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Total Synthesis and Biological Studies of TMC-205 and Analogues as Anticancer Agents and Activators of SV40 Promoter

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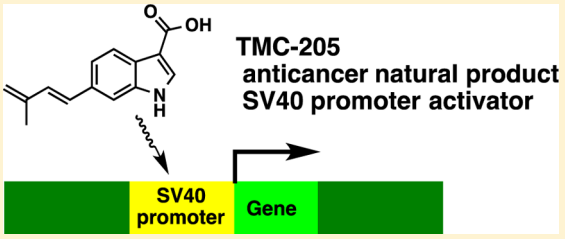
Supporting Information

ABSTRACT: TMC-205 is a natural fungal metabolite with antiproliferative activity against cancer cell lines. The light- and air-sensitivity prevented in-depth exploitation of this novel indole derivative. Herein, we report the first synthesis of TMC-205. On the basis of its reactivity with reactive oxygen species, we developed air-stable analogues of TMC-205. These analogues are 2−8-fold more cytotoxic than TMC-205 against HCT-116 colon cancer cell line. Importantly, at noncytotoxic dose levels, these analogues activated the transcription of luciferase reporter gene driven by simian virus 40 promoter (SV40). Further, these small molecules also inhibit firefly luciferase, presumably by direct interaction.



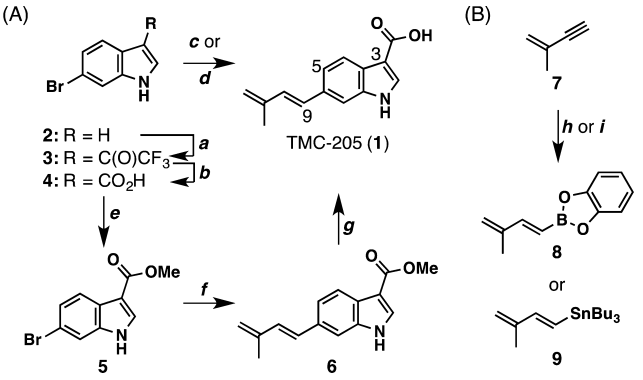
KEYWORDS: *natural product, indole,, TMC-205, simian virus 40 promoter, firefly luciferase, gene activation*

Small molecule-based activators of gene transcription are powerful chemical tools to study the functions of a particular gene through a gain-of-function approach.[1−3] Constitutive promoters, such as SV40 early promoter and cytomegalovirus (CMV) immediate early promoter, are frequently used for driving the expression of transgenes in mammalian cells.[4] Ectopic gene expression assays using an SV40 promoter facilitated the discoveries of natural anticancer agents including azelaic bishydroxamic acid,[5] trichostatin A,[6] romidepsin (FR901228),[7] herboxidiene,[8] and FR901464.[9] FR901464 and herboxidiene are now known as spliceosome inhibitors, while the other three compounds are known as histone deacetylase inhibitors.[10] Romidepsin was approved by the US Food and Drug Administration for the treatment of cutaneous T-cell lymphoma. FR901464 analogues are widely distributed to study splicing and the implication of the splicing factor 3b (SF3b) subcomplex of the spliceosome in diseases; it is currently unclear whether/how this activation of SV40 promoter is related to splicing inhibition.

Using a cell-based luciferase assay, TMC-205 (Scheme 1) was discovered from an unidentified fungal strain, TC 1630, as an activator of the SV40 promoter.[11] This indolebased natural product showed antiproliferative activity against various human cancer cell lines with GI_{50} values in the range of 52−203 μM. The fairly compact structure suggested to us that the potency could be improved through chemical synthesis of its analogues. In addition, TMC-205 was found to be light sensitive, and only 3.3 mg of TMC-205 was isolated, necessitating better access to this natural product to carry out structure−activity relationship (SAR) and biological studies. Herein, we report the first synthesis of TMC-205 and its light- and air-stable analogues and subsequent biological studies.

We first set out to devise a concise synthesis of TMC-205 for subsequent biological studies. An obvious convergent approach

Scheme 1. Synthesis of TMC-205[a]



[a]Reagents and conditions: (a) $(CF_3CO)_2O$ (1.1 equiv), DMF, 0 to 23 °C; (b) 4 M aq. NaOH, reflux, 97% (over 2 steps); (c) **8** (3.0 equiv), $Pd(PPh_3)_4$ (10 mol %), Cs_2CO_3 (3.0 equiv), THF, 80 °C, <1%; (d) **4** (1.0 equiv), **9** (2.0 equiv), $Pd(PPh_3)_4$ (10 mol %), DMF, 10%; (e) $TMSCHN_2$ (4.1 equiv), MeOH, 65% (from **2**); (f) **8** (2.9 equiv), $Pd(PPh_3)_4$ (5 mol %), Cs_2CO_3 (3.0 equiv), THF/MeOH (4:1), 70 °C, 98%; (g) 4 M NaOH, MeOH, 80 °C; 3 M $KHSO_4$, 88%; (h) catecholborane (0.4 equiv), 80 °C; (i) $^iPr_2NH$ (1.2 equiv), ^{n}BuLi (1.2 equiv), $^n Bu_3SnH$ (1.15 equiv), CuCN (2.05 equiv), THF, −40 °C, 58%.

was the C6−C9 bond formation via a crosscoupling reaction. We first examined Stille coupling[12] and Suzuki−Miyaura coupling[13] reactions (Scheme 1). Treatment of 6-bromoindole (**2**) with $(CF_3CO)_2O$ followed by aqueous NaOH afforded carboxylic acid **4** in 97% yield over 2 steps.[14] With the known

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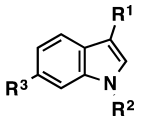
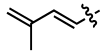
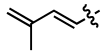
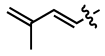
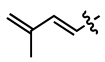
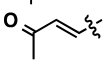
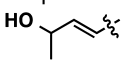
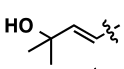
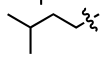
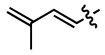
Table 1. Antiproliferative Activity of TMC-205 and Its Analogues against HCT-116 Colon Cancer cells



Analogue	R^1	R^2	R^3	GI_{50} (μM)
1	CO_2H	H		68 ± 3
10	H	H		147 ± 10
11	CH_2OH	H		79 ± 15
12	$C(O)CF_3$	H		39 ± 12
13	$C(O)CF_3$	H		14 ± 4
14	$C(O)CF_3$	H		8 ± 6
15	$C(O)CF_3$	H		>500
16	CO_2H	H		>500
17	$C(O)CF_3$	CH_3		>500

organostannane **9**,[15,16] but not boronic ester **8**,[17] a palladium-catalyzed reaction converted bromide **4** to TMC-205. However, this transformation provided the natural product only in 10% yield, prompting us to seek an alternative approach.

Both of the cross-coupling reactions described above suffered from the poor solubility of carboxylic acid **4** in various solvents (THF, MeOH, DMSO, and 1,4-dioxane). Methyl ester **5** was prepared in 65% overall yield from **2** (Scheme 1A) and was more soluble in these organic solvents. This ester was subjected to the Suzuki–Miyaura coupling conditions with boronic ester **8** to form methyl ester **6** in 98% yield. Finally, treatment of **6** with aqueous NaOH followed by acidification with $KHSO_4$ afforded TMC-205 in 88% yield. Therefore, the total synthesis of TMC-205 was accomplished in 5 steps in the longest linear sequence (6 total steps) from the commercially available bromide **2** in 64% overall yield. The GI_{50} value of the synthetic TMC-205 against HCT-116 tumor cells was 68 ± 3 μM (Table 1), which is in excellent agreement with the literature.[11]

Synthetic TMC-205 was used to determine the cause of its noticeable decomposition in less than 24 h under ambient light and air. The decomposition was not solvent-dependent (Figure S2, Supporting Information). The ^1H NMR spectrum of TMC-205 in CD_3OD (Figure S3, Supporting Information) revealed the presence of an enone group, possibly from enone **I** (Figure 1A) or its derivative produced by an oxidation of the indole ring (carboxylic acid **III**) and a formyl group resulted from the oxidation of the C9–C10 and/or the C2–C3 bond (carboxylic acid **II** and **III**, aldehyde **IV**, or aldehyde **V**; Figure 1A). LC–MS analyses revealed enone **I** and aldehyde **V** as the probable products of TMC-205 when exposed to light and air, based on the limited spectroscopic information obtained.

Indole derivatives are well-known for their antioxidant potential, and the indole substitution patterns influence the reactivity toward ROS.[18] We proceeded to determine which reactive oxygen species (ROS) was responsible for the oxidized byproducts of TMC-205. A qualitative assessment revealed the primary reactivity of synthetic TMC-205 to singlet oxygen, but not superoxide, hydrogen peroxide, or hydroxy radical.[19] In the presence of NaN_3, a widely used singlet oxygen scavenger,[20] the singlet oxygen-mediated oxidation of TMC-205 into **V** was minimized as determined by HPLC analysis (Figures 1B and S4, Supporting Information). Of note, the existence of **I** in the chromatograph indicated the involvement of another ROS in the oxidation of the diene moiety. Ketone **12** was more stable than TMC-205, which is consistent with the literature that had shown that electron-deficient indoles are less reactive toward ROS.[20]

A series of SAR studies were subsequently preformed to TMC-205 and analogues. We first probed the importance of the carboxyl group for the cytotoxicity of TMC-205. The 72 h antiproliferative assays revealed that the decarboxylated analogue **10** (Table 1) was ~3 times less cytotoxic than TMC-205 against HCT-116 colon cancer cells. Alcohol **11** was more sensitive to light, air, and heat than TMC-205, but was equipotent, indicating that the negative charge of the carboxyl

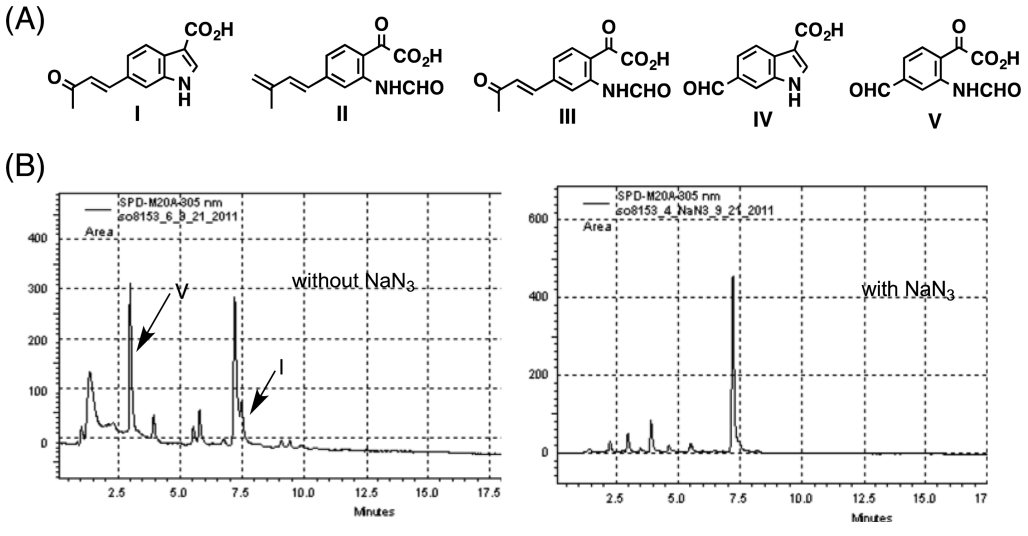


Figure 1. (A) Structures of possible byproducts of decomposed TMC-205. (B) Reaction of TMC-205 with singlet oxygen in the presence or absence of NaN_3.

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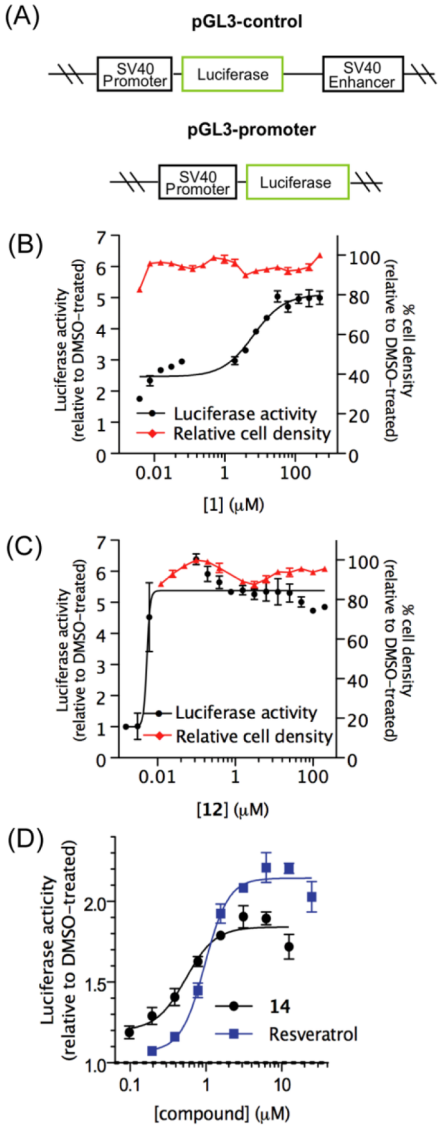

Figure 2. (A) pGL3-control and pGL3-promoter. (B) TMC-205 upregulates expression of luciferase gene in pGL3-control, without loss of cell viability. Data represent mean values ± SD (n = 4). (C) Analogue **12** upregulates expression of luciferase gene in pGL3-control, without loss of cell viability. (D) Upregulation of SV40 promoter-mediated luciferase expression by **14** and resveratrol. Data represent mean values ± SD (n = 2).

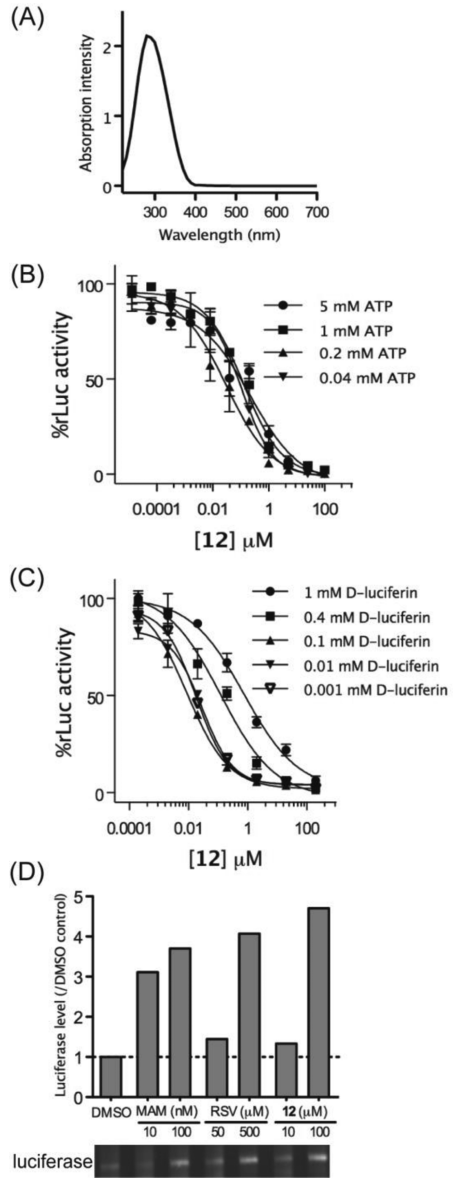

Figure 3. (A) Absorption spectrum of analogue **12** in DMSO. (B,C) Analogue **12** inhibited luciferase. The graph shows that inhibition of luciferase was relieved when the concentrations of D-luciferin (C), but not ATP (B), were increased. Data represent mean values ± SD (n = 2). (D) RTPCR of luciferase expressed in HeLa cells transiently transfected with pGL3-promoter and subsequently treated with DMSO, meayamycin B (MAMB), **12**, and resveratrol (RSV). Activation of luciferase gene from each treatment was presented as the relative intensity of the luciferase fragment from compound-treated compared with DMSO-treated. Data represent results from three independent experiments.

group is not necessary. In order to improve the stability of TMC-205, we introduced an electron-withdrawing 3-trifluoromethyl group to TMC-205, yielding ketone **12**. This ketone was twice as potent as TMC-205, with a GI_{50} value of 39 ± 12 μM.

The oxidation of the diene moiety might occur in cellular environment to yield an enone functionality.[21] We hence speculated that derivatives with this moiety might display superior bioactivity, as the *bona fide* bioactive species in cells. Indeed, enone **13** exhibited a GI_{50} value of 14 ± 4 μM and was 3 and 5 times more potent than **12** and TMC-205, respectively. Thus, we asked whether the electrophilicity was of any significance for the activity of this enone. The secondary allylic alcohol **14** was equipotent to enone **13**, suggesting that **14** might be oxidized to **13**, although this hypothesis warrants further investigation. The tertiary allylic alcohol **15** was far less potent, which implies that the additional methyl group might be too bulky for a putative binding pocket or may prevent the alcohol from being oxidized to the enone. The rigidity of 1,3-diene moiety was crucial as evidenced by a complete loss of activity of the alkane analogue **16**. The *N*-methylated analogue **17** was less cytotoxic than **12**, implying that the N−H group may serve as a hydrogen bonding donor or that a putative binding pocket for this functional group may not tolerate additional steric bulk. Figure S5a (Supporting Information) shows representative data demonstrating the improvement of

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antiproliferative activity against HCT-116 colon cancer cells through the SAR studies.

Previously, TMC-205 was found to activate a luciferase gene driven by an SV40 promoter in an SV40 enhancer-dependent manner using the pGL3-control (Promega). The activation of the luciferase gene driven by the SV40 promoter without the SV40 enhancer (pGL3-promoter, Promega) was negligible in the presence of TMC-205 (Figure 2A).[11] In this study, with 24 h exposure, TMC-205 and **12** activated SV40 promoter in the presence of enhancer in HeLa cells stably transfected with pGL3-control (Figure 2B,C). Interestingly, pGL3-promoter could be activated by TMC-205 as well (Figure S6a,b, Supporting Information), meaning the gene activation was independent of SV40 enhancer. Analogues **12** and **14** also activated the expression of SV40:luciferase without inhibiting cell growth (Figure S6c,d, Supporting Information). It is noteworthy that within the 24 h time frame, before cell growth could be influenced, analogue **12** activated gene transcription in a nanomolar range (Figure 2C and S6c, Supporting Information), demonstrating a property as small-molecule activators for gain-of-function studies in live organisms.

Firefly luciferase is the reporter enzyme in the vectors used both in the original isolation study and our current SAR studies for TMC-205 and analogues. Recently, it was discovered that certain heterocyclic small molecules perturb luciferase-catalyzed production of luminescence either through luciferase binding or compound-specific luminescence absorbance or scattering.[22] In the case of direct binding, these small molecules inhibit luciferase, competitively or noncompetitively, with D-luciferin and/or ATP. Concerns arise when the tested compounds that are active against luciferase may also be active against a target of interest. For example, high throughput screenings for enzymes, such as kinases that are also ATP dependent, would inevitably be susceptible to luciferase inhibitory activity of the hits.[23] For the current study, direct attenuation of luminescence was not a concern for two reasons: first, as shown in Figure 3A, analogue **12** absorbs UV light with a peak at 290 nm, with no absorption in the range of visible light (400−700 nm); second, compound-containing cell medium was thoroughly removed for the luciferase reporter assay.

We decided to examine the interaction between recombinant luciferase and analogue **12** as a representative analogue of TMC-205. Resveratrol was used as a positive control for noncompetitive luciferase inhibition.[24] Substrate competition assays were carried out by varying concentrations of ATP or D-luciferin to acquire the dose−response curves of luciferase inhibition by **12**. As expected, varying the concentration of either ligand did not significantly change the IC_{50} values of resveratrol (Figure S5b,c, Supporting Information). Luciferase inhibition with **12** was perturbed by D-luciferin but not by ATP (Figure 3B,C), indicating that **12** and D-luciferin antagonize each other toward luciferase.

On the basis of these data, we questioned whether the strong inhibition of luciferase enzyme activity underestimated the activation of luciferase expression by **12**. In order to answer this question, we examined the expression of luciferase at the mRNA level in HeLa cells transfected with the pGL3-promoter vector (Figure 3D); in a dose-dependent manner, **12** activated the SV40 promoter, which in turn upregulated luciferase mRNA. Interestingly, resveratrol also activated the SV40 promoter-driven gene (Figure 3D), which is unprecedented and warrants further investigation in the future.

In conclusion, the first total synthesis of TMC-205 has been accomplished. Degradation studies revealed that TMC-205 reacts with singlet oxygen. More oxidation-resistant analogues were up to 8-fold more potent than TMC-205. These compounds bind the D-luciferin binding pocket of luciferase and inhibit the enzymatic activity. Importantly, they activated the expression of an SV40 promoter-driven gene at non-cytotoxic dosage.

■ ASSOCIATED CONTENT

Supporting Information

Compound characterization and methods for syntheses and biological studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

†Y.G. and S.O. contributed equally to this work. Y.G. and S.O. performed experiments. Y.G., S.O., and K.K. interpreted the results and prepared the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CMV, cytomegalovirus; SV40, simian virus 40; SF3b, splicing factor 3b; SAR, structure−activity relationship; GI_{50}, 50% growth inhibitory concentration; MAMB, meayamycin B; Me, methyl; RSV, resveratrol; ROS, reactive oxygen species; RTPCR, reverse transcription-polymerase chain reaction; ATP, adenosine triphosphate; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; HPLC, high-performance liquid chromatography; UV, ultraviolet; SD, standard deviation

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