than their natural precursors. For example, salicylic acid was optimized into aspirin (2, Figure 1), a common and effective pain reliever. Another example is the discovery of paclitaxel (Taxol®) from the bark of Pacific yew tree Taxus brevifolia. Since the initial discovery, structural derivatives such as docetaxel, Abraxane® and cabazitaxel have been developed with improved pharmacological properties.

Among numerous natural products that provide inspirations for drug discovery and development, spiroxin A (3, Figure 3) and angelmarin (40, Figure 7) have been the focus of our research, due to the diverse biological activities of both compounds, such as antitumor and antibacterial activities. Moreover, recent efforts in our laboratory have demonstrated that structural analogs of both natural products are cytotoxic to pancreatic cancer cells.3,4,5

**Pancreatic Cancer**

Pancreatic adenocarcinoma is one of the most lethal human cancers. It is the fourth leading cause of cancer-related deaths in the United States. The American Cancer Society has estimated that there will be 48,960 new cases and 40,560 deaths in 2015. Among all cancers, the 5-year survival rate of pancreatic cancer has remained the only cancer survival rate that is still below 10%.6 Pancreatic cancer is known for its early metastasis and aggressive invasion of surrounding tissues. Due to the lack of early detection methods and the absence of easily-recognizable symptoms, patients are usually diagnosed at late tumor stages without surgical therapy options.7 Between 2004 and 2010, over 80% of pancreatic cancer patients were diagnosed at the regional or distant stage, where the tumor had spread to a different part of the
body or metastasized. Even for patients who were diagnosed at an early stage (or the localized stage), the 5-year relative survival rate was less than 30% (Figure 2). Only 15% – 20% of all patients receive radiotherapy, among whom only 3% – 4% can achieve long-term cure. In addition, pancreatic cancer is highly resistant to conventional chemotherapies such as gemcitabine, Taxol®, 5-fluorouracil and cisplatin. Unfortunately, no effective clinical treatment is currently available to guarantee the complete eradication of pancreatic cancer.

**Figure 2.** Percent of cases and 5-year relative survival by stage at diagnosis for pancreatic cancer. Figure taken from [http://seer.cancer.gov/statfacts/html/pancreas.html](http://seer.cancer.gov/statfacts/html/pancreas.html).

Throughout carcinogenesis, tumor cells are exposed to hypoxia and poor nutrient conditions, because of insufficient vascularization. Metabolic stress triggers angiogenesis – the creation of new blood vessels, which eventually results in tumor progression. Anti-angiogenesis to prevent tumor growth used to be considered the most promising anticancer therapy. However, the role of angiogenesis in pancreatic cancer remains controversial, since pancreatic cancer is mostly hypovascular. Recently, angiogenesis inhibitors were reported ineffective in treating patients with pancreatic cancer. In 2000, Izuishi and coworkers proposed that pancreatic cancer cells might have acquired tolerance to nutrient deprivation throughout tumor
progression. In their study, pancreatic cancer cells and normal human fibroblasts were cultured in nutrient-deprived media lacking glucose, amino acids and serum. Four pancreatic cancer cell lines, including PANC-1, AsPC-1, BxPC-1, and KP-3, survived for 48 h under nutrient deficiency, whereas normal fibroblasts died within 24 h under the same conditions. Since normal tissues seldom encounter nutrient deprivation, the austerity of pancreatic cancer cells has become a novel biochemical target for cancer therapies. Since then, significant effort has been carried out to search for anti-austerity agents.

**Discovery and Synthetic History of the Spiroxins**

In 1999, a marine-derived fungal strain LL-37H248 was collected from a soft orange coral in Vancouver Island, Canada. Bioassay-directed purification of the marine fungus yielded spiroxins A-E (3-7, Figure 3). As members of the bisnaphthospiroketal family, the spiroxins display intriguing structural features – two partially saturated naphthalene rings are connected via a spiroketal and a carbon-carbon (C-C) linkage. The saturated portion of each naphthalene unit, combined with an epoxide, creates a unique octacyclic ring system. Aside from the intricate ring systems, the spiroxins contain six stereogenic centers in a 20-carbon framework. In fact, the absolute stereochemistry of spiroxins A-E (3-7, Figure 3) was not confirmed until 2001.
Figure 3. Structures of spiroxins A-E.

The complex structures of the spiroxins have made them challenging synthetic targets. To date, only one total synthesis has been published for all five spiroxins. In 2003, Miyashita and coworkers reported the total synthesis of $\pm$-spiroxin C via a Suzuki-Miyaura cross-coupling key step activated by tetra-$n$-butylammonium fluoride (TBAF). The simplified synthetic scheme is shown below (Scheme 1). The two naphthalene units 12 and 13 were prepared individually from known compounds (8-11). The key step of the synthesis (blue, Scheme 1) was to bring together the naphthalene units 12 and 13 to create a binaphthyl derivative 14 with two oxygen substituents at both peri-positions, which was achieved via a Pd(0)-catalyzed cross-coupling with the addition of TBAF. The binaphthyl derivative 14 was not only a key intermediate in the synthetic scheme, but it also served as a chiral auxiliary to direct the desired orientation of the basic bisnaphthospiroketal skeleton 17. Introduction of epoxides from a sterically less hindered side of 17 completed the synthesis of spiroxin C (5) with an overall yield of 1.3\%.16
Scheme 1. Simplified total synthesis of (±)-spiroxin C. The key Suzuki-Miyaura cross-coupling step is highlighted in blue.\(^\text{16}\)

However, since the total synthesis of (±)-spiroxin C, only a few attempts have been reported en route to resolving the synthetic challenges posed by the complex structures of the spiroxins, including the work by Nabatame and coworkers. A hexacyclic spiroxin framework 27 was constructed in a ten-step synthesis (Scheme 2), involving key steps such as CuCl-mediated oxidative biaryl coupling from 20 to 21 (blue, Scheme 2), oxidative desymmetrization from 22 to 23 (green, Scheme 2), site-selective acetoxylation from 24 to 25 (pink, Scheme 2), and oxidative cyclization of 26 to the hexacycle 27 (purple, Scheme 2).\(^\text{17}\) Needless to say, the highly oxygenated and strained fused-ring system that all spiroxins share remains a challenging synthetic mission.
Scheme 2. A ten-step synthesis of a hexacyclic spiroxin framework 27. Key steps are colored in blue, green, pink and purple.\textsuperscript{17}

Medicinal Significance of Spiroxin A

Among all five spiroxins, only spiroxin A has been thoroughly investigated and found to possess biological activities. Spiroxin A was reported to show antitumor activity in nude mice against ovarian carcinoma (59\% inhibition after 21 days, 1 mg/kg/dose), and exhibit cytotoxicity against a panel of 25 diverse cell lines with a mean IC\textsubscript{50} value of 0.09 \( \mu \)g/mL. In evaluation of the mechanism of action, it was observed that spiroxin A caused a concentration-dependent nicking of pBR322 DNA, indicating that the cytotoxicity of spiroxin A might be partly exerted through a single-stranded DNA cleavage.\textsuperscript{14} It was also hypothesized that the spiroxins might
have chemically-similar behaviors as quinone epoxides (see Figure 4 for examples of quinone epoxides), which can induce cell death via a variety of mechanisms such as DNA modification, alkylation or oxidation of essential protein thiol groups. Liquid chromatography and mass spectrometry (LC-MS) experiments in this study have demonstrated that spiroxin A formed conjugates when reacting with 2-mercaptoethanol 30 or dithiothreitol 31 (Figure 5). Thus, the exact mechanism of the cytotoxicity of spiroxin A might be more complicated and require a more thorough investigation. Spiroxin A also showed antibacterial activity against Gram-positive bacteria. To date, the biological activities of spiroxins B-E have not been reported.

![Figure 4. Examples of quinone epoxides.](image)

![Figure 5. Structures of 2-mercaptoethanol 30 and dithiothreitol 31.](image)

**Synthesis of the Core Structure of Spiroxin A**

Motivated by the potential therapeutic effects of spiroxin A, our laboratory sought to develop synthetic techniques that could achieve the enantioselective total synthesis of spiroxin A. In 2011, our laboratory reported a novel catalytic asymmetric approach (Scheme 3) to the core structure of spiroxin A, a tertiary naphthoquinol (S)-39, via a tandem oxidation/ring-opening sequence. (S)-33 was obtained by a stereoselective ring-opening of the cyclic ether 32. The
protection of (S)-33 was accomplished using tert-butylidiphenylsilyl chloride (TBDPSCI) and imidazole. Following the generation of a bromide intermediate (S)-35, the vinylarene (S)-36 was obtained via a Stille cross-coupling using trimethyl(phenyl)stannane. Deprotection of (S)-36 with TBAF produced the alcohol (S)-37. A directed m-CPBA epoxidation of (S)-37 generated the epoxyalcohol (S)-38. An oxidation/ring-opening of (S)-38 under Swern conditions afforded the desired chiral tertiary naphthoquinol (S)-39 (Scheme 3). Compared to the existing methods of preparing chiral tertiary naphthoquinols, the synthesis proposed by our group was less synthetically demanding in that there was no need for a chiral auxiliary, and thus it will be more flexible and convenient to construct other substituted tertiary chiral quinols. This approach also represented the first catalytic asymmetric entry to chiral tertiary naphthoquinols.\(^\text{19}\)

**Scheme 3.** Synthesis of enantioenriched tertiary naphthoquinol (S)-39.

Inspired by the antitumor activity of spiroxin A and with ready access to the synthetic intermediates, we have decided to investigate which structural components of spiroxin A could be responsible for its cytotoxicity, leading to an ongoing structure-activity relationship (SAR) study.
Structure-Activity Relationship (SAR) Study

As a major paradigm in pharmacology, SAR studies examine the relationship between the molecular structures and all types of biological activities of chemical compounds. The fundamental assumption behind SAR studies is that the structure of the compound implicitly determines its biological properties. While developing SAR studies, scientists hope to not only pinpoint the structural components relevant to the activity, but also utilize the relationship to design compounds with enhanced biological properties. Residing at the intersection of chemistry, biology and statistics, SAR studies often require a multidisciplinary understanding of the project (Figure 6). A well-established SAR model generally consists of elements such as chemicals of interest, biological activities obtained via experimental/computational techniques, and chemical descriptors or attributes that may help rationalize the connection between structure and activity. Last but not least, researchers ought to exploit appropriate methods of analysis to reach plausible conclusions. Applications of SAR studies can also be found in chemical industries, environmental sciences and toxicology.\textsuperscript{20}

\textbf{Figure 6.} SAR at the intersection of chemistry, biology and statistics.\textsuperscript{20} Figure taken from McKinney \textit{et al.}, 2000.
**Discovery of Angelmarin**

In 2006, a coumarin-based natural product, angelmarin (40, Figure 7), was isolated from the root of the Japanese medicinal plant, *Angelica pubescens*. The 23-carbon structure contains a columbianetin (41, Figure 7) core and a $p$-hydroxycinnamoyl group (blue, Figure 7) connected via an ester linkage. Circular dichroism spectrum of angelmarin revealed a clockwise spatial orientation of the two chromophores. Angelmarin was found to exhibit cytotoxicity against human pancreatic adenocarcinoma cell line PANC-1 preferentially under nutrient-deprived conditions. A concentration of 0.01 µg/mL induced 100% cell death within 24 h of starvation, and the cytotoxicity was more pronounced when the concentration of angelmarin was increased to 10 µg/mL, at which 100% cell death was observed within 6 h. Interestingly, the building blocks of angelmarin, columbianetin and $p$-hydroxycinnamic acid (42, Figure 7) were both inactive in nutrient-rich and nutrient-deprived conditions at even 100 µg/mL, indicating the importance of the unique structure of angelmarin.²¹

![Figure 7. Structures of angelmarin, columbianetin and $p$-hydroxycinnamic acid.](image)

**Novel Isoprenylated Coumarins and their Antitumor Activity**

As the basic structure of angelmarin, coumarins (43, Figure 8) are ubiquitous in a number of pharmaceuticals. Coumarin-containing compounds possess a wide range of physiological
activities such as anticancer, anti-viral, antifungal, anti-inflammatory, antioxidant, anticoagulant, antibacterial, antitubercular, and analgesic activities. In particular, hydroxycoumarin derivatives have been shown to exhibit promising antitumor, anti-inflammatory and anti-viral effects. In particular, hydroxycoumarin derivatives have been shown to exhibit promising antitumor, anti-inflammatory and anti-viral effects. Intrigued by the selective cytotoxicity of angelmarin and the frequent occurrence of coumarins in natural compounds, our laboratory launched SAR studies based on the core coumarin structure of angelmarin.

Figure 8. Structure of coumarin with labeled positions and structure of a geranylgeranylated coumarin derivative 44.

In 2011, our group reported a novel geranylgeranylated ether coumarin derivative (44, Figure 8) that was found to induce PANC-1 cell death at a concentration of 6.25 µM within 24 h, with preferential cytotoxicity under nutrient-deprived conditions. Since then, a library of isoprenylated coumarin derivatives (Table 1), consisting of three series of isoprenylated coumarin ethers with systematic variations in tail length (5, 10 or 15 carbons) and substitution position on the coumarin scaffold (at 3-, 6- or 7-position) has been completed. The preparation of these ether compounds was achieved via a Williamson-ether synthesis using an isoprenyl bromide and an alkoxycoumarin anion pre-generated by treating the corresponding hydroxycoumarin with sodium hydride. The general reaction is outlined in Scheme 4.
<table>
<thead>
<tr>
<th>Series</th>
<th>Compound Number</th>
<th>Structure</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (µM) in ND</th>
<th>LC&lt;sub&gt;50&lt;/sub&gt; (µM) in NR</th>
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<tr>
<td>53</td>
<td></td>
<td></td>
<td>18</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Table 1. *In vitro* cytotoxicity of compounds 45-53 against PANC-1 under nutrient-deprived (ND) and nutrient-rich (NR) conditions. Series 1, 2 and 3 differ by substitution position of the isoprenylated tail. LC<sub>50</sub> values are obtained from Jun et al., 2014.  

Scheme 4. General synthetic approach to isoprenylated coumarin derivatives.  

The entire series was tested *in vitro* against PANC-1 cells. The survival of PANC-1 cells under nutrient-deprived and nutrient-rich conditions within 24 h is summarized in Table 1. The
farnesylated ether 50 at position 6 displayed the greatest cytotoxicity under nutrient-deprived conditions with an LC$_{50}$ value as low as 4 µM. All compounds in this series were inactive under nutrient-rich conditions. To better understand the preferential cytotoxicity of the coumarin derivatives, we have chosen to probe the cellular mechanism of action of compound 50 because of its highest potency. To start off, we will investigate the effect of medium components on the cytotoxic activity of 50.

**Introduction to Investigation**

The research presented herein will be comprised of three parts, first, to validate the preliminary results obtained from a previous investigation, which suggested ($S$)-34 as a potential lead pharmacophore, second, to introduce an ongoing SAR study based on the structure of ($S$)-34, and last but not least, to report the results pertinent to our investigation of medium components and the mechanism of antitumor activity of 50.

1. **Validation of Initial Lead Structure**

As previously described, pancreatic tumor cells are known to be significantly more resistant than normal human cells under nutrient-deprived conditions. Therefore, an ideal selective anti-austerity agent should induce cell death only under nutrient-deprived conditions conferred by the absence of amino acids, glucose, and serum. A previous member of our laboratory had obtained preliminary data regarding the cytotoxicity of the four synthetic intermediates ($S$)-33, ($S$)-34, ($S$)-36 and ($S$)-37 (Scheme 3). In her study, ($S$)-34 was found to be the only compound that demonstrated a preferential cytotoxicity under nutrient-deprived conditions, whereas no PANC-1 cell death was observed under nutrient-rich conditions, suggesting the potential of ($S$)-34 to become a lead structure of an SAR study. The other three intermediates ($S$)-33, ($S$)-36 and ($S$)-37 showed no activity in either medium. In order to
validate this exciting finding, more trials of \textit{in vitro} cytotoxicity assays will be performed with the four synthetic intermediates, as well as the commercially-available epoxy ether 32, to complete the initial search for a lead structure. PANC-1 cells will be exposed to all five compounds under both nutrient-rich and nutrient-deprived conditions for 24 h. Cell survival rates will be reported as an indication of the cytotoxic activity of all compounds.

2. \textit{SAR Study based on (S)-34}

Upon an examination of the structure of (S)-34, we have identified three places of immediate interest: the chiral carbon 1 (orange, Figure 9), the tert-butyldiphenylsilyl (TBDPS) protecting group (pink, Figure 9), and substituents on carbon 4 (purple, Figure 9).

![Figure 9. Expanded structure of (S)-34.](image)

Chirality plays an important role in myriad natural processes, especially in drug-protein interactions. Proteins are usually enantioselective towards their binding partners, and thus a specific stereochemistry is often required for a drug to effectively bind to its molecular target. Additionally, chirality in drug design is addressed in governmental regulations. In 1992, the United States Food and Drug Administration issued guidelines and policies regarding the development of chiral compounds\textsuperscript{23}, emphasizing the importance of chirality in drug design.

In our study, the enantiomer of (S)-34, (R)-34 as well as the racemic mixture, will be included in the SAR compound library, a summary of which is listed in Table 2. The preparation
of \((R)-34\) can be achieved via a two-step process shown in Scheme 5. The racemic mixture will be obtained by combining equal amounts of \((S)-34\) and \((R)-34\). Optical rotation measurements will be performed to confirm the chirality of each compound.

\[
\begin{align*}
\text{Zn, Valproic Acid} & \quad \text{Pd[(S)-binap]Cl}_2 \\
32 & \quad \text{33 (R)} \\
\text{TBDPSCI} & \quad \text{34 (R)}
\end{align*}
\]

Scheme 5. Two-step synthesis to prepare \((R)-34\).

The significant difference in cytotoxicity between \((S)-34\) and \((S)-33\) indicated that the presence of the TBDPS protecting group might play a key role in the selective cytotoxicity observed for \((S)-34\). However, it was unclear whether the TBDPS group alone or the combination of the \((S)-33\) scaffold and the protecting group resulted in the cytotoxicity. Therefore, we are going to test the potency of \textit{tert}-butyldiphenylsilanol (TBDPSOH) as well as a variety of protected \((S)-33\). TBDPSOH will be generated from a hydrolysis reaction of TBDPSCI under basic conditions (Scheme 6). Protecting groups of interest include silyl protecting groups such as \textit{tert}-butyldimethylsilyl (TBDMS) and trimethylsilyl (TMS). Both groups resemble TBDPS in that they are all silicon-containing protecting groups, but TBDMS and TMS are relatively less sterically hindered. In TBDMS, the two phenyl groups are replaced with methyl groups, whereas in TMS, all three bulky substituents are replaced with methyl groups. It will be interesting to see whether the size of the protecting group could cause any effect on the antitumor activity. Besides, several other protected \((S)-33\) with common alcohol protecting groups such as benzyl, tetrahydropyran (THP) and trityl, which do not contain silicon, will also be added to the SAR library (Table 2), to test whether silicon is key to cytotoxicity.
Scheme 6. Synthesis of TBDPSOH from commercially-available TBDPSCl.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Compound Number</th>
<th>Structure</th>
</tr>
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<tr>
<td></td>
<td>(S)-56</td>
<td><img src="structure_image4.png" alt="Structure Image" /></td>
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</table>
Table 2. Proposed SAR compound library based on the lead compound (S)-34.
The inactivity of \((S)-36\), which also contains a TBDPS protecting group, might provide some insight into whether hydrophobicity played a role in the activity observed in this series of compounds. To investigate the correlation between hydrophobicity and cytotoxicity, the octanol-water partition coefficients of various compounds \((\text{Log}P)\) will be calculated using ChemBioDraw\textsuperscript{®} 13.0 (PerkinElmer, Cambridge, MA, USA). \text{Log}P is important for predicting cell membrane penetration\textsuperscript{24} and studies have utilized \text{Log}P values to estimate biological activities of structurally-related compounds. For example, Fratello \textit{et al.} reported a good correlation between the cytotoxicity of halogenated benzenes and their \text{Log}P values\textsuperscript{25}, whereas Sasaki and coworkers re-evaluated the tumor-specific cytotoxicity of mitomycin C, bleomycin and peplomycin based on the \text{Log}P values.\textsuperscript{26} Studies have shown an excellent correlation between ChemDraw\textsuperscript{®} estimated ClogP values and experimentally-measured \text{Log}P values.\textsuperscript{24,27} For example, the validity of \textit{in-silico} predictions of \text{Log}P values was investigated in a recent study by Wolk \textit{et al.} where a comparison was made between experimental and calculated partition coefficients of 154 drugs. In this study, ClogP estimations by ChemDraw\textsuperscript{®} 8.0 (PerkinElmer, Cambridge, MA, USA) and the experimental values demonstrated an excellent correlation \((r^2 = 0.97)\).\textsuperscript{27}

One method to alter the hydrophobicity of the lead structure \((S)-34\) is to modify the substituents on carbon 4 (purple, Figure 9). We have proposed to install a methyl group as an alternate substituent. This modification should in theory decrease the ClogP value of the resulting compound. Besides, by replacing the bulky phenyl group with a simple methyl group, we hope to also test if the steric hindrance at carbon 4 influences the cytotoxicity. The suggested modification can be achieved via an adjustment of reagents in the Stille cross-coupling, an intermediate step in Scheme 3. The proposed reaction is shown in Scheme 7.
3. Effect of Medium Components on Cytotoxicity of 50

To investigate the effect of medium components on the cytotoxic activity of 50, we will prepare six media consisting of different nutrient combinations, a summary of which is shown in Table 3. PANC-1 cells will be cultured in the appropriate medium for 24 h prior to the exposure of 50. Similar studies on medium components have been performed with a variety of pharmaceutical compounds. For example, Izuishi and coworkers showed that troglitazone (62, Figure 10) inhibited PANC-1 cell survival under deprivation of both glucose and serum, whereas LY294002 (63, Figure 10) inhibited PANC-1 cell survival under deprivation of amino acids, irrespective of the presence of glucose or serum. Esumi et al. discovered that pyrvinium pamoate (64, Figure 10) was preferentially toxic when glucose was depleted, irrespective of the presence of serum or amino acids. Awale and coworkers reported cytotoxicity of arctigenin (65, Figure 10) at a concentration of 0.01 µg/mL under glucose-deprived conditions. While we seek to identify the key nutrient(s) that may activate the antitumor activity of 50, we decided to explore whether 50 exhibits universal cytotoxicity against other pancreatic cancer cell lines. Therefore, the cytotoxicity of 50 against two additional pancreatic cancer cell lines BxPc-3 and Capan-2 will also be examined.

Scheme 7. Synthesis of (S)-61 via a Stille cross-coupling reaction.
<table>
<thead>
<tr>
<th>Medium Number</th>
<th>Glucose</th>
<th>Amino Acid</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>6</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3. Six cell culture media prepared via addition of different combinations of nutrients to nutrient-deprived medium.

![Troglitazone](image1)
![LY294002](image2)

![Pyrvinium Pamoate](image3)

![Arctigenin](image4)

Figure 10. Structures of troglitazone, LY294002, pyrvinium pamoate and arctigenin.
RESULTS AND DISCUSSION

Validation of Initial Lead Structure

As previously described, pancreatic tumor cells are known to be significantly more robust than normal human cells under nutrient-deprived conditions.\textsuperscript{11} Therefore, an ideal selective anti-austerity agent should induce cell death only under nutrient-deprived conditions conferred by the absence of amino acids, glucose, and serum. Replicate experiments were conducted for five compounds (32, (S)-33, (S)-34, (S)-36, (S)-37, Scheme 3) \textit{in vitro} to evaluate their cytotoxicity against the human pancreatic adenocarcinoma cell line PANC-1 under both nutrient-rich and nutrient-deprived conditions. The goal of this study was to validate the preliminary results of compounds (S)-33, (S)-34, (S)-36 and (S)-37 obtained by a former lab member\textsuperscript{22}, and identify a lead structure with the additional results for epoxy ether 32. Survival of PANC-1 cells under nutrient-deprived and nutrient-rich conditions within 24 h following exposure to all five compounds is shown in Figure 11. We were pleased to discover that compound (S)-34 did indeed meet our expectation for an ideal anti-austerity agent: it exhibited preferential cytotoxicity under nutrient-deprived conditions with an LC\textsubscript{50} value of 11 µM. Administration of 100 µM of (S)-34 induced 88% cell death under nutrient-deprived conditions, whereas no cytotoxicity was observed under nutrient-rich conditions. Among the other compounds tested, compounds 32, (S)-33 and (S)-36 showed no cytotoxicity in either medium condition, regardless of the concentration of the compound. Compound (S)-37 also did not show appreciable cytotoxicity. It induced 30% cell death only at 100 µM, exhibiting no cytotoxicity at low concentrations.\textsuperscript{5} The preferential cytotoxicity of (S)-34 enabled the identification of a useful pharmacophore, leading to an ongoing SAR study described in the next section.
Figure 11. Survival of PANC-1 cells under nutrient-deprived conditions (red) within 24 h. PANC-1 cell survival under nutrient-rich conditions (blue) was examined as control. (S)-34 exhibited preferential cytotoxicity with an LC$_{50}$ value of 11 µM. All cell viabilities are means of ±SEM, $n = 3$. Concentrations of compounds investigated were 6.25, 12.5, 25, 50 and 100 µM.

**SAR Study based on (S)-34**

Based on the results reported in the previous section, we identified three attributes of (S)-34 that might account for its preferential cytotoxicity, including the chirality of carbon 1 (orange, Figure 9), the TBDPS protecting group (pink, Figure 9), and the substituents on carbon 4 (purple, Figure 9). The proposed structures of the SAR compound library are summarized in Table 2. This section will be divided into two parts: part I will focus on the synthesis of the SAR library and part II will present and discuss the biological evaluations of (S)-34 analogs.
1. Preparation of the SAR Library

Two-step Synthesis of (R)-34 – Asymmetric Ring Opening

The preparation of (R)-34 involved a palladium-catalyzed reductive asymmetric ring opening from the cyclic ether 32 to a chiral homoallylic alcohol (R)-33, followed by the protection of the alcohol using TBDPSCl (Scheme 5).

The palladium-catalyzed ring opening reaction was first reported by Li and coworkers\(^\text{30}\), as a convenient and mild method to stereoselectively prepare 1,2-dihydronaphth-1-ol in one pot from easily accessible starting materials. The reaction was accomplished in the presence of an organic carboxylic acid, zinc metal, and a palladium metal complex with a chiral bidentate ligand, all of which were necessary for a successful reaction.\(^\text{30}\) In our study, the conversion of 32 to (R)-33 was achieved using valproic acid, zinc metal, and Pd[(S)-binap]Cl\(_2\) (Figure 12).

![Figure 12. Structures of valproic acid and Pd[(S)-binap]Cl\(_2\).](image)

A test reaction of 100 mg was first performed following the same procedures described in the published work by our previous lab members.\(^\text{19}\) A first attempt of purification via Preparatory Thin Layer Chromatography (Prep TLC) was not sufficient to yield a pure desired product. The Hydrogen Nuclear Magnetic Resonance (\(^1\)H-NMR) spectrum of the isolated product in acetone-\(d_6\) contained additional signals between 0.5-2 ppm suggesting the presence of some contaminations, while the rest of the spectrum confirmed the structure of (R)-33 (Figure 13).
The contamination was speculated to consist of mainly hydrocarbons due to the upfield position of the corresponding peaks. An $^1$H-NMR spectrum of the same compound was taken in benzene-d$_6$, and an increase of peak intensities within the same region was observed. Upon examination of participating reagents in the reaction mixture, the contamination was confirmed to be residual valproic acid (Figure 14), which contained shielded hydrogens that had NMR signals within 0.5-2 ppm.
Figure 14. $^1$H-NMR spectra of the desired product (R)-33 in acetone-d6 (A) and benzene-d6 (B). There was an increase in peak intensity between 0.5-2 ppm.

Additional extraction to remove the residual valproic acid and purification via Prep TLC were performed, reducing the amount of valproic acid in the product by 75% (Figure 15). The characterization of (R)-33 was completed with $^{13}$C-NMR spectroscopy, High Resolution Mass Spectrometry (HRMS), and polarimetry. $^{13}$C-NMR and HRMS results were in good agreement with the structure, while the optical rotation (+42.9°) was slightly higher than the expected value (+39.3°), since (S)-33 has an optical rotation of -39.3°. The fully characterized (R)-33 was obtained in 18% yield.
Figure 15. $^1$H-NMR spectra of the desired product ($R$)-33 before (A) and after (B) additional purification. Impurity intensity decreased by 75%.

A second scale-up reaction of ($R$)-33 was conducted, followed by column chromatography for purification, which unfortunately did not yield a pure product. Residual valproic acid was detected in the initial $^1$H-NMR spectrum. Therefore, further purification via extraction and Prep TLC was required and performed prior to characterizing the product. In this reaction, ($R$)-33 demonstrated an optical rotation of $+28.0^\circ$ in 48% yield.

While the detailed pathway$^{31}$ of the catalytic transformation was not clear, a general reaction mechanism was proposed based on the reaction conditions (Figure 16). Pd[($S$)-binap]Cl$_2$, an 18-electron Pd(II) species, was first reduced to Pd(0) by zinc metal – a key step to initiate the catalytic cycle, which also explained why no reaction was observed in the absence of Zn. Oxidative addition of an organic acid, or valproic acid in this case, to Pd(0) led to the generation of Pd(II) hydride species (66, Figure 16), where the Pd metal coordinates with the
carbon-carbon double bond of 32 via the endo face. Insertion of the double bond to the Pd center generated the intermediate 67, followed by a β-heteroatom elimination to achieve the intermediate 68. Protonation of 68 yielded the desired product (R)-33 and regenerated the Pd(II) species 69, which could be reduced to Pd(0) by Zn metal to re-enter the catalytic cycle. The stereochemistry of the binaphthalene ligand in the Pd complex played a key role in facilitating the stereoselectivity of the reaction. In fact, when Pd[(R)-binap]Cl2 was used, the Pd metal coordinated the carbon-carbon double bond via the exo face, and subsequent β-elimination and protonation resulted in the deprotected lead structure (S)-33. Therefore, this Pd-catalyzed ring opening method allowed generation of either stereoisomer with high enantioselectivity under mild conditions.30
Two-step Synthesis of (R)-34 – Protection of (R)-33

The protection of the homoallylic alcohol (R)-33 (Scheme 5) was accomplished using TBDPSCl, imidazole and DMAP to generate the TBDPS-protected silyl ether (R)-34 in 64% yield. The identity of (R)-34 was confirmed via $^1$H-NMR, $^{13}$C-NMR and HRMS, a representative of which with correctly assigned peaks is shown in Figure 17. (R)-34 showed an optical rotation of $+76.1^\circ$, slightly higher than the expected value $+62.5^\circ$ ([α]D$^{25}$ for (S)-34 = -62.5$^{19}$).
Figure 17. $^1$H-NMR spectrum of (R)-34 with assigned peak and corrected integrations.

As common reagents in protection of alcohol, imidazole and DMAP facilitated the formation of intermediates in the S$_\text{N}$2-like mechanism (Figure 18). DMAP first targeted the electrophilic silicon to generate a pyridinium cation 70, making silicon more susceptible to nucleophilic attack by alcohol (R)-33. Imidazole extracted a proton from the intermediate 71, generating the desire product (R)-34.
Preparation of Racemic 34

The preparation of the racemic mixture of (R)- and (S)-34 was achieved by simply combining equal masses of each enantiomer. The resulting compound showed no optical rotation. The purity of 34 was briefly checked via TLC, where only one spot with the correct Rf value appeared.

Synthesis of 55 via Hydrolysis of TBDPSCI (54)

Hydrolysis of TBDPSCI occurred under treatment with KOH, H2O and CH3OH (Scheme 6). This reaction followed a basic S_n1 mechanism, where TBDPS cation was created after the departure of the leaving group –Cl, and hydroxide anions performed the nucleophilic attack to install the hydroxyl group (Figure 19). Full characterization of 55 was completed with $^1$H-NMR, $^{13}$C-NMR and HRMS. $^1$H-NMR spectra before and after D2O shake are shown in Figure 20. The intensity of the peak between 5-6 ppm (red, Figure 20) decreased while the peak intensity of water increased after the addition of D2O, which confirmed that the exchangeable proton appeared between 5-6 ppm.
It was discovered by accident that TBDPSCI hydrolyzed on silica gel. When TBDPSCI was loaded on a TLC plate, it interacted with the slightly acidic silica gel to produce the decomposition product TBDPSOH, which was fortunately the desired product. This hypothesis was proven by a 2D TLC experiment, where after a regular TLC experiment, the plate was turned 90° and let develop in the same solvent system. The decomposition product such as TBDPSOH (blue dot, Figure 21, left) would be stable and appear still as one spot, while the residual reactant such as TBDPSCI (red dot, Figure 21, left) would continue being hydrolyzed and appear as two spots, thus the final markings on the TLC plate should resemble those on plate C in Figure 21 (left). The resulting plate indeed demonstrated the expected pattern (Figure 21, right). Therefore, TBDPSCI was proven to decompose on silica-coated TLC plates.
Figure 21. Schematic diagram of a 2D-TLC experiment (left) and the expected pattern was observed on the actual TLC plate (right).

**Synthesis of (S)-60 via Tritylation of (S)-33**

Tritylation is another common method of protecting hydroxyl functional groups, and is usually conducted in a manner analogous to TBDPS protection reactions. (S)-60 was generated in 41% yield by treating (S)-33 with trityl chloride (TrtCl), DMAP and Et$_3$N. Likewise, the reaction underwent an $S_N$2-like mechanism where DMAP replaced imidazole and formed intermediates 72 and 73 (Figure 22). (S)-60 was fully characterized with $^1$H-NMR, $^{13}$C-NMR and HRMS.
Prior to synthesizing other analogs of (S)-34, such as (S)-56 (Table 2), we decided to first add TBDMSOH (75) to our compound library, since it can be easily obtained from hydrolysis of TBDMSCl (74) under treatment with KOH, H\textsubscript{2}O and CH\textsubscript{3}OH (Scheme 8). This reaction followed the exact same mechanism as hydrolysis of TBDPSCl. Since neither 74 nor 75 was UV-active, no chromatography techniques were exploited in isolating the desired product. Characterization of 75 included \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR and GC/MS, and 75 was obtained in 33\% yield.

2. Biological Evaluations of SAR Analogs

Cytotoxicity assays against PANC-1 cells were performed with compounds (R)-33, (R)-34, 34, 55, (S)-60 and 75 under both nutrient-rich and nutrient-deprived conditions. PANC-1 cell
viability within 24 h upon treatment with different compounds is shown in Figure 23 and 25. The first attribute examined was the chirality of carbon 1 (orange, Figure 9). To our surprise, compound (\(R\))-34 exhibited an even better cytotoxic activity than the lead enantiomer, with an LC\(_{50}\) value of 4 \(\mu\)M under nutrient-deprived conditions, about 64\% lower than the LC\(_{50}\) of (\(S\))-34\(^5\) (11 \(\mu\)M, Figure 11). The racemic compound 34 showed an LC\(_{50}\) value of 7 \(\mu\)M under the same conditions, which was approximately an average of the LC\(_{50}\) values of the enantiomers. However, both (\(R\))-34 and racemic 34 showed modest cytotoxicity against PANC-1 under nutrient-rich conditions, which was not observed in the case of (\(S\))-34. Approximately 10-20\% cell death was observed for higher concentrations of (\(R\))-34 and racemic 34 under nutrient-rich conditions, whereas (\(S\))-34 was completely inactive under the same conditions. Additionally, (\(R\))-33 was found to be inactive in either medium condition, similar to the behavior of (\(S\))-33 (Figure 23). Therefore, chirality of carbon 1 (orange, Figure 9) did not seem to be responsible for the preferential cytotoxicity of the lead compound. Although (\(R\))-34 and racemic 34 exhibited better potency inducing PANC-1 cell death, (\(S\))-34 demonstrated more promising specificity toward tumor cells. Despite a likely trade-off between potency and tumor specificity in this series of compounds, a direct reductive epoxide ring-opening method to prepare a racemic combination of the homoallylic alcohol 33 may offer an alternative approach to SAR exploration, since absolute stereochemistry did not significantly impact the antitumor activity. This proposed reaction could be achieved with Pd catalysts\(^{32}\), LiH\(^{33}\) or boron catalysts\(^{34}\), which could greatly reduce the cost of reagents, as the chiral Pd complexes (Pd[(\(S\))-binap]Cl\(_2\) and Pd[(\(R\))-binap]Cl\(_2\)) cost $139.00/gram\(^{35}\) and $806.00/gram\(^{36}\), respectively. Moreover, the symmetry of epoxy ether 32 will also eliminate the concern of regioselectivity.
Figure 23. Survival of PANC-1 cells under nutrient-deprived conditions (red) and nutrient-rich conditions (blue) within 24 h. (R)-34 and racemic 34 showed preferential cytotoxicity with LC$_{50}$ values of 4 µM and 7 µM, respectively. All cell viabilities are means of ±SEM, n = 3. Replicate experiments were performed and similar values were obtained. Concentrations of compounds investigated were 6.25, 12.5, 25, 50 and 100 µM.

Next, we investigated the effect of protecting groups on the cytotoxic activity of (S)-34. Comparing (S)-34 to (S)-33, we hypothesized that the cytotoxicity of (S)-34 was a result of either the TBDPS- protecting group alone or the combination of the naphthalene scaffold and the protecting group. To test the first hypothesis, PANC-1 cells were treated with TBDPSOH, a hydrolysis product derived from TBDPSCI. Interestingly, TBDPSOH exhibited a preferential cytotoxicity against PANC-1 with an LC$_{50}$ value of 48 µM (Figure 25), which was about four times higher than that of the lead structure. Thus, it is clear that the strong potency observed in (S)-34 required both the naphthalene unit and the protecting group. To continue our study, we
replaced the silicon-containing TBDPS group with a trityl group \(((S)-60)\), which is composed of purely hydrogen and carbon atoms, in an effort to explore the impact of silicon on compound potency, while retaining similar steric hindrance posed by the protecting groups. In this case, the removal of silicon demonstrated a slight but not considerable reduction in both antitumor activity and anti-austerity. Compared to \((S)-34\), the tritylated alcohol \((S)-60\) exhibited less cytotoxicity against PANC-1 cells with an LC\(_{50}\) value of 19 \(\mu\)M, while losing the degree of specificity possessed by \((S)-34\). Less than 100% cell viability was seen under nutrient-rich conditions, with 100 \(\mu\)M of \((S)-60\) inducing more than 30% cell death (Figure 25). Nonetheless, it is plausible to conclude from this study that the replacement of silicon with carbon seemed to exert a minor effect on the PANC-1 cell growth inhibitory activity. To account for the 8 \(\mu\)M difference between LC\(_{50}\) values of \((S)-34\) and \((S)-60\), we returned to examine the steric hindrance posed the TBDPS group and the trityl group. Although TBDPS and trityl groups both contain two phenyl substituents, TBDPS contains a \(t\)-butyl group as the third substituent, while the trityl group possesses a third phenyl ring (Figure 24). Phenyl groups, because of their planar geometry, are generally less sterically demanding than \(t\)-butyl groups. Thus, we proposed that the small difference in the cytotoxic activities of \((S)-34\) and \((S)-60\) might be a result from the variation in sterics.

**Figure 24.** Structures of TBDPS and trityl protecting groups.

On the other hand, TBDMSOH (75), another silanol derived from a common alcohol
protecting group, showed no cytotoxicity under either nutrient-deprived or nutrient-rich conditions (Figure 25). Recalling the moderate activity exhibited by TBDPSOH (55), we suspected hydrophobicity to be a key factor in explaining this set of results.

**Figure 25.** Survival of PANC-1 cells under nutrient-deprived conditions (red) and nutrient-rich conditions (blue) within 24 h. 55 and (S)-60 showed preferential cytotoxicity with LC₅₀ values of 48 µM and 19 µM, respectively. (S)-60 also induced more than 30% cell death under nutrient-rich conditions. All cell viabilities are means of ±SEM, n = 3. Replicate experiments were performed and similar values were obtained. Concentrations of compounds investigated were 6.25, 12.5, 25, 50 and 100 µM.

**Hydrophobicity Calculations**

Given the observations mentioned previously and since hydrophobicity was initially included in the three attributes of interest, we performed LogP calculations using ChemBioDraw® 13.0 (PerkinElmer, Cambridge, MA, USA) for all compounds that have been biologically evaluated: 32, (S)-33 (also representing (R)-33), (S)-34 (also representing (R)-34 and
racemic 34), (S)-36 and (S)-37, 55, (S)-60 and 75 (Table 4). In the absence of the TBDPS protecting group, the ClogP values of compounds 32, (S)-33 and (S)-37, were relatively low compared to that of (S)-34, suggesting a possible correlation between increasing hydrophobicity and cytotoxicity. However, the TBDPS protected vinylarene (S)-36, possessing the highest ClogP value of 9.501, did not exhibit any cytotoxic activity. This suggested that even though an increase in hydrophobicity could be associated with cytotoxicity, an optimal range of hydrophobicity might be required to observe the desired activity. This idea was further tested with compounds 55, (S)-60 and 75. Table 4 displays an array of ClogP values ranging from 0 to 10, where only compounds 55, (S)-60 and (S)-34 showed preferential cytotoxicity against PANC-1 cells. A possible optimal range of ClogP values seemed to occur between 4 and 8, within which the larger the ClogP value, the greater the antitumor activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ClogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>0.965</td>
</tr>
<tr>
<td>32</td>
<td>1.656</td>
</tr>
<tr>
<td>(S)-33</td>
<td>1.773</td>
</tr>
<tr>
<td>(S)-37</td>
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<td>(S)-34</td>
<td>7.943</td>
</tr>
<tr>
<td>(S)-36</td>
<td>9.501</td>
</tr>
</tbody>
</table>

Table 4. CLog(P) values of tested compounds. The values were obtained from ChemBioDraw® 13.0. Compounds highlighted in red exhibited preferential cytotoxicity against PANC-1 cells.

Effect of Medium Components on Cytotoxicity of 50

The farnesylated ether 50 was prepared via a Williamson-ether synthesis between a farnesyl bromide 76 and a 6-hydroxycoumarin anion 77 pre-generated from deprotonation of 6-
hydroxycoumarin with sodium hydride (Scheme 9).\(^4\)

![Scheme 9. Synthesis of farnesylated ether 50.](image)

Our previous studies have shown that 50 exhibited selective cytotoxicity against PANC-1 cells under nutrient-deprived conditions, while no activity was observed under nutrient-rich conditions.\(^4\) Based on the different compositions of nutrient-rich and nutrient-deprived media, we hypothesized that lacking certain nutrients (a single nutrient or a combination of two) might account for the preferential cytotoxicity of 50. To identify the key nutrients, we prepared six cell culture media described in Table 3. Compound 50 was tested \textit{in vitro} for its cytotoxic activity against PANC-1 cells under all six medium conditions, with nutrient-deprived and nutrient-rich media included as controls. The LC\(_{50}\) values and percent cell death at 100 µM of 50 are presented in Table 5.

<table>
<thead>
<tr>
<th>Medium Number</th>
<th>Components</th>
<th>LC(_{50}) (µM)</th>
<th>Cell Death at 100 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDM</td>
<td>Nutrient-Deprived</td>
<td>9</td>
<td>&gt;100%</td>
</tr>
<tr>
<td>2</td>
<td>NDM + AA</td>
<td>6</td>
<td>&gt;100%</td>
</tr>
<tr>
<td>4</td>
<td>NDM + AA + Glu</td>
<td>41</td>
<td>55%</td>
</tr>
<tr>
<td>1</td>
<td>NDM + Glu</td>
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<td>36%</td>
</tr>
<tr>
<td>6</td>
<td>NDM + AA + Ser</td>
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<td>24%</td>
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<tr>
<td>3</td>
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<td>&gt;100</td>
<td>18%</td>
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<td>5</td>
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<tr>
<td>NRM</td>
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<td>19%</td>
</tr>
</tbody>
</table>

**Table 5.** Effect of medium components on the preferential cytotoxicity of 50. Compound 50 remained active in the absence of serum and glucose or in the absence of serum only.
In this study, the cytotoxicity was observed in the medium deprived of serum only, and in the medium deprived of a combination of serum and glucose, with LC₅₀ values being 41 µM and 6 µM, respectively. These initial results suggested that the lack of serum was linked to the observed cytotoxic activity, and that the lack of both serum and glucose resulted in maximum cell death when PANC-1 cells were exposed to 50. In all other medium conditions, compound 50 showed no significant activity at 100 µM and displayed LC₅₀ values greater than 100 µM. However, the fetal bovine serum (FBS) used to prepare all media described in Table 5 contained a relatively high concentration of glucose and amino acids. More definitive conclusions obtained from experiments conducted under media 6, 3 and 5 (Table 5) and NRM may require additional experiments replacing FBS with dialyzed serum to afford carefully controlled media conditions devoid of glucose and amino acids. Dialyzed serum, having a lower concentration of glucose and amino acids, is expected to more accurately assess the function of serum in the observed cell growth inhibitory activity of our compounds. Preliminary results from ongoing experiments are suggesting that the lack of glucose may also play a role in inhibiting PANC-1 cell proliferation under the treatment with 50.

The cytotoxicity of 50 was also investigated in vitro against two other pancreatic cancer cell lines, BxPc-3 and Capan-2, in order to examine whether 50 possesses universal antitumor activities against pancreatic cancer. Survival of BxPc-3 and Capan-2 cells under nutrient-deprived and nutrient-rich conditions within 24 h following the exposure to 50 is shown in Figure 26.
Figure 26. Survival of BxPc-3 and Capan-2 cells under nutrient-deprived conditions (red) and nutrient-rich conditions (blue) within 24 h. Compound 50 showed preferential cytotoxicity with \( LC_{50} \) values of 5 \( \mu M \) against both cell lines. All cell viabilities are means of ±SEM, \( n = 3 \). Replicate experiments were performed and similar values were obtained. Concentrations of compounds investigated were 6.25, 12.5, 25, 50 and 100 \( \mu M \).

Similar to PANC-1, both cell lines exhibited a preferential sensitivity to 50 only under nutrient-deprived conditions, with \( LC_{50} \) values of 5 \( \mu M \) against both cell lines (Table 6). \( LC_{50} \) values were consistently greater than 100 \( \mu M \) under nutrient-rich conditions.

<table>
<thead>
<tr>
<th></th>
<th>( LC_{50} ) (( \mu M )) NDM</th>
<th>( LC_{50} ) (( \mu M )) NRM</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANC-1</td>
<td>9</td>
<td>&gt;100</td>
</tr>
<tr>
<td>BxPc-3</td>
<td>5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Capan-2</td>
<td>5</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Table 6. Preferential cytotoxicity of 50 against BxPc-3 and Capan-2.

Deer and coworkers have reported several variances among pancreatic cancer cell lines. One feature that differentiates the three cell lines in our study is the expression of the inducible enzyme cyclooxygenase-2 (COX-2)\(^{38}\), which is often overexpressed in malignant tumors.\(^{39}\) COX-2 promotes angiogenesis by converting arachidonic acid into bioactive molecules that will function as activating factors in angiogenesis.\(^{40}\) Moreover, selective COX-2 inhibitors enhance the curative effect of chemotherapies for pancreatic cancer.\(^{41,42}\) As shown in Figure 27, BxPc-3
demonstrated the greatest level of COX-2, which was essentially undetectable in Capan-2 and PANC-1.\textsuperscript{38} However, since compound 50 exhibited a universal cytotoxicity against all three cell lines, the difference in the level of COX-2 might not be associated with the mechanism of action of 50.

**Figure 27.** Western blot analysis for the basal expression of COX-2 in pancreatic cancer cell lines. Figure taken from Deer \textit{et al.}, 2010.\textsuperscript{38}

**Future Directions**

Current results from the SAR study of spiroxin A precursors suggested small impacts of chirality and protecting group center on the preferential cytotoxicity of the lead compound. However, the sterics of the protecting group as well as the hydrophobicity of the entire structure seemed to play an more important role in explaining the observed antitumor activity of (S)-34. Therefore, we propose to further study these two attributes by expanding our current SAR compound library. Compounds (S)-78 and (S)-79 will be added to evaluate the steric hindrance posed by the protecting group, and alcohols 80, 81 and 82 will be included for hydrophobicity investigations (Table 7). Synthesis and biological evaluations of the updated SAR library will hopefully reveal the structural component(s) responsible for the antitumor activity of (S)-34, and thus provide insight into improving the design of anti-pancreatic cancer agents.
<table>
<thead>
<tr>
<th>Attribute</th>
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<th>Structure</th>
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<td></td>
<td>(S)-78</td>
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</table>
Protecting group center could be either Si or C.

<table>
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<td>OTBDPS</td>
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<tr>
<td>80</td>
<td><img src="image4" alt="Chemical Structure" /></td>
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</table>
For the next stage of the medium component study, our goal is to explore the cytotoxic mechanism of action of compound 50 at a cellular level. We have initiated some early experiments focusing on nutrients that are essential for cell proliferation, the results of which have given rise to new studies to disclose the molecular target of compound 50. We have hypothesized that 50 might inhibit autophagy in PANC-1 cells. Autophagy is a catabolic mechanism that involves degradation of cellular constituents to maintain cellular energy levels, which is important for cells to respond to nutrient stress and survive nutrient starvation. Autophagy is also responsible for removing misfolded or aggregated proteins, clearing damaged organelles and eliminating intracellular pathogens. Current investigations are ongoing to test our hypothesis and hopefully determine whether such mechanism is valid, and if so, identify which stage of autophagy is inhibited by 50.

<table>
<thead>
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<th>compound</th>
<th>structure</th>
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<td><em>Center could be either Si or C.</em></td>
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<tr>
<td>82</td>
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<tr>
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<td><em>Center could be either Si or C.</em></td>
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</table>

*Table 7. Updated SAR compound library.*
CONCLUSION

A spiroxin A precursor (S)-34 was confirmed to be a lead pharmacophore against human pancreatic cancer cell PANC-1, inducing 50% of PANC-1 cell death at 11 µM only under nutrient-deprived conditions, and 100% cell viability was observed under nutrient-rich conditions. The anti-austerity ability of the lead structure has led to an ongoing SAR study to examine the chirality of carbon 1 (orange, Figure 9) and the nature of the protecting group (pink, Figure 9) of (S)-34. Compounds (R)-33, (R)-34, 34, 55, (S)-60 and 75 were prepared in sufficient yields with full characterization, and evaluated against PANC-1 cells. We observed preferential cytotoxicity for compounds (R)-34, 34, 55 and (S)-60, three of which demonstrated less specificity toward nutrient starvation compared to (S)-34. Altering the absolute stereochemistry of carbon 1 (orange, Figure 9) or silicon center in the protecting group resulted in no dramatic changes in the cytotoxicity of the lead structure, whereas varying the hydrophobicity of the entire structure produced a wide spectrum of antitumor activities. In addition, we have included another aspect of the protecting group (steric hindrance) in the list of attributes under investigation. Future work will aim to complete the synthesis and evaluation of the updated SAR library, in order to identify pivotal structural components responsible for the preferential cytotoxicity.

Investigating the effect of medium components on the cytotoxicity of 50 was our first step towards discovering the cellular mechanism of action of 50. In this study, deprivation of a combination of serum and glucose reproduced the cytotoxicity of 50 against PANC-1 cells seen under complete nutrient deprivation. A possible mechanism of action of 50 is the inhibition of autophagy in PANC-1 cells, but future efforts are required to obtain concrete evidence for this hypothesis.
EXPERIMENTAL

General Protocol

All reagents and solvents were purchased commercially from Sigma-Aldrich®, EMD, Acros Organics or TCI America and were used as they were received. All air- and moisture-sensitive reactions were performed in oven-dried glassware, under nitrogen gas, as well as in anhydrous solvents. Thin Layer Chromatography (TLC) was performed on pre-coated glass plates with silica gel 60 F254 purchased from VWR® to monitor reaction progress. Purification of desired products was accomplished via Preparatory Thin Layer Chromatography (Prep TLC) and Flash Column Chromotography. Prep TLC was performed on pre-coated plates with silica gel coated 1000-µm thick from Analchem. Flash column chromatography was performed under a pressure of 4 psi with silica gel 70-230 mesh, 60 Å from Sigma-Aldrich®.

All synthesized compounds were characterized by Nuclear Magnetic Resonance (NMR) Spectroscopy, High-Resolution/Low-Resolution Mass Spectrometry (HR/LRMS), Gas Chromatography-Mass Spectrometry (GC-MS) and Polarimetry when applicable. All 1H- and 13C-NMR data were recorded on a Bruker 300 Fourier transform spectrometer at 300 MHz (1H-NMR) or 75 MHz (13C-NMR). Samples of interest were diluted in acetone-d6, chloroform-d (CDCl3) and benzene-d6 (C6D6). Chemical shifts (δ) were reported in parts per million (ppm) relative to tetramethylsilane (TMS), while all coupling constants were reported in Hz. HRMS and LRMS were performed on a 70-VSE or Q-Tof Ultima mass spectrometer at the University of Illinois at Urbana-Champaign Mass Spectrometry Facility. GC-MS results were abstracted from an HP 5890 Series II Plus gas chromatograph obtained using a crosslinked 5% diphenyl-95% dimethylsiloxane column from Hewlett Packard, and an HP 5972 electrical ionization mass detector. Polarimetry was performed on an Autopol® II Automatic Polarimeter.
PANC-1 cells were obtained from American Type Culture Collection (ATCC). Materials related to cell culture and cytotoxicity assays were purchased from Sigma-Aldrich®, BD Falcon BD Falcon®, BD Syringe, VWR®, Rainin, Corning, Celltreat®, Pall® Corporation and Cellgro®. Cell counting kit was purchased from Dojindo Molecular Technologies. The cell incubator was a product of Sheldon Manufacturing Inc. The microplate reader was a SpectraMax® M3 from Molecular Devices.

**Synthesis of (R)-1,2-dihyronaphthalen-1-ol ((R)-33)**

![Chemical structure](image)

**Trial 1. (100 mg test reaction)**

Pd[(S)-binap]-Cl₂ (26.5 mg, 0.0331 mmol) and zinc powder (251.9 mg, 3.85 mmol) were added to an oven-dried round-bottom flask. The system was evacuated and purged with nitrogen gas three times. Anhydrous toluene (2 mL) was added to the flask via syringe and the mixture was stirred at room temperature under nitrogen for 25 min. Valproic acid (0.32 mL, 2.00 mmol) was added to the flask via syringe and the reaction mixture was stirred at room temperature under nitrogen for 1 h. Within 7 min following the addition of valproic acid, the reaction mixture transitioned from yellow to orange, to red, and finally reached a dark wine color. 7-oxabenzonorbornadiene (32, 98.4 mg, 0.683 mmol) dissolved in anhydrous toluene (1 mL) was added to the reaction mixture, which was allowed to stir under nitrogen at room temperature for 2 hours. Reaction progress was monitored by TLC (4:1 hexanes:ethyl acetate). The solution was stirred in air for an additional 15 minutes, filtered through silica gel, the filter pad washed with
2% (v/v) MeOH in CH$_2$Cl$_2$ and the mixture was concentrated *in vacuo*. The filtrate was diluted with CH$_2$Cl$_2$ and extracted with saturated sodium bicarbonate three times to remove excess valproic acid. The aqueous layers were combined and extracted once with dichloromethane. The organic layers were combined and dried over sodium sulfate. The dried organic fraction was then filtered and concentrated *in vacuo*. The crude mixture was purified via Prep TLC (4:1 hexanes:ethyl acetate). The desired band (bottom band) was isolated, washed with 100 mL 2% (v/v) MeOH in CH$_2$Cl$_2$ and concentrated *in vacuo*. Further extraction with sodium bicarbonate, as well as purification via Prep TLC (4:1 hexanes:ethyl acetate) were performed to minimize residual valproic acid. The desired product (R)-33 (17.5 mg) was isolated in 18% yield. TLC $R_f$ = 0.24 on silica gel (hexanes:ethyl acetate = 4:1). The absolute configuration was assigned by comparison of the optical rotation with the value reported for (S)-33.$^{19}$ $[^{[a]}]D^{25} = +42.9^\circ$ (c = 1.5, CHCl$_3$). $^1$H-NMR (300 MHz, acetone-d$_6$): $\delta$ 2.43 (m, 2H), 4.31 (d, $J = 5.7$ Hz, 1H), 4.84 (m, 1H), 5.99 (m, 1H), 6.52 (d, $J = 9.6$ Hz, 1H), 7.10 (dd, $J = 5.1$ Hz, 3.3Hz, 1H), 7.20 (m, 2H), 7.48 (m, 1H); $^{13}$C-NMR (75 MHz, acetone-d$_6$): $\delta$ 34.1, 68.2, 127.1, 127.2, 127.3, 127.5, 128.3, 128.4, 134.5, 139.7. HRMS (EI) Calc’d for C$_{10}$H$_{10}$O: 146.0732; found 146.0731.

**Trial 2. (500 mg scale-up reaction)**

Pd[(S)-binap]-Cl$_2$ (127 mg, 0.159 mmol) and zinc powder (1.1919 g, 18.2 mmol) were added to an oven-dried round-bottom flask. The system was evacuated and purged with nitrogen gas three times. Anhydrous toluene (13 mL) was added to the flask via syringe and the mixture was stirred at room temperature under nitrogen for 25 min. Valproic acid (1.61 mL, 10.0 mmol) was added to the flask via syringe and the reaction mixture was stirred at room temperature under nitrogen for 1 h. The reaction mixture underwent expected color changes as in trial 1. 7-oxabenzonorbornadiene (32, 493.7 mg, 3.42 mmol) dissolved in anhydrous toluene (6 mL) was
added to the reaction mixture, which was allowed to stir under nitrogen at room temperature for 2 hours. Reaction progress was monitored by TLC (4:1 hexanes:ethyl acetate). The solution was stirred in air for an additional 15 minutes, filtered through silica gel, the filter pad washed with 2% (v/v) MeOH in CH₂Cl₂ and the mixture was concentrated in vacuo. The filtrate was diluted with CH₂Cl₂ and extracted with saturated sodium bicarbonate three times to remove excess valproic acid. The aqueous layers were combined and extracted once with dichloromethane. The organic layers were combined and dried over sodium sulfate. The dried organic fraction was then filtered and concentrated in vacuo. The residue was purified via flash column chromatography with eluents starting as 100% hexanes and gradually increasing in polarity to 4:1 hexanes:ethyl acetate. Further extraction with sodium bicarbonate, as well as purification via Prep TLC (4:1 hexanes:ethyl acetate) were needed to minimize residual valproic acid. The desired product (R)-33 (241.5 mg) was isolated in 48% yield. TLC Rf = 0.24 on silica gel (hexanes:ethyl acetate = 4:1). The absolute configuration was assigned by comparison of the optical rotation with the value reported for (S)-33.\(^\text{19}\) [α]\(_D\)\(^{25}\) = +28.0° (c = 1.5, CHCl₃). Characterization data see trial 1.

**Synthesis of (R)-tert-butyl((1,2-dihydronaphthalen-1-yl)oxy)diphenylsilane ((R)-34)\(^\text{19}\)**

(R)-33 (93.5 mg, 0.640 mmol), imidazole (121.5 mg, 1.78 mmol), and DMAP (6.3 mg, 0.052 mmol) were dissolved in 3.5 mL of anhydrous CH₂Cl₂ in an oven-dried round-bottom flask. TBDBPSCl (185 µL, 0.711 mmol) dissolved in 3.5 mL of anhydrous CH₂Cl₂ was added to the reaction flask via syringe. The reaction was allowed to stir at room temperature under
nitrogen. Reaction progress was monitored by TLC (4:1 hexanes:ethyl acetate). No precipitation was observed, which was concerning because immediate precipitation was formed in the synthesis of (S)-34. After 22 h, it was realized that the TBDPSCl purchased was a 1 M solution instead of pure TBDPSCl. Therefore, the calculation for the amount of TBDPSCl needed was corrected and another 1.1 equivalent of TBDPSCl (0.704 mL, 0.640 mmol) was added to the reaction, upon which immediate white precipitate was observed. The reaction was let stir for another 30 h. The solvent was removed in vacuo and diethyl ether was added to dilute the residue. The precipitate was filtered and washed with diethyl ether. The filtrate was concentrated in vacuo and purified via Prep TLC (4:1 hexanes:ethyl acetate). The desired band (top band) was isolated, washed with 100 mL 2% (v/v) MeOH in CH₂Cl₂ and concentrated in vacuo. The desired product (R)-34 (157.7 mg) was isolated in 64% yield. TLC Rf = 0.77 on silica gel (hexanes:ethyl acetate = 4:1). The absolute configuration was compared to the optical rotation of (S)-34. [α]D²⁵ = +76.1° (c = 1.5, CHCl₃). ¹H-NMR (300 MHz, CDCl₃): δ 1.08 (s, 9H), 2.15 (m, 1H), 2.40 (m, 1H), 4.95 (dd, J = 9.5 Hz, 6.1 Hz, 1H), 5.82 (m, 1H), 6.43 (d, J = 9.6 Hz, 1H), 7.24 (m, 10H), 7.62 (d, J = 7.5 Hz, 2H), 7.73 (d, J = 7.2 Hz, 2H). ¹³C-NMR (75 MHz, CDCl₃): δ 19.3, 26.9, 32.8, 69.9, 126.2, 127.5, 127.6, 129.3, 129.5, 129.8, 130.0, 133.4 133.7, 134.3, 134.8, 135.2, 135.6, 136.1, 137.5. HRMS (EI) Calc’d for C₂₆H₂₈O₅Si: 384.1909; found 384.1905.

**Preparation of tert-butyl((1,2-dihydronaphthalen-1-yl)oxy)diphenylsilane (34)**

(R)-34 (8 mg, 0.0208 mmol) and (S)-34 (8 mg, 0.0208 mmol) were transferred and combined in acetone and then concentrated in vacuo. The desired racemic mixture 34 (15.8 mg,
0.0411 mmol) was obtained in 99% yield. TLC $R_f = 0.80$ on silica gel (hexanes:ethyl acetate = 4:1). $[\alpha]_D^{25} = 0^\circ$ (c = 1.5, CHCl$_3$). Characterization data see (R)-34.

**Synthesis of tert-butyldiphenylsilanol (55)**

![Chemical Structure of tert-butyldiphenylsilanol (55)](attachment:image.png)

**Trial 1.**

Potassium hydroxide (KOH, 48.47 mg, 0.864 mmol), deionized water (260 µL) and methanol (MeOH, 60 µL) were added to a 10 mL round bottom flask, sealed with a rubber septum with a needle for the potential production of HCl gas. The reaction flask was immersed in an ice bath at 5 °C. In a separate vial, 160 µL (0.615 mmol) of tert-butyldichlorodiphenylsilane (54) were added dropwise via syringe to the reaction flask. The mixture was let stir at 5 °C for 10 min. Reaction progress was monitored by TLC (4:1 hexanes:ethyl acetate). Two spots were observed for the starting material 54, resulting in a suspicion of contamination. Thus, the reaction was terminated and stored in the freezer for future analysis.

**Trial 2.**

KOH (57.10 mg, 1.018 mmol), deionized water (260 µL) and MeOH (80 µL) were added to a 10-mL round bottom flask, sealed with a rubber septum with a needle for the potential production of HCl gas. The reaction flask was immersed in an ice bath at 5 °C. In a separate vial, 170 µL (0.654 mmol) of tert-butyldichlorodiphenylsilane (54) were dissolved in 520 µL of diethyl ether. The solution was added dropwise via syringe to the reaction flask. The mixture was let stir at 5 °C and slowly warmed up to room temperature, and reaction from trial 1 was resumed under the same conditions. Reaction progress was monitored by TLC (4:1 hexanes:ethyl acetate). After
2 h, both reactions were treated with an additional equivalent of KOH (57.10 mg, 1.018 mmol) and deionized water (260 µL), and let stir for 26 h in total. Both reaction mixtures were combined and extracted three times with diethyl ether. The organic layers were combined and dried over sodium sulfate. The dried organic fraction was then filtered and concentrated in vacuo. The crude mixture was purified via Prep TLC (4:1 hexanes:ethyl acetate). The desired band (bottom band) was isolated, washed with 100 mL 2% (v/v) MeOH in CH₂Cl₂ and concentrated in vacuo. TLC Rf = 0.78 on silica gel (hexanes:ethyl acetate = 4:1). ¹H-NMR (300 MHz, acetone-d₆): δ 1.03 (s, 9H), 5.54 (s, 1H), 7.40 (m, 3H), 7.80 (m, 2H). ¹³C-NMR (75 MHz, CDCl₃): δ 19.3, 26.5, 127.5, 129.6, 134.6, 134.9. HRMS (EI) Calc’d for C₁₆H₂₀OSi: 256.1283; found 256.1295.

2D-TLC for Decomposition Check of 54.

A small amount of a solution of 54 in acetone was spotted on a TLC plate, which was placed and let develop in a chamber with a mobile phase of 4:1 hexanes:ethyl acetate. The TLC plate was removed from the chamber when the mobile phase was about 1 cm below the top edge of the plate. The migrated spots were marked under a UV lamp. The plate was then rotated 90° and placed back into the chamber, until the mobile phase reached the desired position. The positions of updated spots were marked under a UV lamp.

Synthesis of (S)-1-(trityloxy)-1,2-dihydronaphthalene ((S)-60)⁴⁶
(S)-33 (46.6 mg, 0.319 mmol) was dissolved in 0.3 mL of anhydrous CH₂Cl₂ in an oven-dried round-bottom flask. The solution was let stir at 0 °C under nitrogen. Triethylamine (50 µL, 0.711 mmol), DMAP (3.6 mg, 0.0295 mmol) and trityl chloride (100 mg, 0.359 mmol) were added to the reaction flask. The reaction mixture became orange brown after 1 h of stirring and was allowed to stir at room temperature under nitrogen for 24 h. Reaction progress was monitored by TLC (4:1 hexanes:ethyl acetate and 9:1 hexanes:ethyl acetate). The mixture was diluted with CH₂Cl₂ and deionized water. The organic layer was washed with water and brine three times each. The organic layer was dried over sodium sulfate and filtered. The filtrate was concentrated in vacuo and purified via Prep TLC (4:1 hexanes:ethyl acetate and 9:1 hexanes:ethyl acetate). The desired band was isolated and the starting material band was retrieved. Both bands were washed with 100 mL 2% (v/v) MeOH in CH₂Cl₂ and concentrated in vacuo. The desired product (S)-60 (50.7 mg) was isolated in 41% yield. 22.8 mg of starting material (S)-33 was recovered from Prep TLC purification. TLC Rf = 0.78 on silica gel (hexanes:ethyl acetate = 4:1) and Rf = 0.62 on silica gel (hexanes:ethyl acetate = 9:1) [α]D{sup 25} = -16.6° (c = 1.5, CHCl₃). ¹H-NMR (300 MHz, CDCl₃): δ 1.69 (m, 2H), 4.54 (t, J = 5.6 Hz, 7.3 Hz, 1H), 5.75 (m, 1H), 6.48 (d, J = 9.5 Hz, 1H), 7.06 (d, J = 7.1 Hz, 1H), 7.30 (m, 11H), 7.52 (d, J = 7.4 Hz, 7H); ¹³C-NMR (75 MHz, CDCl₃): δ 30.4, 70.6, 87.5, 126.3, 126.8, 127.3, 127.5, 127.8, 128.1, 128.8, 129.4, 134.6, 136.8. HRMS (ESI) Calc’d for C₂₉H₂₄ONa: 411.1725; found 411.1734.
Synthesis of tert-butyldimethylsilanol (75)$^{45}$

\[
\text{OH} \quad \text{Si} \quad 75
\]

KOH (50.65 mg, 0.904 mmol), deionized water (500 µL) and MeOH (160 µL) were added to a 10-mL round bottom flask, sealed with a rubber septum with a needle for the potential production of HCl gas. The reaction flask was immersed in an ice bath at 5 °C. In a separate vial, 216.0 mg (1.64 mmol) of tert-butylchlorodimethylsilane (TBDMSCl) were dissolved in 1 mL of diethyl ether. The solution was added dropwise via syringe to the reaction flask. The mixture was let stir at 5 °C and slowly warmed up to room temperature. Reaction was let stir for 24 h. Reaction progress was not monitored, since neither reactant nor product was UV-active. The reaction mixture were combined and extracted three times with dichloromethane. The organic layers were combined and dried over sodium sulfate. The dried organic fraction was then filtered and concentrated in vacuo. The desired product was obtained in 33% yield. $^1$H-NMR (300 MHz, CDCl$_3$): δ 0.00 (s, 6H), 0.83 (s, 9H), 2.91 (s, 1H). $^{13}$C-NMR (75 MHz, CDCl$_3$): δ -4.23, 17.0, 24.9. GC-MS: 4.30 min, m/z = 132 (0.02, M), 75 (100, M-t-Bu).

Preparation of complete Dulbecco Modified Eagle’s Medium (DMEM)

One bottle of DMEM powder (Sigma-Aldrich) was dissolved in 800 mL of HPLC grade water. Sodium bicarbonate solution (49.1 mL) and 10 mL of 100x antibiotic-antifungal solution were added to the mixture. The volume was brought to 900 mL and the solution was adjusted to a pH between 7-7.4. Heat-inactivated fetal bovine serum (FBS, 100 mL) were added to the neutralized solution and filtered via a 0.2-µm Corning filter. The medium was stored at 4 °C.
Preparation of Nutrient-Deprived Medium (NDM), Nutrient-Rich Medium (NRM) and Media 1-6

For all medium conditions, the following electrolytes and vitamin solution were added in concentrations as follows: CaCl$_2$ (2H$_2$O), 265 mg/L; Fe(NO$_3$)(9H$_2$O), 0.1 mg/L; KCl, 400 mg/L; MgSO$_4$(7H$_2$O), 200 mg/L; NaCl, 6400 mg/L; NaHCO$_3$, 700 mg/L; NaH$_2$PO$_4$, 125 mg/L; phenol red, 15 mg/L; 25 mM (5.958 g/L) HEPES buffer (pH 7.4); and MEM vitamin solution (1x/L) (Life Technologies, Inc., Rockville, MD), which completed the preparation of the NDM. For NRM, additional nutrients were supplement at concentrations as follows: D-glucose, 1000mg/L; L-glutamine, 2mM; MEM amino acids solution and MEM nonessential amino acids solution (Life Technologies, Inc.), 20 mL and 10 mL respectively for 1-L medium; FBS, 100 mL for 1-L medium. For media 1-6 (Table 3), corresponding combinations of nutrients were added at concentrations identical to those in NRM.

Tumor Cell Culture Procedures

To a cell-containing T-75 culture flask, 2-5 mL aliquot of filtered trypsin-EDTA was added to detach the cells after the aspiration of DMEM and a PBS wash. The trypsin-containing flask was placed in the incubator (37 °C and 5% CO$_2$) for 2-3 minutes and then monitored under a microscope with a 10x amplification. Detached cells were transferred to a 15 mL centrifuge tube and pelleted for 3 minutes at 1000 rpm. The supernatant of the resulting content was aspirated. The cell pellet was re-suspended thoroughly in the complete DMEM and transferred to an appropriately-labeled T-75 flask with additional DMEM to reach a final volume of 25 mL.

General Procedures of Cytotoxicity Assays

*In vitro* cytotoxicity assay: Pancreatic cancer cells (PANC-1, BxPc-3 or Capan-2), ranging from passage number 40-52 for PANC-1 (passage numbers not available for BxPc-3 and
Capan-2), were seeded in 96-well plates at a density of 23,000 cells per well and incubated in a fresh Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) at 37 °C, 5% CO₂ for 24 h. After rinsing with PBS, cells were subjected to the addition of NRM, NDM, or media 1-6. Serially diluted solutions of synthesized compounds (5.5% v/v DMSO in NDM) were added to the cells up to a series of concentrations of 100 µM, 50 µM, 25 µM, 12.5 µM and 6.25 µM, followed by a 24 h incubation at 37 °C, 5% CO₂. Cell morphology was monitored under an inverted microscope. Cytotoxicity was assessed on PBS washed cells by the addition of fresh DMEM containing 10% WST-8 cell counting reagent (Dojindo). Following a 3 h incubation at 37 °C, 5% CO₂, absorbance values were measured with a plate reader at 450 nm, and cell viability was calculated using the equation:

\[
\% \text{ cell viability} = \left[ \frac{\text{Abs}_{\text{test sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}} \right] \times 100.
\]

**Cytotoxicity Assays of Spiroxin A Derivatives against PANC-1**

A replicate experiment for each of the four synthetic intermediates (S)-33, (S)-34, (S)-36 and (S)-37 was performed following the general cytotoxicity assay procedures in NRM and NDM, in order to validate the preliminary data obtained from a former member of our laboratory.²²

Two to three replicate experiments were conducted for each of the following compounds: 32, (R)-33, (R)-34, 34, 55, (S)-60 and 75, following the same assay procedures mentioned above.

**Cytotoxicity Assays of 50 against PANC-1**

A total of 6 assays were performed following the general cytotoxicity assay procedures.⁴⁷ Each assay contained NRM, NDM and two of the six medium conditions in Table 3. Two replicate experiments were conducted for each medium condition in Table 3, and similar results were obtained.
Cytotoxicity Assays of 50 against BxPc-3 and Capan-2

Two replicate assays were performed following the general cytotoxicity assay procedures. Each assay tested the cytotoxicity of 50 against both cell lines in NRM and NDM. Similar results were obtained.
REFERENCES AND NOTES


35. Sigma-Aldrich®

36. Sigma-Aldrich®

37. Experiments performed by Ronghao Zhou '17.


47. Two of the assays were performed by former lab members Maria Jun '14 and Alyssa Bacay '14.

48. With the help of Ronghao Zhou '17.
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16. Replicate cytotoxicity assays for (R)-1,2-dihyronaphthalen-1-ol ($(R)-33$)
17. Replicate cytotoxicity assays for (R)-tert-butyl((1,2-dihyronaphthalen-1-yl)oxy)diphenylsilane ($(R)-34$)
18. Replicate cytotoxicity assays for tert-butyl((1,2-dihyronaphthalen-1-yl)oxy)diphenylsilane (34)
19. Replicate cytotoxicity assays for tert-butylidiphenylsilanol (55)
20. Replicate cytotoxicity assays for (S)-1-(trityloxy)-1,2-dihyronaphthalene ($(S)-60$)
21. Replicate cytotoxicity assays for tert-butylidimethylsilanol (75)
22. Compiled data from cytotoxicity assays under different medium conditions
23. Replicate cytotoxicity assays against BxPc-3
24. Replicate cytotoxicity assays against Capan-2
Dora Carrico-Moniz, FZ-4-11(1)_NaCl  University of Illinois, SCS, Mass Spectrometry Lab

Q-tof_56267c 28 (2.096) AM (Cen,5, 80.00, Ar,15000,0.7,16.46,0.70,LS 3); Sm (SG, 2x5.00); Cm (24:29-5:10x3.000)

1: TOF MS ES+

1.68e4
Blue: Nutrient rich conditions. Red: Nutrient deprived conditions.
TBDPSOH (55)

Cell Viability (%) vs. Concentration (µM)

-20 0 20 40 60 80 100 120

TBDPSOH (55)

Cell Viability (%) vs. Concentration (µM)

-20 0 20 40 60 80 100 120

TBDPSOH (55)