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Synthesis and Biological Evaluation of Subglutinin Analogs for Immunomodulatory Agents

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Abstract

Autoimmune diseases are chronic inflammatory diseases associated with high morbidity and mortality. Treatment options for autoimmune diseases have increased over the past several decades, but they are in general limited in their clinical efficacy due to high toxicity and lack of selectivity. Thus, efforts must be made to identify new immunomodulatory agents that are effective through a novel mechanism to circumvent existing side effects. To define the structural requirements of subglutinins for immunomodulatory activity and to provide guiding principles on future therapeutic development, we prepared and evaluated several subglutinin analogs for their immunomodulatory activities. Our efforts identified a subglutinin analog with reduced structural complexity as a potential lead compound for future autoimmune drug development. Our study will provide an important framework to the design of potent and non-toxic immunomodulating agents derived from subglutinins.

Graphical Abstract

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Supporting Information

Synthesis and characterization of compounds, NMR analysis of **31a**, **31b**, and **41a**, preliminary characterization of subglutinin A analogs, flow cytometry gating strategy for T cell, dendritic cell, and regulatory T cell immune profiling of EAE mice, and attempts for hydrogen atom transfer, Claisen rearrangement, and hydroboration/oxidation reactions

Table of SMILES representations and IC₅₀ values (CSV)

The authors declare no competing financial interest.

INTRODUCTION

Autoimmune diseases are chronic inflammatory diseases of unknown etiology and result from the attack of self-antigens by autoreactive lymphocytes.^{1, 2} There are more than 100 human autoimmune diseases, including multiple sclerosis, ulcerative colitis, rheumatoid arthritis, and type 1 diabetes. Since they affect ~ 8% of the US population, their social and economic impact is enormous.^{3, 4}

In the last decade or so, several new immunosuppressive drugs have been added as options available to treat autoimmune diseases. However, current therapeutic options are in general limited in their clinical efficacy and cause unwanted side effects.⁵ For example, antiproliferative methotrexate and cyclophosphamide are limited due to their non-specific effects on various types of proliferating cells, resulting in serious bone marrow suppression and increased incidence of infections. Cyclosporine A, FK506, and rapamycin act more selectively on various stages of the T- and B-lymphocyte activation cycles, but they show acute neurological toxicity and chronic nephrotoxicity. Moreover, their structural complexity is not quite amenable to medicinal chemistry campaigns for further improvement. Biologic therapies have been gaining in popularity since they have faster onset of action and higher efficacy than small molecule drugs.^{1, 6} But, biologic therapies are also reported to cause severe side effects such as progressive multifocal leucoencephalopathy and idiopathic thrombocytopenic purpura.¹ Thus, efforts must be made to develop new immunomodulatory agents with a novel mechanism to circumvent existing side effects.

Subglutinols A (SubA, **1a**) and B (SubB, **1b**) (Figure 1) are α -pyrone diterpenoids isolated from *Fusarium subglutinans*, an endophytic fungus from the vine *Tripterygium wilfordii*.^{7, 8} Extracts from *T. wilfordii* have been used as therapies for rheumatoid arthritis, psoriasis, lupus associated autoimmune nephrotic syndrome, and autoimmune hepatitis in traditional Chinese medicine.^{9, 10} Clardy and co-workers reported that SubA (**1a**) and SubB (**1b**) are equally potent in a mixed-lymphocyte reaction (MLR) assay ($IC_{50} = 0.1 \mu M$).⁷ Due to the potential for novel immunosuppressive therapeutics, subglutinols have attracted a strong interest across the synthetic chemistry community.^{11–16}

Intrigued by their potent immunomodulatory activity, we established two complementary syntheses (i.e., first- and second-generation syntheses) of SubA (**1a**) and SubB (**1b**) which paved the way for the synthesis of the analogs described in this report.^{11, 12} After completion of the synthesis of **1a** and **1b**, we performed a comprehensive evaluation of the *in vitro* and

in vivo immunosuppressive activity of **1a**.^{11, 12, 17, 18} Our study indicated that overall, in a side-by-side comparison, the immune-suppressive activity profile of **1a** was comparable to that of cyclosporine A. SubA (**1a**) also showed promising *in vivo* efficacy in antigen-induced delayed hypersensitivity.¹⁸ Our preliminary mode of action study suggested that subglutinols may exert their anti-inflammatory effects by affecting mitochondria and T cell metabolism to prevent effector T cell function.¹⁸

Encouraged by the potential of subglutinols as novel therapeutics for autoimmune diseases, we prepared and evaluated several subglutanol analogs to define key pharmacophores important for the immunosuppressive activity of subglutinols. We also aimed to identify non-toxic subglutanol analogs with an excellent therapeutic window since **1a** showed high *in vitro* toxicity.^{7, 8} Herein, we describe our efforts toward the synthesis and biological evaluation of subglutanol analogs to guide the design of chemically tractable analogs for future therapeutic development for autoimmune diseases.

RESULTS AND DISCUSSION

Synthesis of subglutanol analogs.

One of the most critical aspects of drug discovery programs driven by natural products is the identification of structurally simple and chemically tractable compounds. When we designed subglutanol analogs, we sought to reduce the structural complexity by eliminating functional groups and structural motifs that are not essential for immunosuppressive activity. We used potency and non-specific toxicity as guiding parameters for the design of new analogs to define key pharmacophores while determining safety properties. As illustrated in Figure 1, three areas of subglutinols, tetrahydrofuran (THF), decalin, and α -pyrone regions were explored for structure–activity relationship (SAR) studies.

To investigate the role of the THF moiety of subglutinols in the immunosuppressive activity of subglutinols, we designed the hydroxy allyl and decalin-pyrone analogs (**11** and **19**, Schemes 1 and 2) by removing the THF and 2-methylpropenyl moieties of subglutinols. By cutting back the structure of subglutinols, we expected that the structural simplicity of analogs would increase the ligand efficiency, which is a very important metric in lead selection.¹⁹

The synthesis of the hydroxy allyl analog **11** began with ketal deprotection and TBS protection of the known alcohol **2**^{11, 12} (Scheme 1). Following the procedure established by our group,^{11, 12} formylation of **3** with HCO₂Et, ethoxyethyl (EE)-protection, NaBH₄ reduction, and elimination smoothly proceeded to give the α,β -unsaturated aldehyde **5**. NaBH₄ reduction of **5** and stannylmethylation of the resulting allylic alcohol set the stage for the stereocontrolled [2,3]-Wittig rearrangement. Treatment of **6** with *n*-BuLi stereoselectively provided the corresponding homoallylic alcohol as a single diastereomer, which was oxidized to the β,γ -unsaturated aldehyde **7** by Dess–Martin periodinane. Addition of 3-lithio- γ -pyrone, which was in situ generated from **8** and *n*-BuLi, to **7** and Barton–McCombie deoxygenation of the resulting alcohol provided **10**. TBS deprotection, hydrolysis under basic conditions, and γ - to α -pyrone tautomerization finally completed the synthesis of **11**.

The decalin-pyrone analog **19** was prepared in a similar manner (Scheme 2). Briefly, the known (4*aR-trans*)-octahydro-8*a*-methyl-1(2*H*)-naphthalenone (**12**)²⁰ was converted to the corresponding α,β -unsaturated aldehyde **14** via formylation, EE-protection, reduction, and elimination. Following the NaBH₄ reduction of **14**, the stannylmethylation of the resulting alcohol afforded the substrate **15** for the [2,3]-Wittig rearrangement. Treatment of **15** with *n*-BuLi followed by Dess–Martin oxidation gave the β,γ -unsaturated aldehyde **16** in 68% over two steps. Addition of 3-lithio- γ -pyrone, Barton–McCombie deoxygenation, and hydrolysis completed the synthesis of **19**.

As the role of the decalin group of subglutinols is likely to provide conformational rigidity, we hypothesized that the decalin could be replaced by a conformationally rigid scaffold such as naphthalene without significant loss of activity. To test this hypothesis, we designed the naphthalene analog **27** (Scheme 3). The allylation of the known naphthalenol **20**²¹ followed by LiAlH₄ reduction provided the alcohol **21**. Conversion of **21** to the corresponding benzylic bromide, coupling with dimethyl malonate, and decarboxylation²² successfully afforded the desired methyl ester **22** (54%) along with the Claisen rearrangement product **23** (16%). After searching for conditions for the Claisen rearrangement of **22** to **23**, we found that the Claisen rearrangement of **22** in 1,2-dichlorobenzene (150 °C, 24 h) gave the best result (52%, 62% BRSM) (see the Supporting Information for details). The cross-metathesis (CM) reaction of **23** with *cis*-1,4-diacetoxy-2-butene followed by the BF₃·OEt₂-promoted ring-closure gave the desired tetrahydrofuran **24**. After installing the 2-methylpropenyl group to **24** by the cross-metathesis reaction with 2-methylpropene, we chose to use the aldol strategy that we established for the second-generation synthesis of subglutinols in order to introduce the α -pyrone group to **24**.^{11, 12} Aldol condensation of the CM product and **25** followed by Dess–Martin oxidation of the resulting secondary alcohol provided the ketone **26**. Deprotection of the 1,3-dithiane group and subsequent DBU-mediated cyclization completed the synthesis of the naphthalene analog **27**.

As illustrated in Schemes 1 and 2, the installation of the *exo*-methylene group of subglutinols requires multiple steps. Therefore, we sought to replace the *exo*-methylene group with a more synthetically accessible group. To define the role of the *exo*-methylene group in the immunosuppressive activity of subglutinols and further improve the efficiency of analog synthesis, we designed the de-*exo*-methylene analog **34a** (Scheme 4). We envisioned that a stereoselective synthesis of the axial alcohol without relying on the [2,3]-Wittig rearrangement would be the key to the preparation of **34a**. Towards this goal, we explored the hydrogen atom transfer reaction²³ and the S_N2 substitution reaction (see the Supporting Information for details). Unfortunately, none of our attempts for the hydrogen atom transfer reaction resulted in the formation of the desired axial alcohol.

Next, we turned our attention to the stereoselective hydroboration/oxidation reaction of the alkene **29** to directly introduce the requisite axial hydroxymethyl group. Catalytic hydrogenation of **28** followed by Wittig reaction with methyltriphenylphosphonium iodide afforded **29**. The hydroboration of **29** with 9-BBN gave a 3:1 mixture of inseparable diastereomers, but in low yield (39%). Dess–Martin oxidation of **30** gave a mixture of the diastereomeric aldehydes **31a,b** which were separated by SiO₂ column chromatography.

Careful analyses of the ^1H NMR spectra of **31a,b** indicated that the desired axial aldehyde **31a** was the minor isomer of the hydroboration reaction by 9-BBN (see the Supporting Information for details). Use of $\text{BH}_3\cdot\text{THF}$ for the hydroboration step improved the reaction yield to 86% and gave a 1:1 mixture of the two diastereomeric alcohols **30**. Addition of γ -pyrone to **31a** followed by Barton–McCombie deoxygenation provided **33**. The final hydrolysis of **33** gave the desired de-*exo*-methylene analog **34a** in 86% yield. The C6-*epi* aldehyde **31b** was also converted to **34b** via the aldol condensation route (Scheme 4).^{11, 12}

To further reduce the structural complexity of subglutinols, we designed the simplest analog **41a**. The synthesis of **41a** began with a Wittig reaction of the known ketone **12**²⁰ (Scheme 5). To introduce the axial hydroxymethyl group to **41a**, we explored various hydroboration/oxidation conditions.^{11, 12} Disappointingly, none of the conditions we tested gave the desired **38** as the major product (see the Supporting Information for details). $\text{BF}_3\cdot\text{THF}$ complex gave a 1:1 inseparable mixture of the alcohols. Dess–Martin oxidation of **38** followed by addition of 3-lithio- γ -pyrone afforded the alcohol **39**. Barton–McCombie deoxygenation of **39** and final hydrolysis accomplished the synthesis of **41a,b**. The mixture of the diastereomeric decalin-pyrone analogs **41a,b** was separated by SiO_2 column chromatography to give **41a** and **41b**. The stereochemistry of C6 in the product **41a** was established by the nOe experiment (see the Supporting Information for details).

According to our preliminary SAR,^{11,12} the α -pyrone group is essential to the immunosuppressive activity of subglutinols. To validate our initial finding and confirm the role of the α -pyrone group in the immunosuppressive activity of subglutinols, we replaced the α -pyrone group with a phenyl group and prepared the phenyl analog **45** (Scheme 5). Compound **42**^{11, 12} was treated with Dess–Martin periodinane and PhLi to provide **43**. Barton–McCombie deoxygenation of **43** and final CM of **44** with 2-methylpropene accomplished the synthesis of the phenyl analog **45**.

Biological characterization.

As detailed in our previous publication, SubA (**1a**) was effective in abolishing the inflammatory cytokine production by skewed Th1 or Th17 CD4^+ T cells.¹⁸ Self-antigen induced production of $\text{IFN}\gamma$ (by Th1 cells) or IL-17A (by Th17 cells) is a hallmark of autoimmune disease, so blocking the production of these cytokines is a vital component of any immunosuppressive drug. We reasoned that any new analog we developed must efficiently suppress $\text{IFN}\gamma$ or IL-17A production by activated CD4^+ T cells to a level comparable to the suppression mediated by SubA. To assess whether the new analogs affect cytokine production, we polarized naïve OT-II CD4^+ T cells *in vitro* for 4 days under appropriate skewing conditions to produce cells that acquired competence to produce either $\text{IFN}\gamma$ (Th1) or IL-17A (Th17) cytokines. We then treated these effector CD4^+ T cells with control DMSO, SubA, or analogs for an additional 48 h. As previously observed, SubA was effective at reducing the percentage of $\text{IFN}\gamma$ producing cells in the Th1 skewed conditions in a dose dependent manner, with 100 nM and 10 μM dosage of SubA causing complete suppression of $\text{IFN}\gamma$ cytokine production (Figure 2A,B). Similarly, high doses of SubA diminished the ability of Th17 cells to produce IL-17A. Most of the analogs tested were also effective at reducing $\text{IFN}\gamma$ production at high doses, albeit not as potently as SubA, with the

exception of the analog **41b** (Figure 2A). Notably, compounds **34a** and **41a** were the only analogs that were as effective as SubA in suppressing both IFN γ and IL-17A production (Figure 2B,C,D). However, we decided to pursue studies using compound **41a** due to its additional effects in promoting lower cell toxicity, as detailed in the following section of our study.

As important as it is to find a compound that is effective in suppressing inflammatory cytokine development by activated and differentiated Th1 and Th17 cells, it is equally important to make sure that the proposed compound does not cause substantial toxicity to naïve, bystander T cells. As we observed significant naïve T cell toxicity of SubA in our previous study,¹⁸ we examined the toxic effects of the new SubA analogs. We activated either OT-II (primarily CD4 T cells) or OT-I (CD8 T cells) with their cognate peptides *in vitro* and added increasing concentrations of SubA analogs. After 48 h, cell viability was determined by staining using a Live/Dead Viability kit which positively stains dead and dying cells. We normalized the data to DMSO-treated cells and calculated the fold change of T cell death or survival to that elicited by DMSO treatment. Positive fold change values represent reduced toxicity in response to compound addition, while negative values represent heightened toxicity. We did not observe much toxicity of either CD4 or CD8 T cells with lower (100 nM or 10 nM) doses of most analogs (Figure 3). However, as observed previously, SubA exhibited dramatic toxicity in both CD4 and CD8 T cells (Figure 3A,B).

Combining the results of this toxicity data with the immunosuppression data reported above, we decided to focus on the analog **41a**, as it showed almost comparable suppression of IFN γ and IL-17A cytokines with SubA, but a substantial reduction of toxicity of CD4 T cells at both intermediate and high concentrations of the compound. A 10 μ M concentration of SubA resulted in the survival of only 24.4% of CD4 T cells, as compared with 41.5% CD4 T cell survival in the **41a** treatment group (Figure 3C). An intermediate dosage of 100 nM resulted in 46% CD4 T cell survival upon treatment with SubA and 60.6% survival with **41a** treatment. The high compound dose in CD8 T cell treated conditions (7.19% vs 8.85% survival in SubA vs. **41a**, respectively) did not have as dramatic an effect as the intermediate dose (14.5% vs. 42.6% CD8 T cell survival in SubA vs. **41a**, respectively). Taken together, these data highlight the new compound, **41a** may be an effective improvement to SubA as an immunomodulatory agent.

Our preliminary SAR study of subglutinol analogs (**11**, **19**, **27**, **34a**, **34b**, **41a**, **41b**, and **45**) suggested that the α -pyrone (SubA vs. **45**, Supplementary Figure S3) and decalin (SubA vs. **27**, Supplementary Figure S3) groups are essential for the immunosuppressive activity of subglutinols. It is of note that the stereochemistry of the pyrone group (axial vs. equatorial) plays an important role in the biological activity of analogs (**41a** vs. **41b** and **34a** vs. **34b**, Figure 2 and Supplementary Figure S3). The SAR analysis also suggested that the toxicity of subglutinols primarily originates from the THF group.

To test whether **41a** functions in an *in vivo* context, we examined its potential disease-ameliorating roles in experimental autoimmune encephalitis (EAE), the mouse model of multiple sclerosis.²⁴ In this model, self-antigen elicited cytokine production by both Th1 and Th17 cells is pathologic in the development of disease. We hypothesized that since **41a**

strongly suppresses cytokine production by both Th1 and Th17 cells with low toxicity, it could potentially be used as a therapeutic agent in suppressing pathologic cytokine production in the EAE model. In this model, mice were immunized with an emulsion containing the autoantigen, Myelin Oligodendrocyte Glycoprotein (MOG_{35–55}) peptide with Complete Freund's Adjuvant (CFA), *Mycobacterium tuberculosis* (Mtb), as well as two doses of pertussis toxin (Figure 4A). We treated the mice with three doses of DMSO (control) or 4 μ mol of **41a** starting two days after disease induction, and monitored the progression of limb paralysis- the hallmark clinical manifestation of disease- over a course of approximately 50 days. We found that neither DMSO nor **41a** treatment caused toxicity in the mice. While disease symptoms normally peak around 15 days in this model then slowly subside, our animals exhibited a prolonged disease course with clinical symptoms finally reducing in the DMSO-treated animals around 30 days, though not to a significant level compared to the **41a** treated animals (Figure 4B). Interestingly, there was a slight difference in time to disease onset in these animals; mice treated with **41a** exhibited a slightly faster disease onset (average of 15 days) as compared to the DMSO-treated mice (average of 19.5 days), though this disease onset was not statistically significant. This suggests that DMSO-treated mice have a slightly better ability to suppress the early effects of EAE-mediated immune pathology, a phenotype that is a bit at odds with **41a**-mediated immune suppression shown in our *in vitro* experiments.

In addition to this slight increase in time to disease onset mediated by **41a** treatment, we were surprised to find that **41a** did not ameliorate EAE disease in our experiments. To investigate the immune cell subsets that may have been affected by treatment with either DMSO or **41a**, we performed immune phenotyping (Figure 5). EAE disease was induced as previously described, and either DMSO or **41a** compounds were injected at two time-points (days 4 and 6). At day 7 post disease induction, we sacrificed all animals and looked at the composition of their immune cells (T cells, dendritic cells, regulatory T cells, macrophages, neutrophils and granulocytic myeloid derived suppressor cells (gMDSCs) by flow cytometry (Supplementary Figures S4–S6). We found no difference in the composition of most immune cell subsets in these animals. However, we found that **41a** treatment resulted in a significant increase in the percentage of inflammatory neutrophils and a concomitant reduction of the percentage of gMDSCs (* $p < 0.05$). This data is interesting given reports that indicate an increase in inflammatory neutrophils in EAE are particularly pathogenic,^{25, 26} potentially explaining why **41a** treated animals have slightly worse clinical EAE symptoms than DMSO treated animals. Taken together, these data do not rule out the potential use of **41a** as an immunosuppressive drug in autoimmune disease, just that it is not effective in EAE.

CONCLUSION

Subglutinols are novel immunosuppressive natural products with an as yet unknown mode of action that may be unique among other reported immunosuppressive agents. In order to develop subglutinols as potential therapeutics for autoimmune diseases, we prepared and evaluated subglutininol analogs for their immunomodulatory activity. The SAR study of subglutininol analogs provided valuable insights into the structural requirements for the

immunosuppressive activity of subglutinols. Among the analogs prepared, **41a** had significantly reduced structural complexity and showed almost comparable suppression of IFN γ and IL-17A cytokines with SubA, but with a substantial reduction of toxicity of CD4 T cells at both intermediate and high concentrations. The analog **41a** did not ameliorate EAE disease in our experiments, but **41a** caused an increase in the percentage of inflammatory neutrophils and a reduction of the percentage of gMDSCs. These unexpected results warrant further investigation, particularly in diseases where an influx of inflammatory neutrophils may be beneficial for prognosis.

In conclusion, our efforts in synthesis and biological evaluation of subglutininol analogs successfully identified **41a** with significantly reduced structural complexity as a potential lead compound for future autoimmune drug development. We expect that our findings will be an important basis for future design and optimization of drug-like properties of subglutininol analogs for novel therapeutic development for autoimmune diseases.

EXPERIMENTAL SECTION

General Chemistry Procedures.

All reactions were conducted in oven-dried glassware under nitrogen. Unless otherwise stated all reagents were purchased from Sigma–Aldrich, Acros, or Fisher and were used without further purification. All solvents were ACS grade or better and used without further purification except tetrahydrofuran (THF) which was freshly distilled from sodium/benzophenone each time before use. Analytical thin layer chromatography (TLC) was performed with glass backed silica gel (60 Å) plates with fluorescent indication (Whatman). Visualization was accomplished by UV irradiation at 254 nm and/or by staining with *p*-anisaldehyde solution. Flash column chromatography was performed by using silica gel (particle size 230–400 mesh, 60 Å). All ^1H NMR and ^{13}C NMR spectra were recorded with a Varian 400 (400 MHz) and a Bruker 500 (500 MHz) spectrometer in CDCl_3 by using the signal of residual CHCl_3 , as an internal standard. All NMR δ values are given in ppm, and all *J* values are in Hz. Electrospray ionization (ESI) mass spectrometry (MS) were recorded with an Agilent 1100 series (LC/MSD trap) spectrometer and were performed to obtain the molecular masses of the compounds. The purity of all compounds used in bioassays was determined to be 95% pure.

Synthesis of subglutininol analogs.

Details of the synthesis and characterization of intermediates for the preparation of subglutininol analogs are presented in the Supporting Information.

3-(((1*R*,4*aR*,5*S*,6*S*,8*aR*)-5-Allyl-6-hydroxy-5,8*a*-dimethyl-2-methylenedecahydronaphthalen-1-yl)methyl)-4-hydroxy-5,6-dimethyl-2*H*-pyran-2-one (Analog **11**). To a solution of **S4** (16 mg, 0.04 mmol) in THF/MeOH (2/1, 4.5 mL) was added 2 N NaOH (1.5 mL) at 25 °C. The resulting mixture was refluxed for 24 h, cooled to 25 °C, neutralized with saturated aqueous NH_4Cl , and diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The

residue was purified by column chromatography (silica gel, hexanes/EtOAc, 2/1) to afford **11** as a white solid (14 mg, 90%): ^1H NMR (400 MHz, MeOD- d_4) δ 5.60–5.89 (m, 1H), 5.20–5.05 (m, 2H), 4.48 (t, J = 2.3 Hz, 1H), 4.20 (t, J = 2.0 Hz, 1H), 3.54–3.46 (m, 1H), 2.72 (dd, J = 13.0, 11.8 Hz, 1H), 2.54 (dd, J = 13.1, 4.4 Hz, 1H), 2.43 (td, J = 13.3, 5.3 Hz, 1H), 2.33 (dd, J = 14.2, 7.4 Hz, 1H), 2.20 (s, 3H), 2.12 (dd, J = 11.7, 4.5 Hz, 1H), 2.09–2.00 (m, 2H), 1.93 (s, 3H), 1.83–1.61 (m, 4H), 1.42–1.21 (m, 3H), 0.96 (s, 3H), 0.81 (s, 3H); ^{13}C NMR (125 MHz, MeOD- d_4) δ 169.2, 167.6, 156.8, 150.3, 136.0, 118.0, 110.2, 108.7, 104.2, 74.4, 56.4, 43.0, 42.7, 40.4, 38.7, 35.5, 32.2, 28.3, 23.9, 23.5, 22.5, 17.5, 17.3, 10.4; HRMS (ESI) m/z 387.2532 [(M+H) $^+$ calcd for C₂₄H₃₄O₄ 387.2530].

4-Hydroxy-5,6-dimethyl-3-(((1*R*,4*aR*,8*aS*)-8*a*-methyl-2-methylenedecahydronaphthalen-1-yl)methyl)-2*H*-pyran-2-one (Analog **19**). To a solution of **18** (5 mg, 0.02 mmol) in THF/MeOH (2/1, 1.5 mL) was added 2 N NaOH (0.5 mL) at 25 °C. The resulting mixture was refluxed for 15 h, cooled to 25 °C, neutralized with saturated aqueous NH₄Cl, and diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄, and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexanes/EtOAc, 3/1) to afford **19** as a white solid (4 mg, 84%): ^1H NMR (400 MHz, CDCl₃) δ 5.73 (s, 1H), 4.69 (s, 1H), 4.49 (s, 1H), 2.81 (dd, J = 14.7, 3.7 Hz, 1H), 2.52 (dd, J = 14.5, 10.2 Hz, 1H), 2.40 (td, J = 13.6, 5.5 Hz, 1H), 2.20 (s, 3H), 2.16 (d, J = 4.6 Hz, 1H), 1.98 (dd, J = 10.5, 3.8 Hz, 1H), 1.91 (s, 3H), 1.76–1.69 (m, 2H), 1.63–1.54 (m, 2H), 1.49 (dt, J = 13.1, 3.7 Hz, 1H), 1.45–1.34 (m, 2H), 1.34–1.10 (m, 4H), 0.84 (s, 3H); ^{13}C NMR (125 MHz, CDCl₃) δ 165.1, 164.3, 155.9, 150.2, 111.4, 106.1, 103.2, 54.7, 37.8, 37.7, 36.6, 31.4, 30.3, 29.2, 26.8, 22.1, 22.0, 18.9, 17.4, 10.0; HRMS (ESI) m/z 317.2112 [(M+H) $^+$ calcd for C₂₀H₂₈O₃ 317.2111].

4-Hydroxy-5,6-dimethyl-3-((2-(2-methylprop-1-en-1-yl)-1,2-dihydronaphtho[2,1-*b*]furan-6-yl)methyl)-2*H*-pyran-2-one (Analog **27**). [Aldol Reaction] To a solution of diisopropylamine in THF (2 mL) at 0 °C was added dropwise *n*-BuLi (2.5 M in hexanes, 0.4 mL, 0.97 mmol). The resulting solution was stirred for 30 min at 0 °C and for 5 min at 25 °C and then cooled to –78 °C to generate LDA. The ester **S11** (50 mg, 0.16 mmol) in THF (1 mL) was added dropwise to a solution of LDA at –78 °C. After stirring for 30 min at the same temperature, the aldehyde **25** (163 mg, 0.86 mmol) in THF (1 mL) was added dropwise to this solution. The resulting mixture was stirred for additional 30 min at –78 °C. The reaction was quenched by an addition of saturated aqueous NH₄Cl, and the resulting mixture was diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexanes/EtOAc, 4/1) to afford the alcohols. [Dess–Martin Oxidation] To a cooled solution (0 °C) of the alcohols were added Dess–Martin periodinane (109 mg, 0.26 mmol) and NaHCO₃ (58 mg, 0.69 mmol). After stirring for 1 h at 25 °C, the reaction was quenched by additions of saturated aqueous Na₂S₂O₃ and saturated aqueous NaHCO₃. The resulting mixture was stirred vigorously for 1 h. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography

(silica gel, hexanes/EtOAc, 10/1) to afford the thioketal **26** as a colorless oil (42 mg, 52% for two steps). [Thioketal Deprotection] To a solution of **26** (42 mg, 0.08 mmol) in CH₃CN/H₂O (3:1, 5 mL) were added CaCO₃ (169 mg, 1.68 mmol) and MeI (0.1 mL, 1.68 mmol) at 25 °C. After stirring at the same temperature for 60 h, the reaction mixture was diluted with EtOAc and H₂O. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. This crude 1,3-diketone was used in next step without further purification. [Pyrone Formation] To a solution of the crude 1,3-diketone in benzene (6 mL) was added DBU (0.13 mL, 0.84 mmol) at 25 °C. The resulting mixture was stirred under reflux condition for 3 h and cooled to 25 °C. The reaction mixture was neutralized carefully with 1 N HCl at 0 °C and diluted with EtOAc and H₂O. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexanes/EtOAc, 2/1) to afford **27** as a colorless oil (4 mg, 13% for two steps): ¹H NMR (400 MHz, CDCl₃) δ 7.88 (d, *J* = 9.1 Hz, 1H), 7.51 (d, *J* = 8.3 Hz, 1H), 7.37 (dd, *J* = 8.2, 7.1 Hz, 1H), 7.25 (d, *J* = 6.6 Hz, 1H), 7.10 (d, *J* = 9.0 Hz, 1H), 6.64 (s, 1H), 5.66 (q, *J* = 9.0 Hz, 1H), 5.50 (dt, *J* = 8.5, 1.5 Hz, 1H), 4.21 (s, 3H), 3.62 (dd, *J* = 15.4, 9.3 Hz, 1H), 3.16 (dd, *J* = 15.3, 8.3 Hz, 1H), 2.19 (s, 3H), 1.83 (d, *J* = 1.4 Hz, 3H), 1.80 (d, *J* = 1.4 Hz, 3H), 1.77 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 166.7, 165.3, 157.5, 156.7, 138.3, 135.6, 131.8, 127.5, 126.5, 125.5, 124.9, 123.4, 122.5, 119.8, 112.6, 107.2, 100.8, 80.8, 35.7, 28.8, 26.0, 18.5, 17.4, 9.8; HRMS (ESI) *m/z* 399.1568 [(M+Na)⁺ calcd for C₂₄H₂₄O₄ 399.1567].

3-(((2*R*,3*aS*,5*aR*,6*S*,9*aR*,9*bS*)-2-Ethyl-5*a*,9*b*-dimethyldodecahydronaphtho[2,1-*b*]furan-6-yl)methyl)-4-hydroxy-5,6-dimethyl-2*H*-pyran-2-one (Analog **34a**). To a solution of **33** (6 mg, 0.02 mmol) in THF/MeOH (2/1, 1.5 mL) was added 2 N NaOH (0.5 mL) at 25 °C. The resulting mixture was refluxed for 24 h, cooled to 25 °C, neutralized with saturated aqueous NH₄Cl, and diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH₂Cl₂/MeOH, 50/1) to afford **34a** as a white solid (5 mg, 86%): ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.75–3.68 (m, 1H), 2.95 (dd, *J* = 11.2, 3.8 Hz, 1H), 2.60–2.39 (m, 2H), 2.13 (s, 3H), 1.86 (s, 3H), 1.83–1.73 (m, 1H), 1.72–1.54 (m, 4H), 1.49–1.37 (m, 5H), 1.31–1.22 (m, 3H), 1.19 (d, *J* = 13.2 Hz, 1H), 1.10 (d, *J* = 12.8 Hz, 1H), 1.01 (s, 3H), 0.88 (t, *J* = 7.4 Hz, 3H), 0.75 (s, 3H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 165.2, 164.2, 154.9, 106.5, 102.5, 86.3, 78.8, 45.6, 45.4, 45.2, 43.5, 37.6, 34.9, 29.5, 24.6, 23.7, 23.6, 22.1, 21.1, 20.9, 17.3, 17.1, 11.1, 10.4; HRMS (ESI) *m/z* 389.2695 [(M+H)⁺ calcd for C₂₄H₃₆O₄ 389.2686].

3-(((2*R*,3*aS*,5*aR*,6*R*,9*aR*,9*bS*)-2-Ethyl-5*a*,9*b*-dimethyldodecahydronaphtho[2,1-*b*]furan-6-yl)methyl)-4-hydroxy-5,6-dimethyl-2*H*-pyran-2-one (Analog **34b**). [Aldol Reaction] To a solution of diisopropylamine (0.07 mL, 0.47 mmol) in THF (1.5 mL) at 0 °C was added dropwise *n*-BuLi (2.5 M in hexanes, 0.18 mL, 0.47 mmol). The resulting solution was stirred for 30 min at 0 °C and 5 min at 25 °C and then, cooled to –78 °C to generate LDA. The ester **35** (25 mg, 0.08 mmol) in THF (0.7 mL) was added dropwise to a solution of LDA at –78

°C. After stirring for 30 min at the same temperature, the aldehyde **36** (89 mg, 0.62 mmol) in THF (0.7 mL) was added dropwise to this solution. The resulting mixture was stirred for additional 30 min at –78 °C. The reaction was quenched by an addition of saturated aqueous NH₄Cl, and the resulting mixture was diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexanes/EtOAc, 2/1) to afford the alcohols (40 mg, 87%). [Dess–Martin Oxidation] To a cooled solution (0 °C) of the alcohols (40 mg, 0.09 mmol) were added Dess–Martin periodinane (73 mg, 0.17 mmol) and NaHCO₃ (29 mg, 0.34 mmol). After stirring for 2 h at 25 °C, the reaction was quenched by additions of saturated aqueous Na₂S₂O₃ and saturated aqueous NaHCO₃. The resulting mixture was stirred vigorously for 1 h. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexanes/EtOAc, 10/1) to afford **37** as a colorless oil (19 mg, 61%). [Ketal Deprotection] To a solution of **37** (19 mg, 0.04 mmol) in acetone (2 mL) was added Cl₂Pd(CH₃CN)₂ (1.5 mg, 0.004 mmol) at 25 °C. After stirring at the same temperature for 15 h, the solvent was removed *in vacuo*. The residue was purified by column chromatography (silica gel, hexanes/EtOAc, 10/1) to afford the ketone (13 mg, 76%). [Pyrene Formation] To a solution of the ketone (13 mg, 0.03 mmol) in benzene (3 mL) was added DBU (0.05 mL, 0.31 mmol) at 25 °C. The resulting mixture was stirred under reflux condition for 1.5 h and cooled to 25 °C. The reaction mixture was neutralized carefully with 1 N HCl at 0 °C and diluted with EtOAc and H₂O. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CH₂Cl₂/MeOH, 30/1) to afford **34b** as a colorless oil (8 mg, 67%): ¹H NMR (400 MHz, DMSO-*d*₆) δ 3.73–3.65 (m, 1H), 2.95 (dd, *J* = 11.9, 3.5 Hz, 1H), 2.31 (dd, *J* = 13.2, 1.5 Hz, 1H), 2.19 (dd, *J* = 12.6, 10.3 Hz, 1H), 2.13 (s, 3H), 1.85 (s, 3H), 1.74–1.38 (m, 6H), 1.30–1.10 (m, 8H), 0.97 (dd, *J* = 12.4, 1.6 Hz, 2H), 0.93–0.82 (m, 6H), 0.76 (s, 3H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 165.1, 164.2, 154.8, 106.6, 101.5, 86.3, 78.9, 54.7, 49.5, 45.3, 43.6, 37.8, 37.2, 29.5, 26.5, 26.3, 23.7, 23.0, 22.2, 17.2, 17.0, 15.2, 11.1, 10.3.

4-Hydroxy-5,6-dimethyl-3-(((1*S*,4*aR*,8*aS*)-8*a*-methyldecahydronaphthalen-1-yl)methyl)-2*H*-pyran-2-one (Analog **41a**). To a solution of **40** (8 mg, 0.03 mmol) in THF/MeOH (2/1, 1.5 mL) was added 2 N NaOH (0.5 mL) at 25 °C. The resulting mixture was refluxed for 24 h, cooled to 25 °C, neutralized with saturated aqueous NH₄Cl, and diluted with EtOAc. The layers were separated, and the aqueous layer was extracted with EtOAc. The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, hexanes/ CH₂Cl₂/EtOAc, 10/1/1) to afford **41a** as a white solid (2 mg, 21%): ¹H NMR (400 MHz, MeOD-*d*₄) δ 2.67 (dd, *J* = 13.3, 11.0 Hz, 1H), 2.56 (dd, *J* = 12.9, 4.0 Hz, 1H), 2.23 (s, 3H), 1.96 (s, 3H), 1.91 (d, *J* = 13.8 Hz, 1H), 1.78–1.48 (m, 7H), 1.43–1.13 (m, 8H), 0.99 (s, 3H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 165.2, 164.2, 154.8, 106.5, 102.4, 41.7, 37.8, 36.3,

36.2, 29.1, 29.0, 26.5, 23.8, 21.7, 21.1, 20.5, 18.5, 17.1, 10.4; HRMS (ESI) m/z 305.2114 [(M+H)⁺ calcd for C₁₉H₂₈O₃ 305.2111].

4-Hydroxy-5,6-dimethyl-3-(((1*R*,4*aR*,8*aS*)-8a-methyldecahydronaphthalen-1-yl)methyl)-2*H*-pyran-2-one (Analog **41b**). ¹H NMR (400 MHz, MeOD-*d*₄) δ 2.57 (d, *J* = 12.6 Hz, 1H), 2.25 (s, 3H), 2.15 (dd, *J* = 13.3, 9.9 Hz, 1H), 1.97 (s, 3H), 1.93 (d, *J* = 12.5 Hz, 1H), 1.77–1.63 (m, 2H), 1.56–1.50 (m, 3H), 1.43–1.05 (m, 10H), 0.88 (s, 3H).

(2*S*,3*aS*,5*aR*,6*R*,9*aR*,9*bS*)-6-Benzyl-5*a*,9*b*-dimethyl-7-methylene-2-(2-methylprop-1-en-1-yl)dodecahydronaphtho[2,1-*b*]furan (Analog **45**). To a cooled (–78 °C) solution of the alkene **44** (4.3 mg, 0.01 mmol) in 2-methylpropene/CH₂Cl₂ (2:1, 1.5 mL) was added Grubbs' second-generation catalyst (1 mg, 0.0013 mmol). The resulting mixture was refluxed for 12 h. An addition of Grubbs' second-generation catalyst (1 mg, 0.0013 mmol) was repeated twice every 12 h. The reaction mixture was cooled to –78 °C and DMSO (2 drops) was added. The resulting mixture was stirred for 12 h at 25 °C. The solvent was removed *in vacuo*. The residue was purified by column chromatography (silica gel, hexanes/EtOAc, 50/1) to afford **45** as a colorless oil (1.6 mg, 35%): ¹H NMR (400 MHz, CDCl₃) δ 7.28–7.18 (m, 2H), 7.14 (td, *J* = 7.3, 1.5 Hz, 1H), 7.06 (d, *J* = 7.9 Hz, 2H), 5.37 (d, *J* = 8.5 Hz, 1H), 4.71 (td, *J* = 9.5, 3.5 Hz, 1H), 4.52 (d, *J* = 2.0 Hz, 1H), 4.14 (d, *J* = 2.0 Hz, 1H), 3.16–3.07 (m, 1H), 2.96 (dd, *J* = 13.2, 3.5 Hz, 1H), 2.63–2.53 (m, 1H), 2.35–2.24 (m, 1H), 2.19 (dd, *J* = 13.6, 4.6 Hz, 1H), 1.94–1.80 (m, 5H), 1.72 (d, *J* = 8.0 Hz, 6H), 1.61–1.56 (m, 2H), 1.51–1.45 (m, 2H), 1.36–1.32 (m, 1H), 0.98 (s, 3H), 0.87 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 146.8, 141.9, 135.7, 129.1, 128.1, 127.4, 125.6, 111.2, 87.5, 74.3, 60.8, 48.1, 45.8, 44.6, 38.9, 35.3, 33.9, 30.9, 25.9, 25.3, 24.8, 22.6, 18.3, 17.1; HRMS (ESI) m/z 365.2839 [(M+H)⁺ calcd for C₂₆H₃₆O 365.2839].

Mice.

OT-I mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J) and OT-II mice (B6.Cg-Tg(TcraTcrb)425Cbn/J) were purchased from the Jackson Laboratory. All mice were housed under pathogen-free conditions and used between 6 to 10 weeks of age for all experimental procedures in accordance with the Duke University Institutional Animal Care and Use Committee guidelines.

Cell culture.

Splenocytes harvested from OT-I or OT-II mice were emulsified into a single cell suspension and passed through a 70µm cell strainer. T cells were cultured in RPMI-1640 media supplemented with 10% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 50 µM 2-mercaptoethanol in a humidified 37 °C incubator with 7% CO₂.

Cell viability assays.

Naïve OT-I or OT-II splenocytes were activated with 1µM OVA_{257–264} peptide or 1 µM OVA_{323–339} peptide, respectively, together with varying concentrations of drugs or DMSO control. After 48 hours of culture, OT-I or OT-II T cells were assessed for viability using the Live/Dead viability kit (Invitrogen) and the following antibodies were used for staining (all

from Biolegend): anti-mouse CD4-Pacific Blue, anti-mouse CD8 α -APC, and anti-mouse TCR β -FITC. Samples were acquired on the FACSCanto II and data analyzed using the FlowJo software.

T cell differentiation assays.

Naïve OT-II splenocytes were activated with 1 μ M OVA_{323–339} peptide and cultured under Th differentiating conditions for 5 days. For Th1 differentiation, 50 U/mL IL-2 (Biolegend), 50 ng/mL IL-12 (R&D Systems), 10 μ g/mL anti-IL-4 (Biolegend), and 5 ng/mL anti-TGF β (R&D Systems) were added. For Th17 differentiation, 50 ng/mL IL-6 (Biolegend), 4 ng/mL TGF β (R&D Systems), 10 μ g/mL anti-IL-4 (Biolegend), and 10 μ g/mL anti-IFN γ (Biolegend) were added. During the final 3 days of culture, the indicated amounts of drug or DMSO was added. On the final day of culture, OT-II T cells were restimulated for 4 hours with 0.9 nM pDBU (Sigma), 0.5 μ g/mL ionomycin (Sigma), 5 μ g/mL brefeldin A (eBioscience) and 2 μ M monensin A (eBioscience). Viable cells were stained using the Live/Dead viability kit (Invitrogen) before intracellular staining. Intracellular staining was performed after fixing the cells in 2% paraformaldehyde and membrane permeabilization using 0.1% saponin. The following antibodies were used for staining (all from Biolegend): anti-mouse CD4-FITC, anti-mouse TCR β -PerCPy5.5, anti-mouse IFN γ -Pacific Blue, anti-mouse IL-2-APC (for Th1 skew) and anti-mouse IL17A-APC (for Th17 skew). Samples were acquired on the FACSCanto II and data analyzed using the FlowJo software.

Active EAE induction.

Six-week C57B/6 mice (Jackson Labs) were immunized with 200 μ L of a 1:1 mixture of MOG_{35–55} peptide and Complete Freund's Adjuvant including Mtb (Sigma). On day 0, 100 μ g of the emulsified mixture was subcutaneously injected into the flank of the mice. 200 ng of pertussis toxin (Sigma) was intraperitoneally injected into each mouse on days 0 and 2. EAE clinical symptoms were monitored starting at day 7 and were assessed daily thereafter. EAE score was evaluated as follows- 0.5: distal tail limpness, 1: complete tail limpness, 1.5: hind limb weakness, 2: impaired righting reflex, 2.5: paralysis of one hind limb, 3: paralysis of both hind limbs, 3.5: paralysis of both hind limbs and dragging forelimb, 4: paralysis of both hind limbs and partial front leg paralysis, 5: moribund or requiring euthanasia.

Pharmaceutical treatment of mice with EAE.

Active EAE was induced in C57B/6 mice as described above. DMSO or an 80 mM stock of **41a** were diluted 50 $\times$ in PBS and 250 μ L was injected intraperitoneally into the right flank of mice at days 4, 6 and 8. The final concentration of **41a** injected per day was 4,000 nmol. EAE clinical symptoms were monitored starting at day 7 and continuing to day 50. No adverse toxicity was noticed in either of the treatment groups.

Immune phenotyping of EAE mice.

Active EAE was induced in C57B/6 mice as described above. DMSO and **41a** (4,000 nmol per injection) were diluted 50 $\times$ in PBS and 250 μ L was injected intraperitoneally into the right flank of mice at day 4 and day 6. On day 7, all mice were sacrificed and splenocytes harvested for EAE immune phenotyping. Splenocytes were dissociated and filtered using a

70 μ m cell strainer. 3×10^6 splenocytes were separated for each of the immune profiling staining panels. Cells were stained using the Aqua Live/Dead viability kit (Invitrogen) and the following antibodies were used for staining. For the T cell panel (all from Biolegend): anti-mouse TCR β -FITC, anti-mouse CD4-Pacific Blue, anti-mouse CD8 α -PECy7, anti-mouse CD44-APC, and anti-mouse CD62L-APC Cy7. For the macrophage, neutrophil and gMDSC panel (all from Biolegend): anti-mouse CD11b-PE Cy7, anti-mouse F4/80-FITC and anti-mouse GR-1-APC. For the dendritic cell panel (all from Biolegend): anti-mouse CD11b-PE Cy7, anti-mouse CD11c-FITC, anti-mouse I-A/I-E-PE, anti-mouse CD103-Pacific Blue. For the regulatory T cell panel, the Foxp3 staining kit (eBioscience) was used following the manufacturer's suggestions and the following antibodies used for staining: anti-mouse CD4-FITC (Biolegend), anti-mouse CD25-PE Cy7 (Biolegend), anti-mouse Foxp3-PE (eBioscience), anti-mouse CD39-APC (eBioscience). Samples were acquired on the FACSCantoII and data analyzed using the FlowJo software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

CD	cluster of differentiation
EAE	experimental autoimmune encephalomyelitis
GMDCs	granulocytic myeloid derived suppressor cells
CsA	cyclosporine A
IL	interleukin
INF	interferon
MOG	myelin oligodendrocyte glycoprotein
SAR	structure–activity relationship
SubA	subglutinin A
SubB	subglutinin B

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MLR Assay (IC_{50} , μM)	
Subglutinols A & B	0.1
Cyclosporine A	0.1

Figure 1.
Structure and immunosuppressive activity of subglutinols A and B.

Figure 2.

At high concentrations, the analog **41a** suppresses inflammatory cytokine production similar to that elicited by SubA. All new analogs were tested for their ability to inhibit inflammatory cytokine production in Th1 and Th17 cells. CD4⁺ T cells were activated and polarized *in vitro* for 4 days to produce either IFN γ producing Th1 cells (A,B) or IL-17 producing Th17 cells (C,D). Analogs were added for an additional 48 h in increasing concentrations and the percentages of cytokine producing CD4⁺ T cells were determined by intracellular flow cytometry. Representative flow cytometry plots are shown in (B,D). All data presented is representative of three independent experiments. Error bars represent mean with SEM.

Figure 3.

The analog **41a** exhibits less toxicity in both CD4+ and CD8+ T cells as compared to SubA. Naïve CD4+ (A) and CD8+ (B) T cells were activated for 2 days *in vitro* in the presence of varying concentrations of compounds. After 48 h, cell viability was determined by flow cytometry. Fold-change was calculated by normalizing the data to DMSO (vehicle)-treated cells; a positive fold change represents less toxicity in response to drug treatment, and negative fold change values represent increased toxicity. (C) Representative flow cytometry plots clearly show that higher concentrations of **41a** elicit less toxicity particularly in naïve CD4+ T cells as compared to SubA treatment. Data represents a combination of two independent experiments. Error bars represent mean with SEM.

Figure 4.

In vivo treatment of **41a** does not ameliorate the symptoms of experimental autoimmune encephalitis (EAE). Mice were immunized with a mixture of MOG+CFA+Ptx to induce EAE disease and treated with intraperitoneal injections of either **41a** or DMSO control as shown in the experiment schema in (A). Limb paralysis and disease score was monitored over 50 days (B). There was no significant effect of **41a** on moderating the course of disease compared to DMSO treatment. Data is representative of three independent experiments. Error bars represent mean with SEM.

Figure 5.

Treatment of **41a** increases the number of inflammatory neutrophils produced in EAE diseased mice. Immune phenotyping was performed 7 days post EAE disease induction to investigate the immune cell subsets that may have been affected by treatment with either DMSO or **41a**. There was no difference in the percentage of total T cells (A), activated T cells (B), dendritic cells (C) or regulatory T cells (D). The only difference elicited by **41a**-treatment was a significant increase in the percentage of inflammatory neutrophils and a concomitant reduction in the percentage of gMDSCs (E). (F) A representative flow cytometry plot showing the gating strategy of macrophages, inflammatory neutrophils and gMDSCs shows the differences elicited by **41a** treatment. Data represent mean with SEM. * $p < 0.05$ (unpaired t -test).

Scheme 1.

Synthesis of the hydroxy allyl analog **11**.^a

^aReagents and conditions: (a) 1 N HCl, THF, 60 °C, 3 h, 93%; (b) TBSOTf, 2,6-lutidine, CH₂Cl₂, -78 to 0 °C, 10 min, 98%; (c) NaH, HCO₂Et, THF, reflux, 1 h, 88%; (d) ethyl vinyl ether, PPTS, THF, 25 °C, 16 h, 80%; (e) NaBH₄, EtOH, 0 °C, 2 h, 76%; (f) MsCl, Et₃N, CH₂Cl₂, 0 °C, 30 min, 90%; (g) NaBH₄, EtOH, 0 °C, 30 min, 93%; (h) *n*-Bu₃SnCH₂I, KH, 18-c-6, THF, 0 °C, 30 min, 95%; (i) *n*-BuLi, hexanes, -50 to 25 °C, 5 h, 50%; (j) DMP, NaHCO₃, CH₂Cl₂, 25 °C, 1 h, 52%; (k) **8**, *n*-BuLi, THF, -78 °C, 35 min, 76%; (l) NaHMDS, CS₂, THF, -78 °C, 1 h, then MeI, -78 °C, 1 h; (m) *n*-Bu₃SnH, AIBN, toluene, reflux, 1 h, 71% for 2 steps; (n) BF₃•OEt₂, MeCN, 0 °C, 1.5 h, 91%; (o) 2 N NaOH, THF/MeOH, reflux, 24 h, 90%.

Scheme 2.

Synthesis of the decalin-pyrone analog **19**.^a

^aReagents and conditions: (a) NaH, HCO₂Et, THF, reflux, 2 h, 98%; (b) ethyl vinyl ether, PPTS, THF, 25 °C, 1.5 h, 70%; (c) NaBH₄, EtOH, 0 °C, 2 h, 97%; (d) MsCl, Et₃N, CH₂Cl₂, 0 °C, 30 min, 95%; (e) NaBH₄, EtOH, 0 °C, 30 min, 69%; (f) *n*-Bu₃SnCH₂I, KH, 18-c-6, THF, 0 °C, 30 min, 80%; (g) *n*-BuLi, hexanes, -50 to 25 °C, 5 h, 73%; (h) DMP, NaHCO₃, CH₂Cl₂, 25 °C, 1 h, 93%; (i) **8**, *n*-BuLi, THF, -78 °C, 1 h, 86%; (j) NaHMDS, CS₂, THF, -78 °C, 1 h, then MeI, -78 °C, 1 h 55%; (k) *n*-Bu₃SnH, AIBN, toluene, reflux, 2 h, 87%; (l) 2 N NaOH, THF/MeOH, reflux, 15 h, 84%.

Scheme 3.

Synthesis of the naphthalene analog **27**.^a

^aReagents and conditions: (a) $\text{CH}_2=\text{CHCH}_2\text{Br}$, K_2CO_3 , acetone, reflux, 13 h, 99%; (b) LiAlH_4 , THF, 0 °C, 30 min, 83%; (c) PBr_3 , CH_2Cl_2 , 0 °C, 1.5 h, 62%; (d) $\text{CH}_2(\text{CO}_2\text{Me})_2$, NaH, THF, 0 °C, 1.5 h, 96%; (e) LiCl , H_2O , DMSO, 130 °C, 15 h; (f) 1,2-dichlorobenzene, 150 °C, 24 h, 52% (62% BRSM); (g) *cis*-1,4-diacetyxy-2-butene, Grubbs II, CH_2Cl_2 , reflux, 2.5 h, 74%; (h) $\text{BF}_3 \cdot \text{OEt}_2$, CH_2Cl_2 , 0 to 25 °C, 2.5 h, 30%; (i) 2-methylpropene, Grubbs II, CH_2Cl_2 , reflux, 36 h, 83%; (j) **25**, LDA, THF, -78 °C, 1 h; (k) DMP, NaHCO_3 , CH_2Cl_2 , 0 to 25 °C, 1 h, 52% for 2 steps; (l) CaCO_3 , MeI, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, 25 °C, 60 h; (m) DBU, benzene, reflux, 3 h, 13% for 2 steps.

Scheme 4.

Synthesis of the de-*exo*-methylene (**34a**) and its C6-*epi* (**34b**) analogs.^a

^aReagents and conditions: (a) Pd/C, H₂, EtOH, 25 °C, 3 h, 95%; (b) CH₃PPh₃⁺I⁻, LiHMDS, THF, 0 to 25 °C, 15 h, 61% (82% BRSM); (c) BH₃•THF, THF, 0 °C, 3 h then, H₂O₂, 3 N NaOH, 0 °C, 16 h, 86% [dr = 1:1]; (d) DMP, NaHCO₃, CH₂Cl₂, 0 to 25 °C, 2 h, 75%; (e) **8**, *n*-BuLi, THF, -78 °C, 1 h, 84%; (f) NaHMDS, CS₂, THF, -78 °C, 1 h then MeI, -78 °C, 1 h; (g) *n*-Bu₃SnH, AIBN, toluene, reflux, 3 h, 38% for 2 steps; (h) 2 N NaOH, THF/MeOH, reflux, 24 h, 86%; (i) methyl (triphenylphosphoranylidene)acetate, CH₂Cl₂, 0 to 25 °C, 90 h, 82%; (j) Pd/C, H₂, EtOH, 25 °C, 15 h, 99%; (k) **36**, LDA, THF, -78 °C, 1 h, 87%; (l) DMP, NaHCO₃, CH₂Cl₂, 0 to 25 °C, 2 h, 61%; (m) Cl₂Pd(CH₃CN)₂, acetone, 25 °C, 15 h, 76%; (n) DBU, benzene, reflux, 1.5 h, 67%.

Scheme 5.

Synthesis of the de-*exo*-methylene decalin-pyrone (**41a,b**) and phenyl (**45**) analogs.^a

^aReagents and conditions: (a) $\text{CH}_3\text{PPh}_3^+\text{Br}^-$, $\text{KO}t\text{-Bu}$, toluene, 110°C , 3 h, 56%; (b) $\text{BH}_3\cdot\text{THF}$, THF, 0°C , 1.5 h, then H_2O_2 , 3 N NaOH , 0°C , 16 h, 83% [dr = 1:1]; (c) DMP, NaHCO_3 , CH_2Cl_2 , 0 to 25°C , 2 h, 87%; (d) **8**, $n\text{-BuLi}$, THF, -78°C , 35 min, 63%; (e) NaHMDS , CS_2 , THF, -78°C , 1 h, then MeI , -78°C , 1 h; (f) $n\text{-Bu}_3\text{SnH}$, AIBN, toluene, reflux, 1.5 h, 50% for 2 steps; (g) 2 N NaOH , THF/MeOH, reflux, 24 h, 21%; (h) DMP, NaHCO_3 , CH_2Cl_2 , 25°C , 1 h, 70%; (i) PhLi , THF, -78°C , 30 min, 82%; (j) NaHMDS , CS_2 , THF, -78°C , 1 h, then MeI , -78°C , 1 h, 63%; (k) $n\text{-Bu}_3\text{SnH}$, AIBN, toluene, reflux, 2 h, 96%; (l) 2-methylpropene, Grubbs II, CH_2Cl_2 , reflux, 36 h, 35%.