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Article

Developing Gingerol-Based Analogs against *Pseudomonas aeruginosa* Infections

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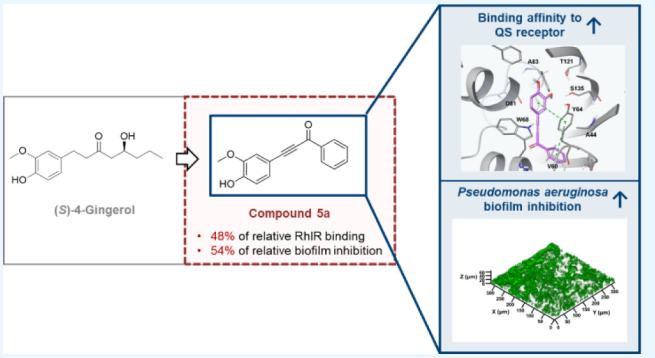
ABSTRACT: *Pseudomonas aeruginosa* (*P. aeruginosa*), a Gram-negative opportunistic pathogen, produces virulent factors and forms biofilms through a quorum sensing (QS) mechanism. Modulating QS networks is considered an effective strategy for treating *P. aeruginosa* infections. Particularly, the *rhl* system, one of the QS networks, can be a potential target in treating patients with chronic infections. We previously discovered that gingerol acts as a RhlR antagonist of *P. aeruginosa*. Based on the chemical structure of gingerol, we have designed and synthesized gingerol derivatives by introducing various functional groups in the middle and tail regions. A comprehensive structure−activity relationship study showed that compound **5a** substituted with phenyl group in the tail region was the most potent in various biological assessments, such as RhlR binding affinity, *rhl* gene expression, and virulence factor production of *P. aeruginosa*. Furthermore, compound **5a** decreased the biofilm formation and pathogenicity of *P. aeruginosa*. Interestingly, compound **5a** also influenced *las* system in addition to the *rhl* system. Taken together, compound **5a** can be utilized as a potent compound for controlling *P. aeruginosa* infection.



INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen that can cause serious infections.[1] In general, *P. aeruginosa* is the leading cause of morbidity and mortality in cystic fibrosis (CF) patients and nosocomial infections worldwide.[2] *P. aeruginosa* becomes resistant to antibiotic treatment by forming thick biofilms. Due to its antibiotic resistance, *P. aeruginosa* can be life-threatening pathogen and is emerging as a challenge to public health. Biofilms are immobile microbial communities associated with about 80% of human bacterial infections. Microorganisms colonize and are embedded within self-produced extracellular polymeric substances (EPS).[3] EPS prevents antibiotics from accessing molecular target sites of microorganisms in biofilms, decreasing antibiotic activities.[4] Bacteria cells within biofilms can become up to 1,000-fold more antibiotic-resistant than planktonic cells.[5]

P. aeruginosa is able to regulate the formation of biofilms and the production of virulence factors through a quorum sensing (QS) system.[6] QS is a bacterial cell-to-cell communication network, which allows bacteria to share information depending on cell density and behave as a community to respond to environmental changes.[7] The intercellular communication network is controlled by interactions between chemical signal molecules and their cognate receptors.[8] In *P. aeruginosa*, three major QS network systems (*las*, *rhl*, and *pqs*) are closely interconnected.[9] *P. aeruginosa* uses *N*-acyl-*L*-homoserine lactones (AHLs) as signal molecules in QS systems.[10] LuxI-type synthetases produce AHLs that are recognized by the LuxR-type receptor in the cytoplasm.[11] *P. aeruginosa* produces *N*-(3-oxo-dodecanoyl)-*L*-homoserine lactone (OdDHL) and *N*-butyryl-*L*-homoserine lactone (BHL) for the *las* and *rhl* systems, respectively.[12] Once *P. aeruginosa* reach a certain population density threshold, AHLs bind to their cognate receptors, affecting gene expression through transcriptional activation.[13] In addition to the *las* and *rhl* systems, the 2-heptyl-3-hydroxy-4(1*H*)-quinolone (*Pseudomonas* quinolone signal, PQS) circuit is the third system regulated by PqsR.[9] These interactive signal-network systems affect biofilm formation, production of virulence factors, and modulation of host immune responses. Therefore, modulating the QS network between signal molecules and their cognate receptors is considered a promising strategy for controlling biofilm formation and the production of virulence factors of *P. aeruginosa*.[14]

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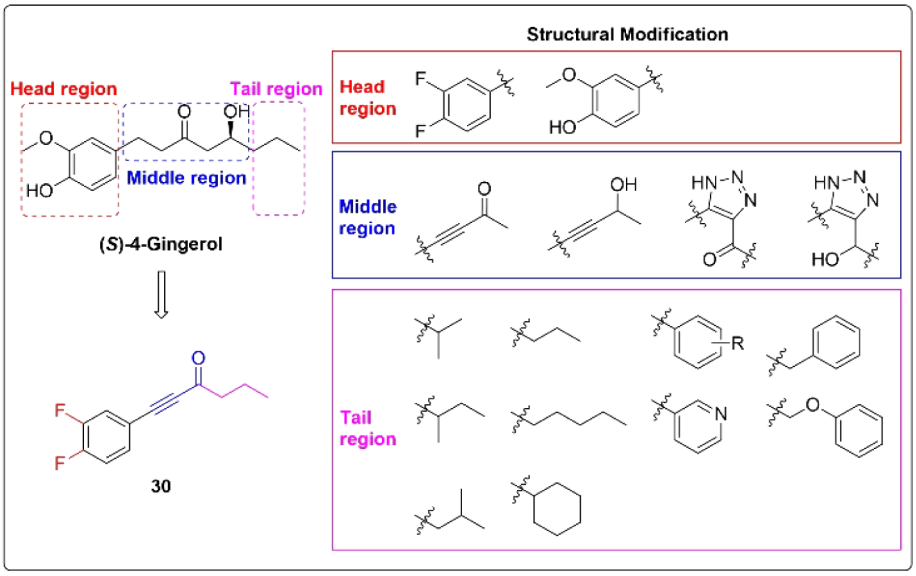

Figure 1. Strategy for structural modification of gingerol-based RhlR antagonists.

Since *las* is at the top of the QS system hierarchy, most studies have focused on the *las* rather than the *rhl* system.[15−17] However, RhlR can be a potential target for treating *P. aeruginosa* chronic infections, particularly in patients with LasR mutants.[18] The presence of LasR mutants constantly increases among environmental and clinical isolates in chronic CF patients,[19,20] because LasR mutants have growth advantages over the wild-type for nutrients in infected lungs.[21] Moreover, RhlR alone can control pathology and biofilm formation in *P. aeruginosa* and regulates the cytotoxicity of lung epithelial cells.[22,23] RhlR antagonists can decrease the mortality of *P. aeruginosa*-infected mouse and mealworm models by modulating the host immune system.[25−27] Further, synergistic combinations with antibiotics improve the therapeutic effects against *P. aeruginosa* infections.[24,28] Overall, the importance of RhlR was highlighted in the treatment of chronic *P. aeruginosa* infections.[24]

There have been several reports on RhlR-targeted agonists and antagonists.[30−33] RhlR-targeted agonists and antagonists were designed based on a BHL structure, a signal molecule of the *rhl* system. Homoserine lactone and homocysteine thiolactone in the head region are commonly found in both RhlR-targeted agonists and antagonists.[25] Replacement of the homoserine lactone with cyclopentanone, cyclopentane, or tetrahydrofuran markedly decreased RhlR activities, suggesting that ring variation in the head region influences the binding affinity for RhlR.[17] In the middle section, the sulfonamide or alkynylketone groups were utilized as surrogates of the amide group for RhlR agonists and antagonists.[30,31] In the tail region, the branched alkyl chains (e.g., isobutyl and isopropyl) and the cycloalkyl rings (e.g., cyclobutanyl and cyclopentanyl) instead of the *n*-propyl group in BHL provided RhlR agonism while 2,4-dichlorophenylmethyl, *p*-substituted phenoxymethyl, and *p*-substituted phenylmethyl groups did RhlR antagonism.[32,33]

To identify new RhlR antagonists, we have designed and synthesized gingerol-based analogs. Our previous structure−activity relationship (SAR) study identified compound **30** as a potential candidate for novel RhlR antagonists.[30] Compound **30** possesses a 3,4-difluorophenyl group in the head region, an alkynylketone in the middle region, and n-propyl in the tail region (Figure 1).[30] Compound **30** strongly inhibited biofilm formation and reduced the production of virulence factors in *P. aeruginosa*. Furthermore, its antibiotic activities were more potent when combined with tobramycin without affecting the cytotoxicity of human lung epithelial cells.[24]

To optimize gingerol-based RhlR antagonists, the head region was maintained with the 3′-methoxy-4′-hydroxyphenyl group and 3′,4′-difluorophenyl group referring to compound **30**, the most potent gingerol-based RhlR antagonist in our previous study.[24]

Various functional groups were introduced in the middle and tail regions. The biological activities of RhlR antagonists were evaluated by determining relative RhlR activity, relative biofilm formation, production of virulence factors in *P. aeruginosa*, and mortality of mealworms (*Tenebrio molitor* larvae). We aimed to investigate the relationship of chemical structure with biological effects of newly synthesized compounds on RhlR antagonism and biofilm inhibition of *P. aeruginosa*.

RESULTS AND DISCUSSION

We designed and synthesized 54 derivatives based on the chemical structure of 4-gingerol and compound **30**. As the 3′-methoxy-4′-hydroxyphenyl group and 3′,4′-difluorophenyl group in the head region of gingerol showed the strongest RhlR antagonism activities in our previous study, modification of the head region was limited to these two moieties.[24] Instead, we introduced various functional groups in the middle and tail regions: 1) variation of the bulkiness in the tail region, 2) introduction of the triazole ring in the middle region, 3) reduction of the ketone functional group to the corresponding alcohol in the middle region, 4) absolute configuration change of the chiral carbon linked to the OH group in the middle region (Figure 1).

Scheme 1 describes the synthesis of gingerol derivatives which have 3′-methoxy-4′-hydroxyphenyl group in the head region with variation in the middle and tail regions. Compounds **1**−**3** were prepared by applying the previously reported procedure.[24] The free phenolic hydroxyl group of vanillin, a starting material, was protected with *tert*-butyldimethylsilyl (TBDMS) group to afford compound **1**.

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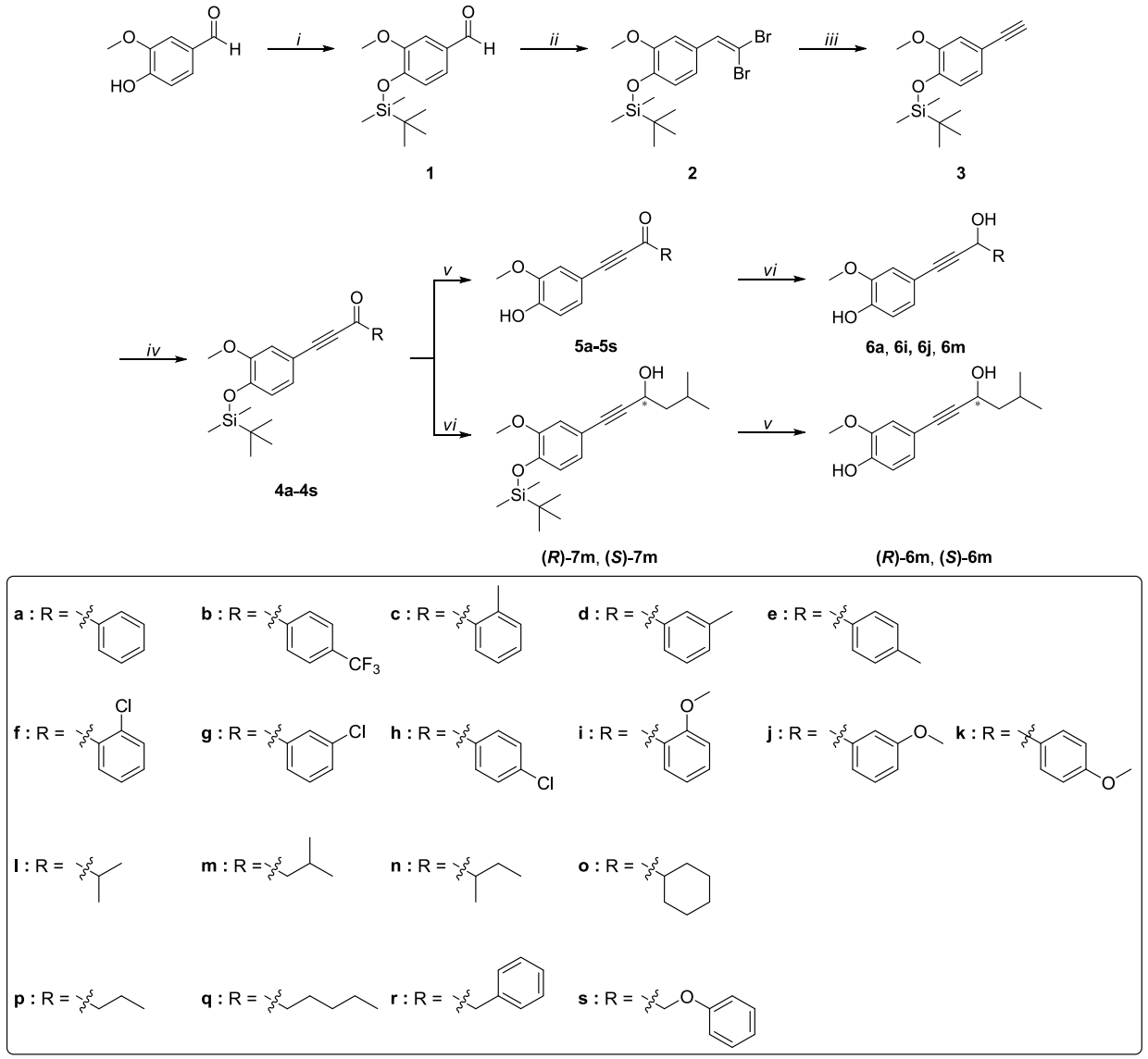
Scheme 1. Synthesis of Gingerol Derivatives with Variation in the Middle and Tail Regions[a]



[a]Reagents and conditions: (i) TBDMSCl, imidazole, DCM, rt, 18 h, 94% yield; (ii) CBr_4, PPh_3, DCM, rt, 3 h, 98% yield; (iii) *n*-BuLi 1.6 M in hexanes, THF, −78 °C, 2 h, 96% yield; (iv) *n*-BuLi (1.6 M in hexanes), appropriate acid chloride or Weinreb amides, THF, −78 °C, 16 h, 5−99% yield; (v) TBAF, THF, rt, 20 min, 32−87% yield; (vi) $NaBH_4$, MeOH, rt, 30 min, 20−87% yield (for compounds **6a**, **6i**, **6j**, **6m**), RuCl[(*R*,*R*)-TsDPEN(mesitylene)], KOH, 2-propanol, 40 °C, 16 h, 28% yield (for compound (*R*)-**7m**), RuCl[(*S*,*S*)-TsDPEN(mesitylene)], KOH, 2-propanol, 40 °C, 16 h, 21% yield (for compound (*S*)-**7m**).

Compound **3** was prepared from compound **1** via the synthetic 2-steps using the Corey−Fuchs reaction. The aldehyde group of **1** was converted to the dibromo alkene group in 98% yield by reacting with CBr_4 and PPh_3 in dichloromethane (DCM). The terminal alkyne group of compound **3** was obtained in 97% yield via elimination and lithiation by treating 3 equiv of *n*-butyllithium (*n*-BuLi) at −78 °C.

The TBDMS-protected ynone analogs (**4a**−**4s**) were obtained in 18−99% yield by reacting **3** with an appropriate acid chloride. In order to introduce bulkiness in the tail region, commercially available acid chlorides were used, including substituted benzoyl chloride and phenoxyacetyl chloride. Compounds **4p**−**4r** were obtained by reacting **3** with Weinreb amide reagents because the corresponding acid chlorides (butyryl chloride, hexanoyl chloride, 2-phenylacetyl chloride) were too reactive to produce byproducts.

The synthetic yield variation in this step might be due to the high reactivity of acid chloride, which was easily decomposed during the reaction. In addition, various byproducts including the tertiary alcohol compound were observed because the further reaction of acetylide ion was proceeded with the produced alkynyl ketone.

The ynone analogs (**5a**−**5s**) were obtained in 30−80% yield via desilylation with tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) at 0 °C for 20−30 min. The purity of compounds **5a**−**5s** was confirmed by analytical HPLC and used to determine *in vitro* RhlR- and biofilm-inhibitory activities. The alkynol analogs (**6a**, **6i**, **6j**, **6m**) were obtained in 20−87% yield by reaction of the ketone compounds with sodium borohydride ($NaBH_4$) in methanol. Compound **6m** was prepared in racemate or in pure enantiomer by using chiral catalysts. Chiral catalysts, such as RuCl[(*R*,*R*)-TsDPEN-

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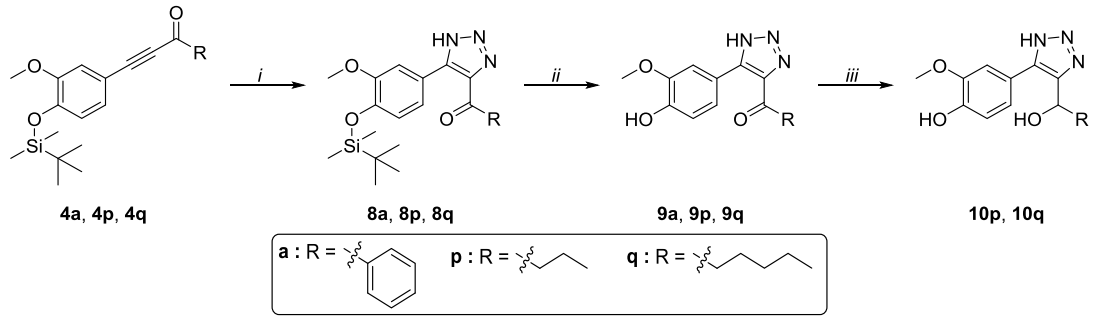
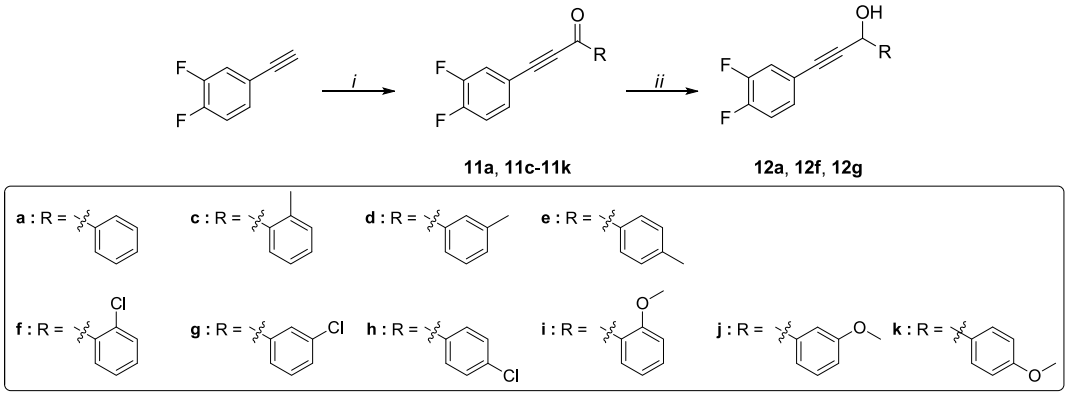
Scheme 2. Synthesis of Triazole Derivatives with Variation in the Middle Region[a]



[a]Reagents and conditions: (i) NaN_3, DMF, rt, 2 h, 61−88% yield; (ii) TBAF, THF, rt, 20 min, 70−80% yield; (iii) $NaBH_4$, MeOH, rt, 30 min, 57−74% yield.

Scheme 3. Synthesis of 3′,4′-Difluorophenyl Derivatives with Variation in the Tail Region[a]



[a]Reagents and conditions: (i) *n*-BuLi (1.6 M in hexanes), appropriate acid chloride, THF, − 78 °C, 16 h, 5−44% yield; (ii) $NaBH_4$, MeOH, rt, 30 min, 52−71% yield.

(mesitylene)] and RuCl[(*S*,*S*)-TsDPEN(mesitylene)], produced the (*R*)-alkynol and (*S*)-alkynol with high enantiomeric excess (*ee*) value via Noyori asymmetric hydrogenation.[34]

The synthesized compounds (**6m**, (*R*)-**6m**, (*S*)-**6m**) were analyzed by chiral reversed-phase HPLC (Supporting Information). Compound **6m**, a racemic mixture, was observed as two distinct peaks at 22.37 and 22.95 min with a ratio of 1:1. Compounds (*R*)-**6m** and (*S*)-**6m** were eluted at 22.31 and 22.95 min, respectively, with the *ee* values of >99%.

During the enantioselective reduction reaction, the free phenolic hydroxyl group disturbed the activation of the chiral catalyst (Figure S54A). Therefore, we first prepared silyl-protected alkynol analog (**7m**). Compounds (*S*)-**6m** and (*R*)-**6m** were obtained in 21−28% by reacting **7m** with chiral catalyst at 40 °C for overnight. In addition, compounds with bulky moieties in the tail region, such as substituted phenyl groups, were more resistant to the reduction by chiral catalysts. The bulky moieties in the tail region were likely to prevent the ynone analogs from reacting with the catalysts (Figure S54B).

The silyl-protected triazole analogs (**8a**, **8p**, **8q**) were obtained in 60−88% yield via Click chemistry using sodium azide (NaN_3) (Scheme 2).[35] Click chemistry is a variant of azide−alkyne 1,3-dipolar cycloaddition. Conventional Click chemistry requires the presence of copper catalysts that is toxic to liver.

Therefore, we used copper-free Click chemistry based on the reaction of a cyclooctyne moiety with an azide-labeled reaction partner, known as rapid [3 + 2] cycloaddition. As a result, we could obtain stable triazoles in high synthetic yield. Compounds **10p** and **10q** were prepared using TBAF, followed by the reduction with $NaBH_4$ in MeOH.

Starting materials, 3′,4′-difluorophenyl derivatives, were easily prepared by commercially available 4-ethynyl-1,2-difluorobenzene. 3′,4′-Difluorophenyl derivatives were synthesized mainly with substituted phenyl group in the tail region (Scheme 3). The 3′,4′-difluorophenyl ynone analogs (**11a**, **11c−11k**) were obtained in 20−44% by reacting 4-ethynyl-1,2-difluorobenzene with appropriate acid chloride. The high reactivity of acid chloride led to variations in the synthesis yield. In addition, due to the instability of starting material in this step, the synthetic yields were relatively low.

The 3′,4′-difluoro alkynol analogs (**12a**, **12f**, **12g**) were obtained in 52−71% by the treatment of $NaBH_4$ in MeOH. Unlike gingerol derivatives, 3′,4′-difluorophenyl derivatives have low electron density of the alkyne group due to the electron-withdrawing effect of 3′,4′-difluoro moiety in the head region. It is noted that ynone analogs may act as a Michael reaction acceptor. Therefore, the reduction of difluoro ynone analogs forms α,β-unsaturated ketone analogs as well as alkynol analogs (Figure S55). Both *cis*- and *trans*-geometrical isomers were purified and identified with *cis*-isomer as the major product.

By using a reporter strain assay, we determined the relative RhlR activity of the synthesized gingerol-based compounds (Schemes 1−3). First, we tested diverse lipophilic alkyl chain or substituted phenyl rings in the tail region of gingerol analogs, as most BHL-based RhlR antagonists were reported to

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be tolerant to variation of bulky moieties in the tail region (Scheme 1).[30]

We tried to identify the most potent substituents for RhlR antagonism and compare the activities of three gingerol compounds (**5a**, **5r**, **5s**) with phenyl group, phenylacetyl group, and phenoxyacetyl group in the tail region, respectively (Table 1) because the three moieties are representative bulky

Table 1. Relative RhlR Activity (%) on *P. aeruginosa* with 10 μM of Synthesized Compounds[a]

Entry	AVG	SD	Entry	AVG	SD	Entry	AVG	SD
C	100	4	5m	81*	4	10p	96	3
6G	84*	5	5n	93	5	10q	93	1
30	75**	2	5o	74**	3	11a	59**	4
5a	52**	6	5p	76**	2	11c	74*	9
5b	67**	8	5q	73*	7	11d	69**	6
5c	64**	7	5s	75*	1	11e	69**	6
5d	76*	9	6a	60**	7	11f	63**	8
5e	62**	6	6i	56**	6	11g	72**	5
5f	62**	11	6j	68**	5	11h	100	2
5g	63**	2	6m	83	10	11i	50**	5
5h	65**	9	(*R*)-6m	72**	3	11j	55**	3
5i	52**	2	(*S*)-6m	74*	8	11k	77*	9
5j	64**	1	9a	75*	3	12a	78**	5
5k	96	7	9p	73**	5	12f	58**	6
5l	91	6	9q	88	7	12g	59**	3

[a]AVG: average value; SD: standard deviation; C: DMSO (negative control); 6G: 6-gingerol (positive control). *, $p < 0.05$; **, $p < 0.005$ versus with the control.

structures showing RhlR antagonism. Compound **5a** showed stronger RhlR-inhibitory activity than the other compounds **5r** and **5s**. Therefore, we introduce diverse substituents on the phenyl ring. Among the substituted phenyl ring compounds (**5c**−**5k**), *o*-substituted and *m*-substituted compounds showed stronger RhlR-inhibitory activity than *p*-substituted compounds. The inductive effect of substituents did not significantly affect the antagonistic activity. In addition, most of the phenyl compounds showed much stronger RhlR-inhibitory activity than lipophilic alkyl chain compounds (**5l**, **5m**, **5n**, **5p**, **5q**). In this series, compound **5a** without any substituent in the phenyl ring showed more potent RhlR-inhibitory activity than the other gingerol derivatives (**5b**−**6m**). To confirm the effect of 3′,4′-difluorophenyl group in the head region, we replaced 3′-methoxy-4′-hydroxyphenyl group with 3′,4′-difluorophenyl group (**11a**, **11c**−**11k**) (Scheme 3). Although the overall trend was similar to the 3′-methoxy-4′-hydroxyphenyl compounds, compounds with 3′,4′-difluorophenyl ring showed reduced RhlR inhibition (Table 1).

Second, we attempted to assess the importance of alkyne group in the middle region by converting the alkyne into a triazole ring in the middle region (Scheme 2). Triazole ring was considered a surrogate for *cis*-alkene moiety. According to the comparison of the ynone compounds (**5a**, **5p**, **5q**) with the corresponding triazole compounds (**9a**, **9p**, **9q**), relative RhlR activity of *P. aeruginosa* at 10 μM treatment was significantly reduced to 75% and 88% for compounds **9a** and **9q** as compared to **5a** and **5q**, respectively (Table 1).

Third, we evaluated the necessity of the carbonyl group and the effect of absolute configuration on the RhlR activities (Schemes 1 and 3). The ynone compounds (**5a**, **11a**) with phenyl group in the tail region showed better RhlR-inhibitory activities than corresponding alkynol compounds (**6a**, **12a**) (Table 1). In contrast, the difluoro ynone analogs (**11f**, **11g**) with substituted phenyl group in the tail region were less potent than corresponding alkynol compounds (**12f**, **12g**). These results suggest that the hydroxyl group could be a surrogate for the carbonyl group. We prepared chirality-controlled alkynol analogs (**6m**, (*R*)-**6m**, (*S*)-**6m**) of the ynone analog **5m**. The (*R*)-stereoisomer was slightly more active than the (*S*)-stereoisomer or the racemate. It is the similar trend to the results reported in the previous studies.[24,36] Compounds **5a**, **5o**, **5s**, **6a**, **11f**, and **11e** displayed remarkable inhibition of *P. aeruginosa* biofilm formation at 10 μM as compared to other compounds (Table 2). Compound **5a** showed high inhibitory

Table 2. Relative Biofilm Formation (%) on *P. aeruginosa* with 10 μM of Synthesized Compounds[a]

Entry	AVG	SD	Entry	AVG	SD	Entry	AVG	SD
C	100	4	5m	68**	7	10p	74**	4
6G	83**	5	5n	80**	5	10q	78**	6
30	66**	6	5o	42**	5	11a	77**	5
5a	46**	6	5p	65**	7	11c	71**	5
5b	66**	4	5q	69**	7	11d	64**	4
5c	75**	7	5s	53**	7	11e	53**	6
5d	77**	8	6a	51**	7	11f	50**	4
5e	74**	8	6i	61**	5	11g	56**	4
5f	76**	6	6j	62**	5	11h	62**	8
5g	67**	4	6m	75**	4	11i	78**	7
5h	65**	9	(*R*)-6m	72**	2	11j	83**	3
5i	70**	6	(*S*)-6m	69**	4	11k	88**	1
5j	65**	4	9a	70**	8	12a	78**	8
5k	73**	1	9p	72**	7	12f	137**	8
5l	76**	9	9q	70**	2	12g	136**	8

[a]AVG: average value; SD: standard deviation; C: DMSO (negative control); 6G: 6-gingerol (positive control). *, $p < 0.05$; **, $p < 0.005$ versus with the control.

activity in the static biofilm formation assay as well as the RhlR reporter strain assay. Interestingly, compound **5o** with lipophilic cyclohexyl group in the tail region showed similar biofilm inhibitory activity with compound **5a**. However, the growth of *P. aeruginosa* decreased by 20% at 100 μM of compound **5o**, whereas significant growth inhibition patterns were not observed in other compounds (Table S1).

In general, there was no distinct correlation between the RhlR reporter strain assay and the static biofilm formation assay. We hypothesized that their inconsistent patterns may be associated with other QS systems in *P. aeruginosa*, especially with the *las* system.[8] The structures of BHL and PQS are not alike, but BHL is structurally similar to OdDHL, except for the carbonyl group in the middle and the length of the alkyl chain in the tail region (Figure S56).[9] It indicates that structural similarity between BHL and OdDHL may affect LasR selectivity in addition to RhlR.[29]

To validate the hypothesis, we selected some compounds (**30**, **5a**, **5i**, **5j**, **5m**, **11f**, **11g**, **11i**, **11j**) based on the SAR results and evaluated additional biological activities. We compared receptor or binding affinity, gene expression, and virulence factor production regarding QS systems (*las*, *rhl*, *pqs*) in *P. aeruginosa* under the same experimental conditions of the RhlR reporter strain assay and the static biofilm formation assay. The selected compounds are categorized into several specific criteria (Figure 2). We expected to confirm the appropriate

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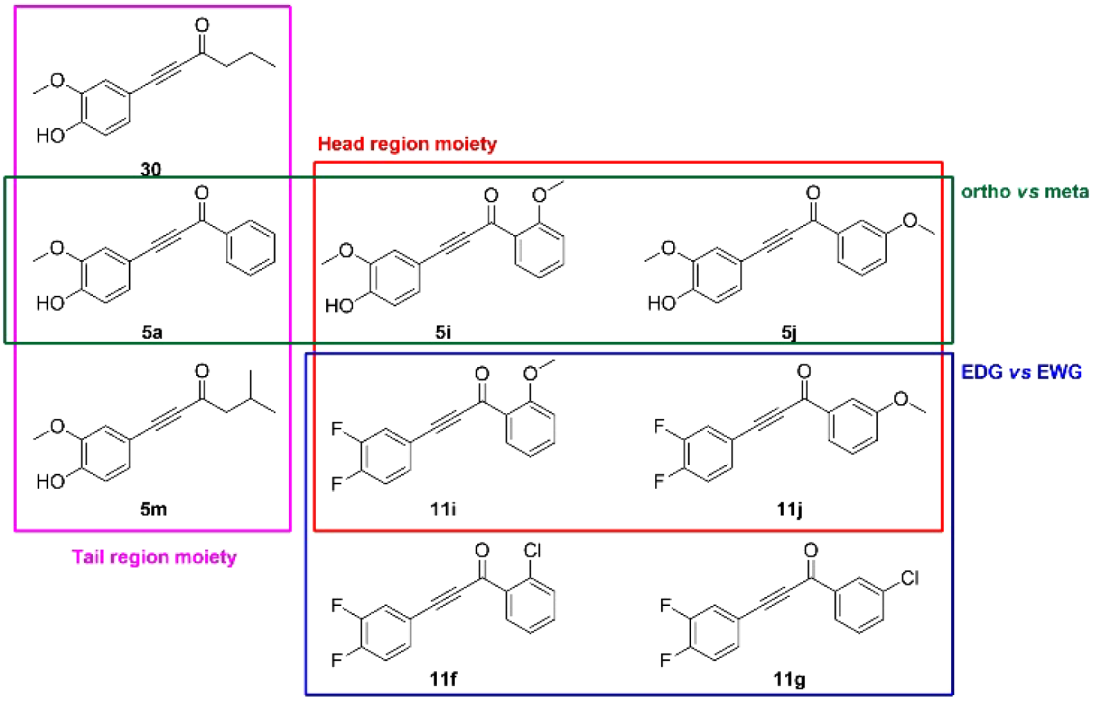

Figure 2. Selected compounds for further biological studies.

moiety between the phenyl group and the lipophilic alkyl chain in the tail region by comparing compounds **30**, **5a**, and **5m**. By comparing compound **5a** with **5i** and **5j**, we determined the positional effect of the substituent on QS systems.

Additionally, compounds **11i** and **11j**, which showed the strong RhlR-inhibitory activity, were compared with the corresponding compounds **5i** and **5j** to identify the effect of the substituents in the head region. Lastly, we determined the inductive effect of the substituent in the tail region with compounds **11i**, **11j**, **11f**, and **11g**.

Overall, the phenyl compound without any substituent in the tail region (**5a**) showed the strongest RhlR-inhibitory activity with an 18 μM of IC_{50} (Figure 3, Tables 3, and S3). There was no significant difference in the positional effect of the substituent in phenyl ring (**5i**, **5j**).

Compounds with 3′,4′-difluorophenyl group in the head region (**11i**, **11j**) and difluoro ynone group with substituted phenyl ring in the tail region (**11f**, **11g**) showed decreased RhlR activity.

None of the synthesized compounds exhibited agonism activity in RhlR including LasR and PqsR (Figure S57). Surprisingly, all selected compounds are not bound to PqsR. In contrast, they mostly showed LasR-inhibitory activity (Figure 3 and Table S2). In particular, compound **5a** with a phenyl group in the tail region showed strong LasR-inhibitory activity, whereas this was not the case for compound **5m** with a branched alkyl chain in the tail region. Interestingly, our previous gingerol-based RhlR antagonist without any functional group in the tail region, compound **30**, displayed only RhlR antagonism but not LasR and PqsR.[30] From these results, we expected that the phenyl group in the tail region of compound **5a** may act as a dual-antagonism against LasR and RhlR.

The plausible binding modes of compound **5a** in LasR and RhlR were predicted by *in silico* molecular docking studies using the Schrödinger Maestro program (ver. 13.4). The X-ray

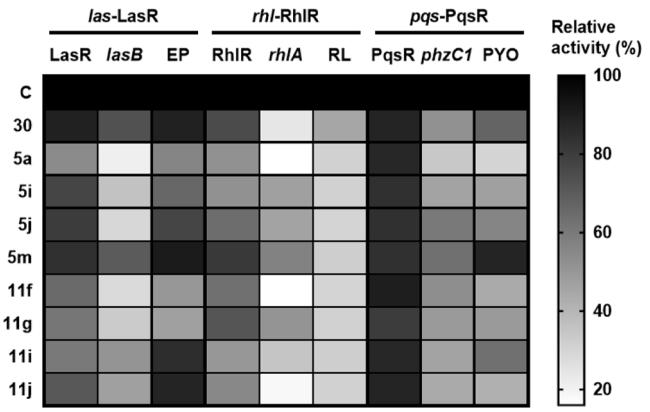

Figure 3. Heatmap of relative biological activities of selected compounds on QS systems (*las*, *rhl*, *pqs*). The binding affinity of cognate receptors (LasR, RhlR, PqsR) was assessed at 1:1 ratios of signal molecules (OdDHL, BHL, PQS) and compounds. Representative gene expression levels (*lasB*, *rhlA*, *phzC1*) and production of virulence factors (exoprotease, rhamnolipid, pyocyanin) were evaluated using *P. aeruginosa* treated with 10 μM compounds. EP: exoprotease; RL: rhamnolipid; PYO: pyocyanin.

crystal structures of LasR (PDB ID: 2UV0) in complex with OdDHL and RhlR (PDB ID: 7R3J) in complex with BHL were downloaded from the RCSB Protein Data Bank. As shown in Figure 4, the best-docked pose of compound **5a** made hydrogen-bonding interactions with Ser 129 and Tyr47 (Figure 4B). Furthermore, the phenyl group in the tail region formed $\pi-\pi$ stacking interactions with Tyr56 and Trp88. Therefore, the introduction of phenyl group in the tail region seems to affect additional LasR activity. In contrast, no PqsR-inhibitory activity was observed regardless of the molecular size and the substituents in the tail region because PQS is structurally different from OdDHL and BHL (Figures 3 and S56, Table S2). In the case of the docking studies with RhlR, the best-docked pose of compound **5a** showed that the two

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Table 3. IC_{50} (Half Maximal Inhibitory Concentration) Values (μM) of Selected Compounds on *P. aeruginosa* RhlR Binding and Biofilm Formation[a]

Entry	RhlR binding		Biofilm formation	
	AVG	SD	AVG	SD
6G	>100		46	3
30	78	5	25	2
5a	18	2	9	1
5i	18	1	21	2
5j	47	4	16	1
5m	>100		17	1
11f	41	5	10	1
11g	>100		14	1
11i	14	1	33	2
11j	71	3	41	1

[a]AVG: average value; SD: standard deviation; 6G: 6-gingerol.

phenyl groups in the head and tail regions formed $\pi-\pi$ stacking interactions with Tyr64 while BHL made hydrogen-bonding interaction with Tyr64 (Figure 4C,D). Overall, compound **5a** interacted with the key amino acids in the active site identified in the X-ray crystal structures of LasR and RhlR, respectively.

The selected compounds significantly affected *rhl*-associated gene expression and virulence factor production with an average inhibitory activity of 67% compared to the *las* (42%) and *pqs* (48%) systems (Figure 3 and Table S2). We selected genes and virulence factors representing the QS system as follows: *lasB* and exoprotease for *las* system, *rhlA* and rhamnolipid for *rhl* system, and *phzC1* and pyocyanin for *pqs* system.[30] In detail, 27−79% of *lasB*, 43−84% of *rhlA*, and 37−66% of *phzC1* were down-regulated in *P. aeruginosa* biofilm cells treated with the selected compounds. The compounds reduced virulence factor production by 9−53% of exoprotease, 55−70% of rhamnolipid, and 12−70% of pyocyanin in *P. aeruginosa*. Interestingly, although there was no

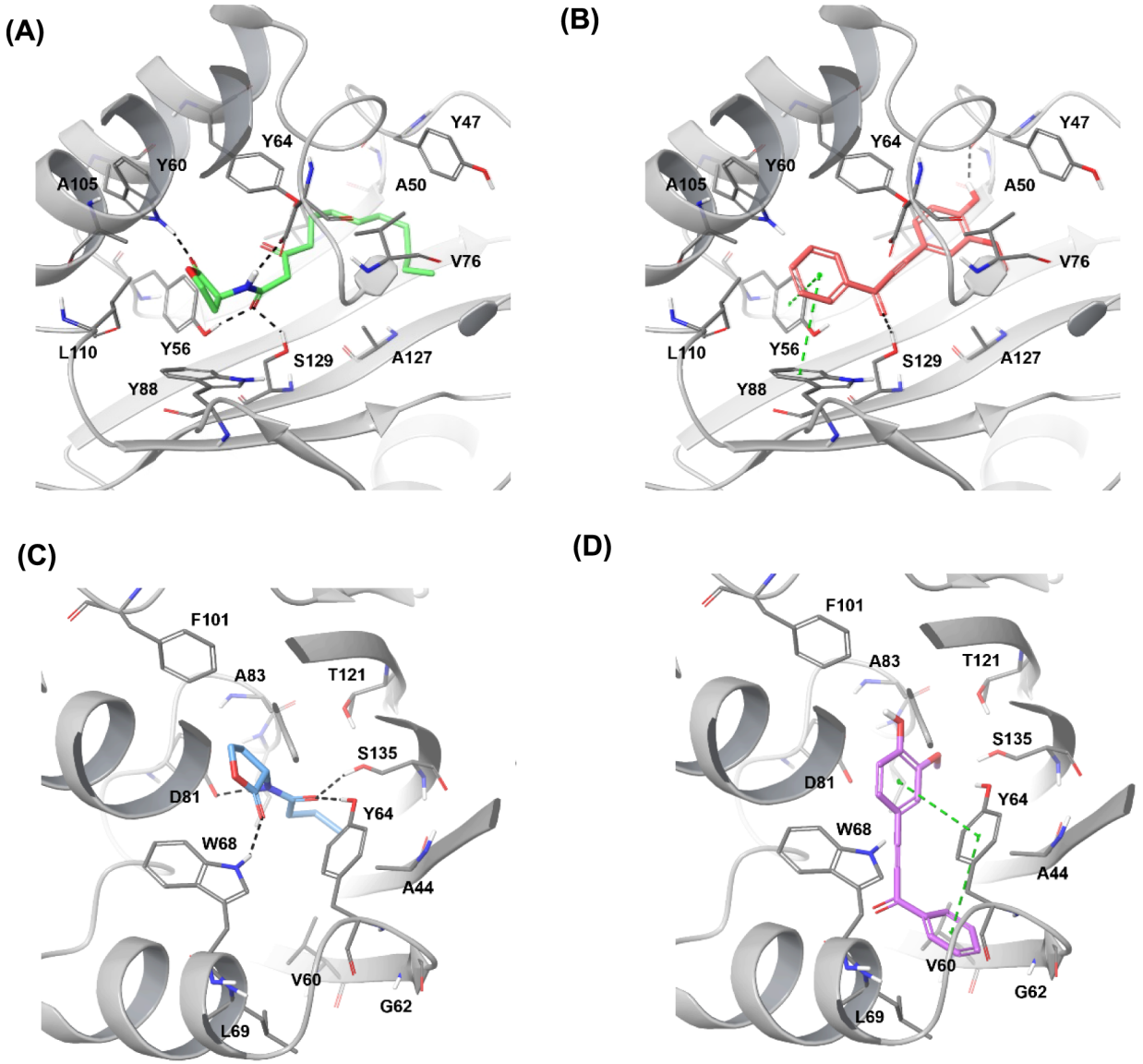

Figure 4. (A) OdDHL with LasR (PDB ID: 2UV0). (B) Compound **5a** with LasR. (C) BHL with RhlR (PDB ID: 7R3J). (D) Compound **5a** with RhlR.

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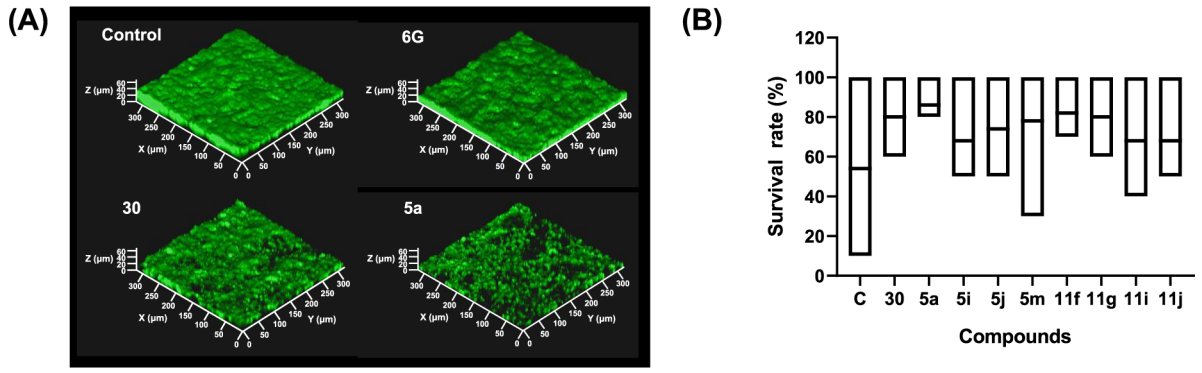

Figure 5. Biofilm formation and pathogenicity of *P. aeruginosa* treated with 10 μM compounds. (A) Biofilm formation under flow conditions. (B) Mortality of mealworms (*Tenebrio molitor* larvae). Floating bars indicate the range (min to max) of survival rate for 4 days. Lines located in the middle of the bars indicate average survival rate. The survival curve is statistically significant with 0.0295 and 0.0326 of *p* values by Log-rank and Gehan-Breslow-Wilcoxon tests, respectively.

interaction between the selected compounds and PqsR binding, the compounds inhibited *phzC1* expression and pyocyanin production. The *pqs* system is activated by the *las* system, while it is inhibited by the *rhl* system.[9]

Accordingly, Blackwell and coworkers developed an RhlR agonist to inhibit pyocyanin production by suppressing *pqs* signaling.[37] Other researchers found that *rhlR* mutants insignificantly affect *las*- or *pqs*-related system genes.[38] Some studies even revealed that phzA1-G1 gene cluster and pyocyanin production were decreased in *rhlR* mutants.[23] The QS system is complicated and can be changed easily due to various variables.

Although explaining the impact of the QS system accurately is complex, the interactive systems would have influenced *pqs*-related gene expression and the production of virulence factors.

Among the selected compounds, compound **5a**, a LasR and RhlR dual antagonist, showed the strongest disturbance capability in various QS systems (Figure 3 and Table S2). Encouraged by the results, we analyzed the effects of compound **5a** on the biofilm formation and pathogenicity of *P. aeruginosa*. Biofilm formation under the dynamic conditions was observed with 6-gingerol and compound **30** as positive controls (Figure 5A).[31,36] Biofilm formation treated with compound **5a** appeared loose and porous compared to control condition (i.e., compound-untreated biofilm). The volume of biofilm treated with compound **5a** decreased by 1.5- and 3.4-folds compared to 6-gingerol or compound **30**-treated biofilms, respectively (Figure S58). In contrast, there were little differences in thickness among them (35−46%). Under the static conditions, IC_{50} of 6-gingerol, **30**, and **5a** were determined as 46, 25, and 9 μM, respectively (Table 3), confirming that compound **5a** was the most potent biofilm inhibitor.

Furthermore, compound **5a** effectively reduced the mortality rate of mealworms infected with *P. aeruginosa* (Figure 5B). Mealworms started to die from the beginning, and only 20% of them survived after 4 days. Compound **5a** significantly increased the survival rate of mealworms *in vivo* by 80% compared to the other compounds (Figure 5B). Only 20% of mealworms died when injected *P. aeruginosa* treated with compound **5a**, while 30−70% of mealworms were affected by other compounds (Figure 5B). Our previous study reported that survival rate of mealworms injected with *rhlR* mutants of *P. aeruginosa* enhanced compared to those with wild-type *P. aeruginosa*: 90% for *rhlR* mutants and 40% for wild-type.[30] This indicates that a close correlation of the *rhl* system with pathogenicity of *P. aeruginosa* and their pathogenicity was successfully alleviated by compound **5a**.

In addition to PA14, 6-gingerol and its analogs affected other clinical *P. aeruginosa* PAO1. Biofilm turned loose compared to the control, with the following inhibition ratios: 27% for 6-gingerol, 36% for compound **30**, and 50% for compound **5a** (Figure S59A). Especially, compound **5a** reduced the mortality of PAO1-injected mealworms by 1.3−1.5 folds at the end of the incubation when comparing positive controls (6-gingerol and compound **30**) (Figure S59B). Overall, they showed similar inhibition effects on biofilm formation and pathogenicity between PA14 and PAO1, which indicates that compound **5a** can be effective against other clinical *P. aeruginosa*.

This potent therapeutic efficiency of compound **5a** may be explained by the dual RhlR-LasR targeting strategy. Dual inhibitors were known from some studies,[26,39] but their synergistic mechanism remains to be elucidated due to the complexity of QS systems.[8,9] Soto-Aceves and coworkers found that individual elimination of either *lasR* or *rhlR* partially affects QS-related virulence factors.[40] In contrast, their double mutants completely attenuated *P. aeruginosa* infections. Furthemore, QS mutants were isolated from CF patients at a frequency of 3 to 50%, although the possibility of isolation of *rhlR* mutants was relatively lower than that of *lasR* mutants.[18−20,41] Overall, a dual targeting strategy may be another attractive approach to treat *P. aeruginosa* infections in CF patients.

CONCLUSIONS

RhlR is an important QS transcription factor and may be a potential target for the treatment of chronic *P. aeruginosa* infection. Based on the structure of compound **30**, identified as the most potent gingerol-based RhlR antagonist in our previous study, 55 compounds were synthesized by introducing various functional groups in the middle and tail regions. Among the synthesized compounds, compound **5a** substituted with the phenyl group in the tail region showed the most potent inhibitory activities in the RhlR strain assay (52% relative RhlR activity at 10 μM) as well as the static biofilm formation assay (46% relative biofilm formation at 10 μM). Furthermore, compound **5a** showed LasR antagonism through a phenyl ring in the tail region. This dual targeting strategy against RhlR and LasR would have resulted in strong inhibition

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of QS gene expression levels, production of virulence factors, and pathogenicity in *P. aeruginosa*. Therefore, compound **5a** inhibits not only the *rhl* system but also the *las* system, suggesting its potential use as a dual inhibitor of RhlR and LasR to manage *P. aeruginosa* infections.

EXPERIMENTAL REGION

General. All commercial chemicals and solvents were used without further purification. The reaction was monitored using thin layer chromatography (TLC) on precoated $60F_{254}$ (Merck; Darmstadt, Germany) silica gel plates and visualized under UV light at 254 nm. Column chromatography was conducted using silica gel (Silica gel 60; 230−400 mesh ASTM, Merck, Darmstadt, Germany). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Ultrashield 600 MHz Plus spectrometer (^{1}H, 600 MHz; ^{13}C, 150 MHz). All chemical shifts of NMR spectra are reported in parts per million (ppm) from tetramethylsilane (δ = 0) or measured relative to the deuterated solvent in which the sample was dissolved ($CDCl_3$: δ 7.26 for ^{1}H NMR, δ 77.16 for ^{13}C NMR; MeOH-d_4: δ 3.31 for ^{1}H NMR, δ 49.0 for ^{13}C NMR). High-resolution mass spectra (HRMS) were obtained on an Agilent 6530 Accurate Mass Q-TOF LC/MS spectrometer. All final compounds were purified and their purity determined by analytical reverse-phase (RP) HPLC on an Agilent 1260 Infinity and a Phenomenex C18 column (150 mm × 4.6 mm, 3 μm, 110 Å). RP-HPLC was performed using the following isocratic conditions: for method A, mobile phase was acetonitrile and water (60:40, *v*/*v*); for method B, mobile phase was acetonitrile and water (55:45, *v*/*v*); for method C, mobile phase was acetonitrile and water (50:50, *v*/*v*); for method D, mobile phase was acetonitrile and water (40:60, *v*/*v*); for method E, mobile phase was acetonitrile and water (30:70, *v*/*v*); for method F, mobile phase was acetonitrile and water (20:80, *v*/*v*). All compounds were eluted with a flow rate of 1 mL/min and monitored by the UV detector (254 nm). The purity of the final compounds for biological testing was >95%.

Chemical Synthesis. Compounds **1**−**3** and Weinreb amides (*N*-methoxy-*N*-methylbutyramide, *N*-methoxy-*N*-methylhexamide) were prepared by applying the synthetic procedure reported previously.[25] *N-methoxy-N-methyl-2-phenylacetamide* was synthesized as described below.

To a solution of *N*,*O*-dimethylhydroxylamine hydrochloride (585 mg, 6 mmol, 1.2 equiv) and phenyl acetyl chloride (0.66 mL, 5 mmol) in DCM (20 mL) was added dropwise pyridine (0.89 mL, 2.2 equiv) for 5 min at 0 °C under argon. The reaction mixture was stirred at room temperature for 1 h, followed by washing with 0.1 N HCl (2 × 20 mL), distilled water (2 × 20 mL), and saturated $NaHCO_3$ (2 × 20 mL). The combined organic layer was washed with brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to furnish *N-methoxy-N-methyl-2-phenylacetamide* (915 mg, 97%) as yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.33−7.27 (m, 4H), 7.26−7.22 (m, 1H), 3.77 (s, 2H), 3.60 (s, 3H), 3.19 (s, 3H).

*General Procedure for Compounds **4a**−**4s**.* To a solution of appropriate ethynylbenzene (300 mg) in THF (30 mL) was added *n*-BuLi (1.5 eq., 1.6 M in hexanes) at −78 °C. The solution was stirred for 30 min under argon at the same temperature. The corresponding acid chloride (1.3 equiv) or Weinreb amide (1.3 equiv) was added dropwise to the solution. The reaction mixture was stirred at the same temperature for 12−16 h. The reaction was quenched with aqueous NH_4Cl (10 mL), extracted with EtOAc (3 × 25 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and purified by column chromatography on silica gel (Hexane/EtOAc = 80/1 to 10/1, *v*/*v*) to provide compounds **4a**−**4s**.

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-phenylprop-2-yn-1-one (**4a**).* Compound **4a** (362 mg, 0.99 mmol) was prepared in 88% yield as a yellow oil, by following the same method as described in the general procedure with benzoyl chloride. R_f = 0.58 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.22 (d, *J* = 7.2 Hz, 2H), 7.63 (t, *J* = 7.2 Hz, 1H), 7.52 (t, *J* = 7.8 Hz, 2H), 7.24 (dd, *J* = 8.1 and 1.8 Hz, 1H), 7.16 (d, *J* = 1.8 Hz, 1H), 6.87 (d, *J* = 8.4 Hz, 1H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-(4-(trifluoromethyl)phenyl)prop-2-yn-1-one (**4b**).* Compound **4b** (244 mg, 0.56 mmol) was prepared in 50% yield as a yellow oil, by following the same method as described in the general procedure with 4-(trifluoromethyl)benzoyl chloride. R_f = 0.65 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.32 (d, *J* = 8.1 Hz, 2H), 7.79 (d, *J* = 8.2 Hz, 2H), 7.26−7.23 (m, 1H), 7.16 (d, *J* = 1.7 Hz, 1H), 6.88 (d, *J* = 8.1 Hz, 1H), 3.86 (s, 3H), 1.00 (s, 9H), 0.19 (s, 6H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-(o-tolyl)prop-2-yn-1-one (**4c**).* Compound **4c** (387 mg, 1.02 mmol) was prepared in 89% yield as a yellow oil, by following the same method as described in the general procedure with *o*-toluoyl chloride. R_f = 0.54 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.27 (d, *J* = 7.6 Hz, 1H), 7.46 (t, *J* = 7.1 Hz, 1H), 7.36 (t, *J* = 7.5 Hz, 1H), 7.27 (d, *J* = 7.6 Hz, 1H), 7.19 (dd, *J* = 8.1 and 1.8 Hz, 1H), 7.13 (d, *J* = 1.7 Hz, 1H), 6.85 (d, *J* = 8.1 Hz, 1H), 3.84 (s, 3H), 2.67 (s, 3H), 1.00 (s, 9H), 0.18 (s, 6H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-(m-tolyl)prop-2-yn-1-one (**4d**).* Compound **4d** (295 mg, 0.78 mmol) was prepared in 68% yield as a yellow oil, by following the same method as described in the general procedure with *m*-toluoyl chloride. R_f = 0.46 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.03 (d, *J* = 7.4 Hz, 1H), 8.01 (s, 1H), 7.46−7.38 (m, 2H), 7.23 (dd, *J* = 8.1 and 1.8 Hz, 1H), 7.16 (d, *J* = 1.6 Hz, 1H), 6.87 (d, *J* = 8.1 Hz, 1H), 3.85 (s, 3H), 2.45 (s, 3H), 1.00 (s, 9H), 0.19 (s, 6H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-(p-tolyl)prop-2-yn-1-one (**4e**).* Compound **4e** (233 mg, 0.61 mmol) was prepared in 54% yield as a yellow oil, by following the same method as described in the general procedure with *p*-toluoyl chloride. R_f = 0.50 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.11 (d, *J* = 8.1 Hz, 2H), 7.31 (d, *J* = 8.0 Hz, 2H), 7.23 (dd, *J* = 8.1 and 1.7 Hz, 1H), 7.16 (d, *J* = 1.5 Hz, 1H), 6.87 (d, *J* = 8.1 Hz, 1H), 3.85 (s, 3H), 2.45 (s, 3H), 1.00 (s, 9H), 0.19 (s, 6H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-(2-chlorophenyl)prop-2-yn-1-one (**4f**).* Compound **4f** (314 mg, 0.78 mmol) was prepared in 68% yield as a yellow oil, by following the same method as described in the general procedure with 2-chlorobenzoyl chloride. R_f = 0.40 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.06 (d, *J* = 7.6 Hz, 1H), 7.51−7.43 (m, 2H), 7.40 (t, *J* = 7.1 Hz, 1H), 7.19 (d, *J* = 8.1 Hz, 1H), 7.12 (s, 1H), 6.85 (d, *J* = 8.1 Hz, 1H), 3.83 (s, 3H), 0.99 (s, 9H), 0.18 (s, 6H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-(3-chlorophenyl)prop-2-yn-1-one (**4g**).* Compound **4g** (454 mg, 1.13 mmol) was prepared in 99% yield as a yellow oil, by

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following the same method as described in the general procedure with 3-chlorobenzoyl chloride. R_f = 0.63 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.17 (s, 1H), 8.10 (d, *J* = 7.7 Hz, 1H), 7.59 (dd, *J* = 7.9 and 1.0 Hz, 1H), 7.46 (t, *J* = 7.8 Hz, 1H), 7.26−7.24 (m, 1H), 7.16 (d, *J* = 1.6 Hz, 1H), 6.88 (d, *J* = 8.1 Hz, 1H), 3.85 (s, 3H), 1.00 (s, 9H), 0.19 (s, 6H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-(4-chlorophenyl)prop-2-yn-1-one (**4h**).* Compound **4h** (432 mg, 1.08 mmol) was prepared in 94% yield as a yellow oil, by following the same method as described in the general procedure with 4-chlorobenzoyl chloride. R_f = 0.53 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.15 (d, *J* = 8.4 Hz, 2H), 7.49 (d, *J* = 8.4 Hz, 2H), 7.23 (dd, *J* = 8.1 and 1.4 Hz, 1H), 7.15 (s, 1H), 6.87 (d, *J* = 8.1 Hz, 1H), 3.85 (s, 3H), 1.00 (s, 9H), 0.19 (s, 6H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-(2-methoxyphenyl)prop-2-yn-1-one (**4i**).* Compound **4i** (89 mg, 0.22 mmol) was prepared in 18% yield as a yellow oil, by following the same method as described in the general procedure with 2-methoxybenzoyl chloride. R_f = 0.13 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.08 (dd, *J* = 7.7 and 1.6 Hz, 1H), 7.56−7.51 (m, 1H), 7.17 (dd, *J* = 8.1 and 1.8 Hz, 1H), 7.13 (d, *J* = 1.6 Hz, 1H), 7.06 (t, *J* = 7.5 Hz, 1H), 7.02 (d, *J* = 8.4 Hz, 1H), 6.84 (d, *J* = 8.1 Hz, 1H), 3.97 (s, 3H), 3.83 (s, 3H), 1.00 (s, 9H), 0.18 (s, 6H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-(3-methoxyphenyl)prop-2-yn-1-one (**4j**).* Compound **4j** (357 mg, 0.90 mmol) was prepared in 76% yield as a yellow oil, by following the same method as described in the general procedure with 3-methoxybenzoyl chloride. R_f = 0.39 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.86 (d, *J* = 7.6 Hz, 1H), 7.70 (dd, *J* = 2.2 and 1.4 Hz, 1H), 7.43 (t, *J* = 7.9 Hz, 1H), 7.23 (dd, *J* = 8.1 and 1.9 Hz, 1H), 7.19−7.16 (m, 1H), 7.16 (d, *J* = 1.7 Hz, 1H), 6.87 (d, *J* = 8.1 Hz, 1H), 3.89 (s, 3H), 3.85 (s, 3H), 1.00 (s, 9H), 0.19 (s, 6H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-(4-methoxyphenyl)prop-2-yn-1-one (**4k**).* Compound **4k** (199 mg, 0.50 mmol) was prepared in 44% yield as a yellow oil, by following the same method as described in the general procedure with 4-methoxybenzoyl chloride. R_f = 0.23 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.19 (d, *J* = 8.9 Hz, 2H), 7.22 (dd, *J* = 8.1 and 1.9 Hz, 1H), 7.15 (d, *J* = 1.7 Hz, 1H), 6.99 (d, *J* = 8.9 Hz, 2H), 6.86 (d, *J* = 8.1 Hz, 1H), 3.90 (s, 3H), 3.84 (s, 3H), 1.00 (s, 9H), 0.18 (s, 6H).

*1-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-4-methylpent-1-yn-3-one (**4l**).* Compound **4l** (229 mg, 0.69 mmol) was prepared in 63% yield as a yellow oil, by following the same method as described in the general procedure with isobutyryl chloride. R_f = 0.54 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.11 (dd, *J* = 7.8 and 1.8 Hz, 1H), 7.05 (s, 1H), 6.83 (d, *J* = 8.1 Hz, 1H), 3.81 (s, 3H), 2.78−2.70 (m, 1H), 1.26 (d, *J* = 6.9 Hz, 6H), 0.99 (s, 9H), 0.17 (s, 6H).

*1-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-5-methylhex-1-yn-3-one (**4m**).* Compound **4m** (179 mg, 0.52 mmol) was prepared in 45% yield as a yellow oil, by following the same method as described in the general procedure with isovaleryl chloride. R_f = 0.50 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.11 (dd, *J* = 8.1 and 1.9 Hz, 1H), 7.04 (d, *J* = 1.8 Hz, 1H), 6.82 (d, *J* = 8.1 Hz, 1H), 3.81 (s, 3H), 2.53 (d, *J* = 7.1 Hz, 2H), 2.36−2.29 (m, 1H), 1.01 (s, 3H), 1.00 (s, 3H), 0.99 (s, 9H), 0.17 (s, 6H).

*1-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-4-methylhex-1-yn-3-one (**4n**).* Compound **4n** (334 mg, 0.96 mmol) was prepared in 84% yield as a yellow oil, by following the same method as described in the general procedure with 2-methylbutyryl chloride. R_f = 0.52 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.12 (dd, *J* = 8.1 and 1.8 Hz, 1H), 7.05 (d, *J* = 1.6 Hz, 1H), 6.83 (d, *J* = 8.1 Hz, 1H), 3.82 (s, 3H), 2.62−2.56 (m, 1H), 1.91−1.83 (m, 1H), 1.60−1.55 (m, 1H), 1.23 (d, *J* = 7.0 Hz, 3H), 0.99 (s, 9H), 0.98−0.94 (m, 3H), 0.17 (s, 6H).

*3-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-cyclohexylprop-2-yn-1-one (**4o**).* Compound **4o** (290 mg, 0.78 mmol) was prepared in 68% yield as a yellow oil, by following the same method as described in the general procedure with cyclohexanecarbonyl chloride. R_f = 0.55 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.12 (dd, *J* = 8.1 and 1.8 Hz, 1H), 7.05 (d, *J* = 1.8 Hz, 1H), 6.82 (d, *J* = 8.1 Hz, 1H), 3.82 (s, 3H), 2.52−2.48 (m, 1H), 2.05 (dd, *J* = 13.4 and 2.8 Hz, 2H), 1.85−1.79 (m, 2H), 1.71−1.66 (m, 1H), 1.53−1.45 (m, 2H), 1.38−1.29 (m, 2H), 1.28−1.19 (m, 1H), 0.99 (s, 9H), 0.17 (s, 6H).

*1-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-hex-1-yn-3-one (**4p**).* Compound **4p** (287 mg, 0.86 mmol) was prepared in 76% yield as a colorless oil, by following the same method as described in the general procedure with *N*-methoxy-*N*-methylbutyramide. R_f = 0.65 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.11 (dd, *J* = 8.1 and 1.9 Hz, 1H), 7.05 (*d*, J = 1.8 Hz, 1H), 6.82 (d, *J* = 8.1 Hz, 1H), 3.81 (s, 3H), 2.63 (t, *J* = 7.3 Hz, 2H), 1.81−1.72 (m, 2H), 1.01−0.97 (m, 12H), 0.17 (s, 6H).

*1-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-oct-1-yn-3-one (**4q**).* Compound **4q** (322 mg, 0.89 mmol) was prepared in 78% yield as a colorless oil, by following the same method as described in the general procedure with *N*-methoxy-*N*-methylhexamide. R_f = 0.75 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.11 (dd, *J* = 8.2 and 1.5 Hz, 1H), 7.05 (d, *J* = 1.5 Hz, 1H), 6.82 (d, *J* = 8.1 Hz, 1H), 3.81 (s, 3H), 2.64 (t, *J* = 7.5 Hz, 2H), 1.78−1.70 (m, 2H), 1.40−1.30 (m, 4H), 0.99 (s, 9H), 0.91 (t, *J* = 6.7 Hz, 3H), 0.17 (s, 6H).

*4-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-phenylbut-3-yn-2-one (**4r**).* Compound **4r** (54 mg, 0.14 mmol) was prepared in 12% yield as a yellow oil, by following the same method as described in the general procedure with *N-methoxy-N-methyl-2-phenylacetamide* (167 mg, 0.93 mmol). R_f = 0.61 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.38 (t, *J* = 7.5 Hz, 2H), 7.34−7.30 (m, 3H), 6.98 (dd, *J* = 8.2 and 1.8 Hz, 1H), 6.89 (d, *J* = 1.6 Hz, 1H), 6.79 (d, *J* = 8.2 Hz, 1H), 3.91 (s, 2H), 3.78 (s, 3H), 0.98 (s, 10H), 0.16 (s, 6H).

*4-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1-phenoxybut-3-yn-2-one (**4s**).* Compound **4s** (24 mg, 0.06 mmol) was prepared in 5% yield as a yellow oil, by following the same method as described in the general procedure with phenoxyacetyl chloride (205 μL, 1.49 mmol). R_f = 0.39 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.32 (t, *J* = 8.0 Hz, 2H), 7.07 (dd, *J* = 8.1 and 1.8 Hz, 1H), 7.01 (t, *J* = 7.3 Hz, 1H), 6.98−6.94 (m, 3H), 6.82 (d, *J* = 8.1 Hz, 1H), 4.80 (s, 2H), 3.79 (s, 3H), 0.99 (s, 9H), 0.17 (s, 6H).

*General Procedure for Compounds **5a**−**5s**, **9a**, and **9p**−**9q**.* To a solution of the silyl-protected compound (0.02 M) in THF was added tetrabutylammonium fluoride solution (1 M in THF, 2.0 equiv) at 0 °C. The reaction mixture was stirred for 20 min at the same temperature. The reaction mixture was

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quenched with saturated aqueous NH_4Cl (10 mL) and extracted with EtOAc (3 × 25 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and the crude residue was purified by column chromatography on silica gel (Hexane/EtOAc = 10/1 to 6/1, *v*/*v* for **5a**−**5s**, DCM/MeOH = 30/1, *v*/*v* for **9a**, **9p**−**9q**) to provide compounds **5a**−**5s**, **9a**, and **9p**−**9q**.

*3-(4-Hydroxy-3-methoxyphenyl)-1-phenylprop-2-yn-1-one (**5a**).* Compound **5a** (120 mg, 0.48 mmol) was prepared in 61% yield as a yellow powder, by following the same method as described in the general procedure with **4a** (274 mg, 0.75 mmol). R_f = 0.13 (Hexane/EtOAc = 5/1, *v*/*v*). m.p: 126−128 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.21 (s, 2H), 7.63 (t, *J* = 7.2 Hz, 1H), 7.52 (t, *J* = 7.8 Hz, 2H), 7.30 (d, *J* = 8.4 Hz, 1H), 7.17 (s, 1H), 6.96 (d, *J* = 8.4 Hz, 1H), 3.95 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 178.2, 148.9, 146.6, 137.1, 134.1, 129.6, 128.7, 128.2, 115.3, 115.1, 111.4, 94.9, 86.5, 56.2. HRMS *m*/*z* calculated for $C_{16}H_{12}O_3$ $[M-H]^-$: 251.0713; found: 251.0723. >95% purity (as determined by RP-HPLC, method D, t_R = 9.96 min).

*3-(4-Hydroxy-3-methoxyphenyl)-1-(4-(trifluoromethyl)-phenyl)prop-2-yn-1-one (**5b**).* Compound **5b** (84 mg, 0.26 mmol) was prepared in 49% yield as a yellow powder, by following the same method as described in the general procedure with **4b** (232 mg, 0.54 mmol). R_f = 0.17 (Hexane/EtOAc = 5/1, *v*/*v*). m.p: 146−148 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.32 (d, *J* = 8.1 Hz, 2H), 7.79 (d, *J* = 8.2 Hz, 2H), 7.31 (dd, *J* = 8.2 and 1.6 Hz, 1H), 7.17 (d, *J* = 1.6 Hz, 1H), 6.97 (d, *J* = 8.2 Hz, 1H), 6.02 (s, 1H), 3.96 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 176.8, 149.2, 146.6, 139.6, 135.5, 135.2, 135.0, 134.8, 129.8, 128.5, 125.8, 125.8, 125.8, 125.7, 124.6, 122.8, 115.4, 115.2, 111.0, 96.3, 86.4, 56.3. HRMS *m*/*z* calculated for $C_{17}H_{11}F_3O_3$ $[M-H]^-$: 319.0587; found: 319.0597. >95% purity (as determined by RP-HPLC, method C, t_R = 14.43 min).

*3-(4-Hydroxy-3-methoxyphenyl)-1-(o-tolyl)prop-2-yn-1-one (**5c**).* Compound **5c** (147 mg, 0.53 mmol) was prepared in 58% yield as an orange powder, by following the same method as described in the general procedure with **4c** (341 mg, 0.90 mmol). R_f = 0.13 (Hexane/EtOAc = 5/1, *v*/*v*). m.p: 92−94 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.27 (dd, *J* = 7.7 and 0.72 Hz, 1H), 7.46 (dt, *J* = 7.5 and 1.1 Hz, 1H), 7.38−7.33 (m, 1H), 7.29−7.26 (m, 1H), 7.26−7.22 (m, 1H), 7.14 (d, *J* = 1.7 Hz, 1H), 6.93 (dd, *J* = 8.2 and 3.3 Hz, 1H), 3.93 (s, 3H), 2.67 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 180.1, 148.8, 146.6, 140.4, 136.08, 133.1, 132.8, 132.3, 128.0, 126.0, 115.2, 115.1, 111.6, 93.5, 88.0, 56.2, 22.0. HRMS *m*/*z* calculated for $C_{17}H_{14}O_3$ $[M-H]^-$: 265.0870; found: 265.0882. >95% purity (as determined by RP-HPLC, method C, t_R = 9.51 min).

*3-(4-Hydroxy-3-methoxyphenyl)-1-(m-tolyl)prop-2-yn-1-one (**5d**).* Compound **5d** (144 mg, 0.54 mmol) was prepared in 70% yield as an orange powder, by following the same method as described in the general procedure with **4d** (292 mg, 0.77 mmol). R_f = 0.16 (Hexane/EtOAc = 5/1, *v*/*v*). m.p: 102−104 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.03 (d, *J* = 7.4 Hz, 1H), 8.01 (s, 1H), 7.37−7.46 (m, 2H), 7.29 (dd, *J* = 8.2 and 1.7 Hz, 1H), 7.17 (d, *J* = 1.5 Hz, 1H), 6.96 (d, *J* = 8.2 Hz, 1H), 3.95 (s, 3H), 2.45 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 178.4, 148.9, 146.6, 138.6, 137.2, 135.0, 130.0, 129.0, 128.2, 127.2, 115.3, 115.1, 111.6, 94.5, 86.6, 56.3, 21.5. HRMS *m*/*z* calculated for $C_{17}H_{14}O_3$ $[M-H]^-$: 265.0870; found: 265.0880. >95% purity (as determined by RP-HPLC, method C, t_R = 9.19 min).

*3-(4-Hydroxy-3-methoxyphenyl)-1-(p-tolyl)prop-2-yn-1-one (**5e**).* Compound 5e (60 mg, 0.23 mmol) was prepared in 59% yield as a yellow powder, by following the same method as described in the general procedure with **4e** (146 mg, 0.38 mmol). R_f = 0.16 (Hexane/EtOAc = 5/1, *v*/*v*). m.p: 118−120 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.11 (d, *J* = 7.7 Hz, 2H), 7.35−7.27 (m, 3H), 7.16 (s, 1H), 6.95 (d, *J* = 8.1 Hz, 1H), 6.03 (s, 1H), 3.94 (s, 3H), 2.45 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.9, 148.8, 146.6, 145.2, 134.9, 129.8, 129.8, 129.4, 128.1, 115.3, 115.1, 111.6, 94.2, 86.5, 56.3, 22.0. HRMS *m*/*z* calculated for $C_{17}H_{14}O_3$ $[M-H]^-$: 265.0870; found: 265.0882. >95% purity (as determined by RP-HPLC, method C, t_R = 7.93 min).

*1-(2-Chlorophenyl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-yn-1-one (**5f**).* Compound **5f** (175 mg, 0.61 mmol) was prepared in 87% yield as an orange powder, by following the method as described in the general procedure with **4f** (282 mg, 0.70 mmol). R_f = 0.10 (Hexane/EtOAc = 5/1, *v*/*v*). m.p: 88−90 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05 (d, *J* = 7.4 Hz, 1H), 7.51−7.44 (m, 2H), 7.43−7.37 (m, 1H), 7.25 (dd, *J* = 8.2 and 1.6 Hz, 1H), 7.13 (d, *J* = 1.3 Hz, 1H), 6.93 (d, *J* = 8.2 Hz, 1H), 3.93 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.0, 149.1, 146.6, 136.3, 133.5, 133.3, 132.5, 131.6, 128.4, 126.9, 115.3, 115.1, 111.3, 95.8, 88.1, 56.3. HRMS *m*/*z* calculated for $C_{16}H_{11}ClO_3$ $[M-H]^-$: 285.0324; found: 285.0332. >95% purity (as determined by RP-HPLC, method C, t_R = 7.74 min).

*1-(3-Chlorophenyl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-yn-1-one (**5g**).* Compound **5g** (129 mg, 0.45 mmol) was prepared in 71% yield as an orange powder, by following the same method as described in the general procedure with **4g** (258 mg, 0.64 mmol). R_f = 0.13 (Hexane/EtOAc = 5/1, *v*/*v*). m.p: 114−116 °C. ^{1}H NMR (600 MHz, $CDCl_3$) ? 8.20−8.15 (m, 1H), 8.10 (d, *J* = 7.8 Hz, 1H), 7.59 (dd, *J* = 7.9 and 1.0 Hz, 1H), 7.47 (t, *J* = 7.8 Hz, 1H), 7.30 (dt, *J* = 11.5 and 5.8 Hz, 1H), 7.17 (d, *J* = 1.6 Hz, 1H), 6.96 (d, *J* = 8.2 Hz, 1H), 3.95 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 176.7, 149.2, 146.7, 138.7, 135.0, 133.9, 130.1, 129.5, 128.5, 127.7, 115.4, 115.2, 111.1, 95.8, 86.3, 56.3. HRMS *m*/*z* calculated for $C_{16}H_{11}ClO_3$ $[M-H]^-$: 285.0324; found: 285.0337. >95% purity (as determined by RP-HPLC, method C, t_R = 12.28 min).

*1-(4-Chlorophenyl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-yn-1-one (**5h**).* Compound **5h** (162 mg, 0.57 mmol) was prepared in 70% yield as a yellow powder, by following the same method as described in the general method with **4h** (325 mg, 0.81 mmol). R_f = 0.24 (Hexane/EtOAc = 5/1, *v*/*v*). m.p: 104−106 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.17−8.11 (m, 2H), 7.51−7.46 (m, 2H), 7.27 (dd, *J* = 8.2 and 1.8 Hz, 1H), 7.14 (d, *J* = 1.7 Hz, 1H), 6.94 (d, *J* = 8.2 Hz, 1H), 3.92 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 176.8, 149.2, 146.7, 140.6, 135.5, 130.9, 129.1, 128.3, 115.3, 115.2, 111.1, 95.4, 86.3, 56.3, 56.3, 56.2. HRMS *m*/*z* calculated for $C_{16}H_{11}ClO_3$ $[M-H]^-$: 285.0324; found: 285.0329. >95% purity (as determined by RP-HPLC, method C, t_R = 12.34 min).

*3-(4-Hydroxy-3-methoxyphenyl)-1-(2-methoxyphenyl)-prop-2-yn-1-one (**5i**).* Compound **5i** (51 mg, 0.18 mmol) was prepared in 82% yield as a yellow oil, by following the same method as described in the general procedure with **4i** (89 mg, 0.22 mmol). R_f = 0.11 (Hexane/EtOAc = 3/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.08 (dd, *J* = 7.7 and 1.8 Hz, 1H), 7.56−7.51 (m, 1H), 7.22 (dd, *J* = 8.2 and 1.8 Hz, 1H), 7.13 (d, *J* =

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1.7 Hz, 1H), 7.07−7.03 (m, 1H), 7.02 (d, J = 8.3 Hz, 1H), 6.92 (d, J = 8.2 Hz, 1H), 3.97 (s, 3H), 3.92 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.0, 159.9, 148.6, 146.5, 135.0, 132.7, 128.0, 127.1, 120.4, 115.4, 115.0, 112.3, 112.1, 93.2, 88.7, 56.3, 56.1. HRMS m/z calculated for $C_{17}H_{14}O_4$ $[M-H]^-$: 281.0819; found: 281.0811. >95% purity (as determined by RP-HPLC, method D, t_R = 9.76 min).

*3-(4-Hydroxy-3-methoxyphenyl)-1-(3-methoxyphenyl)-prop-2-yn-1-one (**5j**).* Compound **5j** (215 mg, 0.76 mmol) was prepared in 85% yield as a yellow powder, by following the same method as described in the general procedure with **4j** (357 mg, 0.90 mmol). R_f = 0.21 (Hexane/EtOAc = 3/1, v/v). m.p: 92−94 °C.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.85 (d, J = 7.6 Hz, 1H), 7.70 (dd, J = 2.4 and 1.6 Hz, 1H), 7.43 (dd, J = 10.1 and 5.7 Hz, 1H), 7.31−7.27 (m, 1H), 7.19−7.15 (m, 2H), 6.95 (dd, J = 8.2 and 3.5 Hz, 1H), 6.01 (s, 1H), 3.94 (s, 3H), 3.89 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.9, 159.9, 148.9, 146.6, 138.5, 129.7, 128.3, 122.9, 120.9, 115.3, 115.1, 113.0, 111.5, 94.7, 86.6, 56.3, 55.6. HRMS m/z calculated for $C_{17}H_{14}O_4$ $[M-H]^-$: 281.0819; found: 281.0815. >95% purity (as determined by RP-HPLC, method D, t_R = 18.31 min).

*3-(4-Hydroxy-3-methoxyphenyl)-1-(4-methoxyphenyl)-prop-2-yn-1-one (**5k**).* Compound **5k** (89 mg, 0.32 mmol) was prepared in 69% yield as a white powder, by following the same method as described in the general procedure with **4k** (184 mg, 0.46 mmol). R_f = 0.15 (Hexane/EtOAc = 3/1, v/v). m.p: 124−126 °C.^{1}H NMR (600 MHz, $CDCl_3$) δ 8.21−8.17 (m, 2H), 7.28 (dd, J = 8.2 and 1.8 Hz, 1H), 7.16 (d, J = 1.7 Hz, 1H), 7.01−6.97 (m, 2H), 6.95 (d, J = 8.2 Hz, 1H), 5.97 (s, 1H), 3.94 (s, 3H), 3.91 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 176.9, 164.5, 148.7, 146.6, 132.0, 132.0, 132.0, 130.5, 128.0, 115.3, 115.0, 114.0, 114.0, 111.8, 93.9, 86.4, 56.3, 55.7. HRMS m/z calculated for $C_{17}H_{14}O_4$ $[M-H]^-$: 281.0819; found: 281.0808. >95% purity (as determined by RP-HPLC, method D, t_R = 14.94 min).

*1-(4-Hydroxy-3-methoxyphenyl)-4-methylpent-1-yn-3-one (**5l**).* Compound **5l** (121 mg, 0.56 mmol) was prepared in 81% yield as a yellow powder, by following the same method as described in the general procedure with **4l** (229 mg, 0.69 mmol). R_f = 0.13 (Hexane/EtOAc = 5/1, v/v). m.p: 72−74 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.17 (dd, J = 8.2 and 1.6 Hz, 1H), 7.06 (d, J = 1.4 Hz, 1H), 6.91 (d, J = 8.2 Hz, 1H), 5.95 (s, 1H), 3.91 (s, 3H), 2.78−2.70 (m, 1H), 1.26 (d, J = 7.0 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 192.4, 148.7, 146.5, 128.1, 115.3, 115.0, 111.5, 93.1, 86.4, 56.2, 43.1, 18.3. HRMS m/z calculated for $C_{13}H_{14}O_3$ $[M-H]^-$: 217.0870; found: 217.0873. >95% purity (as determined by RP-HPLC, method D, t_R = 9.66 min).

*1-(4-Hydroxy-3-methoxyphenyl)-5-methylhex-1-yn-3-one (**5m**).* Compound **5m** (97 mg, 0.42 mmol) was prepared in 60% yield as a yellow powder, by following the same method as described in the general procedure with **4m** (238 mg, 0.69 mmol). R_f = 0.32 (Hexane/EtOAc = 3/1, v/v). m.p: 74−76 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.17 (dd, J = 8.2 and 1.8 Hz, 1H), 7.06 (d, J = 1.8 Hz, 1H), 6.91 (d, J = 8.2 Hz, 1H), 5.95 (s, 1H), 3.91 (s, 3H), 2.53 (d, J = 7.1 Hz, 2H), 2.38−2.27 (m, 1H), 1.01 (d, J = 6.7 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 188.1, 148.7, 146.5, 128.2, 115.3, 115.0, 111.4, 92.0, 87.7, 56.2, 54.5, 25.5, 22.6. HRMS m/z calculated for $C_{14}H_{16}O_3$ $[M-H]^-$: 231.1026; found: 231.1024. >95% purity (as determined by RP-HPLC, method D, t_R = 15.45 min).

*1-(4-Hydroxy-3-methoxyphenyl)-4-methylhex-1-yn-3-one (**5n**).* Compound **5n** (94 mg, 0.40 mmol) was prepared in 32% yield as a yellow oil, by following the same method as described in the general procedure with **4n** (444 mg, 1.28 mmol). R_f = 0.32 (Hexane/EtOAc = 3/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.17 (d, J = 8.2 Hz, 1H), 7.06 (s, 1H), 6.91 (d, J = 8.2 Hz, 1H), 5.95 (s, 1H), 3.91 (s, 3H), 2.59 (dt, J = 13.7 and 6.8 Hz, 1H), 1.91−1.83 (m, 1H), 1.60−1.52 (m, 1H), 1.23 (d, J = 6.9 Hz, 3H), 0.97 (t, J = 7.4 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 192.5, 148.7, 146.5, 128.1, 115.3, 115.0, 111.4, 92.94, 86.5, 56.2, 50.0, 26.1, 15.8, 11.6. HRMS m/z calculated for $C_{14}H_{16}O_3$ $[M-H]^-$: 231.1026; found: 231.1017. >95% purity (as determined by RP-HPLC, method D, t_R = 14.58 min).

*1-Cyclohexyl-3-(4-hydroxy-3-methoxyphenyl)prop-2-yn-1-one (**5o**).* Compound **5o** (117 mg, 0.45 mmol) was prepared in 44% yield as a yellow powder, by following the same method as described in the general procedure with **4o** (386 mg, 1.04 mmol). R_f = 0.28 (Hexane/EtOAc = 3/1, v/v). m.p: 92−94 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.18 (dd, J = 8.2 and 1.8 Hz, 1H), 7.06 (d, J = 1.7 Hz, 1H), 6.91 (d, J = 8.2 Hz, 1H), 5.95 (s, 1H), 3.91 (s, 3H), 2.52−2.46 (m, 1H), 2.08−2.02 (m, 2H), 1.85−1.78 (m, 2H), 1.72−1.65 (m, 1H), 1.54−1.45 (m, 2H), 1.38−1.30 (m, 2H), 1.28−1.22 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 191.8, 148.6, 146.5, 128.1, 115.3, 115.0, 111.5, 92.9, 86.7, 56.2, 52.3, 28.5, 25.9, 25.6. HRMS m/z calculated for $C_{16}H_{18}O_3$ $[M-H]^-$: 257.1183; found: 257.1184. >95% purity (as determined by RP-HPLC, method C, t_R = 10.83 min).

*1-(4-Hydroxy-3-methoxyphenyl)hex-1-yn-3-one (**5p**).* *Compound* **5p** (55 mg, 0.25 mmol) was prepared in 79% yield as a white powder, by following the same method as described in the general procedure with **4p** (108 mg, 0.32 mmol). R_f = 0.20 (Hexane/EtOAc = 5/1, v/v). m.p: 80−82 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.16 (dd, J = 8.2 and 1.8 Hz, 1H), 7.05 (d, J = 1.7 Hz, 1H), 6.91 (d, J = 8.2 Hz, 1H), 5.97 (s, 1H), 3.91 (s, 3H), 2.63 (t, J = 7.3 Hz, 2H), 1.81−1.72 (m, 2H), 0.99 (t, J = 7.4 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 188.4, 148.7, 146.5, 128.2, 115.3, 115.0, 111.5, 92.0, 87.4, 56.2, 47.4, 17.9, 13.7. HRMS m/z calculated for $C_{13}H_{14}O_3$ $[M-H]^-$: 217.0870; found: 217.0881. >95% purity (as determined by RP-HPLC, method D, t_R = 10.25 min).

The synthesis of compound **5q** was reported as previously.[25]

*4-(4-Hydroxy-3-methoxyphenyl)-1-phenylbut-3-yn-2-one (**5r**).* Compound **5r** (17 mg, 0.06 mmol) was prepared in 77% yield as a yellow oil, by following the same method as described in the general procedure with **4r** (31 mg, 0.08 mmol). R_f = 0.26 (Hexane/EtOAc = 3/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.37 (t, J = 7.5 Hz, 2H), 7.34−7.28 (m, 3H), 7.03 (dd, J = 8.2 and 1.6 Hz, 1H), 6.89 (d, J = 1.4 Hz, 1H), 6.87 (d, J = 8.2 Hz, 1H), 3.91 (s, 2H), 3.87 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 185.5, 149.0, 146.5, 133.8, 130.1, 128.8, 128.3, 127.4, 115.4, 115.0, 111.1, 94.9, 87.4, 56.2, 52.1. HRMS m/z calculated for $C_{17}H_{14}O_3$ $[M-H]^-$: 265.0870; found: 265.0871. >95% purity (as determined by RP-HPLC, method D, t_R = 14.95 min).

*4-(4-Hydroxy-3-methoxyphenyl)-1-phenoxybut-3-yn-2-one (**5s**).* Compound **5s** (14 mg, 0.05 mmol) was prepared in 82% yield as a yellow oil, by following the same method as described in the general procedure with **4s** (24 mg, 0.06 mmol). R_f = 0.25 (Hexane/EtOAc = 3/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.32 (dd, J = 8.5 and 7.5 Hz, 2H), 7.12 (dd, J = 8.2 and 1.8 Hz, 1H), 7.01 (t, J = 7.4 Hz, 1H), 6.98−6.94 (m, 3H), 6.90 (d, J = 8.2 Hz, 1H), 5.99 (s, 1H), 4.80 (s, 2H), 3.89 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 183.6, 158.0, 149.0,

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146.5, 129.8, 128.6, 121.9, 115.4, 115.1, 114.9, 110.7, 97.1, 85.5, 73.3, 56.2 HRMS m/z calculated for $C_{17}H_{14}O_4$ $[M-H]^-$: 281.0819; found: 281.0822. >95% purity (as determined by RP-HPLC, method D, t_R = 15.22 min).

*(5-(4-Hydroxy-3-methoxyphenyl)-1H−1,2,3-triazol-4-yl)-(phenyl)methanone (**9a**).* Compound **9a** (36 mg, 0.12 mmol) was prepared in 77% yield as a yellow powder, by following the same method as described in the general procedure with **8a** (66 mg, 0.16 mmol). R_f = 0.55 (DCM/MeOH = 10/1, v/v). m.p: 132−134 °C. ^{1}H NMR (600 MHz, MeOD) δ 7.97 (d, J = 7.4 Hz, 2H), 7.60 (t, J = 7.4 Hz, 1H), 7.47 (t, J = 7.8 Hz, 2H), 7.38 (d, J = 1.7 Hz, 1H), 7.22 (dd, J = 8.2 and 1.9 Hz, 1H), 6.84 (d, J = 8.2 Hz, 1H), 3.83 (s, 3H); ^{13}C NMR (150 MHz, MeOD) δ 190.2, 149.3, 148.9, 139.0, 134.3, 131.4, 129.4, 123.1, 116.3, 113.7, 56.4. HRMS m/z calculated for $C_{16}H_{13}N_3O_3$ $[M + H]^+$: 296.1030; found: 296.1027. >95% purity (as determined by RP-HPLC, method E, t_R = 8.45 min).

*1-(5-(4-Hydroxy-3-methoxyphenyl)-1H-1,2,3-triazol-4-yl)-butan-1-one (**9p**).* Compound **9p** (68 mg, 0.26 mmol) was prepared in 70% yield as a colorless oil, by following the same method as described in the general procedure with **8p** (140 mg, 0.37 mmol). R_f = 0.38 (DCM/MeOH = 10/1, v/v). ^{1}H NMR (600 MHz, MeOD) δ 7.59 (d, J = 1.8 Hz, 1H), 7.33 (dd, J = 8.2 and 1.9 Hz, 1H), 6.87 (d, J = 8.2 Hz, 1H), 3.91 (s, 3H), 3.09 (t, J = 7.3 Hz, 2H), 1.78−1.69 (m, 2H), 0.99 (t, J = 7.4 Hz, 3H); ^{13}C NMR (150 MHz, MeOD) δ 197.5, 149.5, 148.7, 141.9, 123.4, 120.1, 116.1, 114.0, 56.5, 43.6, 18.8, 14.1. HRMS m/z calculated for $C_{13}H_{15}N_3O_3$ $[M-H]^-$: 260.1040; found: 260.1052. >95% purity (as determined by RP-HPLC, method E, t_R = 7.52 min).

*1-(5-(4-Hydroxy-3-methoxyphenyl)-1H-1,2,3-triazol-4-yl)-hexan-1-one (**9q**).* Compound **9q** (67 mg, 0.23 mmol) was prepared in 80% yield as a colorless oil, by following the same method as described in the general procedure with **8q** (118 mg, 0.29 mmol). R_f = 0.33 (DCM/MeOH = 10/1, v/v). ^{1}H NMR (600 MHz, MeOD) δ 7.59 (d, J = 1.6 Hz, 1H), 7.33 (dd, J = 8.2 and 1.7 Hz, 1H), 6.87 (d, J = 8.2 Hz, 1H), 3.92 (s, 3H), 3.11 (t, J = 7.4 Hz, 2H), 1.75−1.67 (m, 2H), 1.40−1.33 (m, 4H), 0.92 (t, J = 6.8 Hz, 3H); ^{13}C NMR (150 MHz, MeOD) δ 197.7, 149.5, 148.7, 141.9, 123.4, 116.1, 114.0, 56.5, 41.6, 32.6, 25.2, 23.6, 14.3. HRMS m/z calculated for $C_{15}H_{19}N_3O_3$ $[M-H]^-$: 288.1353; found: 288.1367. >95% purity (as determined by RP-HPLC, method D, t_R = 8.86 min).

*General Procedure for Compounds **6a, 6i, 6j, 6m, 12a, 12f, and 12g**.* To a solution of alkynyl ketone (30 mg) in MeOH (5 mL) was added $NaBH_4$ (1.5 equiv) at 0 °C. The reaction mixture was stirred for 20 min under argon at the same temperature. The reaction mixture was concentrated and then extracted with EtOAc (3 × 10 mL). The organic layer was washed with brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and the crude residue was purified by column chromatography on silica gel (Hexane/EtOAc = 3/1, v/v for **6a**, **6i**, **6j**, **6m**, Hexane/Ether = 3/1, v/v for **12a**, **12f**, **12g**) to provide alkynyl alcohol compounds.

*4-(3-Hydroxy-3-phenylprop-1-yn-1-yl)-2-methoxyphen-ol (**6a**).* Compound **6a** (27 mg, 0.11 mmol) was prepared in 87% yield as a yellow powder, by following the same method as described in the general procedure with **5a** (30 mg, 0.12 mmol). R_f = 0.16 (Hexane/EtOAc = 3/1, v/v). m.p: 93−95 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.62 (d, J = 7.3 Hz, 2H), 7.43−7.39 (m, 2H), 7.37−7.33 (m, 1H), 7.03 (dd, J = 8.2 and 1.8 Hz, 1H), 6.96 (d, J = 1.8 Hz, 1H), 6.86 (d, J = 8.2 Hz, 1H), 5.76 (s, 1H), 5.69 (s, 1H), 3.88 (s, 3H), 2.30 (s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 146.6, 146.3, 141.0, 128.8, 128.6, 126.9, 125.9, 114.6, 114.1, 114.0, 87.0, 87.0, 65.3, 56.1. HRMS m/z calculated for $C_{16}H_{14}O_3$ $[M-H]^-$: 253.0870; found: 253.0873. >95% purity (as determined by RP-HPLC, method E, t_R = 17.08 min).

*4-(3-Hydroxy-3-(2-methoxyphenyl)prop-1-yn-1-yl)-2-methoxyphenol (**6i**).* Compound **6i** (6 mg, 0.02 mmol) was prepared in 20% yield as an orange powder, by following the same method as described in the general procedure with **5i** (30 mg, 0.11 mmol). R_f = 0.10 (Hexane/EtOAc = 3/1, v/v). m.p: 138−140 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.65 (dd, J = 7.5 and 1.6 Hz, 1H), 7.33 (dt, J = 8.1 and 1.7 Hz, 1H), 7.04 (dd, J = 8.2 and 1.7 Hz, 1H), 7.01 (dt, J = 7.5 and 0.8 Hz, 1H), 6.98 (d, J = 1.7 Hz, 1H), 6.94 (d, J = 8.2 Hz, 1H), 6.85 (d, J = 8.2 Hz, 1H), 5.93 (d, J = 6.1 Hz, 1H), 5.72 (s, 1H), 3.93 (s, 3H), 3.88 (s, 3H), 3.03 (d, J = 6.1 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 157.0, 146.5, 146.2, 129.9, 129.1, 128.2, 125.9, 121.1, 114.6, 114.4, 114.2, 111.0, 86.5, 86.4, 61.8, 56.1, 55.8. HRMS m/z calculated for $C_{17}H_{16}O_4$ $[M-H]^-$: 283.0976; found: 283.0966. >95% purity (as determined by RP-HPLC, method E, t_R = 19.13 min).

*4-(3-Hydroxy-3-(3-methoxyphenyl)prop-1-yn-1-yl)-2-methoxyphenol (**6j**).* Compound **6j** (23 mg, 0.08 mmol) was prepared in 76% yield as a yellow oil, by following the same method as described in the general procedure with **5j** (30 mg, 0.11 mmol). R_f = 0.10 (Hexane/EtOAc = 3/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.32 (t, J = 7.9 Hz, 1H), 7.21−7.16 (m, 2H), 7.03 (dd, J = 8.2 and 1.7 Hz, 1H), 6.96 (d, J = 1.7 Hz, 1H), 6.89 (dd, J = 8.2 and 2.4 Hz, 1H), 6.85 (d, J = 8.2 Hz, 1H), 5.74 (s, 1H), 5.66 (d, J = 6.0 Hz, 1H), 3.88 (s, 3H), 3.84 (s, 3H), 2.24 (d, J = 6.2 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 156.0, 146.6, 146.3, 142.5, 129.8, 126.0, 119.1, 114.6, 114.1, 114.0, 112.3, 86.9, 86.9, 65.2, 56.1, 55.4. HRMS m/z calculated for $C_{17}H_{16}O_4$ $[M-H]^-$: 283.0976; found: 283.0964. >95% purity (as determined by RP-HPLC, method E, t_R = 18.52 min).

*4-(3-Hydroxy-5-methylhex-1-yn-1-yl)-2-methoxyphenol (**6m**).* Compound **6m** (17 mg, 0.07 mmol) was prepared in 58% yield as a white powder, by following the same method as described in the general procedure with **5m** (30 mg, 0.13 mmol). R_f = 0.22 (Hexane/EtOAc = 3/1, v/v). m.p: 88−90 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.98 (dd, J = 8.2 and 1.7 Hz, 1H), 6.92 (d, J = 1.7 Hz, 1H), 6.84 (d, J = 8.2 Hz, 1H), 5.77 (s, 1H), 4.63 (t, J = 7.1 Hz, 1H), 3.88 (s, 3H), 1.95−1.84 (m, 2H), 1.76−1.60 (m, 2H), 0.97 (dd, J = 8.5 and 6.7 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 146.4, 146.3, 125.8, 114.6, 114.4, 114.1, 88.6, 85.1, 61.7, 56.1, 47.2, 25.0, 22.8, 22.6. HRMS m/z calculated for $C_{14}H_{18}O_3$ $[M-H]^-$: 233.1183; found: 233.1189. >95% purity (as determined by RP-HPLC, method E, t_R = 20.66 min).

*3-(3,4-Difluorophenyl)-1-phenylprop-2-yn-1-ol (**12a**).* Compound **12a** (18 mg, 0.07 mmol) was prepared in 59% yield as a colorless oil, by following the same method as described in the general procedure with **11a** (30 mg, 0.12 mmol). R_f = 0.33 (Hexane/Ether = 3/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.59 (d, J = 7.4 Hz, 2H), 7.42 (t, J = 7.5 Hz, 2H), 7.37 (t, J = 7.3 Hz, 1H), 7.30−7.26 (m, 1H), 7.23−7.19 (m, 1H), 7.11 (dd, J = 18.3 and 8.4 Hz, 1H), 5.67 (d, J = 2.9 Hz, 1H), 2.26 (d, J = 4.7 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 151.8, 151.7, 151.0, 150.9, 150.1, 150.0, 149.3, 149.2, 140.4, 129.0, 128.8, 128.6, 128.6, 128.6, 128.5, 126.8,

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120.9, 120.8, 119.4, 119.4, 119.3, 119.3, 117.7, 117.6, 89.4, 84.6, 65.2. >95% purity (as determined by RP-HPLC, method C, t_R = 8.78 min).

*1-(2-Chlorophenyl)-3-(3,4-difluorophenyl)prop-2-yn-1-ol (**12f**).* Compound **12f** (21 mg, 0.08 mmol) was prepared in 71% yield as a colorless oil, by following the same method as described in the general procedure with **11f** (30 mg, 0.11 mmol). R_f = 0.31 (Hexane/Ether = 3/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.78 (dd, *J* = 7.6 and 1.6 Hz, 1H), 7.41 (dd, *J* = 7.8 and 1.1 Hz, 1H), 7.35 (dt, *J* = 7.5 and 1.1 Hz, 1H), 7.31 (dd, *J* = 7.7 and 1.7 Hz, 1H), 7.30−7.27 (m, 1H), 7.22−7.19 (m, 1H), 7.13−7.08 (m, 1H), 6.02 (d, *J* = 3.9 Hz, 1H), 2.53 (d, *J* = 4.9 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 151.8, 151.7, 151.0, 150.9, 150.2, 150.1, 149.3, 149.2, 137.7, 132.9, 130.0, 130.0, 128.7, 128.6, 128.6, 128.5, 128.4, 127.5, 121.0, 120.9, 119.3, 119.2, 119.2, 119.1, 117.7, 117.6, 88.3, 84.6, 62.5, 62.4. HRMS *m*/*z* calculated for $C_{15}H_9ClF_2O$ $[M + H]^+$: 279.0383; found: 279.0935. >95% purity (as determined by RP-HPLC, method B, t_R = 9.93 min).

*1-(3-Chlorophenyl)-3-(3,4-difluorophenyl)prop-2-yn-1-ol (**12g**).* Compound **12g** (16 mg, 0.06 mmol) was prepared in 52% yield as a colorless oil, by following the same method as described in the general procedure with **11g** (30 mg, 0.11 mmol). R_f = 0.30 (Hexane/Ether = 3/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.58 (s, 1H), 7.47−7.45 (m, 1H), 7.35−7.33 (m, 2H), 7.30−7.26 (m, 1H), 7.23−7.19 (m, 1H), 7.14−7.09 (m, 1H), 5.65 (s, 1H), 2.37 (s, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 151.9, 151.8, 151.0, 150.9, 150.2, 150.1, 149.3, 149.2, 142.3, 134.8, 130.2, 128.8, 128.7, 128.7, 128.6, 128.6, 127.0, 124.9, 121.0, 120.9, 119.1, 119.1, 119.0, 119.0, 117.8, 117.7, 88.7, 85.0, 85.0, 85.0, 64.4. HRMS *m*/*z* calculated for $C_{15}H_9ClF_2O$ $[M + H]^+$: 279.0383; found: 279.0921. >95% purity (as determined by RP-HPLC, method B, t_R = 10.81 min).

*(R)-1-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphen-yl)-5-methylhex-1-yn-3-ol ((R)-**7m**).* To a solution of **4m** (130 mg, 0.38 mmol) in 2-propanol (3.75 mL) was added KOH (0.13 mg, 2.25 μmol) and RuCl[(*R*,*R*)-TsDPEN(mesitylene)] (1.17 mg, 1.88 μmol) with the ratio of ynone: Ru catalyst: KOH = 200:1:1.2. The reaction mixture was stirred for 18 h at 35 °C under argon and concentrated under reduced pressure. The crude residue was purified by column chromatography on silica gel (Hexane/EtOAc = 8/1, *v*/*v*) to provide compound (*R*)-**7m** (36.3 mg, 28%) as a colorless oil. R_f = 0.30 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.94−6.90 (m, 2H), 6.77 (d, *J* = 8.0 Hz, 1H), 4.62 (dd, *J* = 12.2 and 6.9 Hz, 1H), 3.79 (s, 3H), 1.95−1.87 (m, 1H), 1.77 (d, *J* = 5.4 Hz, 1H), 1.75−1.62 (m, 2H), 1.00−0.95 (m, 15H), 0.14 (s, 6H).

*(S)-1-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphen-yl)-5-methylhex-1-yn-3-ol ((S)-**7m**).* To a solution of **4m** (130 mg, 0.38 mmol) in 2-propanol (3.75 mL) was added KOH (0.13 mg, 2.25 μmol) and RuCl[(*S*,*S*)-TsDPEN(mesitylene)] (1.17 mg, 1.88 μmol) with the ratio of ynone: Ru catalyst: KOH = 200:1:1.2. The reaction mixture was stirred for 18 h at 35 °C under argon and concentrated under reduced pressure. The crude residue was purified by column chromatography on silica gel (Hexane/EtOAc = 8/1, *v*/*v*) to provide compound (*S*)-**7m** (27.6 mg, 21%) as a colorless oil. R_f = 0.30 (Hexane/EtOAc = 8/1, *v*/*v*). ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.94−6.90 (m, 2H), 6.76 (d, *J* = 8.0 Hz, 1H), 4.62 (dd, *J* = 12.2 and 6.9 Hz, 1H), 3.78 (s, 3H), 1.95−1.87 (m, 1H), 1.76 (d, *J* = 5.4 Hz, 1H), 1.75−1.62 (m, 2H), 1.00−0.95 (m, 15H), 0.14 (s, 6H).

*(R)-4-(3-Hydroxy-5-methylhex-1-yn-1-yl)-2-methoxyphenol ((R)-**6m**).* To a solution of (*R*)-**7m** (36.3 mg, 0.10 mmol) in THF (5 mL) was added tetrabutylammonium fluoride solution (1 M in THF, 2.0 equiv) at 0 °C. The reaction mixture was stirred for 20 min at the same temperature. The mixture was quenched with aqueous NH_4Cl (5 mL) and then extracted with EtOAc (3 × 10 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and the crude residue was purified by column chromatography on silica gel (Hexane/EtOAc = 3/1, *v*/*v*) to provide compound (*R*)-**6m** (14.4 mg, 59%) as a white powder. R_f = 0.22 (Hexane/EtOAc = 3/1, *v*/*v*). m.p: 88−90 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.97 (dd, *J* = 8.2 and 1.6 Hz, 1H), 6.91 (d, *J* = 1.5 Hz, 1H), 6.84 (d, *J* = 8.2 Hz, 1H), 4.62 (t, *J* = 7.2 Hz, 1H), 3.87 (s, 3H), 1.95−1.87 (m, 1H), 1.75−1.62 (m, 2H), 0.97 (dd, *J* = 8.6 and 6.7 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 146.4, 146.3, 125.8, 114.6, 114.3, 114.1, 88.6, 85.0, 61.7, 56.1, 47.2, 25.0, 22.8, 22.6. HRMS *m*/*z* calculated for $C_{14}H_{18}O_3$ $[M-H]^-$: 233.1183; found: 233.1175. The *ee* value was determined by the chiral HPLC analysis on a chiral column (CHIRALPAK IG, 5% ethanol in hexane)

*(S)-4-(3-Hydroxy-5-methylhex-1-yn-1-yl)-2-methoxyphenol ((S)-**6m**).* To a solution of (*S*)-**7m** (27.6 mg, 0.08 mmol) in THF (5 mL) was added tetrabutylammonium fluoride solution (1 M in THF, 2.0 equiv) at 0 °C. The reaction mixture was stirred for 20 min at the same temperature. The mixture was quenched with aqueous NH_4Cl (5 mL) and then extracted with EtOAc (3 × 10 mL). The organic layer was washed with brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and the crude residue was purified by column chromatography on silica gel (Hexane/EtOAc = 3/1, *v*/*v*) to provide compound (*S*)-**6m** (8.9 mg, 48%) as a white powder. R_f = 0.22 (Hexane/EtOAc = 3/1, *v*/*v*). m.p: 88−90 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.97 (dd, *J* = 8.2 and 1.7 Hz, 1H), 6.92 (d, *J* = 1.5 Hz, 1H), 6.84 (d, *J* = 8.2 Hz, 1H), 4.62 (t, *J* = 7.2 Hz, 1H), 3.88 (s, 3H), 1.95−1.87 (m, 1H), 1.75−1.62 (m, 2H), 0.97 (dd, *J* = 8.5 and 6.7 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 146.4, 146.3, 125.8, 114.6, 114.4, 114.1, 88.6, 85.1, 61.7, 56.1, 47.2, 25.0, 22.8, 22.6 HRMS *m*/*z* calculated for $C_{14}H_{18}O_3$ $[M-H]^-$: 233.1183; found: 233.1179. The *ee* value was determined by the chiral HPLC analysis on a chiral column (CHIRALPAK IG, 5% ethanol in hexane)

*General Procedure for Compounds **8a**, **8p** and **8q**.* To a solution of the silyl-protected compound in DMF was added sodium azide (1.2 equiv). The reaction mixture was stirred for 2 h at room temperature under argon. The reaction mixture was concentrated under reduced pressure and extracted with DCM (3 × 25 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and was purified by column chromatography on silica gel (DCM/MeOH = 100/1, *v*/*v*) to provide compounds **8a**, **8p**, and **8q**.

*(5-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxyphenyl)-1H-1,2,3-triazol-4-yl)(phenyl)methanone (**8a**).* Compound **8a** (66 mg, 0.16 mmol) was prepared in 70% yield as a yellow oil, by following the same method as described in the general procedure with **4a** (87 mg, 0.23 mmol). R_f = 0.52 (DCM/MeOH = 10/1, *v*/*v*). ^{1}H NMR (600 MHz, MeOD) δ 7.96 (d, *J* = 7.5 Hz, 2H), 7.60 (t, *J* = 7.4 Hz, 1H), 7.46 (t, *J* = 7.8 Hz, 2H), 7.36 (s, 1H), 7.22 (dd, *J* = 8.2 and 2.0 Hz, 1H), 6.87 (d, *J* = 8.2 Hz, 1H), 3.78 (s, 3H), 1.00 (s, 9H), 0.16 (s, 6H).HRMS

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m/z calculated for $C_{22}H_{27}N_3O_3Si$ $[M-H]^-$: 408.1749; found: 408.1762.

*1-(5-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxypheny-L)-1H-1,2,3-triazol-4-yl)butan-1-one (**8p**).* Compound **8p** (140 mg, 0.37 mmol) was prepared in 61% yield as a colorless oil, by following the same procedure as described in the general procedure with **4p** (185 mg, 0.61 mmol). R_f = 0.52 (DCM/MeOH = 10/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.61 (s, 1H), 7.36 (brs, 1H), 6.91 (d, J = 8.2 Hz, 1H), 3.89 (s, 3H), 3.14 (brs, 2H), 1.81−1.73 (m, 2H), 1.02−0.99 (m, 12H), 0.18 (s, 6H).

*1-(5-(4-((tert-Butyldimethylsilyl)oxy)-3-methoxypheny-L)-1H-1,2,3-triazol-4-yl)hexan-1-one (**8q**).* Compound **8q** (118 mg, 0.29 mmol) was prepared in 88% yield as a colorless oil, by following the same method as described in the general procedure with **4q** (118 mg, 0.33 mmol). R_f = 0.44 (DCM/MeOH = 10/1, v/v). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.60 (s, 1H), 7.35 (brs, 1H), 6.91 (d, J = 8.2 Hz, 1H), 3.89 (s, 3H), 3.15 (brs, 2H), 1.80−1.68 (m, 2H), 1.40−1.30 (m, 4H), 1.00 (s, 9H), 0.90 (t, J = 7.0 Hz, 3H), 0.18 (s, 6H). HRMS m/z calculated for $C_{21}H_{33}N_3O_3Si$ $[M-H]^-$: 402.2218; found: 402.2241.

*4-(4-(1-Hydroxybutyl)-1H-1,2,3-triazol-5-yl)-2-methoxyphenol (**10p**).* To a solution of **9p** (55 mg, 0.21 mmol) in MeOH (3 mL) was added $NaBH_4$ (1.5 equiv) at 0 °C. The reaction mixture was stirred for 20 min under argon at the same temperature. The reaction mixture was concentrated and then extracted with EtOAc (3 × 10 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and purified by column chromatography on silica gel (DCM/MeOH = 10/1, v/v) to provide compound **10p** (41 mg, 74%) as a colorless oil. R_f = 0.31 (DCM/MeOH = 10/1, v/v). ^{1}H NMR (600 MHz, MeOD) δ 7.33 (d, J = 1.8 Hz, 1H), 7.15 (dd, J = 8.1 and 1.9 Hz, 1H), 6.89 (d, J = 8.2 Hz, 1H), 4.94 (t, J = 7.1 Hz, 1H), 3.90 (s, 3H), 1.96−1.84 (m, 2H), 1.42−1.33 (m, 1H), 1.32−1.22 (m, 1H), 0.88 (t, J = 7.4 Hz, 3H); ^{13}C NMR (150 MHz, MeOD) δ 149.2, 148.3, 122.2, 116.5, 113.0, 66.1, 56.5, 56.5, 56.4, 39.4, 20.1, 14.1. HRMS m/z calculated for $C_{13}H_{17}N_3O_3$ $[M-H]^-$: 262.1197; found: 262.1208. >95% purity (as determined by RP-HPLC, method F, t_R = 14.10 min).

*4-(4-(1-Hydroxyhexyl)-1H-1,2,3-triazol-5-yl)-2-methoxyphenol (**10q**).* To a solution of **9q** (50 mg, 0.17 mmol) in MeOH (3 mL) was added $NaBH_4$ (1.5 equiv) at 0 °C. The reaction mixture was stirred for 20 min under argon at the same temperature. The reaction mixture was concentrated and then extracted with EtOAc (3 × 10 mL). The organic layer was washed with brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and the crude residue was purified by column chromatography on silica gel (DCM/MeOH = 10/1, v/v) to provide compound **10q** (28 mg, 57%) as a colorless oil. R_f = 0.29 (DCM/MeOH = 10/1, v/v). ^{1}H NMR (600 MHz, MeOD) δ 7.33 (d, J = 1.6 Hz, 1H), 7.15 (dd, J = 8.1 and 1.5 Hz, 1H), 6.89 (d, J = 8.1 Hz, 1H), 4.93 (t, J = 7.0 Hz, 1H), 3.90 (s, 3H), 1.94−1.86 (m, 2H), 1.30−1.16 (m, 6H), 0.86−0.81 (m, 3H); ^{13}C NMR (150 MHz, MeOD) δ 149.2, 148.3, 122.2, 116.5, 113.0, 66.4, 56.5, 37.2, 32.7, 26.6, 23.6, 14.3. HRMS m/z calculated for $C_{15}H_{21}N_3O_3$ $[M-H]^-$: 290.1510; found: 290.1530. >95% purity (as determined by RP-HPLC, method E, t_R = 7.62 min).

*General Procedure for Compounds **11a** and **11c−11k**.* To a solution of 4-ethynyl-1,2-difluoronbenzene (256 μL) in THF (30 mL) was added n-BuLi (1.5 equiv) at −78 °C. After the solution was stirred for 30 min at the same temperature, appropriate acid chloride (1.3 equiv) was dropwise added to the solution. The reaction mixture was stirred for 12−16 h at the same temperature. The reaction mixture was quenched with saturated aqueous NH_4Cl (10 mL) and then extracted with EtOAc (3 × 25 mL). The combined organic layer was washed with brine, dried over $MgSO_4$ and filtered. The filtrate was concentrated under reduced pressure and purified by column chromatography on silica gel (Hexane/Ether = 120/1 to 60/1, v/v) to provide compounds **11a** and **11c−11k**.

*3-(3,4-Difluorophenyl)-1-phenylprop-2-yn-1-one (**11a**).* *Compound* **11a** (118 mg, 0.49 mmol) was prepared in 22% yield as a white powder, by following the same method as described in the general procedure with benzoyl chloride (327 μL, 2.82 mmol). R_f = 0.53 (Hexane/Ether = 6/1, v/v). m.p: 96−98 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.19 (dd, J = 8.2 and 1.1 Hz, 2H), 7.67−7.63 (m, 1H), 7.53 (t, J = 7.8 Hz, 2H), 7.51−7.48 (m, 1H), 7.48−7.43 (m, 1H), 7.23 (dt, J = 9.8 and 8.3 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.8, 153.2, 153.1, 151.5, 151.4, 151.2, 151.1, 149.5, 149.4, 136.8, 134.5, 130.3, 130.3, 130.2, 130.2, 129.7, 128.9, 122.2, 122.1, 118.3, 118.2, 117.2, 117.1, 117.1, 117.1, 90.3, 90.3, 90.3, 87.1. HRMS m/z calculated for $C_{15}H_8F_2O$ $[M + H]^+$: 243.0616; found: 243.0607. >95% purity (as determined by RP-HPLC, method A, t_R = 8.24 min).

*3-(3,4-Difluorophenyl)-1-(o-tolyl)prop-2-yn-1-one (**11c**).* Compound **11c** (133 mg, 0.52 mmol) was prepared in 24% yield as a yellow powder, by following the same method as described in the general procedure with *o*-toluoyl chloride (368 μL, 2.82 mmol). R_f = 0.53 (Hexane/Ether = 6/1, v/v). m.p: 62−64 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.25 (dd, J = 7.8 and 1.1 Hz, 1H), 7.50−7.44 (m, 2H), 7.44−7.40 (m, 1H), 7.37 (dd, J = 11.3 and 3.9 Hz, 1H), 7.29 (d, J = 7.6 Hz, 1H), 7.21 (dt, J = 9.9 and 8.3 Hz, 1H), 2.67 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ d179.4, 153.1, 153.0, 151.4, 151.3, 151.1, 149.5, 149.4, 140.8, 135.5, 133.3, 133.3, 132.4, 130.1, 130.1, 130.0, 130.0, 126.1, 122.1, 121.9, 118.2, 118.1, 117.4, 117.4, 117.3, 117.3, 89.1, 88.5, 22.1, 22.1. HRMS m/z calculated for $C_{16}H_{10}F_2O$ $[M + H]^+$: 257.0773; found: 257.0760. >95% purity (as determined by RP-HPLC, method A, t_R = 13.92 min).

*3-(3,4-Difluorophenyl)-1-(m-tolyl)prop-2-yn-1-one (**11d**).* Compound **11d** (170 mg, 0.66 mmol) was prepared in 31% yield as a yellow powder, by following the same method as described in the general procedure with *m*-toluoyl chloride (372 μL, 2.82 mmol). R_f = 0.54 (Hexane/Ether = 6/1, v/v). m.p: 86−88 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.00 (d, J = 7.6 Hz, 1H), 7.98 (s, 1H), 7.53−7.48 (m, 1H), 7.48−7.44 (m, 2H), 7.42 (t, J = 7.6 Hz, 1H), 7.23 (dt, J = 9.9 and 8.3 Hz, 1H), 2.46 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.9, 153.2, 153.1, 151.5, 151.4, 151.2, 151.1, 149.5, 149.4, 138.8, 136.8, 135.3, 130.2, 130.2, 130.2, 130.2, 129.9, 128.7, 127.3, 122.2, 122.0, 118.3, 118.2, 117.2, 117.2, 117.2, 117.2, 90.1, 87.2, 21.5. HRMS m/z calculated for $C_{16}H_{10}F_2O$ $[M + H]^+$: 257.0773; found: 257.0762. >95% purity (as determined by RP-HPLC, method A, t_R = 13.85 min).

*3-(3,4-Difluorophenyl)-1-(p-tolyl)prop-2-yn-1-one (**11e**).* Compound **11e** (121 mg, 0.47 mmol) was prepared in 22% yield as a white powder, by following the same method as described in the general procedure with *p*-toluoyl chloride (373 μL, 2.82 mmol). R_f = 0.40 (Hexane/Ether = 6/1, v/v). m.p: 88−90 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.08 (d, J =

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8.1 Hz, 2H), 7.52−7.47 (m, 1H), 7.47−7.42 (m, 1H), 7.32 (d, J = 8.0 Hz, 2H), 7.23 (dt, J = 16.8 and 8.3 Hz, 1H), 2.46 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.4, 153.1, 153.0, 151.4, 151.3, 151.1, 151.1, 149.5, 149.4, 145.7, 134.5, 130.2, 130.2, 130.2, 130.1, 129.8, 129.6, 122.1, 122.0, 118.3, 118.2, 117.3, 117.3, 117.2, 117.2, 89.8, 87.2, 22.0. HRMS m/z calculated for $C_{16}H_{10}F_2O$ $[M + H]^+$: 257.0773; found: 257.0761. >95% purity (as determined by RP-HPLC, method A, t_R = 10.93 min).

*1-(2-Chlorophenyl)-3-(3,4-difluorophenyl)prop-2-yn-1-one (**11f**).* Compound **11f** (47 mg, 0.17 mmol) was prepared in 8% yield as a white powder, by following the same method as described in the general procedure with 2-chlorobenzoyl chloride (357 μL, 2.82 mmol). R_f = 0.31 (Hexane/Ether = 6/1, v/v). m.p: 86−88 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04 (d, J = 7.7 Hz, 1H), 7.50 (d, J = 3.7 Hz, 2H), 7.49−7.44 (m, 1H), 7.41 (dt, J = 9.1 and 4.3 Hz, 2H), 7.22 (dt, J = 16.7 and 8.3 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ d176.6, 153.3, 153.2, 151.6, 151.5, 151.2, 151.1, 149.5, 149.4, 135.7, 133.8, 132.6, 131.8, 130.35, 130.3, 130.3, 130.3, 127.0, 122.2, 122.1, 118.3, 118.2, 117.1, 117.1, 117.0, 116.9, 91.2, 91.2, 91.2, 88.5. HRMS m/z calculated for $C_{15}H_7ClF_2O$ $[M + H]^+$: 277.0226; found: 277.0223. >95% purity (as determined by RP-HPLC, method A, t_R = 10.12 min).

*1-(3-Chlorophenyl)-3-(3,4-difluorophenyl)prop-2-yn-1-one (**11g**).* Compound **11g** (43 mg, 0.16 mmol) was prepared in 7% yield as a white powder, by following the same method as described in the general procedure with 3-chlorobenzoyl chloride (361 μL, 2.82 mmol). R_f = 0.41 (Hexane/Ether = 6/1, v/v). m.p: 106−108 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.14 (t, J = 1.8 Hz, 1H), 8.09−8.05 (m, 1H), 7.64−7.60 (m, 1H), 7.54−7.44 (m, 3H), 7.27−7.21 (m, 1H), 1.54 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 176.3, 153.4, 153.3, 151.7, 151.6, 151.2, 151.1, 149.6, 149.5, 138.2, 135.2, 134.4, 130.4, 130.4, 130.4, 130.4, 130.2, 129.5, 127.8, 122.3, 122.2, 118.4, 118.3, 116.8, 116.8, 116.8, 116.7, 91.1, 86.7. HRMS m/z calculated for $C_{15}H_7ClF_2O$ $[M + H]^+$: 277.0226; found: 277.0227. >95% purity (as determined by RP-HPLC, method A, t_R = 13.73 min).

*1-(4-Chlorophenyl)-3-(3,4-difluorophenyl)prop-2-yn-1-one (**11h**).* Compound **11h** (262 mg, 0.95 mmol) was prepared in 44% yield as a white powder, by following the same method as described in the general procedure with 4-chlorobenzoyl chloride (362 μL, 2.82 mmol). R_f = 0.50 (Hexane/Ether = 6/1, v/v). m.p: 134−136 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.15−8.10 (m, 2H), 7.53−7.48 (m, 3H), 7.47−7.43 (m, 1H), 7.26−7.20 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 176.4, 153.2, 151.5, 151.2, 151.1, 149.4, 141.2, 135.2, 130.9, 130.3, 130.3, 130.3, 130.3, 129.3, 122.3, 122.1, 118.4, 118.3, 116.9, 90.8, 86.8. HRMS m/z calculated for $C_{15}H_7ClF_2O$ $[M + H]^+$: 277.0226; found: 277.0212. >95% purity (as determined by RP-HPLC, method A, t_R = 13.53 min).

*3-(3,4-Difluorophenyl)-1-(2-methoxyphenyl)prop-2-yn-1-one (**11i**).* Compound **11i** (27 mg, 0.10 mmol) was prepared in 5% yield as a yellow powder, by following the same method as described in the general procedure with 2-methoxybenzoyl chloride (420 μL, 2.82 mmol). R_f = 0.27 (Hexane/EtOAc = 6/1, v/v). m.p: 78−80 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.04 (dd, J = 7.7 and 1.5 Hz, 1H), 7.58−7.53 (m, 1H), 7.47−7.41 (m, 1H), 7.41−7.36 (m, 1H), 7.20 (dd, J = 18.1 and 8.4 Hz, 1H), 7.06 (t, J = 7.5 Hz, 1H), 7.03 (d, J = 8.4 Hz, 1H), 3.97 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 176.4, 160.1, 153.0, 152.9, 151.3, 151.2, 151.1, 151.0, 149.5, 149.4, 135.4, 132.7, 130.1, 130.1, 130.0, 130.0, 126.6, 122.0, 121.9, 120.6, 118.2, 118.1, 117.8, 117.8, 117.7, 117.7, 112.4, 89.4, 88.9, 56.1. HRMS m/z calculated for $C_{16}H_{10}F_2O_2$ $[M + H]^+$: 273.0722; found: 273.0720. >95% purity (as determined by RP-HPLC, method A, t_R = 6.53 min).

*3-(3,4-Difluorophenyl)-1-(3-methoxyphenyl)prop-2-yn-1-one (**11j**).* Compound **11j** (113 mg, 0.42 mmol) was prepared in 19% yield as a yellow powder, by following the same method as described in the general procedure with 3-methoxybenzoyl chloride (396 μL, 2.82 mmol). R_f = 0.45 (Hexane/EtOAc = 6/1, v/v). m.p: 78−80 °C.^{1}H NMR (600 MHz, $CDCl_3$) δ 7.82 (d, J = 7.6 Hz, 1H), 7.67 (dd, J = 2.3 and 1.6 Hz, 1H), 7.52−7.47 (m, 1H), 7.47−7.41 (m, 2H), 7.25−7.17 (m, 2H), 3.89 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 177.5, 160.0, 153.2, 153.1, 151.5, 151.4, 151.1, 151.1, 149.5, 149.4, 138.1, 130.3, 130.2, 130.2, 130.2, 129.9, 123.0, 122.2, 122.1, 121.3, 118.3, 118.2, 117.1, 117.1, 117.1, 117.0, 112.9, 112.9, 90.2, 87.1, 55.6. HRMS m/z calculated for $C_{16}H_{10}F_2O_2$ $[M + H]^+$: 273.0722; found: 273.0718. >95% purity (as determined by RP-HPLC, method A, t_R = 9.31 min).

*3-(3,4-Difluorophenyl)-1-(4-methoxyphenyl)prop-2-yn-1-one (**11k**).* Compound **11k** (63 mg, 0.23 mmol) was prepared in 11% yield as a white powder, by following the same method as described in the general procedure with 4-methoxybenzoyl chloride (382 μL, 2.82 mmol). R_f = 0.27 (Hexane/EtOAc = 6/1, v/v). m.p: 126−126 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.18−8.13 (m, 2H), 7.51−7.46 (m, 1H), 7.46−7.41 (m, 1H), 7.22 (dt, J = 9.8 and 8.3 Hz, 1H), 7.02−6.97 (m, 2H), 3.91 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 176.4, 164.9, 153.1, 152.9, 151.4, 151.3, 151.2, 151.1, 149.5, 149.4, 132.2, 130.2, 130.1, 130.1, 130.1, 130.1, 122.1, 122.0, 118.3, 118.2, 117.4, 117.4, 117.4, 117.3, 114.1, 89.6, 87.2, 55.8. HRMS m/z calculated for $C_{16}H_{10}F_2O_2$ $[M + H]^+$: 273.0722; found: 273.0721. >95% purity (as determined by RP-HPLC, method A, t_R = 8.19 min).

Chiral HPLC Method. Enantiomeric excess (*ee*) value was determined by the chiral HPLC analyses using a chiral column (CHIRALPAK IG (4.6 i.d × 250 mm)). Chromatographic analyses were performed on high-performance liquid chromatography (Agilent 1260 Infinity). The method was applied over 30 min at a flow rate of 1 mL/min with an isocratic of 5% ethanol (0.1% TFA) in hexane (v/v). The autosampler and the column compartment temperature were set at 25 °C. UV detection was carried out at a wavelength of 254 nm. One μL of the samples was injected.

QS Reporter Strain Assay. The binding affinity of compounds toward cognate QS receptors (LasR, RhlR, PqsR) was evaluated by measuring luminescence using specific reporter strains. The reporter strains were cotransformed with QS expression and *lacZ* fusion plasmids as follows: pJN105L (LasR expression plasmid)—pSC11 (*lasI*::*lacZ* fusion plasmid) for LasR, pJN105R (RhlR expression plasmid)—pECP60 (*rhlA*::*lacZ* fusion plasmid) for RhlR, and pJN105P (PqsR expression plasmid)—pJN301 (*pqsA*::*lacZ* fusion plasmid) for PqsR.[34−36]

200 μL of overnight cultured reporter strain (Optical density (OD) at 595 nm = 0.3) diluted in Luria−Bertani medium (Difco, Detroit, MI, USA) were treated with compounds (0−10 μM), and signal molecules (0.01 μM OdDHL for LasR. Ten μM BHL for RhlR, and 1 μM PQS for PqsR). The treated reporter strain was incubated with 0.4% arabinose (Sigma-Aldrich) at 37 °C for 1.5 h. 100 μL of suspension were

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measured by OD at 595 nm using a Victor X5 multimode plate reader (PerkinElmer, Waltham, MA, USA). The remains were mixed with 10% chloroform (Sigma-Aldrich) by vortexing for 15 min. Ten μL of the upper layer was transferred to a 96-well white polystyrene microplate (Corning, New York, USA). Luminescence was measured by the manufacturer's instruction of Tropix-Galacto-Light luminescent kit (Applied Biosystems, CA, USA). The luminescence was normalized by OD at 595 nm to evaluate the binding affinity between compounds and cognate receptors.[42−44]

Static Biofilm Formation Test. The dilution of the overnight cultured PA14 (OD at 595 nm = 0.05) in AB medium (300 mM NaCl, 50 mM $MgSO_4$, 0.2% casamino acids, 10 mM potassium phosphate, 1 mM L-arginine, and 1% glucose, pH 7.5) treated with compounds (0−10 μM) was incubated in a 96-well plate (Sigma-Aldrich) at 37 °C for 24 h without agitation. OD of the suspension was measured at 595 nm using a microplate reader for analyzing bacterial growth. Biofilm cells attached to the plate were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.2) twice, followed by staining with 0.1% crystal violet (CV) for 10 min. After washing stained biofilm cells with deionized water two times, purple colors were eluted with 100% ethyl alcohol from CV. Colors were measured with OD at 545 nm using the microplate reader and then normalized with OD at 595 nm.[45−48]

RT-qPCR. The dilution of overnight cultured PA14 (OD at 595 nm = 0.05) in AB medium treated with 0−10 μM compounds was incubated in borosilicate bottles at 37 °C for 24 h without agitation. Biofilm cells attached to the bottles were scrapped and washed with PBS. Total RNA was extracted from the cells following the manufacturer's instructions using TRI reagent (Molecular Research Center, OH, USA). Two μL of extracted RNA (100 ng/μL) was reacted with 10 μL of SYBR Premix Ex Taq (TaKaRa, Shiga, Japan), 1 μL of 10 μM forward and reverse primers, and RNase-free water to generate a final volume of 20 μL. The primer sequence used in this study was presented in our previous research.[33] RT-qPCR was performed at 95 °C for 10 s for initial denaturation, 95 °C for 10 s for denaturation, 60 °C for 10 s for annealing, and 63 °C for 34 s for extension. Fluorescence signal intensity was measured at the end of the extension step using a CFX-96 real-time system (Bio-Rad, Hercules, CA, USA). The relative fold change of gene expression was analyzed using $2^{-\Delta\Delta Ct}$ methods and normalized with *proC* as a housekeeping gene.[37,38]

Virulence Production Test. The production of exoprotease and rhamnolipid was evaluated by colorimetric methods, whereas pyocyanin was measured by HPLC analysis. The overnight cultured PA14 dilution (OD at 595 nm = 0.01) in AB medium treated with 0−10 μM compounds was incubated at 37 °C for 24 h with shaking conditions (200 rpm). The culture was centrifuged at 8,000 rpm for 5 min, and the supernatant was filtered through a 0.22 μm Millex filter (Car Roth, Karlsruhe, Germany).

For exoprotease production test, 250 μL of supernatant reacted with 0.5% (*w*/*v*) azocasein (Sigma-Aldrich) was incubated at 37 °C for 2 h. After adding 500 μL of 10% trichloroacetic acid (Sigma-Aldrich), the reactant was cooled in ice for 30 min. The supernatant obtained by centrifuging at 10,000 rpm for 10 min was mixed with 500 μL of 1 M NaOH. The reactant was measured at OD 440 nm by the microplate reader.

For rhamnolipid production test, 500 μL of supernatant was mixed with 100% diethyl ether (Sigma-Aldrich). The upper layer transferred to a microtube was evaporated to extract rhamnolipid from the supernatant. The extracted rhamnolipid was reacted with 0.19% orcinal solution (Sigma-Aldrich), followed by boiling at 80 °C for 30 min and cooling at 25 °C for 15 min. The reactant was measured at OD at 421 nm by the microplate reader.

Pyocyanin was oxidized in 5 mL of supernatant by mixing 30 μL of 50% trifluoroacetic acid (TFA; Sigma-Aldrich) at 25 °C for 1 h. 1260 Infinity II Prep-HPLC system (Agilent Technologies, Santa Clara, CA, USA) and an EC-C18 column (4.6 × 150 mm; Agilent Technologies) were used to analyze the oxidized pyocyanin. HPLC was operated under the 99:1 water/TFA (*v*/*v*) mobile phase, 10 mL injection volume, 25 °C temperature, and 0.5 mL/min flow rate. The height of the pyocyanin peak at a retention time of 20 min was detected with a UV−visible detector.

***In Silico* Docking Study Method.** All compounds were generated as 2D structures using ChemDraw Ultra (ver. 12.0.2) and as 3D using Schrödinger Maestro (ver. 13.4), respectively. The protein structures LasR (PDB ID: 2UV0) and RhlR (PDB ID: 7R3J) in the PDB format were downloaded from RCSB protein data bank. All water molecules were removed from the structure and the rest was prepared using the Protein Preparation Workflow with standard settings. The binding site of the structure was generated with the Receptor Grid Generation Tool. Molecular docking was performed by the Glide Docking Wizard in XP mode with default settings.

Dynamic Biofilm Formation Test. A drip-flow reactor (DFR-110; BioSurface Technologies Corporation, Bozeman, MT, USA) equipped with glass slides was used to imitate flow conditions for making biofilm formation. The dilution of the overnight cultured PA14 (OD at 595 nm = 0.01) in AB medium treated with 0−10 μM compounds was supplied continuously using a peristaltic pump (Masterflex C/L tubing pumps; Cole-Parmer, Vernon Hills, IL, USA) at 0.3 mL/min of flow rate. After forming biofilms at 37 °C for 48 h, the slides were removed from the reactor. Biofilm cells were washed with PBS and stained with 2 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI; Carl Roth, Karlsruhe, Germany) for 10 min. The stained biofilm cells were observed using confocal laser scanning microscopy (CLSM; LSM 700, Carl Zeiss, Jena, Germany) by blue-fluorescent light (excitation wavelength: 350 nm, emission wavelength: 470 nm) with a 20× objective lens [W N-Achroplan 20×/0.5 W (DIC) M27]. 3D stack images were analyzed in ImageJ calculator (National Institutes of Health, Bethesda, MD, USA).[39] The volume and thickness of the fluorescent biofilm cells were calculated by Comstat-2 mode.

Mortality Experiment of Mealworms. The bacterial supernatant for injection to mealworms was prepared in the same method of virulence production test. Ten μL of the supernatant was injected directly into mealworms with 1−1.5 cm length using a microsyringe needle. After injection, the mealworms were kept in a terrarium containing wheat bran at 25 °C for 4 days. The numbers of live and dead mealworms were counted daily to calculate the survival rate. The statistical significance was calculated using Log-rank and Gehan-Breslow-Wilcoxon tools in Prism 6 (GraphPad Software, MA, USA).[49]

Statistical Analysis. *P*-values were estimated by Student's *t* test (Excel software, Microsoft, Redmond, WA, USA).

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c06281.

Analytical data (^{1}H NMR and ^{13}C NMR) on key intermediates and final compounds (PDF)

Molecular formula strings and biological data for final compounds (CSV)

PDB coordinate of LasR in complex with compound **5a** (PDB)

PDB coordinate of RhlR in complex with compound **5a** (PDB)

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Author Contributions

[▽]T.L. and S.H. contributed equally. T.L., S.H., and H.-D.P., Y.B. were involved in the initial stages of the project, including the design of gingerol analogs and the development of biological experiments. T.L., J.Y., and H.L. synthesized and analyzed gingerol analogs. S.H. and H.-S.K. performed biological experiments. T.L., S.H., H.-S.K., J.Y., H.L, H.-D.P., and Y.B. analyzed the data and wrote the paper. All authors contributed to editing the final manuscript.

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Notes

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■ ABBREVIATIONS

QS, quorum sensing; SAR, structure−activity relationship; CF, cystic fibrosis; EPS, extracellular polymeric substances; AHL, acyl-*L*-homoserine lactone; BHL, *N*-butyryl-*L*-homoserine lactone; TBDMS, *tert*-butyldimethylsilyl; DCM, dichloromethane; TBAF, tetrabutylammonium fluoride; THF, tetrahydrofuran; OD, optical density; PBS, phosphate-buffered saline; CV, crystal violet; TFA, trifluoroacetic acid; PDB, protein data bank; DAPI, 4′,6-diamidino-2-phenylindole; CLSM, laser scanning microscopy

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