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Synthesis of Rocaglamide Hydroxamates and Related Compounds as Eukaryotic Translation Inhibitors: Synthetic and Biological Studies

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Abstract

The rocaglates/ rocaglamides are a class of natural products known to display potent anticancer activity. One such derivative, silvestrol, has shown activity comparable to taxol in certain settings. Here, we report the synthesis of various rocaglamide analogs and identification of a hydroxamate derivative (-)-**9** having activity similar to silvestrol *in vitro* and *ex vivo* for inhibition of protein synthesis. We also show that (-)-**9** synergizes with doxorubicin *in vivo* to reduce Eμ-Myc driven lymphomas.

Keywords

silvestrol; rocaglamide; hydroxamate; lymphoma; eIF4A; translation initiation; Myc

A wealth of biologically active complex natural products have been isolated from the genus *Aglaia*, including the cyclopenta[*b*]benzofurans silvestrol (**1**) and methyl rocaglate (**2**), shown in Chart 1.¹ Silvestrol shows potent anti-proliferative activity against lung, breast, and prostate cancer cell lines *in vitro*^{1,2} comparable to the cancer chemotherapeutic agent taxol.³ The compound reverses chemoresistance in an engineered murine lymphoma model by re-sensitizing tumor cells to standard-of-care agents such as doxorubicin,⁴ dramatically suppresses tumor growth as a single agent in breast cancer and prostate cancer xenografts, and is well tolerated in animal models.^{2, 5} Previous characterization of silvestrol has shown that it inhibits the ribosome recruitment step of eukaryotic translation initiation through modulation of the RNA helicase, eukaryotic initiation factor (eIF) 4A.⁴ Silvestrol has been the target of synthetic studies by both the Porco and Rizzacasa groups in which a convergent strategy was employed to join the cyclopenta[*b*]benzofuran and dioxanyl fragments.⁶ Given the synthetic challenge of preparing silvestrol, our group has endeavored to prepare

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Author Contributions: C.M.R. prepared compounds (+)-**9** and (-)-**9**, **11-17** and **22-28**, and composed the manuscript. R.C. performed eukaryotic translation assays, RNA filter and affinity pull down assays, animal studies, and composed the manuscript. S.P.R. prepared compounds **7-10**. J.P. and J.A.P. conceived and directed the research and composed the manuscript.

Supporting Information. Experimental procedures, characterization data, pharmacologic data, biological methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

cyclopenta[*b*]benzofuran analogs lacking the dioxanyl moiety present in silvestrol and to assess the resulting molecules as inhibitors of translation. A similar strategy has been adopted by Desaubry and coworkers, who reported synthetic cyclopenta[*b*]benzofurans that are active against various human cancer cell lines *in vitro*.⁷

As part of a previous investigation of the substrate scope of the [3+2] photocycloaddition to construct the cyclopenta[*b*]benzofuran core, various analogs were prepared with substitutions of the C2 position, and (+/-)-rocaglamide hydroxamate **3**⁸ was identified as having eukaryotic translation inhibition activity.⁹ The features of the hydroxamate moiety present in **3** that relate to its properties as an inhibitor of translation may be due to its ability to act as a hydrogen bond acceptor or to chelate metals in the target binding site as depicted in **4** in Figure 1.¹⁰ In a general sense, the carbonyl oxygen and atom “Y” in **5** and **6** may serve as a bidentate chelating moiety when Y is an atom with non-bonded electrons. This Lewis basic moiety may bind amino acid side chains or Lewis acidic metals represented as “A”. In this regard, hydroxamate esters have been characterized as MEK inhibitors in which case they appear to serve as hydrogen bond acceptors of lysine residues.¹¹

Using hydroxamate **3** as a lead structure, hydroxamates **7-9** were prepared with varying substitution of the *N*- and *O*- positions of the hydroxamate (Chart 2). *N*-Methoxy amide **9** was identified as the most potent derivative in translation assays (see below) prompting the preparation of additional hydroxamate esters **10-13** from rocaglaic acid, prepared from the hydrolysis of methyl rocaglate (**2**).

Additional rocaglamides bearing the *N*-methoxyamide functionality were prepared in an effort to further establish SAR and optimize potency. Accordingly, methyl rocaglate (**2**) was investigated as a substrate for electrophilic aromatic substitution of the electron-rich phloroglucinol ring. It was anticipated that regioselectivity in the halogenation reactions could be observed based on a report suggesting the reaction of aromatics with NBS and AuCl₃ proceeded through an arylgold intermediate.¹² As shown in Table 1, quantitative conversion of methyl rocaglate was observed when reacted with NBS in THF for 50 minutes yielding **14c** (60%) and an inseparable 1:2 mixture of **14a** and **14b**. Addition of AuCl₃ had no effect on the regioselectivity. Reaction of **2** with NCS, AuCl₃ (10 mol %) in THF at -78 °C proceeded to partial conversion yielding a separable mixture of products **14d** (23%) and **14e** (29%), along with recovered **2** (47%), as proceeding to full conversion led to an inseparable mixture of **14e** and **14f**. The uncatalyzed reaction proceeded at a slower rate, but no changes in regioselectivity were observed. Dichlorinated product **14f** was prepared by reaction of 2 equiv of NCS in THF and proceeded in quantitative yield. Reaction of methyl rocaglate with Selectfluor in methanol formed product **14h** (40%) in addition to an inseparable mixture of **14g** and numerous undesired side products. Nitration of methyl rocaglate with Cu(NO₃)₂ in Ac₂O yielded **14i** in <5% yield, and 26% of regioisomer **14j**. The halogenated and nitrated methyl esters **14** were carried forward to the corresponding hydroxamates **15** by hydrolysis with lithium hydroxide in aqueous dioxane and subsequent *N*-methoxyamide formation with EDCI and HOBt.

For further biological studies, an enantiopure sample of the lead hydroxamate **9** was needed to compare to the enantiopure natural product, silvestrol (**1**). We have previously reported the asymmetric synthesis of methyl rocaglate (-)-**2**.¹³ In the current study, we wished to prepare both natural and unnatural enantiomers of hydroxamate **9** for biological evaluation (Scheme 1). To achieve this goal, methyl rocaglate (+/-)-**2** was hydrolyzed and was esterified using (-)-menthol, DCC, and DMAP to yield menthyl esters **16** and **17**.¹⁴ The diastereomers were resolved by flash column chromatography and hydrolyzed to give both enantiomers of rocaglaic acid (>98% ee). Each enantiomer of rocaglaic acid was carried

forward to *N*-methoxyamides (-)-**9** and (+)-**9** using methoxylamine hydrochloride, EDCI, HOBT, and Et₃N in CH₂Cl₂ in 65-67% yield.

To investigate the hypothesis that the hydroxamate moiety may serve as a chelating group to metals or as a hydrogen bond acceptor, a number of heterocyclic aromatic ketones and amides with the appropriate heteroatoms were prepared.¹⁵ Encouraged by a report that 2-lithiobenzothiazole undergoes single addition to esters due to the formation of a stable tetrahedral intermediate,¹⁶ we anticipated that heterocyclic aromatic ketone analogs may be prepared directly from methyl rocaglate (Chart 3). Indeed, 2-lithiobenzothiazole (3.1 equiv) was prepared and reacted with methyl rocaglate at -78 °C for 1 h to yield the desired benzothiazole ketone **25** in 50% yield based on recovered starting material (BRSM). In a similar manner, 2-lithiopyridine was reacted with methyl rocaglate and the desired pyridyl ketone **26** was formed in 40% yield (BRSM). Efforts to react other lithio heterocycles with methyl rocaglate were unsuccessful, and unreacted methyl rocaglate was recovered in most cases.

Weinreb amide **3** was also investigated as a substrate for ketone formations and was found to react with a range of lithiated heterocycles including 2-lithiothiophene, 2-lithiofuran, and 2-lithiothiazole (Chart 4). Additional furan derivatives **27** -**28** were prepared by reaction of the lithiofurans with hydroxamate **3**.

Testing of all derivatives for translation inhibition activity revealed that only hydroxamate (-)-**9** inhibited eukaryotic protein synthesis to the same extent as silvestrol *in vitro* (Figure 2a, b). Hydroxamate (+)-**9** showed no activity and the racemic mixture displayed partial activity (Figure 2a). Of the derivatives from series **10** -**13**, hydroxamates **11** -**13** also inhibited protein synthesis, with **11** showing similar activity to **9** (Figure 2a). Other derivatives such as furyl ketone **27** showed moderate activity, indicating that the furyl ketone can function as a hydroxamate replacement (*c.f.* Figure 1). We therefore prepared (-)-**11** to compare its activity to (-)-**9**. *Ex vivo* testing of these active hydroxamates displayed a similar rank order for inhibition of translation (Figure 2c). Derivatives **12**-**13** appeared to be less potent *in vitro* and *ex vivo* (Figure 2a, c). The target of silvestrol has been previously identified by Pelletier and coworkers to be eIF4AI and eIF4AII, two RNA helicases required to load 40S ribosomes onto mRNA templates during the initiation phase of translation.⁴ eIF4AI and eIF4AII (collectively referred to as eIF4AI/II) are 90% identical and functionally interchangeable. Silvestrol stimulates nonproductive binding of eIF4AI/II to RNA, sequestering it from the eIF4F complex, where it usually resides and from which it is delivered to mRNA templates to participate in ribosome recruitment.⁴ In a filter binding assay designed to retain eIF4AI:³²P-RNA complexes, eIF4AI shows very little RNA binding activity (Figure 2d). eIF4AI RNA binding is increased in a dose dependent manner by both silvestrol^{2b} and (-)-**9**. This property is inhibited by hippuristanol, a selective RNA binding inhibitor of eIF4AI/II.¹⁷ The net effect of increased binding of eIF4A to RNA by silvestrol and (-)-**9** is depletion of eIF4AI/II from the eIF4F complex (Figure 2d).

Hydroxamate (-)-**9** and silvestrol displayed similar potencies towards inhibiting translation *ex vivo* in BJAB lymphoma cells that ranged from an IC₅₀ ~ 20 nM when cells were exposed to compound for one hour to an IC₅₀ ~ 0.5 nM when cells were exposed to compound for 72 h (Figure 3a). The IC₅₀ for inducing cell death in BJAB cells was found to be ~ 1.5 nM following 72 h exposure, indicating that the observed effects on protein synthesis are not secondary consequences of cell death (Figure 3b). We have previously used engineered Pten^{+/-} Eμ-myc lymphoma cells to show that silvestrol and doxorubicin can synergize to extend tumor free-survival in this chemoresistant model⁴. We took advantage of this pre-clinical model and utilized Eμ-Myc(Myf)Akt tumors (with constitutive AKT activation) to show that (-)-**9** synergized with doxorubicin (DOX) to extend tumor-free survival to the

same extent as silvestrol (Figure 3c). Hence, we have demonstrated that silvestrol is capable of synergizing with DOX against Eμ-Myc(Myf)Akt lymphomas and that (-)-**9** shows activity indistinguishable from silvestrol.

In vitro pharmacokinetic properties of hydroxamate (-)-**9** were also evaluated (see Supporting Information). The solubility of the compound was assessed to be high (>150 mg/mL) at three physiologically relevant pH levels (5.0, 6.2, and 7.4). The compound exhibited moderate to high permeability as measured by the Parallel Artificial Membrane Permeation Assay (PAMPA) at pH 5.0, 6.2, and 7.4. The compound was significantly bound by human plasma proteins (82-84% bound after 4 h exposure), showed excellent stability (100% remaining after 3 h) in human plasma, and exhibited significant hepatic stability in the presence of human and murine liver microsomes (>95% compound remaining after 1 h exposure). These data suggest that hydroxamate (-)-**9** should exhibit significant metabolic stability *in vivo*. *In vivo*, hydroxamate (-)-**9** showed little toxicity towards the pre-B/B cell (B220+), myeloid (Ly6G+), and T cell (CD11b and CD4) populations from mice treated for 5 consecutive days (See Supporting Information; Section III). These results indicate that at the doses used here, (-)-**9** does not lead to general depletion of normal cells of the hematopoietic system. The significant effects of eIF4A inhibition on BJAB viability (Fig. 3b) as well as on global protein synthesis (Fig. 2c) is more pronounced than has been described for suppression of eIF4E, another subunit of the eIF4F complex.¹⁸ These results are consistent with global translation being more dependent on eIF4A activity than on eIF4E.²

In conclusion, various hydroxamates and heterocyclic ketone derivatives of cyclopenta[*b*]benzofurans (rocaglates) were prepared that could function as hydrogen bond acceptors or as metal chelators. Hydroxamate (-)-**9** was identified in this study as a potent eukaryotic translation inhibitor (Figures 2 and 3). Further characterization revealed that the compound is capable of synergizing with doxorubicin in extending tumor-free survival in a preclinical murine lymphoma model.

Experimental Section

General Methods

Proton NMR spectra were recorded at 500 MHz at ambient temperature in the specified solvents. High-resolution mass spectra were obtained using a Waters Q-TOF API-US mass spectrometer. Analytical thin layer chromatography was performed using 0.25 mm silica gel 60-F plates. Flash chromatography was performed using 200-400 mesh silica gel (Scientific Absorbents, Inc.). Purities for all compounds were determined to be >95% by UPLC-MS. Methyl rocaglate (**2**) and hydroxamate **3** were prepared according to the methods of Porco and coworkers.⁹ All reactions were carried out in oven-dried glassware under an argon atmosphere unless otherwise noted.

N-Methoxy rocaglamide (-)-**9**

A round bottom flask was charged with (-)-rocaglaic acid (27.0 mg, 0.06 mmol), CH₂Cl₂ (4 mL), EDCI (16 mg, 0.08 mmol), HOBt (11 mg, 0.07 mmol), methoxylamine hydrochloride (24 mg, 0.28 mmol), and triethylamine (39 μL, 0.28 mmol), and was stirred at room temperature for 12 h. The reaction was quenched with 1M HCl, concentrated, and purified by flash chromatography (gradient elution 50% to 100% ethyl acetate in hexanes) to afford a (-)-**9** as white solid (14 mg, 49%). ¹H NMR (500 MHz, CD₃ OD) δ 7.15 (d, *J* = 9.0 Hz, 2H), 7.09 – 6.99 (m, 3H), 6.94 (d, *J* = 6.9 Hz, 2H), 6.64 (d, *J* = 9.0 Hz, 2H), 6.32 (d, *J* = 1.9 Hz, 1H), 6.21 (d, *J* = 1.9 Hz, 1H), 4.79 (d, *J* = 6.2 Hz, 1H), 4.32 (d, *J* = 14.2 Hz, 1H), 3.87 (s, 1H), 3.85 (s, 1H), 3.72 (dd, *J* = 14.2, 6.2 Hz, 1H), 3.69 (s, 1H), 3.60 (s, 1H); HRMS calcd

for $[\text{C}_{28}\text{H}_{29}\text{NO}_8\text{Na}]^+$ requires m/z 530.1791; found 530.1772 (ES+).; $[\alpha]_{\text{D}}^{20} = -38.4$ (c 0.5, CH_3OH , at >98% ee).

Furan 24

In a flame-dried round bottom flask, furan (28 mL, 0.39 mmol) was dissolved in tetrahydrofuran (0.5 mL) and was cooled to 0 °C. A solution of *n*-BuLi (175 uL, 1.86 mmol, 2.1 M in hexanes) was added dropwise. The solution was stirred for 30 minutes at 0 °C and then a solution of hydroxamate **3** (40 mg, 0.08 mmol) in tetrahydrofuran (300 uL) was added and was stirred at 0 °C for 1 h. The reaction was quenched with water at 0 °C and was brought to room temperature. The reaction was concentrated and purified by flash column chromatography (gradient elution 20% to 60% ethyl acetate in hexanes) to afford the desired product **24** as a white solid (27 mg, 60%). $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 7.66 (dd, $J = 1.7$, 0.7 Hz, 1H), 7.30 – 7.24 (m, 1H), 7.20 (d, $J = 9.0$ Hz, 2H), 7.07 – 6.96 (m, 3H), 6.92 – 6.83 (m, 2H), 6.72 (d, $J = 9.0$ Hz, 2H), 6.58 (dd, $J = 3.6$, 1.7 Hz, 1H), 6.30 (d, $J = 2.0$ Hz, 1H), 6.10 (d, $J = 2.0$ Hz, 1H), 5.27 (dd, $J = 6.6$, 1.1 Hz, 1H), 4.69 (dd, $J = 13.8$, 6.6 Hz, 1H), 4.55 (d, $J = 13.9$ Hz, 1H), 3.84 (s, 3H), 3.83 (s, 3H), 3.72 (s, 3H), 3.49 (br s, 1H), 1.85 (br s, 1H); **HRMS** calcd for $[\text{C}_{31}\text{H}_{28}\text{O}_8\text{Na}]^+$ requires m/z 551.1682; found 551.1697 (ES+).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

DCC	<i>N,N</i> -dicyclohexylcarbodiimide
DMAP	4-(dimethylamino)pyridine
EDCI	<i>N</i> -(3-dimethylaminopropyl)- <i>N'</i> -ethylcarbodiimide hydrochloride
HOBt	1-hydroxybenzotriazole hydrate
MEK	mitogen activated protein kinase kinase

NBS	<i>N</i> -bromosuccinimide
NCS	<i>N</i> -chlorosuccinimide
THF	tetrahydrofuran
DMSO	dimethylsulfoxide
eIF4A	eukaryotic initiation factor 4A
eIF4E	eukaryotic initiation factor 4E
eIF4F	eukaryotic initiation factor 4F

Figure 1. Possible coordination modes of 4, 5, and 6

Figure 2.

Hydroxamate (-)-9 is equipotent to silvestrol *in vitro*. **(a)** Effect of hydroxamates and related compounds on cap-dependent *in vitro* translation. The activity relative to DMSO is presented. Results are the average of duplicates with the error of the mean shown. **(b)** Hydroxamate (-)-9 inhibits cap-dependent *in vitro* translation in a dose dependent manner. The activity relative to DMSO is presented. Values represent the average of duplicates with the error of the mean shown. **(c)** Inhibition of protein synthesis in HeLa cells by the most potent analogs tested in (a). Relative ³⁵S-Met incorporation is presented with values standardized against total protein content and plotted relative to vehicle (DMSO). Results are the average of duplicates with the error of the mean shown. **(d)** Hydroxamate (-)-9 stimulates binding of eIF4AI to radiolabeled RNA. The average of three experiments with the standard error of the mean is presented. **(e)** Hydroxamate (-)-9 depletes eIF4AI/II from the eIF4F complex. Eluents after m⁷GTP affinity purification were resolved on a 10% SDS-PAGE gel followed by Western blot analysis for eIF4E and eIF4AI/II.

Figure 3.

Hydroxamate (-)-9 is equipotent to silvestrol *ex vivo* and *in vivo*. **(a)** Inhibition of protein synthesis by hydroxamate (-)-9 in human Burkitt's lymphoma BJAB cells. Relative ^{35}S -Met incorporation is presented with values standardized to total protein content and plotted relative to vehicle (DMSO). Results are the average of duplicates with the error of the mean shown. **(b)** Effect on cell viability. BJAB cells treated for 72 h with the indicated concentrations of silvestrol or (-)-9, followed by propidium iodine staining and flow cytometry analysis. Results are the average of three experiments and plotted against vehicle controls. **(c)** Hydroxamate (-)-9 synergizes with doxorubicin *in vivo*. Kaplan Meier plot showing tumor free survival of mice bearing E μ -myc/(myr)Akt tumors following treatment with doxorubicin (Dxr, n=5), silvestrol (n=5), (-)-9 (n=5), silvestrol and doxorubicin (Silvestrol+Dxr, n=5), or (-)-9 and doxorubicin ((-)-9+Dxr, n=5). Treatment studies were performed on 6-8 week old C57Bl/6 mice that had received 10^6 E μ -myc/(myr)Akt lymphoma cells intravenously. When tumors were palpable, mice were treated with silvestrol (0.2 mg/kg daily for 5 d), hydroxamate (-)-9 (0.2 mg/kg daily for 5 d), or doxorubicin (once at 10 mg/kg), or combinations thereof. Compounds were administered in PEG 400/ 5.2% Tween 80 via intraperitoneal (i.p.) injection. For combination studies, mice were treated with silvestrol or hydroxamate (-)-9 for five consecutive days, with doxorubicin being delivered once on the second day. $p < 0.001$ for combination vs. single agent treatments.

Chart 1. Silvestrol (1), Methyl Rocaglate (2) and the Related Hydroxamate 3

Chart 2. Hydroxamate Derivatives 7-13^a

^aConditions: (a) LiOH, 1,4-dioxane, H₂O, 60 °C, 5 h. (b) EDCI, HOBT, R¹ONR²H·HCl, Et₃N, CH₂Cl₂.

Chart 3. Tetrahedral Intermediates From Reaction of Lithio-Heterocycles with Ester 2 and Hydroxamate 3

Chart 4. Synthesis of Ketones 22-28^a

^aConditions: (a) (i) *n*-BuLi, THF, -78 °C or 0 °C, 30 min-1 h; (ii) **2** or **3**, THF, -78 °C to rt, 2 h.

Scheme 1. Preparation of Both Enantiomers of Hydroxamate 9^a

^aConditions: (a) LiOH, 1,4-dioxane, H₂O, 60 °C, 5 h. (b) (-)-menthol, DCC, DMAP, CH₂Cl₂, 12 h, 60% combined for **16** and **17** (2 steps). (c) LiOH, 1,4-dioxane, H₂O, 60 °C, 5 h. (d) EDCI, HOBT, MeONH₂·HCl, Et₃N, CH₂Cl₂, 65%. (e) LiOH, 1,4-dioxane, H₂O, 60 °C, 5 h. (f) EDCI, HOBT, MeONH₂·HCl, Et₃N, CH₂Cl₂, 67%

Table 1
Conversion of Methyl Rocaglate to Halogenated and Nitrated *O*-Methylhydroxamates 15a-j^a

2	conditions	14a-j	15a-j	f, g			
compound	R ¹	R ²	conditions	compound	R ¹	R ²	conditions
a	Br	Br	a	f	Cl	Cl	c
b	Br	H	a	g	H	F	d
c	H	Br	a	h	F	H	d
d	H	Cl	b	i	H	NO ₂	e
e	Cl	H	b	j	NO ₂	H	e

^a Conditions: (a) NBS, THF, rt, 50 min. (b) NCS, AuCl₃ (10 mol %), THF, -78 °C, 5 h. (c) NCS, THF, rt, 6 h. (d) Selectfluor, methanol, rt. (e) Cu(NO₃)₂, Ac₂O, 6 h. (f) LiOH, 1,4-dioxane, H₂O, 60 °C, 5 h. (g) EDCI, HOBT, MeONH₂-HCl, Et₃N, CH₂Cl₂.