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SYNTHESIS AND STRUCTURE−ACTIVITY RELATIONSHIP STUDY OF INTERVENOLIN, AN ANTITUMOR AND ANTI-*HELICOBACTER PYLORI* QUINOLONE NATURAL PRODUCT

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Abstract – This review article overviews the chemistry and biology of intervenolin, a quinolone natural product bearing unusual substituents. Intervenolin was isolated as a modulator of tumor–stroma interactions, communication between tumor cells and surrounding normal cells to regulate tumor-associated events including proliferation. The synthesis of intervenolin by the author employs thiocyanate–isothiocyanate rearrangement and Suzuki–Miyaura cross coupling as key reactions, which was utilized in structure–activity relationship studies. The SAR studies generated analogs that are effective in vivo. Moreover, several intervenolin and analogs also showed anti-*Helicobacter pylori* activity. Mechanistic aspects of these biological activities, and synthetic study on structurally related natural products by Spring and co-workers are also discussed.

CONTENTS

1. INTRODUCTION

Humans have been striving to cure cancer through chemotherapy, expecting that this life-threatening disease will be made manageable like the common cold. History has highlighted the importance of natural products as a source of anticancer agents, which were mostly cytotoxic substances in the early days of research. It is believed that the differences in the metabolic profiles of tumor and normal cells can rationalize the efficacy of anticancer agents toward the inhibition of tumor cell growth in the presence of normal cells in the human body. However, the use of cytotoxic agents to treat cancer frequently causes detrimental effects on normal cells, thereby resulting in severe side effects.

Recently, targeted therapy using drugs that affect the tumor-relevant machinery of cellular signal transductions has become available to improve the efficacy of chemotherapy for some types of tumors.[1] However, targeted therapy has intrinsic drawbacks arising from the genetic instability of tumor cells, which cause mutations at the molecular target of these drugs and subsequent relapse after treatment completion.

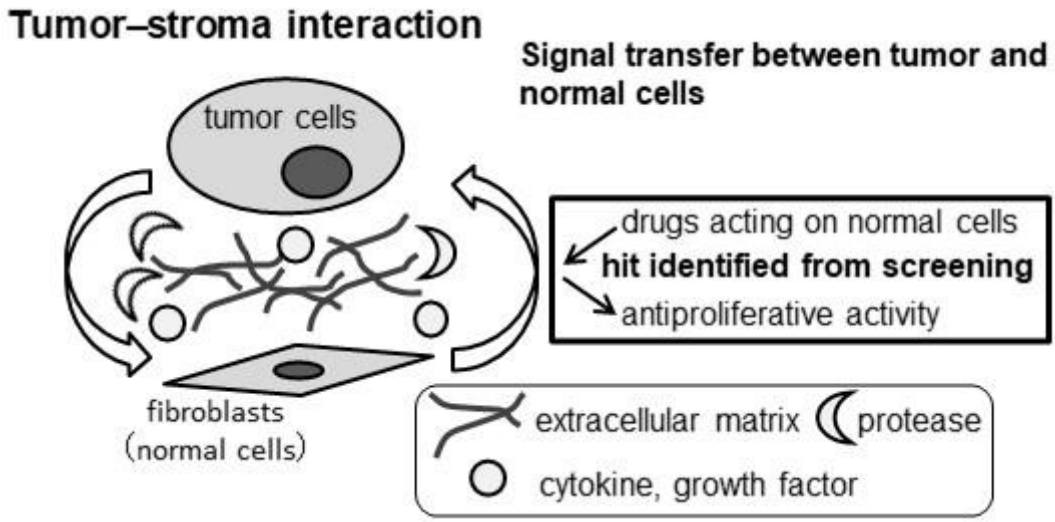


Figure 1. Tumor–stroma interaction

Currently, it is well-recognized that tumor tissues consist of tumor cells and stromal cells, such as fibroblasts, which are normal cells capable of controlling the growth of neighboring tumor cells positively and/or negatively through secreted factors.[2] Kawada elaborated a paradoxical strategy seeking for antitumor seeds acting on normal cells (stromal cells) from natural resources to establish an assay system to pick compounds that can suppress the growth of tumor cells co-cultured with the corresponding stromal cells more potently than monocultured conditions.[3] The hit compounds obtained from the screening campaign are summarized in Figure 1.

The first hit compound was phthoxazolin A (**1**),[4] which was followed by the identification of atpenin derivatives NBRI23477 A (**2**) and B (**3**).[5] Later, atpenins A5 (**4**) and B (**5**)[6] were found to intervene in the

tumor–stroma interaction. NBRI16716 A and B (**6** and **7**, respectively)[7] are other examples of hit compounds.

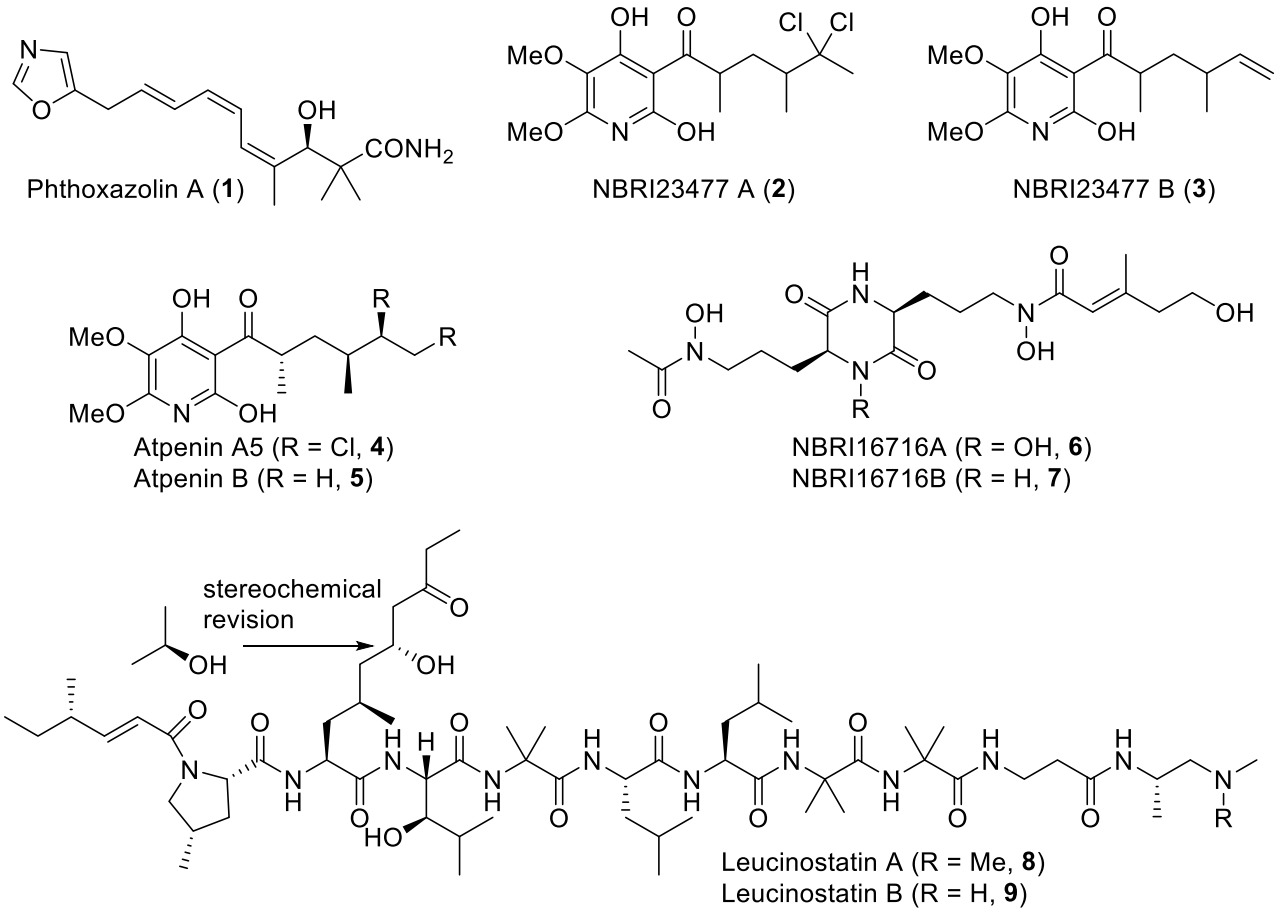


Figure 2. Structures of tumor–stroma interaction modulators with natural origins

Leucinostatin A (**8**) and leucinostatin B (**9**) have been demonstrated to have desirable biological activity.[6] These natural products bearing a nonapeptide backbone containing five unusual amino acids have been particularly investigated on the basis of an assay using prostate tumor cells (DU-145) and their corresponding stromal cells (PrSC). Compound **8** was originally discovered in 1973 by Arai and co-workers[8] as antibacterial[9] and cytotoxic agents,[8,10] which was followed by the investigation of its various biological activities such as phytotoxic,[11] immunosuppressant,[12] and antitrypanosomal[13] activities. X-Ray crystallography studies by Cerrini and co-workers showed that the hydroxy group located on the side chain of the 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMOD) residue has an *S*-configuration.[14] Aiming at establishing an efficient synthetic route that can be applied to investigate the structure–activity relationship (SAR), our group accomplished the total synthesis of **8** under four catalytic asymmetric reaction conditions investigated by Shibasaki and co-workers.[15] During the course of their study, the hydroxy group within the AHMOD residue was determined to exhibit *R* stereochemistry upon comparison of the spectral data, HPLC profiles, and biological activities of synthetic and naturally

occurring samples. It is known that **8** is an inhibitor of complex V in the respiratory chain,[16] which has been recently demonstrated as one of the main factors affecting the tumor–stroma interaction in prostate tumor cells.[17] A SAR study involving alanine scanning identified the importance of each amino acid residue, and reduced biological activity of truncated peptides clearly demonstrates the requirement of the full length of the nonapeptide skeleton.[18]

The latest hit compound obtained from the assay conducted by Kawada and co-workers investigating the role of intervenolin (**10**) as a tumor–stroma interaction modulator (Figure 3).[19] This review provides an overview of the chemistry of intervenolin (**10**), which is supported by a description of its biological background that has been clarified to date.

2. STRUCTURE AND SYNTHESIS OF INTERVENOLIN

2-1. Structure of intervenolin (**10**)

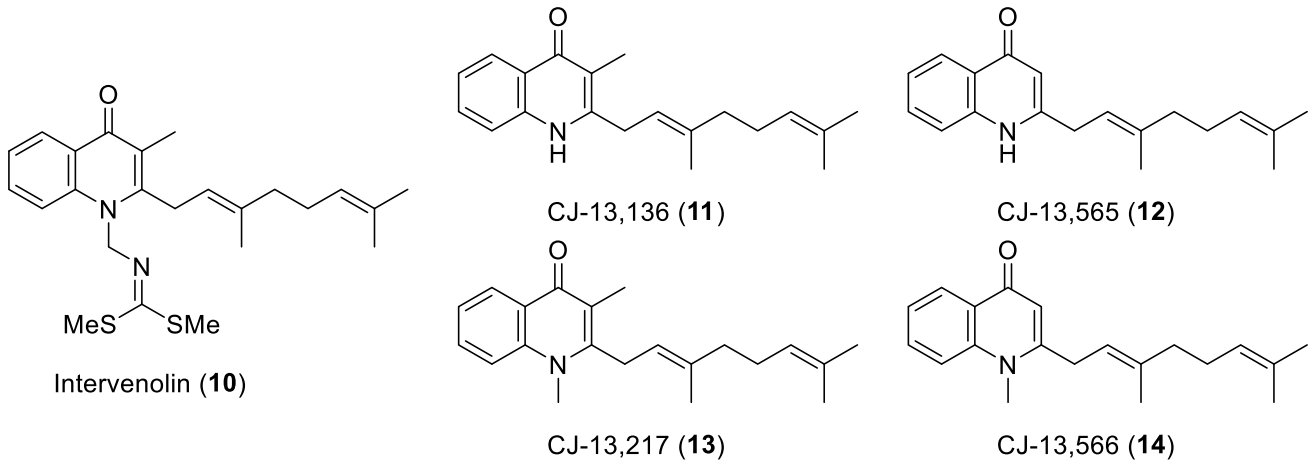
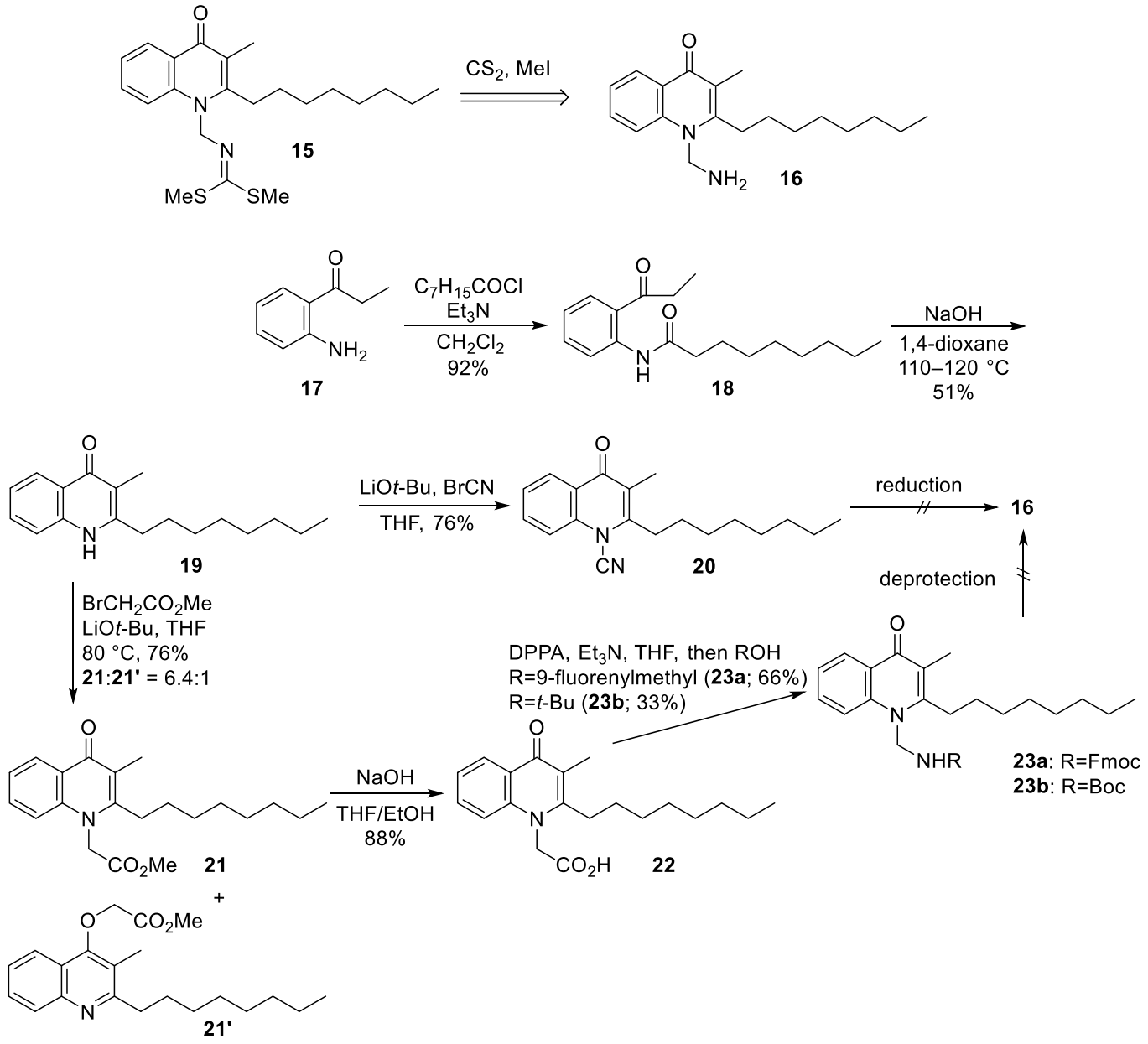


Figure 3. Intervenolin (**10**) and its related compounds

Intervenolin is a hit compound obtained from an assay employing MKN-74 gastric tumor cells and HS738 gastric stromal cells. This natural product was produced by *Nocardia* sp. ML96-86F2, which was found in the culture broth. A conventional isolation protocol and thorough spectroscopic studies have clarified the structure displayed in Figure 3;[19] the 4-quinolone core was substituted and a pendant *N*-[bis(methylthio)methylene]aminomethyl (BMMA) group at the 1-position,[20] a geranyl group at the 2-position, and a methyl group at the 3-position. The BMMA moiety is unfamiliar to natural product chemists. In fact, this partial structure is unknown among any of the natural products isolated to date. This substructure has not been frequently reported in the literature, even in the synthetic field of research. However, it has been reported as a special protecting group for primary amines, thiazole precursors, and β-lactams.[21] At the beginning of this research study, the most common way to construct this structurally characteristic functional group was to treat an amino group with CS_2, followed by *S*-methylation with

MeI. It is also known that the BMMA moiety is labile under acidic conditions. Unexpectedly, geranyl-substituted 4-quinolone compounds are not widely known; the only examples reported to date were discovered during the isolation study of CJ-13,136 (**11**) and three more compounds (**12**–**14**) by a research group at Pfizer in 1998.[22] No synthetic example could be found at the beginning of this study. The only example of structurally related substances has been reported in a patent, which described a series of prenylated derivatives.[23] Limited chemical knowledge of this type of compound made our group realize that the development of an efficient procedure that can be used to furnish 1- and 2-substituents should be key to the successful synthesis of intervenolin.

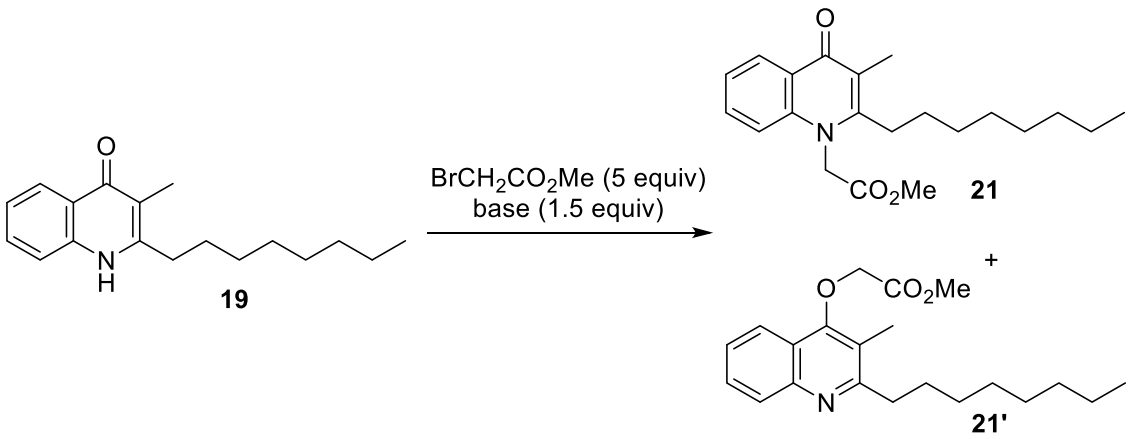
2-2. Synthesis of intervenolin (**10**) by Shibasaki and Watanabe



Scheme 1. Unsuccessful approaches to introduce the BMMA group

Because the establishment of a method to install the geranyl moiety of intervenolin was a major task in the present synthesis, screening for conditions to introduce the BMMA group was carried out using structurally more simplified substrates such as **16** bearing a saturated aliphatic side chain at the 2-position (Scheme 1). It was anticipated that applying the above-mentioned reported procedures[21] to the synthesis of **16** should enable access to **15** bearing a BMMA group in a straightforward manner. Commercially available **17** was acylated under conventional conditions to afford **18**. A base-promoted annulation reaction was then carried out to form the quinolone skeleton and gave **19** (Camps quinolone synthesis).[24] From this intermediate, two approaches were examined to install the aminomethyl group at the 1-position (**16**). Initially, a cyanation reaction was carried out upon treatment with BrCN[25] after the deprotonation of **19** using LiO*t*-Bu to give **20** in good yield. However, various reduction conditions failed to give **16**, only resulted in decomposition.

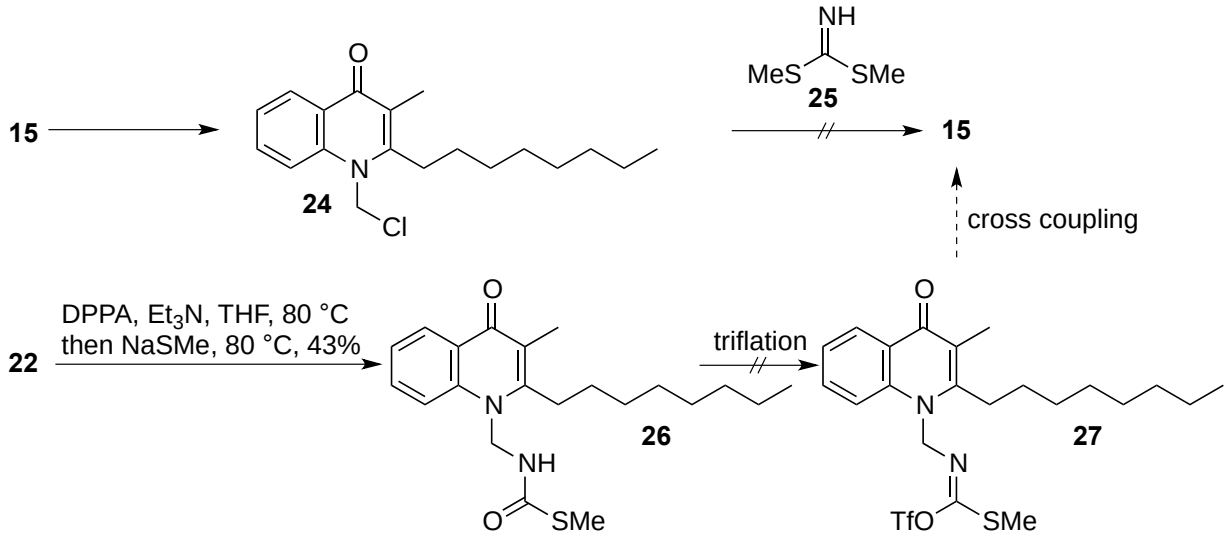
Table 1. Optimization of alkylation conditions for **19**



Entry	Base	Solvent	Temperature (°C)	Yield (%)	**21/21'**
1	LiO*t*-Bu	THF	80	88	6.4:1
2	K_2CO_3	DMF	80	66	1:1.6
3	KO*t*-Bu	DMF	80	59	1:4.8
4	NaH	THF	rt	33	1:3.0
5	LHMDS	THF	rt	12	1.8:1
6	LiH	THF	80	<10	2.0:1
7	*n*-BuLi	THF	–78	trace	nd
8	$Mg(Ot\text{-}Bu)_2$	THF	rt	trace	nd

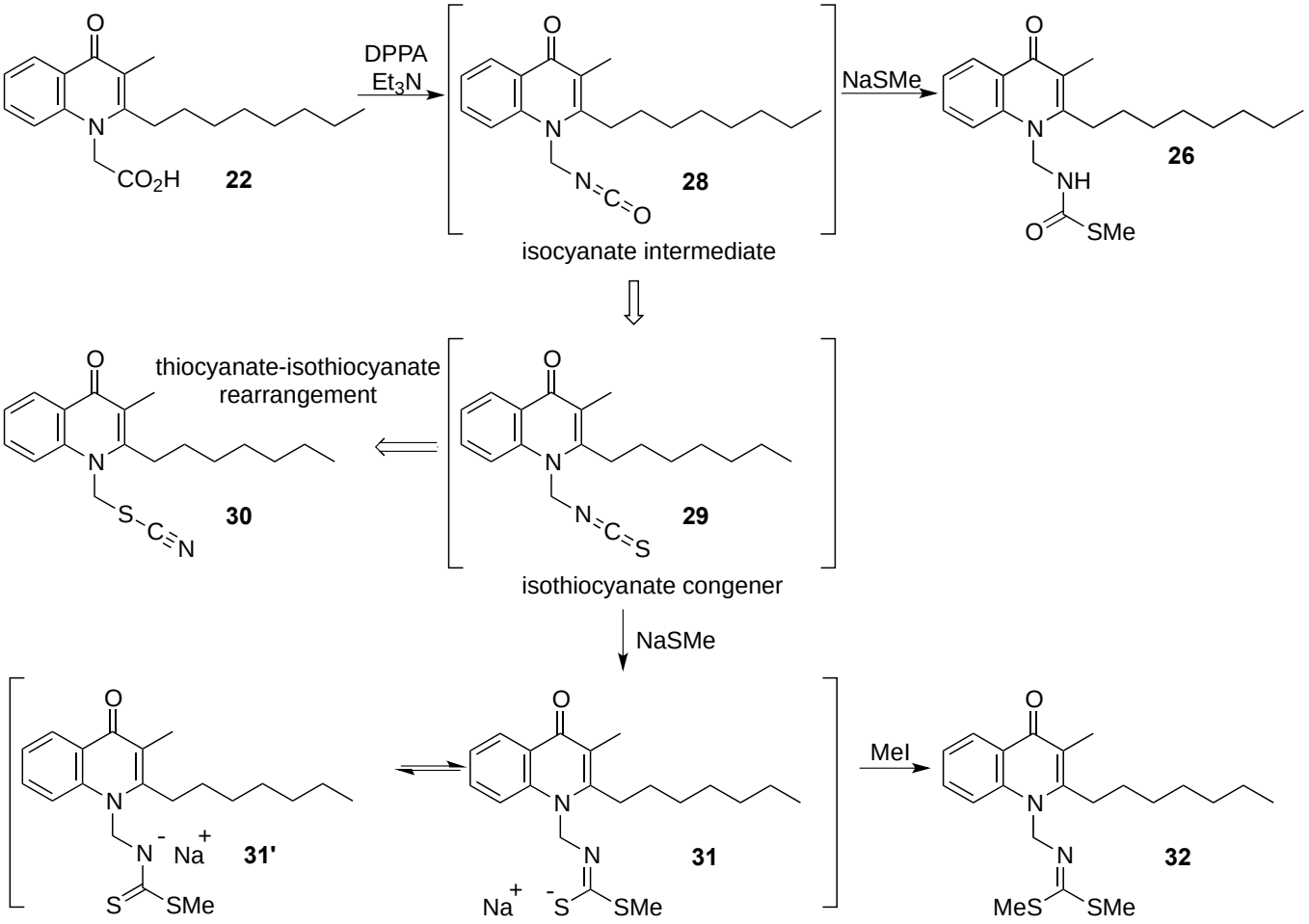
nd: not determined

An alternative stepwise procedure also gave unsatisfactory results. In fact, the acetate unit was successfully introduced on the nitrogen atom of **19** using methyl bromoacetate and LiO*t*-Bu after conducting screening studies under various *N*-selective alkylation conditions (Table 1). According to the literature,[26] the occurrence of *N*-selectivity during the base-promoted alkylation of quinolone compounds or *O*-alkylation depends on the affinity of the counter cation to the carbonyl oxygen atom; *N*-alkylation predominates when a more intimate cation is involved. LHMDS and LiH tend to produce the *N*-alkylated product (**21**) as the major product (1.8:1 (entry 5) and 2.0:1 (entry 6), respectively). However, the use of *n*-BuLi (entry 7) resulted in a trace amount of the desired product. In the same context, Mg(II) as a counter cation prefers functionalization at the oxygen atom, as reported by Fang,[27] although no reaction was observed in the present system. In contrast, the use sodium and potassium bases clearly result in *O*-selectivity, as shown in entries 2–4 of Table 1 (1:1.6 using K_2CO_3, 1:4.8 using KO*t*-Bu, and 1:3.0 using NaH). LiO*t*-Bu was demonstrated to be the optimal base for the preparation of **21**.[27] These optimized alkylation conditions were also used for the synthesis of various intervenolin-related derivatives, which were used in our subsequent SAR studies. However, deprotection of **23a** and **23b** derived from **21** did not proceed. After failing to construct the BMMA moiety using the reported procedure, two alternative approaches were examined (Scheme 2).

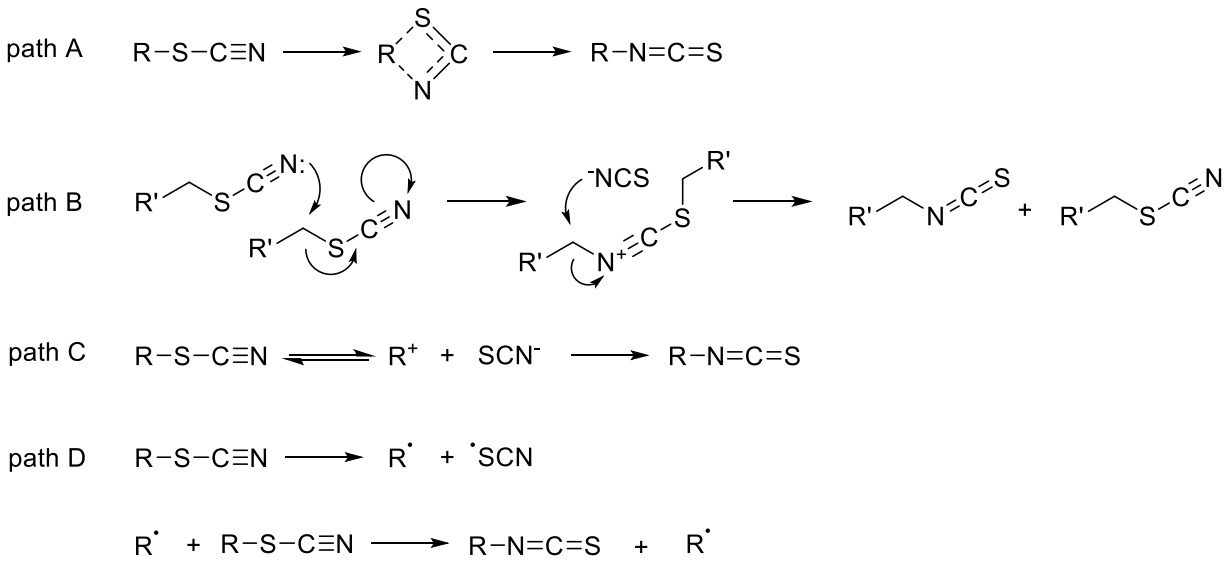


Scheme 2. Unsuccessful approaches to introduce the BMMA group

A simple BMMA unit (**25**) is commercially available. If the direct substitution of the chlorine atom leaving group in **24** with this substance is successful, the desired substructure can be constructed. The chloromethyl group was installed using NaH and $ClCH_2OP{=}O(Ot\text{-}Bu)_2$ to afford **24**,[28] which was then treated with **25**. Unfortunately, none of the desired S_N2 substitution product was observed. Another approach via triflate **27**, followed by an addition–elimination process to incorporate the methylthio group was also unsuccessful. In fact, the triflation step did not proceed.



Scheme 3. Strategy used to construct the BMMA group from isothiocyanate

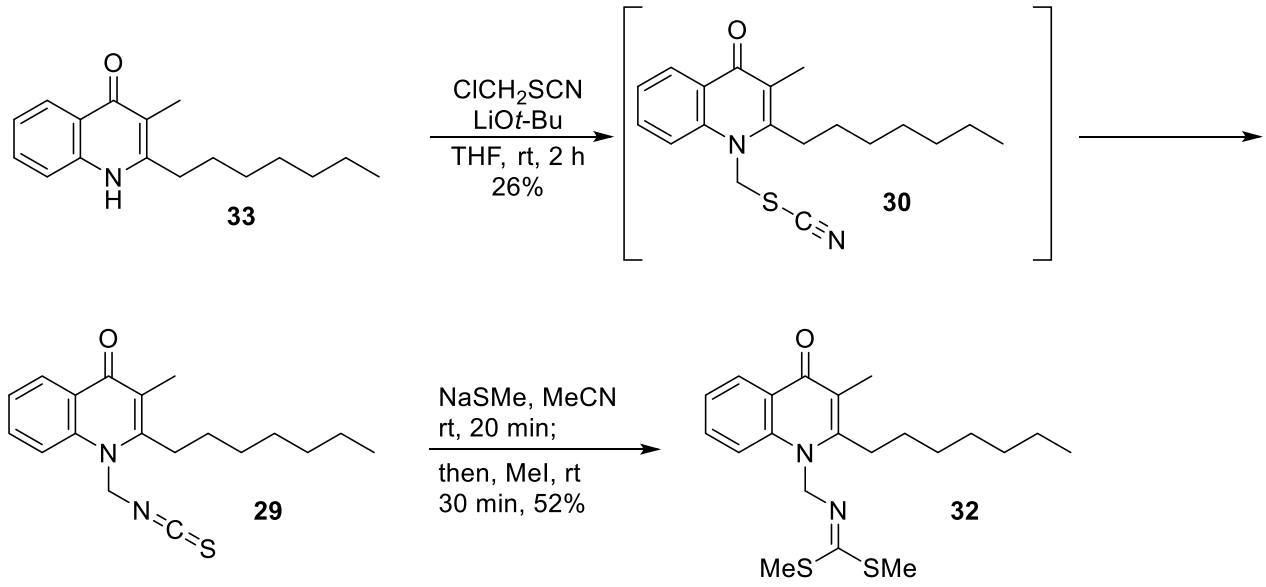


Scheme 4. Postulated mechanism of the thiocyanate–isothiocyanate rearrangement

Although the anticipated reaction schemes displayed above were not unsuccessful, the feasibility of the Curtius rearrangement from **22** gave us another synthetic strategy utilizing isocyanate **28** (Scheme 3). It is well known that the Curtius rearrangement proceeds via this type of intermediate, which is followed by the addition of a nucleophile such as thiolate to give **26**. If the isothiocyanate congener (**29**) is accessible, **31** generated upon the addition of NaSMe can react with electrophiles such as MeI to give BMMA-bearing product **32**. Retrosynthetically, thiocyanate **30** is a possible precursor to **29**.

The transformation of a thiocyanate such as **30** to isothiocyanate **29** is known as the thiocyanate–isothiocyanate rearrangement—a classical thermal rearrangement reaction. Four plausible pathways have been postulated for this reaction, as shown in Scheme 4.[29]

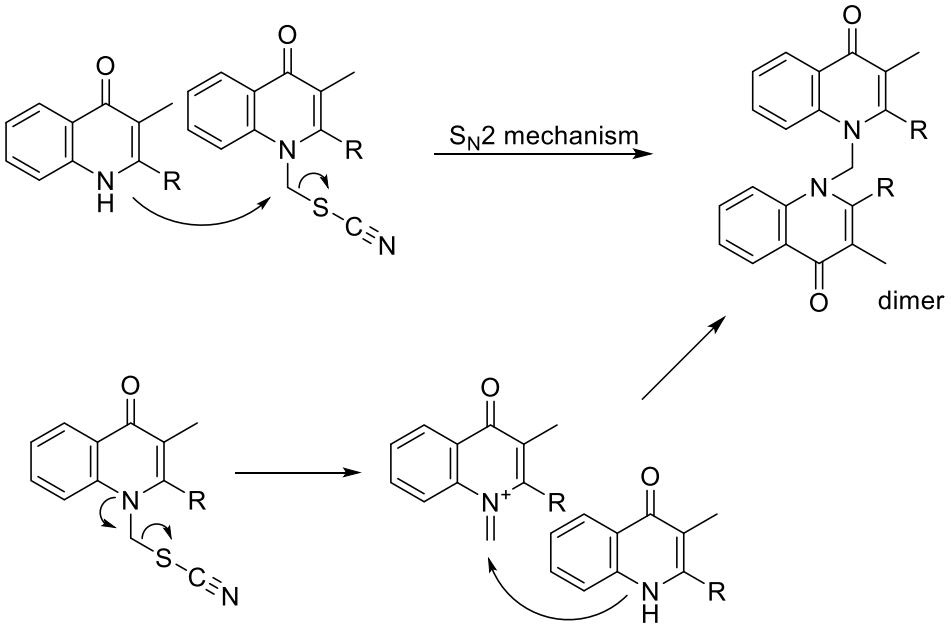
Path A is a concerted mechanism proceeding via a four-membered cyclic transition state. The others are stepwise processes. Path B involves a thiocyanate ion acting as a leaving group upon the nucleophilic addition of the nitrogen atom located at the periphery of another thiocyanate molecule, which is followed by a second nucleophilic substitution reaction by the resultant thiocyanate ion. Overall, one molecule of the isothiocyanate product is produced and one molecule of thiocyanate is recovered. Path C starts with the heterolytic thermal cleavage of thiocyanate to give rise to carbocation and thiocyanate anions. This process is reversible. However, the recombination of the cation and anion is accompanied by C–N bond formation to give the isothiocyanate product, which is no longer under equilibrium. The beginning of path D is a thermal homolysis of thiocyanate, which undergoes a chain reaction process, in which the alkyl radical attacks the nitrogen atom of another thiocyanate molecule.



Scheme 5. Synthesis of an intervenolin analog bearing a saturated aliphatic side chain

The actual synthesis of an intervenolin analog bearing a heptyl side chain at the 2-position is summarized in Scheme 5. The pendant structure at the 1-position of **30** was constructed via the *N*-selective alkylation

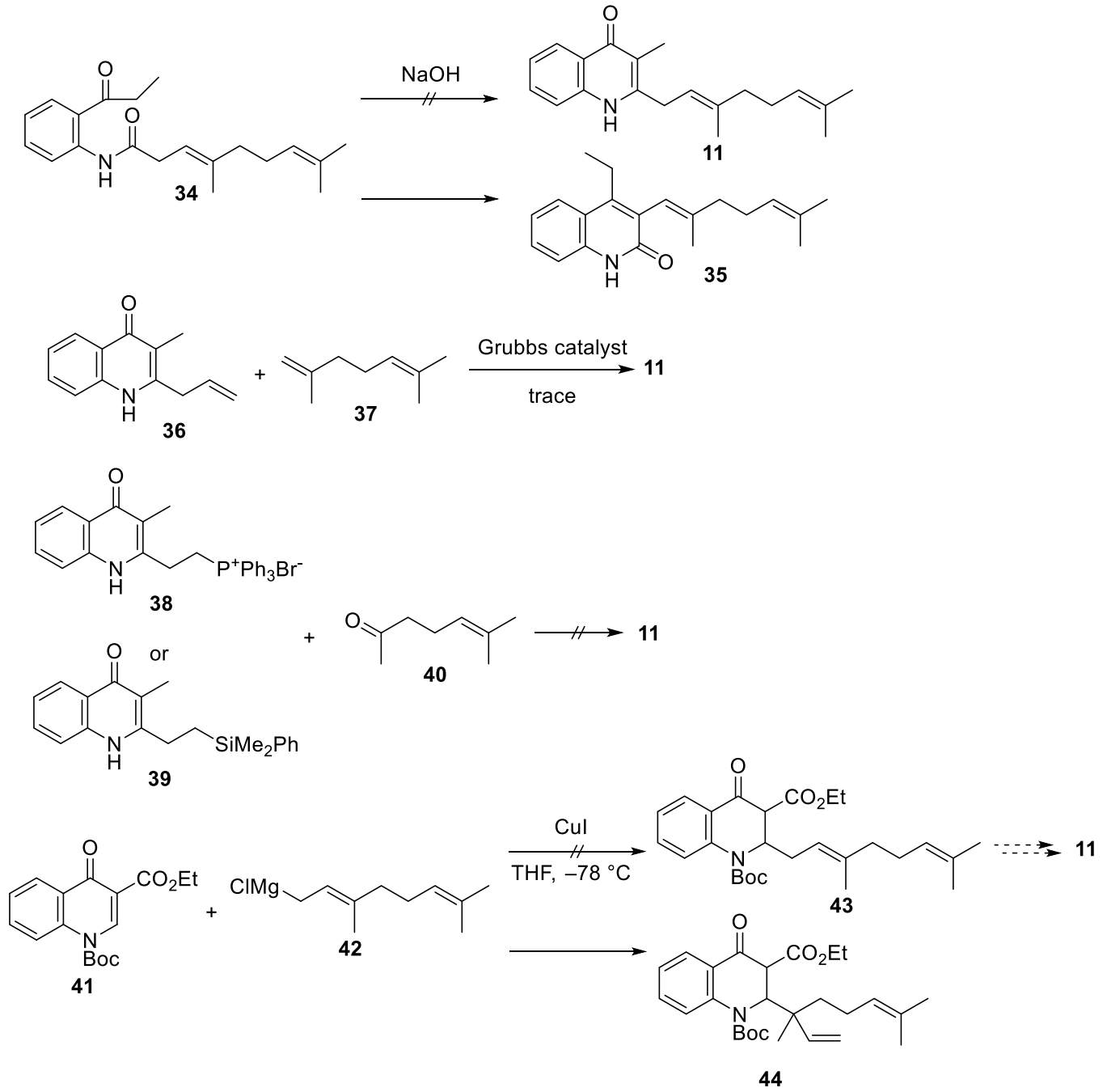
of **33** (whose preparation is described in a later section) employing chloromethylthiocyanate and LiO*t*-Bu as the electrophile and base, respectively. The thiocyanate intermediate (**30**) was not isolated and the rearrangement to thiocyanate **29** occurred spontaneously. The reaction was terminated within 20 min, even at ambient temperature. The isolated yield of **29** was 26%, which was attributed to the formation of the dimer of **29** (Scheme 6), and was in accordance to that reported by Spring et al.[30] This side reaction can proceed via the direct S_N2 reaction between the quinolone starting material (**33** in Scheme 5) and thiocyanate (**30** in Scheme 5), in which the thiocyano group acts as a leaving group. Another possibility is the formation of an iminium intermediate,[30] which is followed by capture by the starting quinolone. Although definitive evidence to determine which is the most plausible pathway has not been obtained, this type of by-product formation can be envisioned to occur during the synthesis of other structurally related analogs. To date, no alternative method to form the BMMA structure has been developed, which is one of the most important future tasks for the synthesis of intervenolin and its related substances.



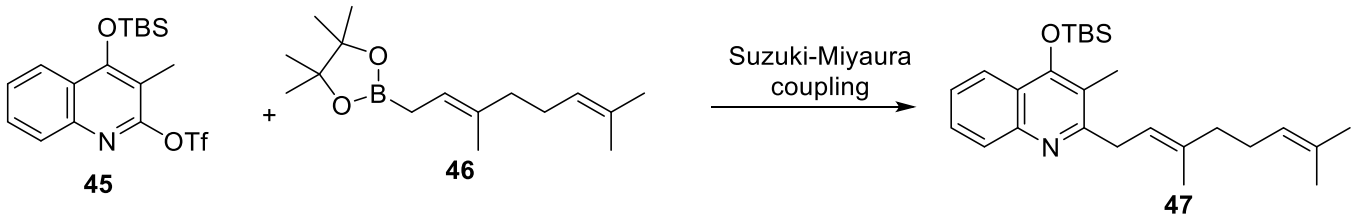
Scheme 6. Dimerization during thiocyanate–isothiocyanate rearrangement

Since the potential strategy to construct the BMMA group in intervenolin was established, the remaining task was to introduce the geranyl side chain at the 2-position. The unsuccessful approaches for this sake are summarized in Scheme 7. The most reasonable strategy appears to be the annulation reaction used in the synthesis of **19**[24] bearing a saturated aliphatic side chain. Substrate **34** was easily prepared and treated with NaOH. However, the basicity of the allylic position of the amide was substantially high and prompted deprotonation to occur at this carbon atom. As a result, the desired 1,4-quinolone compound (**11**) was not observed. Instead, 1,2-quinolone **35** was obtained as the major product. Subsequently, olefin metathesis between **36** and **37** over various Grubbs catalysts was examined. However, only trace amounts

of the condensation products were observed. Conventional olefination processes such as the Wittig and Peterson reactions using **38** and **39**, respectively, with ketone **40** showed no reactivity. The 1,4-addition reaction of Grignard reagent **42** to activated olefin **41** mostly gave γ-addition product **44** (~65% yield) and only a small amount of the desired product (**43**), which was expected to be converted to **11** via oxidation and manipulation of the functional groups.



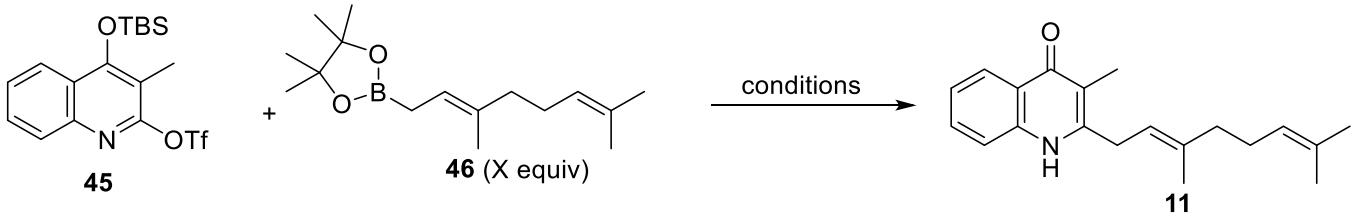
Scheme 7. Unsuccessful approaches to introduce the geranyl side chain at the 2-position of the quinolone core



Scheme 8. Strategy to introduce the geranyl side chain via a Suzuki–Miyaura coupling reaction

Our next approach was the utilization of the Suzuki–Miyaura coupling reaction, as shown in Scheme 8. Because boronate ester **46** has already been reported in the literature,[31] the successful preparation of its triflate counterpart (**45**) bearing a quinoline core (vide infra) will allow the screening of the cross-coupling reaction conditions (Table 2). The deprotection of the corresponding coupling product (**47**) should be uneventful when establishing a protocol for the formation of the geranyl moiety in intervenolin.

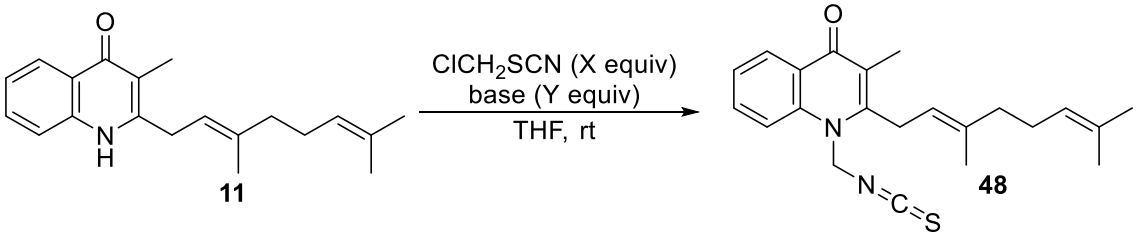
Table 2. Optimization of the Suzuki–Miyaura coupling reaction used to introduce the geranyl side chain



Entry	X	Conditions	Time (h)	Yield (%)
1	1.5	$Pd(PPh_3)_4$ (6 mol%), 2 M $NaHCO_3$, 90 °C, toluene/EtOH (2.5/1)	2	66
2	3.0	$Pd(PPh_3)_4$ (10 mol%), Na_2CO_3 (5.0 equiv), 90 °C, THF/H_2O (2/1)	2	0
3	3.0	$Pd(PPh_3)_4$ (10 mol%), 2 M $NaHCO_3$, reflux, THF/EtOH (2.5/1)	5	10
4	1.3	PEPPSI-IPr (10 mol%), *t*-BuOK (1.3 equiv), rt, 2-propanol	5	0
5	1.5	$Pd(dppf)Cl_2$ (5 mol%), 1 M Na_2CO_3, 90 °C, toluene	8	40
6	1.5	$Pd(dppf)Cl_2$ (5 mol%), 1 M Na_2CO_3, 90 °C, 1,4-dioxane	3	27
7	3.0	$Pd(PPh_3)_2Cl_2$ (10 mol%), 2 M $NaHCO_3$, 90 °C, toluene/EtOH (5/1)	4	70

The reaction did not stop during the coupling process with desilylation taking place to afford the coupling product containing the desired quinolone structure (**11**). For the combination of substrates used in the present study, **45** and **46** prefer $NaHCO_3$ as the base and a mixed solvent system consisting of toluene and EtOH (entries 1 and 7). Pd(0) ($Pd(PPh_3)_4$, entry 1) and Pd(0) species generated in situ from Pd(II) ($Pd(PPh_3)_2Cl_2$, entry 7) resulted in almost the same isolated yield (66% and 70%, respectively) although the former required less boronate ester (1.5 vs. 3.0 equiv) and a reduced reaction time (2 vs. 4 h). The choice of the solvent system was critical even for the reaction system based on the same component as those of entry 1 (entry 3); THF/EtOH gave the desired product in only 10% yield even when using 3 equiv of **46** and prolonging the reaction time (5 h). Other solvent systems and catalysts displayed varying product yields.

Table 3. Fine-tuning of the thiocyanate–isothiocyanate rearrangement used for the synthesis of intervenolin (**10**)

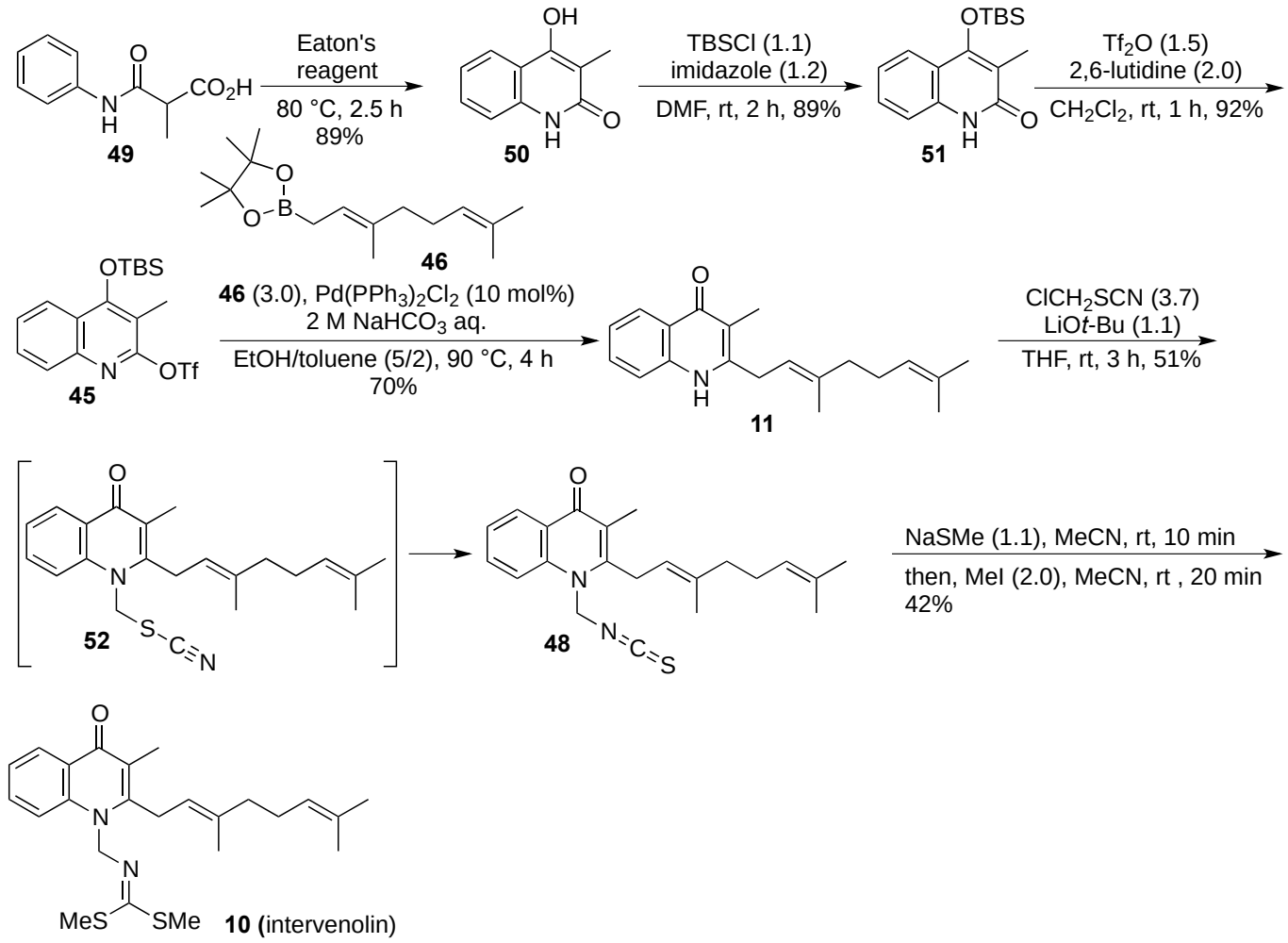


Entry	X (equiv)	Base, Y (equiv)	Time (h)	Yield (%)
1	5.0	LiO*t*-Bu, 1.5	3	50
2	1.5	LiO*t*-Bu, 2.0	4	23
3	4.0	LiO*t*-Bu, 1.5	2	50
4	3.0	LiO*t*-Bu, 1.5	2	36
5	3.7	LiO*t*-Bu, 1.5	2	51
6	3.7	LiO*t*-Bu, 1.5	6	32
7[a]	3.7	LiO*t*-Bu, 1.5	6	18
8	3.7	LiO*t*-Bu, 1.1	3	51
9	3.7	LiH, 1.5	2	15

[a] run at 0 °C

With the geranyl-substituted quinolone intermediate (**11**) in hand, fine-tuning of the thiocyanate–isothiocyanate rearrangement was carried out. Entry 1 in Table 3 was performed under exactly the same reaction conditions to those used for model substrate **19** (5 equiv of chloromethylthiocyanate and 1.5 equiv of LiO*t*-Bu at ambient temperature for 2 h). In the present case, the isolated yield of **48** was still

moderate, but increased to 50%. The use of a higher amount of the base compared to that of the electrophile (2.0 vs. 1.5 equiv) lowered the conversion (entry 2, 23% yield). Entry 3 clearly shows that a reduced amount of chloromethylthiocyanate (4 equiv) and shorter reaction time (2 h) essentially gave the same product yield (50%), whereas 3 equiv of thiocyanate negatively affected the conversion (entry 4, 36% yield). Reaction conditions employing an excess amount of thiocyanate were required because of the presence of the competing dimerization side reaction shown in Scheme 6. Finally, 3.7 equiv was found to be the optimal amount of thiocyanate used in the reaction (entry 5, 51% yield). Prolonging the reaction time up to 6 h resulted in a lower yield because of product decomposition (entry 6, 32% yield). Decreasing the reaction temperature to 0 °C decreased the reaction rate, and 50% of the unreacted substrate (**11**) was recovered (entry 7). Moreover, a small excess of LiO*t*-Bu (1.1 equiv) gave the product in 51% yield over 3 h (entry 8). Once again, a detrimental effect was observed when a different base with a lithium counter cation (LiH) was used (entry 9). From this study, the reaction conditions described in entry 8 were considered to be the most appropriate.



Scheme 9. Synthesis of intervenolin (**10**) by Watanabe and Shibasaki

The establishment of the two key reactions to introduce the unusual characteristic structural units in intervenolin will enable the completion of the first chemical synthesis of intervenolin (Scheme 9). The half amide of 2-methylmalonic acid[32] **49** was subjected to the intramolecular Friedel-Crafts reaction using Eaton's reagent[33] (a methanesulfonic acid solution of P_2O_5) to construct the 2-quinolone core in **50**. The hydroxy group of **50** was protected as a TBS ether found in **51**, which was followed by triflation to afford **45**. The introduction of geranyl and isothiocyanate side chains was completed as described above. The BMMA group was furnished using a two-step one-pot procedure: the addition of the nucleophile (NaSMe) and the subsequent substitution with MeI. Both steps were completed almost instantaneously to accomplish the synthesis of intervenolin (**10**) in 42% yield.[34]

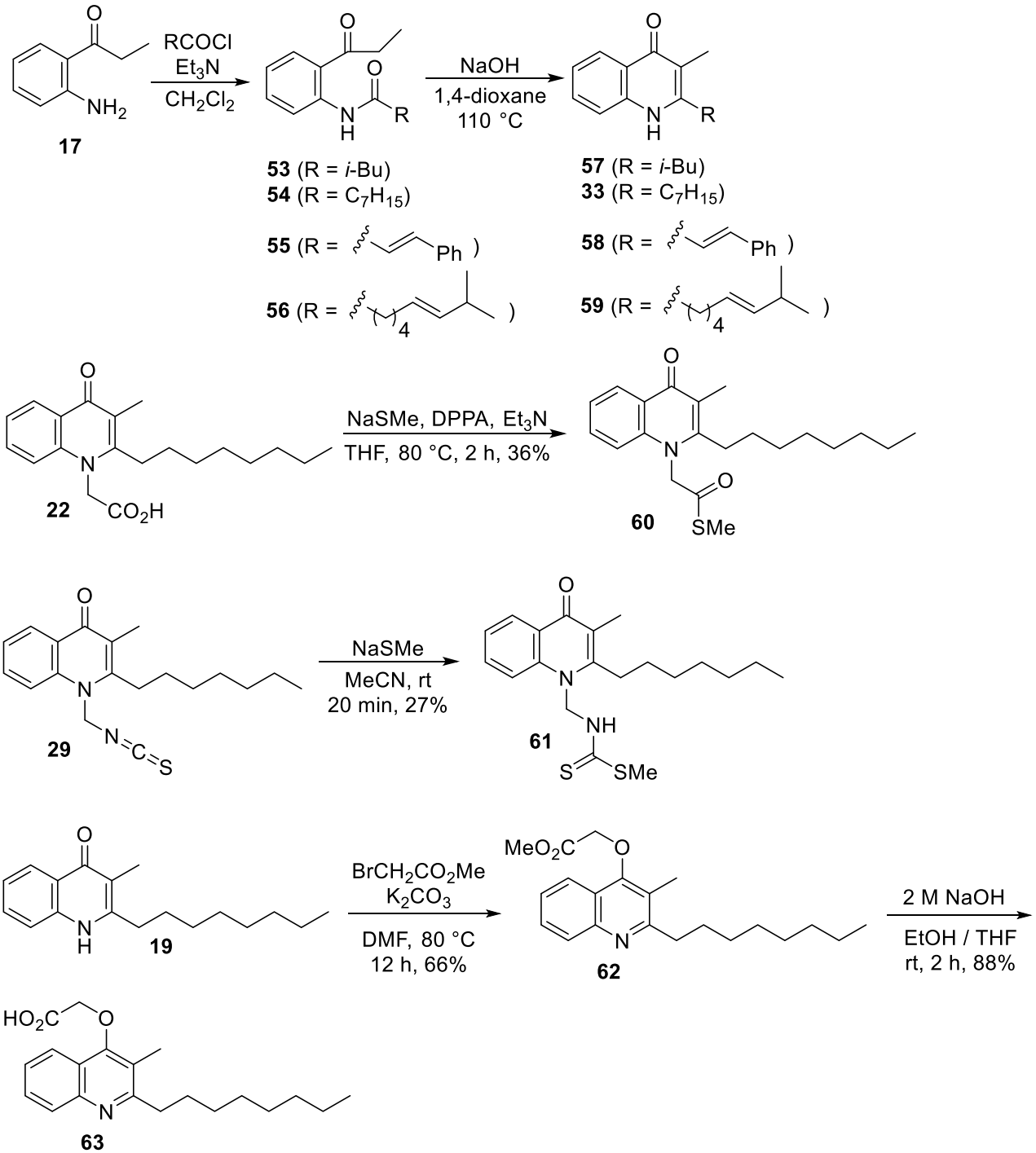
It is noteworthy that the synthetic protocol is reproducible and is not limited to the synthesis of a small amount of material. To date, the protocol has been used to obtain 893 mg of intervenolin, which showed no significant issues during the scale-up of each reaction step. Moreover, various biological properties have become apparent after establishing the above-mentioned preparative method to afford a substantial amount of intervenolin. The biological activities of the natural and synthetic samples were determined to be identical; the IC_{50} value of 0.17 μg/mL was observed for the growth inhibition of human gastric tumor cells (MKN-74) in the presence of gastric stromal cells (Hs738).

3. STRUCTURE–ACTIVITY RELATIONSHIP STUDY OF INTERVENOLIN-RELATED COMPOUNDS FOR INVESTIGATING THEIR ANTIPROLIFERATIVE AND ANTITUMOR ACTIVITIES

Efforts toward generating an array of structurally related intervenolin analogs are potentially important, through which lead compounds used for the development of antitumor medicines with improved physicochemical characteristics and comparable or even more potent biological activities are expected to be obtained. At the time of starting this SAR study, only limited knowledge on the chemical and metabolic stability, toxicity, and in vivo activity was available and all parts of the structure of intervenolin were targets for structural modification in a manner similar to random screening. In particular, the chemical instability of the BMMA group was highly suspected and surrogates for this pendant structure were investigated. Moreover, the optimization of the side chains at the 2- and 3-positions appeared feasible. Consequently, a first-generation SAR study was carried out focusing on these two structural moieties.[35]

To this end, several analogs were synthesized, as shown in Scheme 10. For structural variants with substituents at the 2-position, the methyl group at the 3-position was fixed, which led to the formation of **57**, **33** (Scheme 5), **58**, and **59**. The synthetic route was essentially identical to that used for the preparation of octyl analog **19** (Scheme 1). When this SAR study was undertaken, the protocol for

installing the BMMA moiety had not been completely established. Therefore, analogs without this substituent were included in our biological assay. In addition to these synthesized compounds, known quinolones **64–67** (Figure 4) were obtained according to the literature and applied in our biological assay.



Scheme 10. Synthesis of various intervenolin analogs

In our SAR study, the pendant structure at the 1-position using substituents bearing carbonyl or thiocarbonyl, and methylthio groups separated from the quinolone core by one carbon atom (**60** and **61**)

was also considered. Compound **60** was obtained from **22** via thioester formation with DPPA, as shown in Scheme 10. Many synthetic intermediates that are classified as methylthio-bearing analogs were also tested for their biological activities. Moreover, changing the base to one with potassium as the counter cation during the introduction of the acetate unit resulted in an *O*-alkylated analog (**62**), as expected. The alkaline hydrolysis of the ester functionality smoothly proceeded to afford carboxylic acid **63**. A comparison of the biological activities of the *N*- and *O*-alkylated congeners will clarify the importance of the quinolone core in intervenolin (**10**).

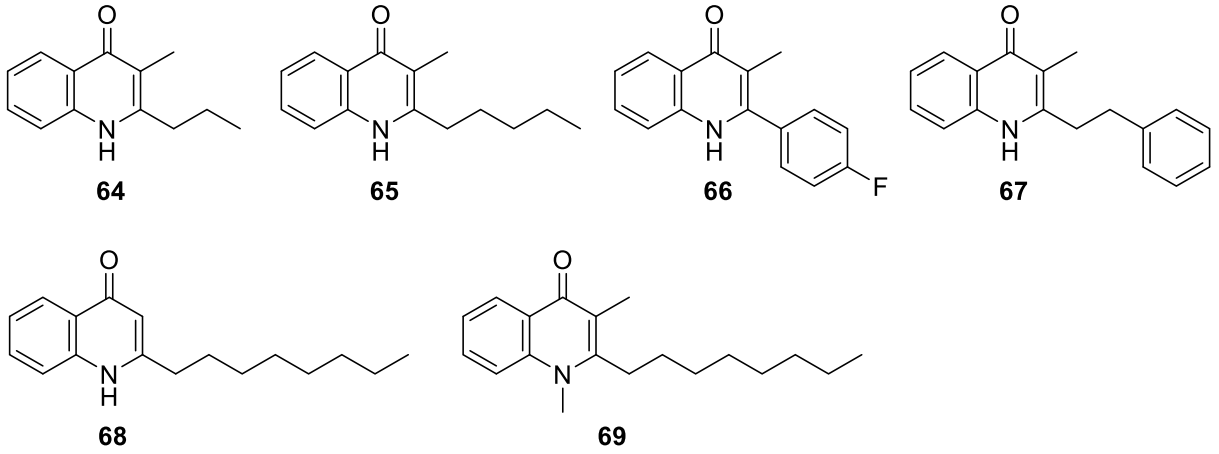


Figure 4. Reported quinolone compounds examined in this study

We next examined the antiproliferative activity of these analogs using human gastric cancer cells (MKN-74) in the presence (co-culture) or absence (monoculture) of their corresponding stromal cells (Hs738) and compared it with that of intervenolin (**10**) (Table 4). In addition, the acute toxicity of these analogs in mice was investigated (MTD = maximum tolerated dose). Most of the compounds found in Table 4 do not have the BMMA moiety because of the difficulties in developing our synthetic method to introduce this group. Therefore, CJ-13,136 (**11**) was set as the tentative mother compound in this SAR study. In fact, CJ-13,136 displayed ~10-fold higher potent activity under both co-culture and monoculture conditions than intervenolin (**10**). Analogs **64**, **65**, **33**, and **69** clearly showed the influence of the chain length of the aliphatic substituent; the octyl derivative was the most potent under the co-culture conditions, which was comparable to CJ-13,136 (**11**) bearing a geranyl side chain. Compound **64** (propyl) displayed 1000-fold lower potency than **11**, and the IC_{50} values of the pentyl (**65**) and heptyl (**33**) analogs were ~30- and ~70-fold higher under the co-culture conditions than those under monoculture conditions; the length of the side chain has a positive impact on the activity of the analogs tested in this study. Another three-carbon aliphatic (isopropyl) substituent (**57**) was proven to be preferable, which was 10 times more potent than **64**.

At this point, it is worth noting that analogs with saturated aliphatic side chains were selective inhibitors under the co-culture conditions compared to intervenolin (**10**) and CJ-13,136 (**11**) bearing an unsaturated alkyl moiety (geranyl group); the former exhibits no antiproliferative activity under the monoculture conditions, which may suggest that the growth signals within the tumor cells are insensitive to the presence of analogs containing saturated side chains. In contrast, substantial acute toxicity (although not too high) was observed for the above-mentioned analogs.

Table 4. Antiproliferative activity and acute toxicity of intervenolin (**10**) and its related analogs

Compound	IC_{50} (μg/mL)		MTD
	cocultured	monocultured	(mg/kg)
10 (intervenolin)	0.17	3.0	>50
11 (CJ-13,136)	0.010	0.25	2.5
64	14.13	>100	NT
57	1.32	>100	25
65	0.37	>100	12.5
33	0.71	>100	6.25
19	0.011	>100	12.5
66	>100	>100	6.25
67	4.30	>100	12.5
58	>100	>100	12.5
59	0.012	>100	1.56
68	0.21	>100	6.25
69	0.21	2.36	>50
20	0.007	>100	6.25
21	0.53	>100	6.25
60	1.40	5.84	>50
22	4.15	71.0	>50
26	0.27	5.84	>50
29	0.12	90.0	6.25
61	2.91	40.0	>50
32	0.78	1.36	>50
62	0.35	28.1	>50
63	0.37	2.95	>50

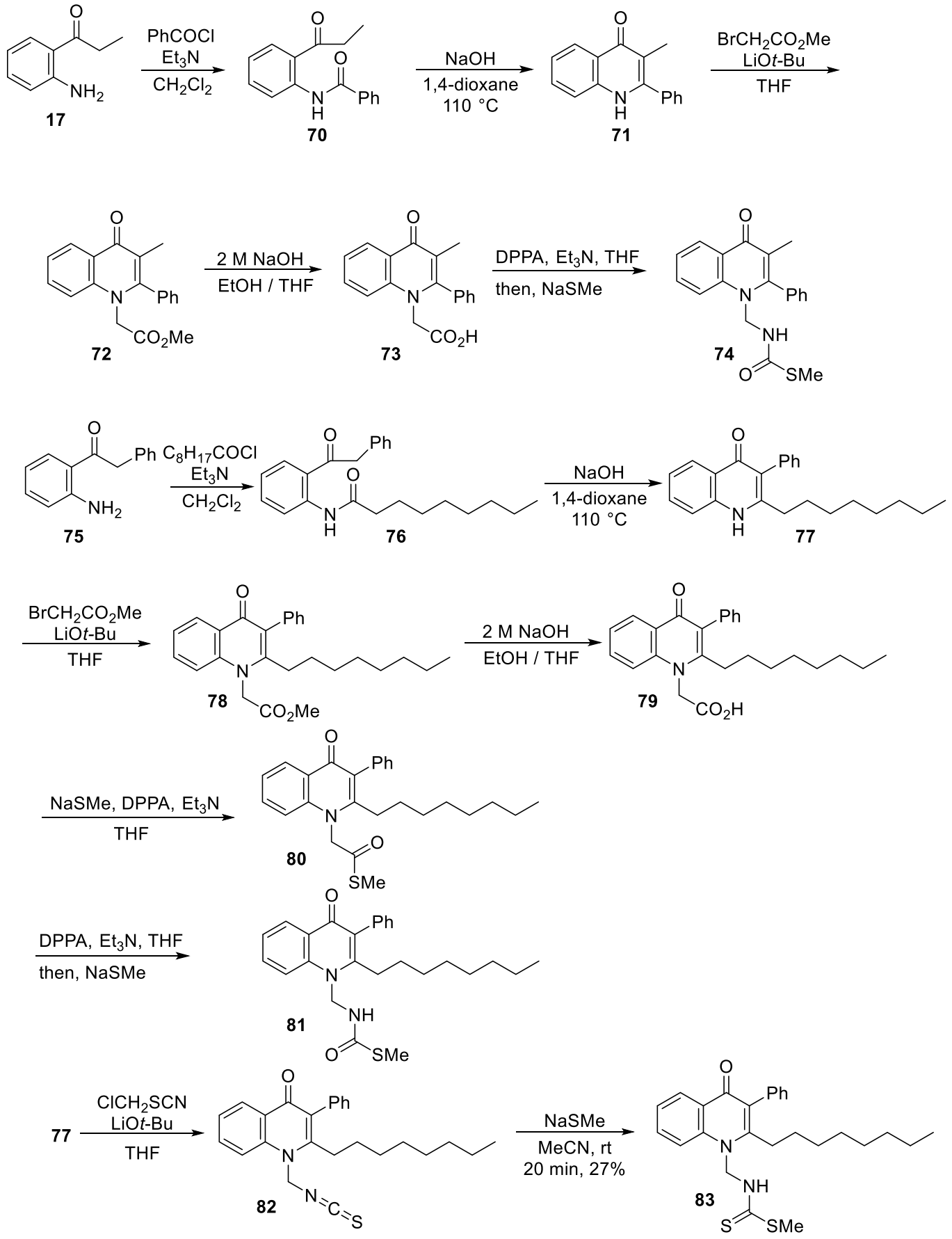
p-Fluorophenyl (**66**), homobenzyl (**67**), and styryl (**58**) groups were introduced to clarify whether it was possible to incorporate an aromatic system at the 2-position, which resulted in a substantial loss of antiproliferative activity.[36] The effect of aromatic substituents was more thoroughly examined in a different set of SAR studies (vide infra). In contrast, unsaturation far from the quinoline core appears to be tolerated; analog **59** was found to be a potent inhibitor of tumor cell growth under the co-culture conditions, which exhibited almost the same activity that CJ-13,136 (**11**) and its octyl analog (**19**) showed.

Consequently, we focused our SAR study toward investigating the effect of the presence of the methyl group at the 3-position and pendant structures located on the nitrogen atom of the quinoline core. For analogs bearing an octyl side chain, the removal of the methyl group (**68**) and methylation at the quinolinone nitrogen atom (**69**) reduced the activity by 1/20th of that of intervenolin (**10**). In many cases, the introduction of an acetate unit at the 1-position drastically changed the biological properties of the resulting analogs. In fact, the installation of the thioester moiety (**60**) reduces the antiproliferative activity under the co-culture conditions (by ~1/130th of that of **19**), however, the lowered acute toxicity was observed. This trend was consistent for the free carboxylic acid (**22**) and thiocarbamate (**26)** analogs, whereas the methyl ester derivative (**21**) retained the properties observed for the former analogs.

Heptyl analogs bearing dithiocarbamate (**61**) and BMMA (**62**) groups also exhibited reduced growth-inhibitory activity, compromised selectivity between culture conditions, and disappearance of acute toxicity. Different trends were observed for analogs bearing relatively reactive substituents on the nitrogen atom including cyano (**20**) and isothiocyanate (**29**) groups, which exhibit potent activity and relatively high acute toxicity.

It was also surprising that the analogs with a quinoline core (**62** and **63**) exhibited biological properties similar to those of the above-mentioned *N*-substituted analogs, which included compromised growth inhibition, reduced selectivity, and low toxicity. This type of compounds having a different core structure is sometimes important as a back-up compound during drug development.

In the second part of our SAR study, we focused on introducing aromatic substituents at the 2- and 3-positions. The synthetic protocol used to prepare these analogs is summarized in Scheme 11, which is essentially the same as that shown in Scheme 10. In our previous study, the *p*-fluorophenyl group at the 2-position results in a complete loss of biological activity.



Scheme 11. Synthesis of intervenolin analogs bearing phenyl groups

In the present assay (Table 5), even phenyl substituents at 2-position did not exhibit any antiproliferative activity in the presence of any pendant structure on the nitrogen atom (**72–74**). The introduction of a phenyl group at the 3-position was more favorable compared to the use of its 2-position congeners; the reduction in growth inhibition remained at ~1/10th of that observed for intervenolin (**10**) depending on the substituent at the 1-position. Acetate units with methyl ester (**78**) and dithiocarbamate (**81** and **83**) motifs were allowed. However, the selectivity toward the co-culture conditions was marginal (see data from analog **81**). Acute toxicity was not observed for these six derivatives. Overall, based on our SAR study on the 3-substituent, intervenolin analogs with favorable characteristics can still be afforded, which should be the subject of future investigation.

Table 5. Antiproliferative activity and acute toxicity of intervenolin (**10**) and its structurally related analogs bearing aromatic substituents

Compound	IC_{50} (μg/mL)		MTD
	cocultured	monocultured	(mg/kg)
10 (intervenolin)	0.52	3.9	>50
72	>10	>10	>50
73	>10	>10	NT
74	>10	>10	>50
77	>10	>10	NT
78	5.9	>10	>50
79	>10	>10	>50
80	>10	>10	NT
81	4.3	9.3	>50
83	5.9	~10	NT

Having established the large-scale applicability of the synthetic method for the preparation of intervenolin and analogs, we next investigated the antitumor activities of the synthetic compounds by using a mouse model inoculated with tumor cells. Five-week-old female BALB/c mice were inoculated with human gastric cancer cells (MKN-74). Then, 12.5 mg/kg of synthetic intervenolin (**10**) was administered for 3 weeks with the intervals depicted in the left panel of Figure 5. The tumors were surgically dissected and their volumes were measured and compared to that of the control.

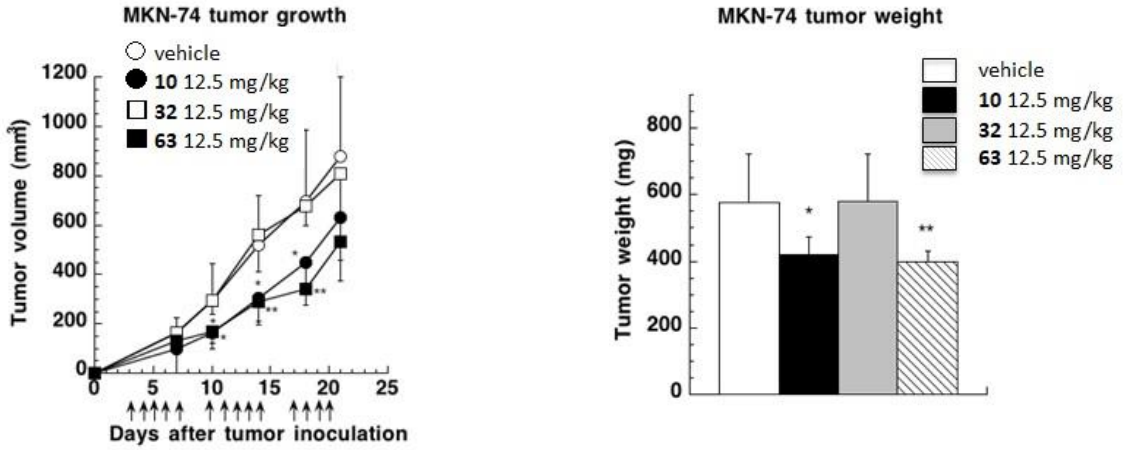


Figure 5. Antitumor activity of synthetic intervenolin (**10**) in a mouse model

As shown in the right panel of Figure 5, an ~30% reduction in the tumor volume was detected, which clearly demonstrated that intervenolin (**10**) exhibits moderate in vivo antitumor activity. However, no in vivo antitumor activity was observed intervenolin analog substituted with a heptyl chain (**32**), which may reflect the reduction of the antiproliferative activity to 1/4th of that observed for **10**, as shown in Table 4. Once again, it is surprising that an intervenolin analog with a quinoline core and a methyl acetate unit linked to the oxygen atom exerted comparable antitumor activity in mice. These results support the applicability of **63** as a back-up compound for developmental studies on antitumor agents.

Naturally, the antiproliferative activity of these compounds toward cell lines derived from diverse tumors in the absence of their corresponding stromal cells was investigated using selected examples shown in Figure 6. Other than MKN-74 cells with or without Hs738 cells described above,[37] the data for BxPC-3 pancreatic cancer cells (and PS pancreatic stromal cells), DU-145 human prostate cancer cells (and PrSC prostate stromal cells), BSY-1 human breast cancer cells (and Hs371 breast stromal cells), HCT-15 human colorectal cancer cells (and CCD-18Co colorectal stromal cells), and A549 human lung cancer cells (and NHLF lung stromal cells) are also shown in Figure 6.

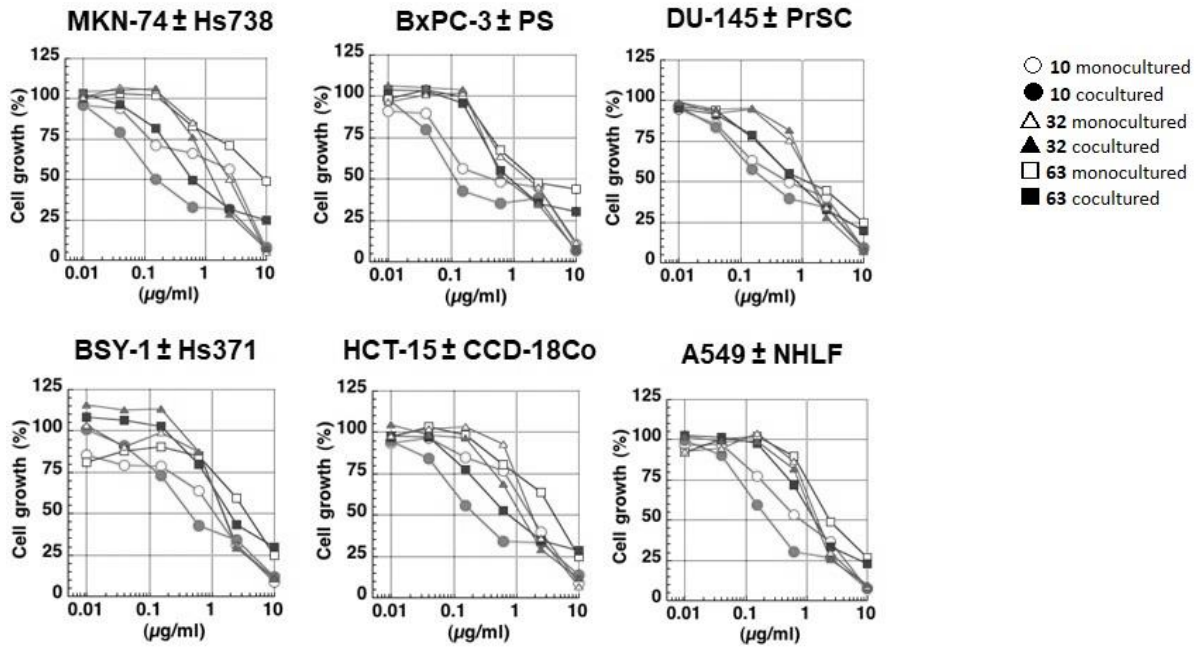


Figure 6. Antiproliferative activity of intervenolin (**10**) and its analogs toward selected cell lines from various types of tumors in the presence or absence of their corresponding stromal cells

The antiproliferative activity was investigated using the intervenolin analog bearing a saturated heptyl side chain (**32**) and the quinoline-based analog bearing an octyl side chain (**63**). For all the tested cell lines, intervenolin (**10**) exhibited the most potent response. Among these, IC_{50} values comparable to those observed with MKN-74 cells were recorded in the experiments using BxPC-3 pancreatic cancer cells although the selectivity toward the co-culture over monoculture conditions was not high. Even so, pancreatic cancer is typically diagnosed at an advanced stage because of its ambiguous symptoms and a complete cure is rarely attained, which necessitates the development of chemotherapeutic agents that are effective for this type of cancer. The next-generation SAR studies should start from this compound and should be guided by the evaluation of the growth inhibition of BxPC-3.

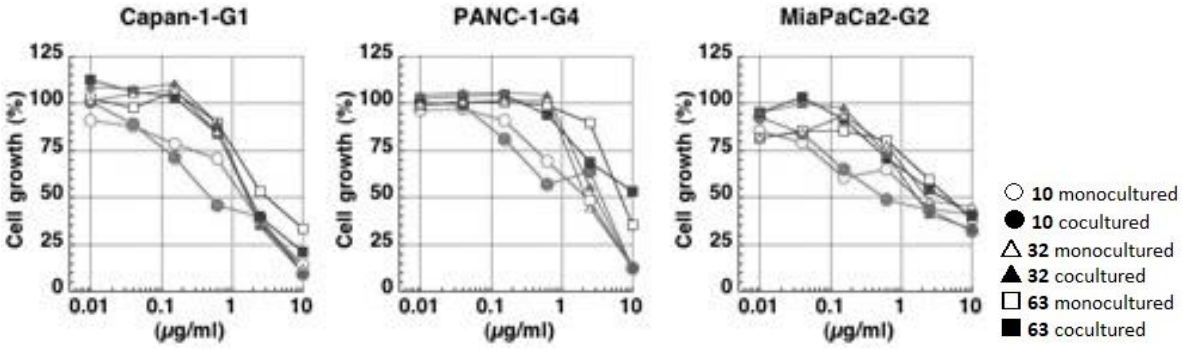


Figure 7. Antiproliferative activity of intervenolin (**10**) and its analogs toward selected cell lines from various pancreatic cancers in the presence or absence of their corresponding stromal cells (PS cells)

At this point, three more cell lines from pancreatic cancers (Capan1-G1, PANC-1-G4, and MiaPaCa2-G2) co-cultured with PS stromal cells were used in the assay to evaluate **10**, **32**, and **63** in detail (Figure 7). Only intervenolin (**10**) exhibited an IC_{50} value of <1 μg/mL for all the studied cell lines. However, the selectivity toward the co-culture conditions was consistently low. Even for **32** and **63**, the IC_{50} values for these cell lines were only slightly higher than those obtained for the system employing MKN-74 cells. Overall, intervenolin (**10**) and its analogs are promising lead compounds for long-awaited effective clinical medicines used to treat pancreatic cancer.

For the DU-145 + PrSC, BSY-1 + Hs371, HCT-15 + CCD-18Co, and A549 + NHLF systems, the same trend in the inhibitory activity of tumor growth in the presence of stromal cells was observed (Figure 6); **10** was the most potent, followed by **63**. The selectivity toward the co-culture conditions was dependent on the cell type used. A combination of colorectal-cancer-derived HCT-15 cells and stromal CCD-18Co cells showed good selectivity, whereas the others exerted closer or essentially the same IC_{50} values with or without stromal cells.

Although the data are not shown in this review article, some other cell types have been found to be sensitive to intervenolin (**10**). In particular, gastric cancer cells should potentially be one of the best therapeutic targets; MKN-1, MKN-7, MKN-28, and MKN-45 exhibit similar or even higher activity than MKN-74. Among prostate cancer cells, PC-3 cells were insensitive to this class of compounds. Breast cancer cells such as HCB-4, HCB-5, and HTB-26 and lung cancer cell lines such as NCI-H460, NCI-H522, DMS114, and DMS273 did not show any positive responses. In contrast, colorectal-cancer-derived DLD-1 cells exhibit a good response toward the compounds examined in this study.

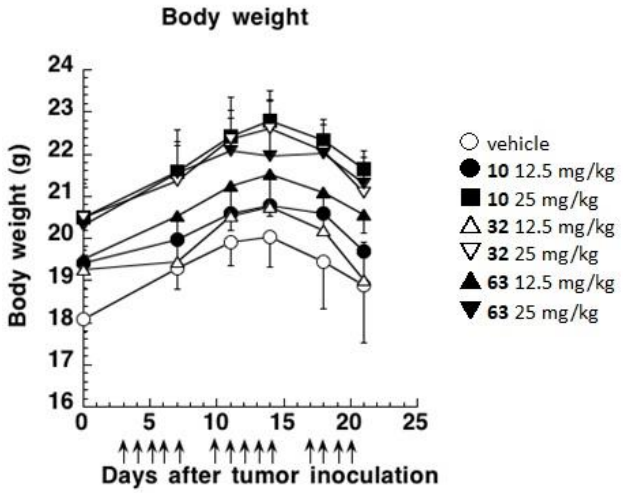


Figure 8. Body weight of mice administered with intervenolin (**10**), and its analogs (**32** and **63**)

Finally, the inertness of intervenolin (**10**) and its analogs in mice is emphasized (Figure 8). As demonstrated in Table 4, the MTD of **10**, **32**, and **64** in mice was >50 mg/kg; this value is higher than the dose in our in vivo assay. As expected, the change in body weight was similar to that observed in the control experiment (Figure 8). Encouraged by the promising in vivo data obtained, ADME studies on related compounds are currently under way in our laboratory.

The mechanistic aspects of intervenolin (**10**) and its derivatives upon exerting antiproliferative and in vivo antitumor activities are intriguing.[38] After the incubation of Hs738 gastric stromal cells with intervenolin (**10**), the resultant conditioned medium can negatively regulate the growth of MKN-74 gastric cancer cells (Figure 9, left panel). Although the data are not shown in this review, other gastric cancer cell lines, such as MKN-7, exhibit similar responses. Proteomic analysis implied that thrombospondin-1 (TSP-1)[39] could be a secreted factor responsible for this phenomenon; recombinant TSP-1 actually inhibits the proliferation of MKN-74 cells (Figure 9, right panel) and neutralizes antibodies for this signal molecule recovered in the cell growth media (data not shown in this article). Unfortunately, the primary molecular targets within stromal cells are currently under investigation. As described in the introduction, knowledge on this issue is currently being accumulated, such as mitochondrial complex V as a molecular target of leusinosiatin A that exhibits the tumor–stroma-interaction. In the near future, the physiological mechanisms underlying the antitumor effects of intervenolin are expected to be unveiled.

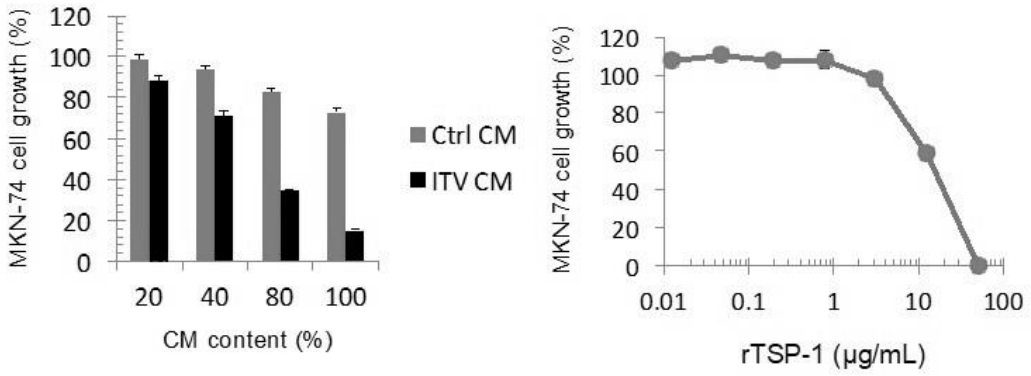


Figure 9. Possible candidate for the secreted factor obtained from Hs738 gastric stromal cells that regulates the growth of MKN-74 cells from gastric cancer

4. ANTI-*HELICOBACTER PYLORI* ACTIVITY OF INTERVENOLIN (10) AND ITS ANALOGS

As already described, CJ-13,136 (**11**) without a substituent on the nitrogen atom bearing a geranyl side chain like intervenolin has been reported as an anti-*Helicobacter pylori* (*H. pylori*) natural product. Therefore, it is logical to envision that intervenolin (**10**) also exhibits the same type of antibacterial activity (Table 6).[35]

Some analogs, such as **33**, **19**, **66**, **59**, **29**, and **61**, exhibit potent anti-*H. pylori* activity, which is comparable to that of clarithromycin, a positive control drug. Among these, **33**, **19**, **59**, and **66** have no substituent on the nitrogen atom and their acute toxicity cannot be ignored, as discussed previously. In this regard, isothiocyanate-bearing analog **29** was also categorized as a toxic compound. Gratifyingly, analog **61** with the dithiocarbamate and BMMA group at the 1-position, respectively, was identified as "hit" compound in this assay because they did not exhibit acute toxicity in mice.

Table 6. Antibacterial activity of intervenolin (**10**) and its analogs in our primary SAR study (MIC: μg/mL)

Test organisms Compound	*Helicobacter pylori* JCM 12093	*H. pylori* JCM 12095	*Staphylococcus aureus* FDA209P	*Enterococcus faecalis* JCM5803	*Escherichia coli* K-12	*Haemophilus influenza* T-196	*H. influenza* ARD476
10	0.0156	0.0078	64	>128	>128	64	64
57	0.5	0.25	128	>128	128	64	128
33	0.0156	0.0156	>128	>128	>128	>128	>128
19	0.0078	0.0078	>128	>128	>128	>128	>128
66	0.0156	0.0156	>128	>128	128	128	128
67	2	1	128	>128	>128	128	>128
58	1	0.5	>128	>128	>128	64	64
59	0.0078	0.0156	>128	>128	>128	>128	>128
68	0.25	0.5	>128	>128	>128	>128	>128
21	2	2	>128	>128	>128	>128	>128
22	1	0.25	128	>128	128	128	128
29	0.0156	0.0156	4	>128	>128	>128	>128
61	0.0312	0.0625	>128	>128	>128	>128	>128
32	2	2	4	>128	>128	>128	>128
63	1	0.5	>128	>128	128	64	64
CAM	0.0078	0.0078	<0.125	0.5	16	8	4
ABPC	0.25	0.125	<0.125	0.5	4	0.5	64

CAM clarithromycin, **ABPC** ammoxicillin

It is also noteworthy that these analogs, including **61**, were extremely selective, in particular, anti-*H. pylori* substances. In fact, all strains from both gram-negative and gram-positive bacteria examined in this study remained viable after the addition of 128 μg/mL of the test samples. As

widely recognized, gastric cancers caused by *H. pylori* are still predominantly occur in particular areas of the world, such as the Eastern Asia region. One of the standard protocols used to address this issue is the eradication of this bacteria in the stomach using antibiotics. This regimen is typically applied to healthy persons as a prophylactic measure, which requires antibacterial agents with a narrow spectrum of activity.[40] The analogs found in this study, especially **61**, meet this requirement and should be used in developmental studies as medicines. The feasibility of the synthesis can also be considered when selecting **61** (AS-1934) as a lead compound for advanced biological studies. In contrast, quinoline-based analog **63** exhibited only moderate anti-*H. pylori* activity, which supports this analog should be used as a back-up compound in the development of anticancer drugs.

Intervenolin analogs bearing aromatic groups at the 2- or 3-position apparently have less potential as anti-*H. pylori* compounds (Table 7) although the potency of their 2-phenyl analogs was slightly higher than that of the 3-phenyl congeners. However, there was no incentive to take any of them as a lead instead of the compounds listed in Table 6, such as **61**.[36]

Table 7. Antibacterial activity of intervenolin and its analogs in the secondary SAR study (MIC: μg/mL)

Test organisms / Compound	*Helicobacter pylori* JCM 12093	*H. pylori* JCM 12095	*Campylobacter coli* 2529	*Staphylococcus aureus* FDA209P	*Enterococcus faecalis* JCM5803	*Escherichia coli* K-12
10	0.125	0.0625	32	128	>128	>128
72	0.25	0. 5	64	128	>128	128
73	1	2	64	128	>128	128
74	0.5	1	64	128	>128	128
77	32	64	>128	128	128	>128
78	4	8	>128	4	>128	128
79	32	0.5	128	>128	>128	128
80	32	32	64	128	>128	>128
81	8	16	64	>128	>128	>128
83	16	16	64	>128	>128	>128
CAM	0.0625	0.0312	4	<0.125	0.25	16

CAM clarithromycin

Next, we focused on investigating the mechanistic background of these anti-*H. pylori* compounds. After extensive research, dihydroorotate dehydrogenase (DHODH) was identified as a molecular target. Figure 10 shows DHODH is a key enzyme for catalyzing a late determining step in the

de novo biosynthesis of pyrimidine and the oxidation of dihydroorotate to provide orotate.[41] This enzyme has long been recognized as a promising target for generating anti-*H. pylori* medicines because *H. pylori* lacks a salvage pathway to synthesize pyrimidines, as shown at the bottom of Figure 10;[42] the inhibition of the de novo route to pyrimidine is believed to cause the death of *H. pylori*.

A preliminary study has shown that intervenolin (**10**) did not inhibit the activity of complex I, complex II, and malate-quinone oxidoreductase from the membrane of the ATCC 49053 strain of *H. pylori*, whereas the activity of the DHODH + complex III and DHODH itself was inhibited. Compounds **61** and **19** were also shown to inhibit the formation of DHODH, with IC_{50} values of 11.1, 4.55, and 0.06 μmol/mL obtained for intervenolin (**10**), **19**, and **61**, respectively. At this stage, the evaluation of the antibacterial activity of intervenolin (**10**), **19**, and **61** (AS-1934) has been carried out (Table 8).

An antibacterial panel consisting of a variety of strains of bacterial species such as aerobic, anaerobic, and bacteria was tested. Once again, **19** showed a tendency to show more potent anti-*H. pylori* activity toward all the tested strains, including clarithromycin-resistant (CAM-R, ATCC 700684) and metronidazole-resistant (MNZ-R, ATCC 43504) strains. Nevertheless, **61** is still an anti-*H. pylori* seed, considering its low acute toxicity and most selective antibacterial effect against *H. pylori.* We then decided to perform an in vivo study to evaluate the clinical potential of **61** using mice infected by the SS-1 strain.

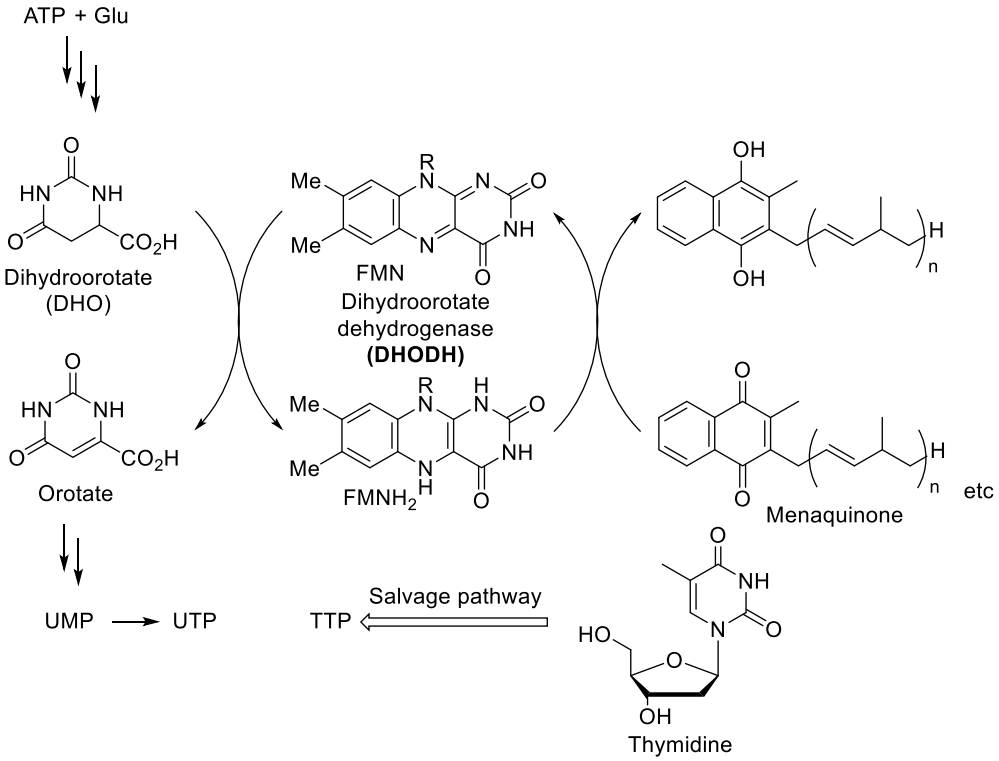


Figure 10. DHODH: molecular target of intervenolin and its analog for anti-*H. pylori* activity

Table 8. Evaluation of the antibacterial activity of intervenolin (**10**) and its analogs (MIC: μg/mL)

Bacteria	Strain	**Intervenolin**	**19**	**61**	**CAM**	**ABPC**	**MNZ**
Helicobacter pylori	JCM 12093	0.0156	0.0312	0.125	0.0156	0.0078	0.25
	JCM 12095	0.0156	0.0312	0.25	0.0156	0.0156	ND
	SS-1	0.125	0.0312	0.125	0.0312	0.125	ND
	ATCC 49503	0.125	0.0312	0.125	0.0156	0.0078	8
	ATCC 700684[a]	0.25	0.0156	0.125	64	0.0625	1
	ATCC 43504[b]	1	0.0625	0.5	0.0625	0.0312	64
Aerobic and anaerobic bacteria							
Staphylococcus aureus	FDA 209P	>64	>64	>128	0.125	0.0625	
	Smith	128	>64	>128	0.125	0.0625	
	MS9610 (MDR)	128	>64	>128	>128	8	
	MRSA No. 5 (MRSA)	128	>64	>128	>128	128	
	TY-04282 (MRSA)	>128	>64	>128	>128	64	
	Mu50 (VISA)	>128	.>64	>128	>128	16	
Micrococcus luteus	FDA 16	16	32	>64	0.063	0.125	
	IFO 3333	16	64	>64	0.063	0.125	
	PCI 1001	16	32	>64	<0.0312	<0.0312	
Bacillus subtilis	NRRL B-558	16	>64	>128	0.125	<0.0312	
	PCI 219	64	>64	>128	0.125	<0.0312	
	ATCC 10702	>64	>64	>128	0.25	2	
Corynebacterium bovis	1810	16	>64	>64	<0.0312	0.25	
Enterococcus faecalis	JCM 5803	>64	>64	>128	0.5	0.5	
	NCTC12201 (VRE)	>64	>64	>128	128	1	
	NCTC12203 (VRE)	>64	>64	>128	128	1	
	JCM 5804	>64	>64	>128	2	0.5	
	NCTC12202 (VRE)	>64	>64	>128	128	16	
	NCTC12204 (VRE)	>64	>64	>64	>64	16	
Escherichia coli	NIHJ	>64	>64	>64	16	0.5	
	K-12	>64	>64	>128	32	2	
	K-12 ML1629	>64	>64	>64	64	8	
	BEM11	>64	>64	>64	1	4	
	BE1121	>64	>64	>64	1	4	
	BE1186	>64	>64	>128	2	1	
Shigella dysenteriae	JS11910	>64	>64	>64	16	0.5	
Salmonella enteritidis	1891	>64	>64	>64	4	0.5	
Proteus vulgaris	OX19	>64	>64	>64	>64	0.5	
Proteus mirabilis	IFM OM-9	>64	>64	>128	>64	0.25	
Serratia marcescens	B-0524	>64	>64	>64	64	128	

Pseudomonas aeruginosa	A3	>64	>64	>64	16	>128
Klebsiella pneumoniae	PCI 602	>64	>64	>64	4	32
Candida albicans	3147	>64	>64	>64	32	>128
Mycobacterium smegmatis	ATCC 607	>64	>64	128	0.5	>128
Enteric bacteria						
Bacteroides distasonis	JCM 5825	32	64	>64	0.25	1
Bacteroides fragilis	JCM 11019	>64	>64	>64	0.25	16
Bacteroides merdae	JCM 9497	>64	64	>64	0.25	1
Bacteroides ovatus	JCM 5824	>4	64	>64	1	32
Bacteroides thetaiotaomicron	JCM 5827	>64	>64	>64	1	32
Bacteroides uniformis	JCM 5828	>64	>64	>64	0.5	2
Bacteroides vulgatus	JCM 5826	>64	>64	>64	0.5	2
Bacteroides eggerthii	JCM 12986T	>64	>64	>64	0.5	0.125
Bifidobacterium adolescentis	JCM 1251	>64	>64	>64	<0.0312	0.125
Bifidobacterium angulatum	JCM 7096	>64	>64	>64	0.0625	0.25
Bifidobacterium bifidum	JCM 1209	>64	>64	>64	0.5	0.5
Bifidobacterium breve	JCM 1192	>64	>64	>64	<0.0312	0.5
Bifidobacterium infantis	JCM 1222	16	>64	>64	<0.0312	0.25
Collinsella aerofaciens	JCM 10188	>64	>64	>64	<0.0312	0.125
Eggerthella lenta	JCM 9979	64	>64	>64	<0.0312	1
Eubacterium limosum	JCM 6421	8	>64	64	<0.0312	0.25
Eubacterium rectale	JCM 17463	64	64	>128	16	0.25
Fusobacterium varium	JCM 6320T	>64	>64	>64	>64	2
Lactobacillus acidophilus	JCM 1132	>64	>64	>64	0.0625	0.5
Lactobacillus fementum	JCM 1173	>64	>64	>64	0.0625	0.125
Lactobacillus gasseri	JCM 1131	>64	>64	>64	0.0625	0.125
Lactobacillus plantarum	JCM 1148	>64	>64	>64	0.125	0.125
Megasphaera elsdenii	JCM 1772T	32	>64	>64	0.0625	0.25
Peptostreptococcus anaerobis	JCM 1769	32	>64	>64	0.0625	0.25
Blautia productus	JCM 1471T	16	>64	>64	0.125	0.5
Blautia hydrogenotrophicus	JCM 14656T	8	>64	>64	0.125	0.25
Blautia hansenii	JCM 14655T	8	>64	64	0.0625	1
Veillonella parvula	JCM 12972T	>64	>64	>64	8	0.25
Enterococcus faecalis	JCM 5803	>128	>128	>128	1	0.5
	NCTC12201	>128	>128	>128	128	0.5
	NCTC12203	>128	>128	>128	128	0.5
Enterococcus faecium	JCM 5804	>128	>128	>128	2	0.25
	NCTC12202	>128	>128	>128	128	8
	NCTC12204	>128	>128	>128	128	8

Clostridium bifermentans	JCM 1386T	16	>64	>64	0.125	0.25
Clostridium butyricum	JCM 1391T	64	>64	>64	0.0625	0.125
Blautia coccoides	JCM 1395T	16	>64	64	0.125	0.5
Clostridium difficile	JCM 1296	>64	>64	>64	0.25	2
	BAA-1382	64	>64	>64	0.5	4
Clostridium indolis	JCM 1380T	32	>64	>64	0.25	1
Clostridium innocuum	JCM 1292T	64	>64	>64	0.25	0.5
Clostridium limosum	JCM 1427T	32	>64	>64	0.125	<0.0312
Clostridium perfringens	PB6K	32	>64	>64	0.5	<0.0312
Clostridium ramosum	JCM 1298T	>64	>64	>64	0.25	0.25
Escherichia coli	K-12	>64	>64	>64	16	2
Klebsiella pneumoniae	PCI602	>64	>64	>64	8	8
Klebsiella oxytoca	GN17031	>64	>64	>64	>64	>64

CAM clarithromycin, **ABPC** amoxicillin, **MNZ** metronidazole

[a] clarithromycin resistant strain (CAM-R), [b] metronidazole resistant strain (MNZ-R); ND, not derermined

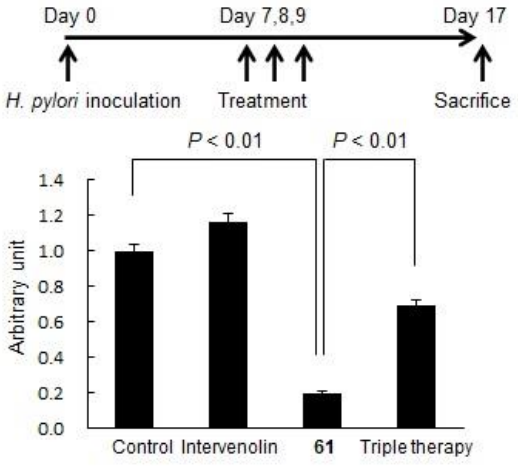


Figure 11. In vivo therapeutic experiments using intervenolin (**10**) and AS-1934 (**61**) and comparison with the clinically employed triple therapy regimen

Therapeutic experiments were carried out in this study, as shown in Figure 11;[43] C57BL/6 was used and inoculated with 1.5 × 10^8 CFU of *H. pylori*,[44] followed by a 3-dose administration on days 7, 8, and 9. On day 17, the mice were sacrificed and the colonies of *H. pylori* on the stomach tissues were counted. Intervenolin (**10**) and AS-1934 (**61**) were administered at a dose of 25 mg/kg each time. The results were compared with those obtained on the basis of a "triple therapy" regimen,[45] which uses a cocktail of two antibiotics [clarithromycin (14.3 mg/kg) and amoxicillin (28.5 mg/kg)] combined with a proton pump inhibitor [omeprazole (400 μmol/kg)].[46] This regimen is clinically used as an eradication therapy for humans infected with *H. pylori*, and a proton pump inhibitor (PPI) is

co-administered to prevent the antibiotics from degrading by reducing acid secretion in the stomach.[47] Pleasantly, AS-1934 (**61**) reduced the numbers of the colonies by >80% when compared to the untreated control (Figure 11). It is more effective than the triple therapy regimen, which exhibited ~40% reduction of the *H. pylori* colonies. Obviously, **61** clearly showed advantages over this clinically used regimen; only a single medicine is required to sequester the pathogen and no ancillary drug to ensure the stability of the antibiotic in the stomach is needed.

In contrast, intervenolin (**10**) displayed no efficacy in the present in vivo experiments, which raised suspicion of the instability of intervenolin under highly acidic conditions. To investigate this issue, a chemical stability study was performed (Figure 12).

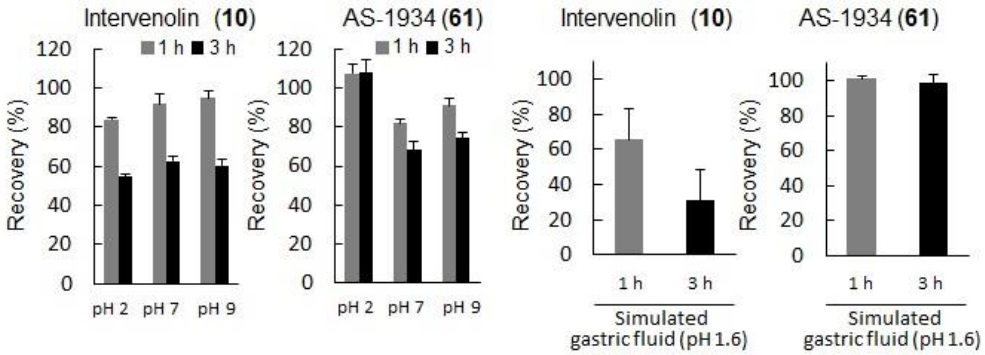


Figure 12. Chemical stability of intervenolin (**10**) and AS-1934 (**61**) in acidic media

The pH of the aqueous solution of each compound was adjusted to 2 and 9 using HCl and NaOH, respectively, and was left unchanged at pH 7 (left two panels of Figure 12). Intervenolin (**10**) was decomposed in acidic, neutral, and basic media, (~40% of the sample was degraded in 3 h), whereas **61** remained intact, especially at pH 2 (in neutral and basic solutions, ~30% of intervenolin was degraded in 3 h). These experiments indicate that the derivatization of intervenolin to **61** enhanced its stability under acidic conditions; **61** was more stable in an acidic solution than in neutral and basic solutions, which makes this analog a perfect lead compound for drugs acting in the stomach. To confirm the suitability of **61** in the stomach, another set of experiments were performed. The recovery after treatment with fasted-state simulated gastric fluid (FaSSGF, pH = 1.6) was analyzed (right two panels in Figure 12). In these experiments, the outstanding stability of **61** was observed, with no recognizable reduction in **61** being observed, whereas the recovery of intervenolin (**10**) decreased with time and ~70% of **61** was degraded over 3 h. Moreover, PPI administration is not required, which is another positive effect.

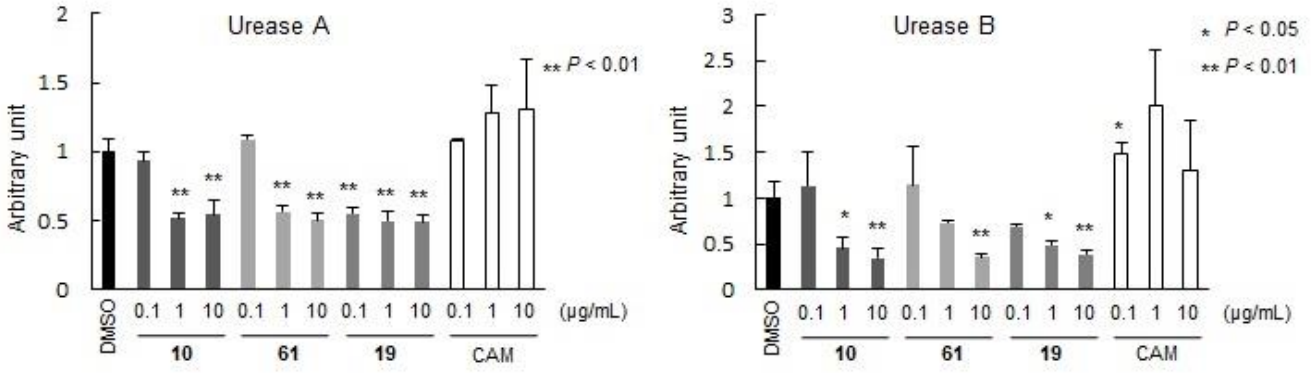


Figure 13. Change in the mRNA level of ureases in *H. pylori* after treatment with intervenolin (**10**) and its analogs (**61**; AS-1934 and **19**)

The tolerance of *H. pylori* to acidity in the stomach is partially attributed to the secretion of ureases, which catalyze the degradation of urea to produce ammonia.[48] The resultant ammonia quenches the acid surrounding *H. pylori* to increase the local pH level, which renders the bacteria viable; lack of ureases is lethal to *H. pylori*. Figure 13 shows to what extent intervenolin (**10**), AS-1934 (**61**), and **19** inhibit the production of mRNA for both urease A and B. Although the data are not shown in this review, these compounds had no effect on the expression of CagA,[49] which strongly suggests that intervenolin (**10**) and its analogs are not general inhibitors of transcription events. In contrast, clarithromycin exhibits no such activity. These results suggest that because of the inhibitory activity toward ureases of *H. pylori*, intervenolin (**10**) and its analogs can impart in vivo antibacterial effects.

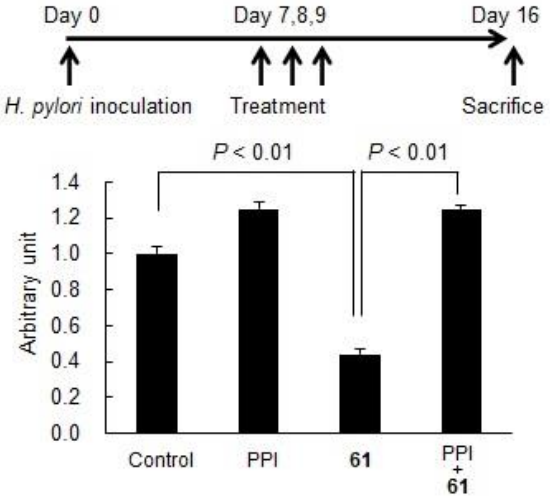
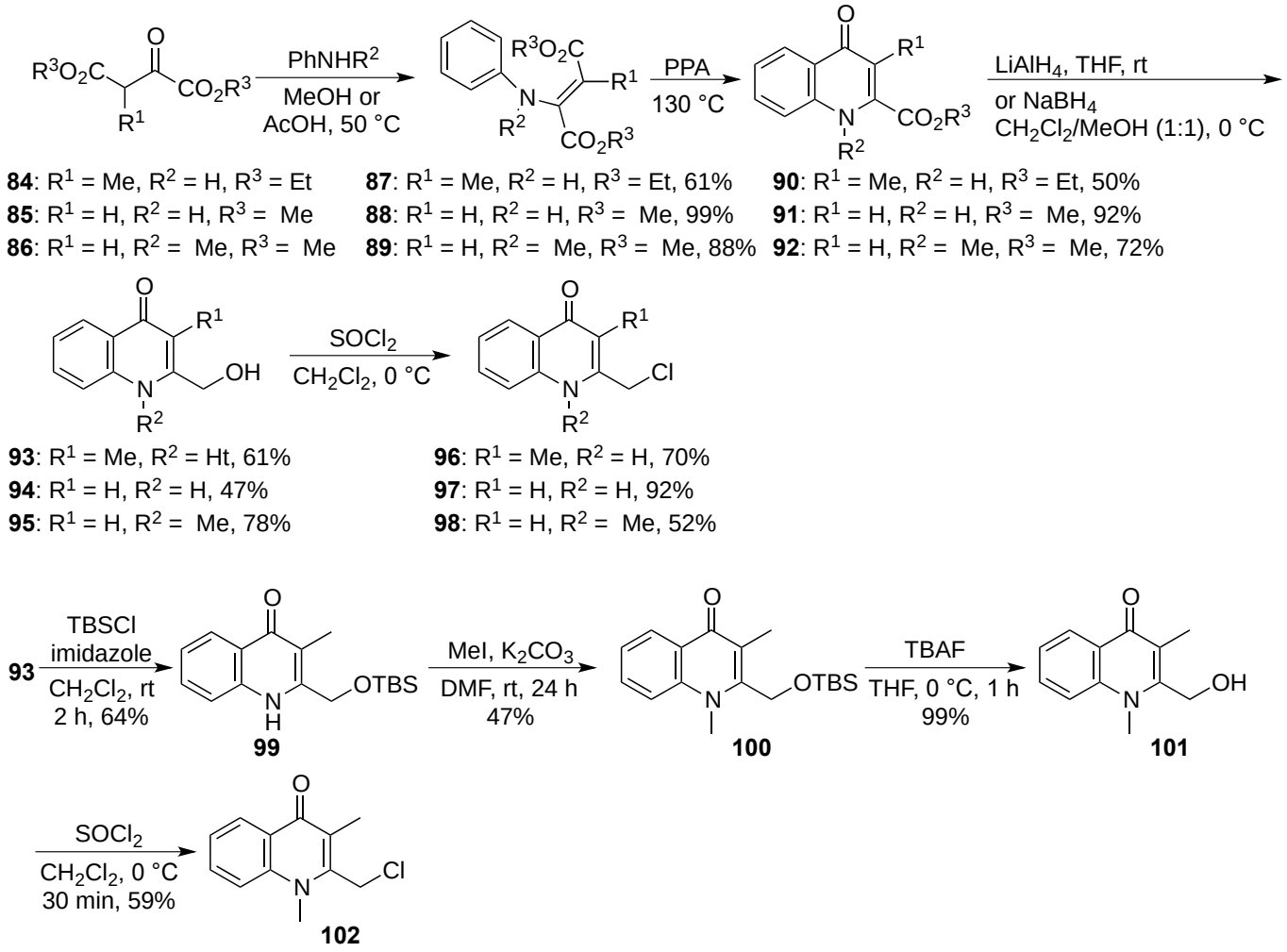


Figure 14. Compromised anti-*H. pylori* activity of AS-1934 (**61**) upon co-administration with a PPI (conditions in the main text)

As implied by the above description, the PPI can behave as a double-edged weapon for anti-*H. pylori* activity in the triple therapy regimen, wherein the PPI prevents antibiotics from acidic degradation. However, the PPI supports the viability of the pathogen by increasing the pH. In fact, Figure 14 displays the negative effect of PPIs; the therapeutic effects of 25 mg/kg of AS-1934 (**61**) were completely cancelled in the presence of 400 μmol/kg of omeprazole. All experimental results shown above emphasize that anti-*H. pylori* agents with anti-urease activity are ideal lead compounds for the development of clinical medicines in future research.

5. OTHER SYNTHETIC STUDIES (BY SPRING AND CO-WORKERS)



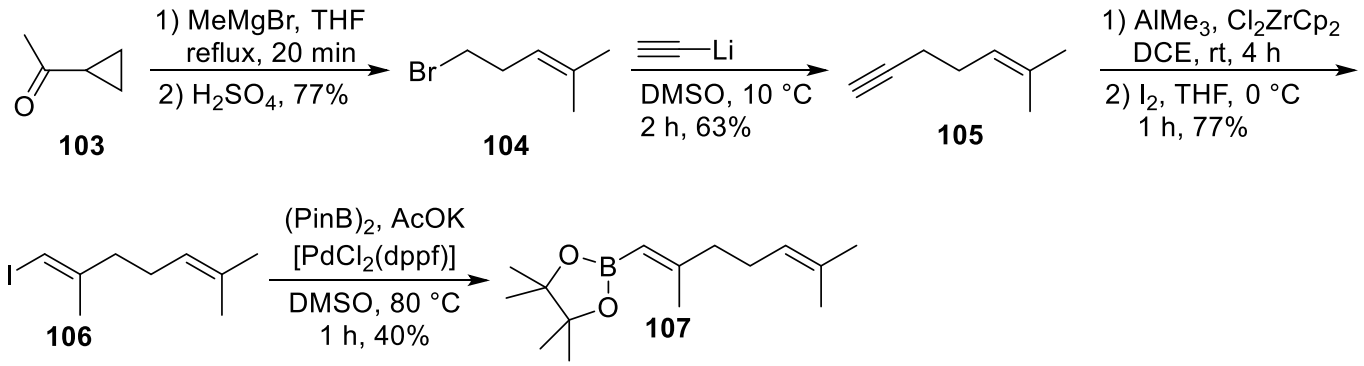
Scheme 12. Synthesis of the quinolone core of CJ-13,136 and its related natural products by Spring and co-workers

Quinolone derivatives bearing a diverse range of substituents have been synthesized and the biological activities of these compounds have been evaluated. However, geranyl-substituted derivatives functionalized at the 2-position have only been reported in the literature during the isolation of natural

products. After the first disclosure of the synthesis of intervenolin (**10**) and CJ-13,136 (**11**), Spring and co-workers reported the synthesis of **11** and its related compounds.[22]

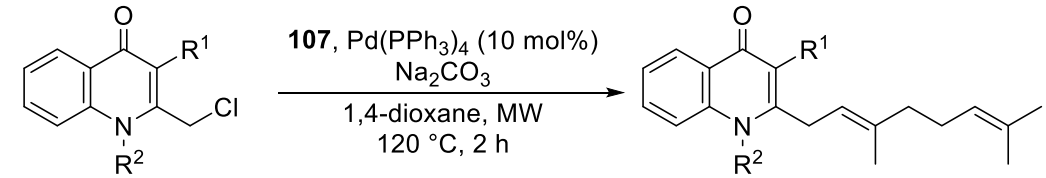
According to their first paper,[50] Spring et al. used the disconnections between C2-C3 within the quinolone core and the double bond embedded in the geranyl group, which did not yield satisfactory results because of the same reasons encountered by our group—the formation of the undesired regioisomer, pyridine, and unsuccessful Wittig chemistry used to furnish the geranyl substructure, respectively. The strategy was then converged to construct intermediates with a quinolone core, followed by the introduction of the side chain via a cross-coupling reaction (Scheme 12). Indeed, this strategy also involved the Suzuki–Miyaura coupling reaction, but a vinylic boronate ester (Scheme 13), not its allylic congener, was utilized, as described by our group.

The synthesis of quinolone building blocks **96–98** and **102** started from malonic esters **84–86**, which underwent a dehydrogenative condensation reaction with a series of aniline derivatives. The R^1 group in **84–86** corresponds to the substructure at the 3-position of the quinolone products, whereas the R^2 group in the aniline derivatives was maintained at the nitrogen atom of the quinolone skeleton. Exposure of the resultant enamines **87–89** to acidic media under heating resulted in an intramolecular Friedel-Crafts-type reaction, which gave quinolone derivatives **90**–**92**.[51] The subsequent reduction of the ester groups in these products was performed upon treatment with LAH or $NaBH_4$; the low chemoselectivity observed for substrate **92** forced the use of the latter as the reducing agent. The resulting primary alcohols were converted to their corresponding chlorides (**96–98**) under conventional conditions. Another chlorinated substrate for the cross-coupling reaction, **102**, was prepared from intermediate **93** using a cascade of functional and protecting group manipulations. Overall, all of the steps described above had no critical problems, although only moderate isolated yields were obtained.



Scheme 13. Preparation of the boronate building block used for synthesis of CJ-13,136 and its related natural products by Spring and co-workers

The synthesis of the other component used in the cross-coupling reaction commenced with a ketone bearing a cyclopropane structure (**103**), which was subjected to a Grignard reaction (Scheme 13). The resultant intermediate underwent the cleavage of the 3-membered ring with concomitant capture Br^- in a dehydrative manner to afford homoprenyl bromide **104**.[52] The introduction of an alkynyl group via an S_N2 substitution reaction,[53] followed by Zr-catalyzed carboalumination and metal-iodine exchange,[54] affords vinylic iodide (**106**), which was transformed to its corresponding boronate (**107**).



96: R^1 = Me, R^2 = H
97: R^1 = H, R^2 = H
102: R^1 = Me, R^2 = Me
98: R^1 = H, R^2 = Me

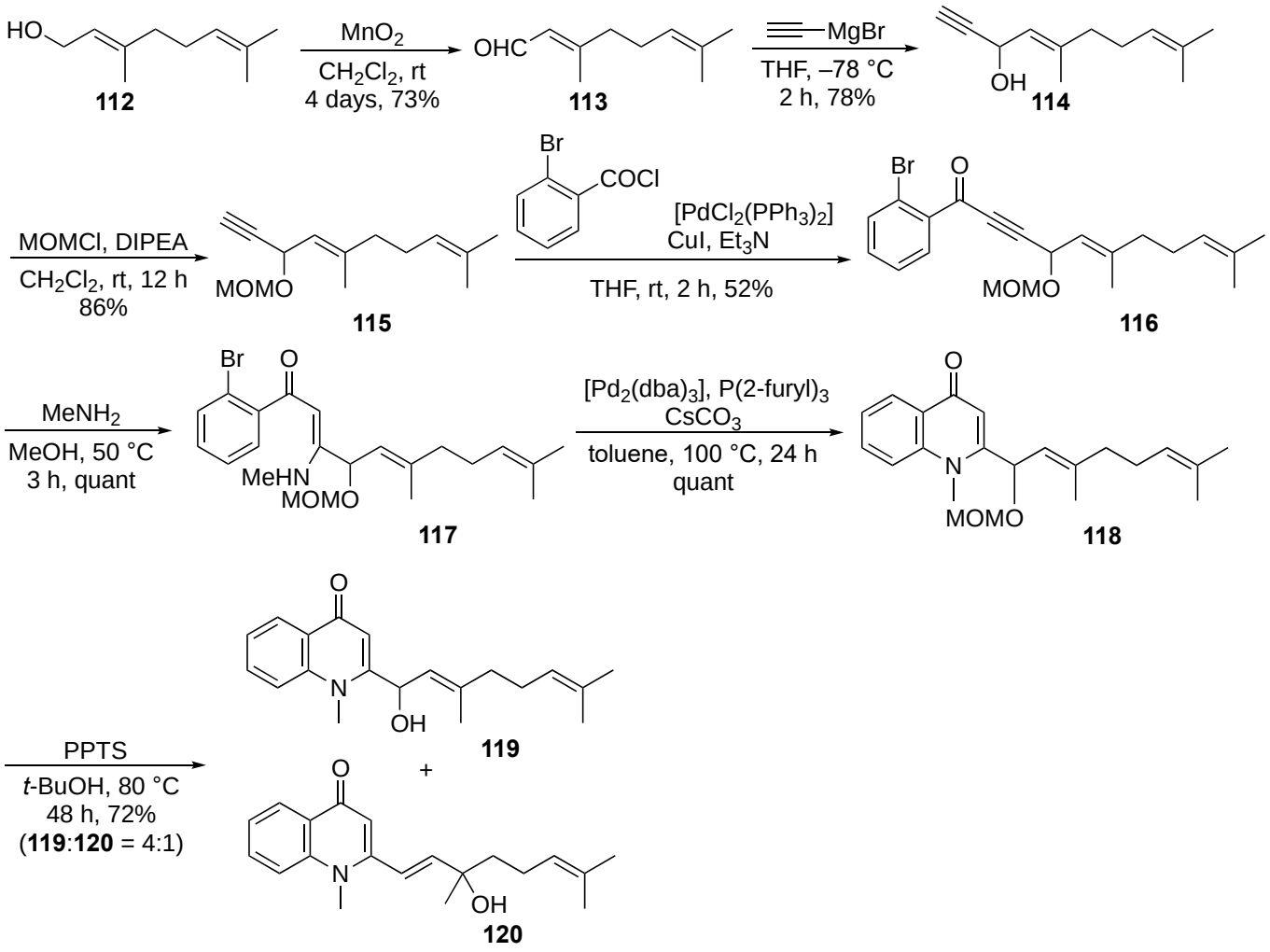
11: R^1 = Me, R^2 = H, 47%
12: R^1 = H, R^2 = H, 23%
13: R^1 = Me, R^2 = Me, 82%
14: R^1 = H, R^2 = Me, 37%

Scheme 14. Completion of the synthesis of CJ-13,136 and its related natural products by Spring and co-workers

The key transformation of this synthetic route, the Suzuki–Miyaura cross-coupling of **96–98** and **102** with **107**, was accomplished, as shown in Scheme 14. Moderate-to-high isolated yields were reported for this reaction employing 10 mol% of $Pd(PPh_3)_4$ as the catalyst and Na_2CO_3 as the base under microwave irradiation. In this transformation, the *E*-isomer of the olefin was exclusively formed. The relatively low isolated yield of **12** was attributed to the difficulties in its purification and not the low conversion rate.

It has been discovered that quinolone compounds play a significant role in the intercellular communication of bacteria, which is known as quorum sensing.[55] Indeed, pathogenic *Pseudomonas aeruginosa* produces many types of quinolone compounds,[56] which was followed by findings on the biosynthesis of quinolones by different specimens of bacteria.[57] Accordingly, Spring et al. were interested in investigating the antibacterial activity of the compounds synthesized in their study. In fact, the effect of these natural products on RqsR (called MvfR), the receptor on *P. aeruginosa* that mediates these signals,[58] has been investigated, but no activity was observed. Subsequently, their interest moved to the investigation of the antibacterial activity toward various species independent of RqsR. Antibacterial assays were conducted for *P. aeruginosa* PA01, *Staphylococcus aureus* 25923, and *Escherichia coli* ESS. No noticeable activity was found on *P. aeruginosa* PA01; however, **11**–**14** were found to cause a delay in the growth of *S. aureus* 25923. Similar effects were observed for **11**, **12**, and **14** on *E. coli* ESS. The reduction of the growth signal was not seen upon treatment with bacteriostatic or bacteriolytic antibiotics; the control drug, gentamycin, completely blocked the growth of bacteria over 8 h. Considering that a

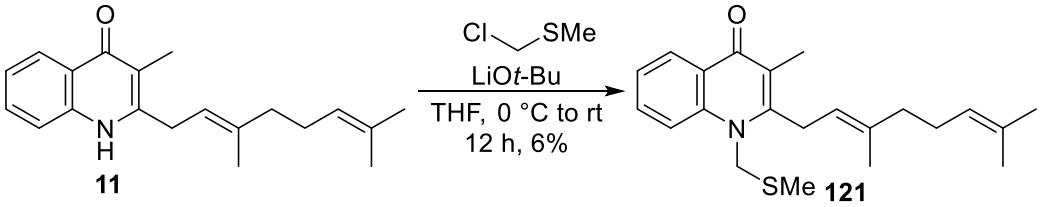
previous report describing a quinolone-based signaling molecule resulted in the deferred growth of several types of gram-positive and gram-negative bacteria, which acted on their respiratory chain,[59] Spring suggested that the natural products shown herein intervene in the same electron transport system.[60] It was also argued that the lack of activity toward *P. aeruginosa* could be due to the complex nature of quinolone uptake[61] when compared to the other three species that utilize passive diffusion. Accordingly, the efficiency of the uptake may be substantially different.



Scheme 15. Synthesis of natural products related to CJ-13,136 bearing an allylic alcohol moiety by Spring and co-workers

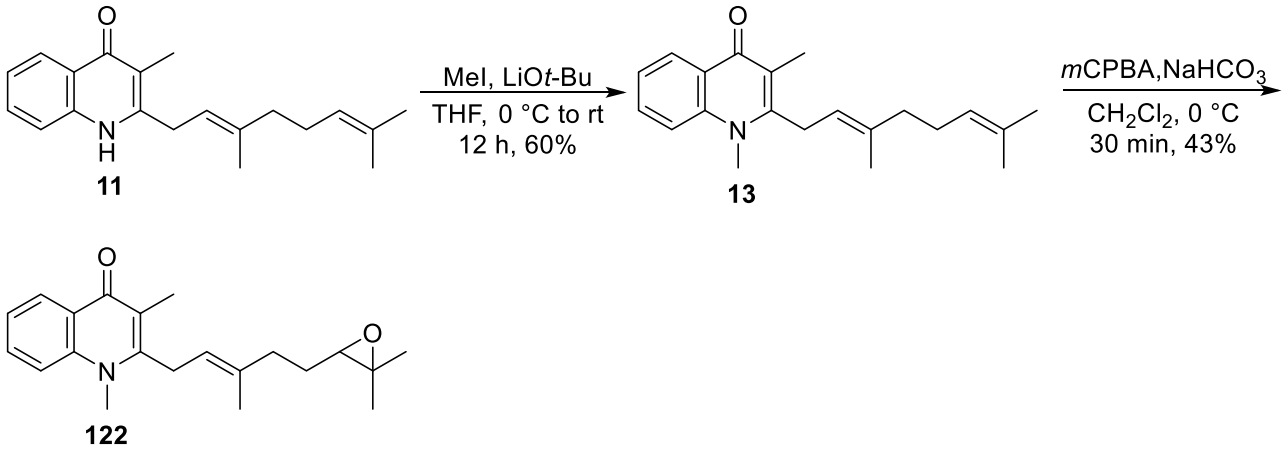
In the same year (2016), Spring and co-workers reported four more geranylated quinolone compounds (**119**–**122**).[22] The synthetic protocol used in this study has a desirable feature (Scheme 15); the diversification of the structure was accomplished at a late stage in the route. Indeed, a convergent strategy is ideal for the preparation of a series of substances with close structural relationships, such as a SAR study. The synthesis started with the oxidation of geraniol (**112**),[62] followed by the addition of a Grignard reagent to the resultant aldehyde (**113**). The resulting propargylic alcohol (**114**) was protected, followed

by the Sonogashira-type reaction[63] to afford **116**. The subsequent Michael addition reaction proceeded quantitatively, which was followed by the Buchwald-Hartwig type amination reaction[64] to construct the quinolone core in **118**. Weakly acidic conditions facilitated the simple removal of the protecting group and the concomitant migration of the double bond and hydroxy group to afford **119** and **120**, respectively. In this transformation, **119** was obtained as the major product (**119**:**120** = 4:1). Although **120** was not obtained in a selective manner, it can be utilized in the extensive preparation of many types of structurally related compounds.



Scheme 16. Synthesis of a methylthiomethyl analog of CJ-13,136

The synthesis of a methylthiomethyl-bearing compound (Scheme 16, **121**) was achieved from CJ-13,136 (**11**). However, the yield of **121** was low (6%). In this transformation, the dimerization pathway discussed in Scheme 6 will occur, in which the resulting dimer will be hydrolyzed under the acidic work-up conditions to regenerate **11**.



Scheme 17. Completion of the synthesis of natural products related to CJ-13,136 (**11**) bearing an epoxide moiety by Spring and co-workers

The remaining natural product, **122**, was accessible from another natural product (**13**) via epoxidation using *m*CPBA in moderate yield in a highly regioselective manner (Scheme 17). The preparation of oxygenated substances such as **120–122** is important not only for natural product synthesis but also investigation of the metabolic fate of this class of compounds in the human body.

6. CONCLUSIONS

In this review article, the chemistry and biology of intervenolin, a quinolone natural product bearing BMMA and geranyl groups at the 1- and 2-positions, respectively, are summarized. Intervenolin was discovered as a modulator of tumor–stroma interactions; the intercellular signal transduction between tumor cells and normal cells such as fibroblasts, which controls the behavior of the former including proliferation in tumor tissues. This article highlights the research activity of our group, a brief overview of the tumor–stroma interaction and naturally occurring modulators of this system, the synthesis and SAR study of intervenolin and its structurally related analogs, and the biological activities such as antitumor and anti-*H. pylori* activities of these compounds.

Moreover, the synthetic study of this class of natural products reported by Spring and co-workers is summarized. The SAR study on the intervenolin-generated analogs exhibiting efficacy toward cells from hard-to-treat cancers, e.g., pancreatic cancer, and their in vivo antitumor activity in mice is investigated. Some of the analogs show potent and almost specific antibacterial activity toward *H. pylori*, which was reflected in their good therapeutic index during their in vivo examination in an infected mouse model, and details of their mode of action are clarified. Modular syntheses of CJ-13,136 and its structurally related analogs bearing no BMMA group by Spring et al. provide another synthetic strategy, which will expand the scope of the SAR study on this type of substance to pursue investigation of its preferable biological activities.[65]

The developmental study on medicines based on the information provided in this review to satisfy unmet clinical needs is in high demand and currently underway in our laboratory. The importance of biologically active substances with low molecular weight as drug seeds has received considerable attention in terms of their biologics in the field of medicinal science. However, natural products that pass through biosynthetic processes under physiological conditions may be generally expected to have characteristics similar to those found in the human body. Although the current developmental study aimed at generating novel clinical medicine is still ongoing, the promising results described in this review have encouraged our group to believe in the potential of these natural products as a source of drug seeds.

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(BIKAKEN) for evaluating the antibacterial activity and detailed evaluation of the anti-*H. pylori* properties, respectively, and that of Dr. J. Yoshida for the investigation of secreted factors from gastric stromal cells as a possible regulator of growth of gastric tumor cells. The author appreciates fruitful collaboration with Dr. K. Kita and D. K. Inaoka (Nagasaki University), Drs. A. Nomoto, H. Adachi, Mrs. T. Masuda, S.-i. Ohba, and H. Inoue (BIKAKEN), and technical support from Drs. R. Sawa and K. Iijima, and Mss. Y. Kubota, C. Hayashi, and Y. Takahashi (BIKAKEN). The author thanks the Project for Cancer Research and Therapeutics Evolution (P-CREATE) of the Japan Agency for Medical Research and Development (AMED) for financial support (PI: Dr. M. Kawada).

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