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Total Synthesis and Pharmacological Characterization of Solomonsterol A, a Potent Marine Pregnane-X-Receptor Agonist Endowed with Anti-Inflammatory Activity

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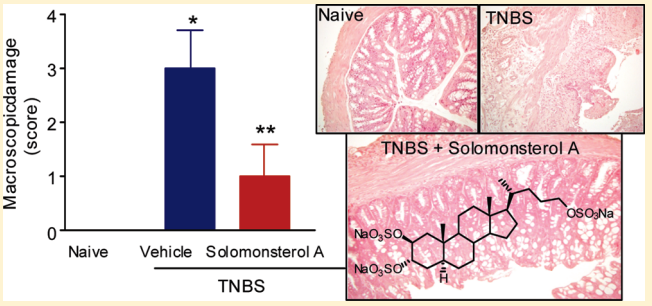
ABSTRACT: Recently, we reported the identification of a novel class of pregnane-X-receptor (PXR) agonists, solomonsterols A and B, isolated from the marine sponge *Theonella swinhoei*. Preliminary pharmacological studies demonstrated that these natural compounds are potential leads for the treatment of human disorders characterized by dysregulation of innate immunity. In this article, we describe the first total synthesis of solomonsterol A and its in vivo characterization in animal models of colitis. Using transgenic mice expressing the human PXR, we found that administration of synthetic solomonsterol A effectively protects against development of clinical signs and symptoms of colitis and reduced the generation of TNFα, a signature cytokine for this disorder. In addition, we have provided the first evidence that solomonsterol A might act by triggering the expression of TGFβ and IL-10, potent counter-regulatory cytokines in inflammatory bowel diseases (IBD). Finally, we have shown that solomonsterol A inhibits NF-κB activation by a PXR dependent mechanism. In summary, solomonsterol A is a marine PXR agonist that holds promise in the treatment of inflammation-driven immune dysfunction in clinical settings.



INTRODUCTION

Inflammatory bowel diseases (IBD), encompassing Crohn's disease (CD) and ulcerative colitis (UC), are two progressive and self-destructive disorders of the small intestine and colon arising from a genetically driven dysregulation of innate and adaptive immunity. In recent years, a growing body of evidence has been accumulated, indicating that dysregulation of innate immunity plays a role in IBD development and self-perpetuation. The innate immune system has dual roles in host defense, providing direct and immediate response against microbial invaders as well as playing an instructive role in influencing the nature of adaptive immunity. This instructive role of the innate immune system is served by a subset of intestinal mucosal immune cells, the antigen presenting cells (APCs), which includes macrophages and dendritic cells,[1,2] suggesting that attenuation of unbalanced generation of pro-inflammatory mediators by intestinal macrophages might be effective in protecting against IBD development.[1] At least 27 nuclear receptors (NRs) are expressed in the colon and intestine[3] including steroid and xenobiotic receptor (SXR), also known as pregnane-X-receptor (PXR), a transcription factor important for xenobiotic metabolism.[4] Following ligand binding, PXR forms a heterodimer with the retinoid-X-receptor (RXR) that binds to specific PXR response elements (PXREs), located in the 5′-flanking regions of PXR target genes, resulting in their transcriptional activation.[5−7] Ligands for PXR, such as rifaximin,[5,7] a broad-spectrum semisynthetic rifamycin derivative, have been shown effective in treating human IBDs. Thus, rifaximin is efficient in inducing clinical remission of moderately active Crohn's.[8] In addition, rifaximin could contribute to the maintenance of the intestinal barrier integrity by regulating the metabolism of xenobiotics by increasing the expression and activity of PXR and PXR-regulated genes.[7,9] The preventive and therapeutic role of rifaximin has been demonstrated in rodent models of colitis using transgenic mice that express the human PXR.[10]

Steroids have always attracted considerable attention because of being a fundamental class of biological signaling molecules. Their profound biological, scientific, and clinical importance is now well validated.[11,12] They can regulate a variety of biological processes and thus have the potential to be developed as drugs for the treatment of a large number of diseases[13,14] including cardiovascular,[15] autoimmune diseases,[16] and cancer.[17] In recent years, a variety of steroids with unusual and interesting structures have been isolated from a wide range of marine organisms, particularly from sponges and echinoderms.[18]

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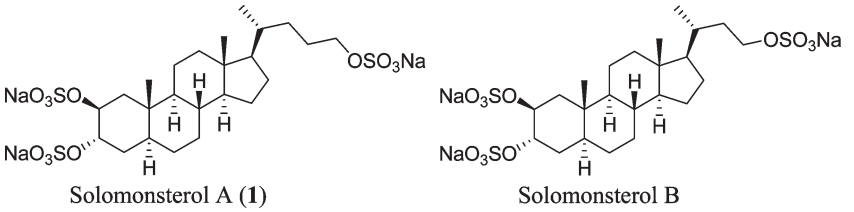
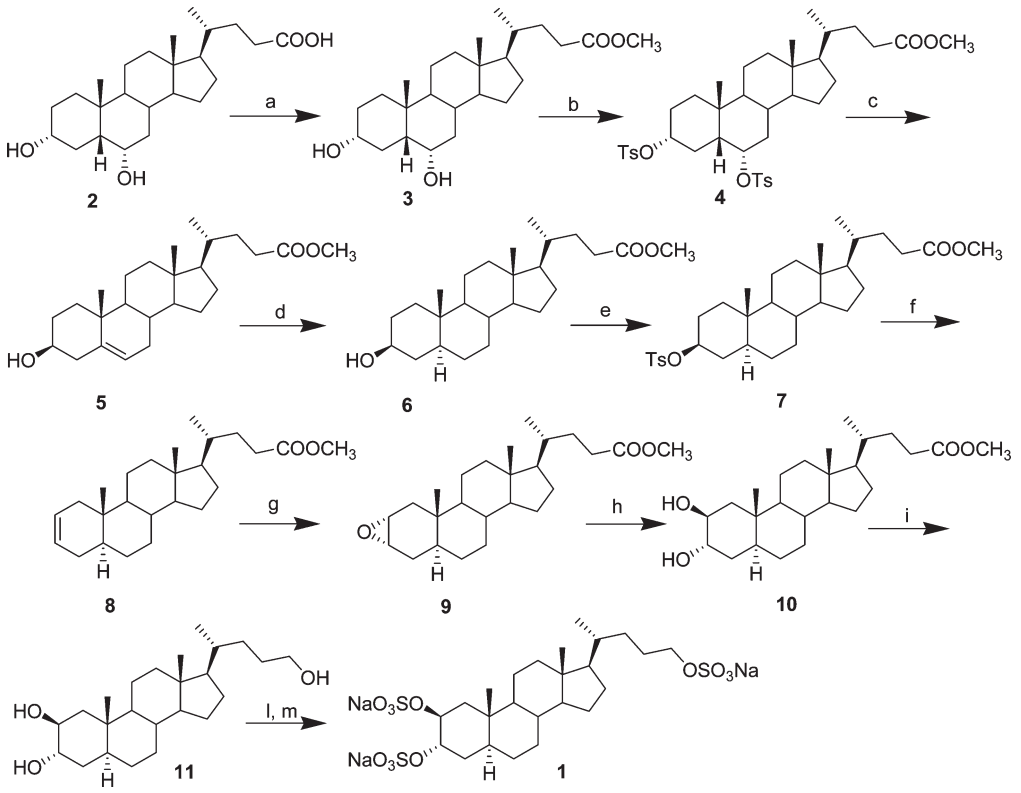


Figure 1. Solomonsterols A and B from *Theonella swinhoei*.

Scheme 1[a]



[a] Reagents and conditions: (a) CH_2N_2, quantitative; (b) *p*-TsCl, pyridine, quantitative; (c) CH_3COOK, DMF/H_2O 7:1, reflux, 78%; (d) H_2 (1 atm), Pd/C, THF/MeOH 1:1, 80%; (e) *p*-TsCl, pyridine; (f) LiBr, $LiCO_3$, DMF, reflux, 83% over two steps; (g) mCPBA, Na_2CO_3, CH_2Cl_2/H_2O 1:0.7; (h) H_2SO_4 1N, THF, 73% over two steps; (i) $LiBH_4$, MeOH/THF, 0 °C, 92%; (l) $Et_3N.SO_3$, DMF, 95 °C; (m) Amberlite CG-120, sodium form, MeOH, 90% over two steps.

In the course of our interest in marine natural products as NR ligands,[19] we found the sponge *Theonella swinhoei* as an extraordinary source of bioactive compounds.[20−24] Among these, the cyclic peptides, perthamides[20] and solomonamides,[21] are endowed with anti-inflammatory activity whereas solomonsterols A and B,[22] represent the first example of natural marine PXR agonists (Figure 1).

Effectively, in our previous paper,[22] we demonstrated that solomonsterols were potent inducers of PXR transactivation in a human hepatocyte cell line (HepG2 cells) boosting the receptor activity by 4−5-fold and were at least as potent as rifaximin. In addition, both agents stimulated the expression of CYP3A4 and MDR1 (cytochrome P450 3A4 and multidrug resistance 1), two well characterized PXR responsive genes in the same cell line.[25] Therefore, solomonsterols A and B are potential leads for the treatment of human disorders characterized by inflammation and dysregulation of innate immunity. Unfortunately, due to the scarcity of biological material isolated from the marine sponge *Theonella swinhoei*, further in vivo experiments were hampered. In this article, we report the first total synthesis of solomonsterol A (**1**) and its in vivo characterization in animal models of colitis.

RESULTS AND DISCUSSION

Chemistry. Key structural features of **1** are the presence of a truncated C24 side chain, and three sulfate groups at C2, C3, and C24. We envisaged that the commercially available hyodeoxycholic acid (**2**) could be a suitable starting material to set up a robust route to prepare solomonsterol A in large amounts. Thus, the total synthesis of solomonsterol A (**1**) started with **2**, which was methylated with diazomethane and treated with tosyl chloride in pyridine to give the corresponding 3,6-ditosylate (**4**) in nearly quantitative yield (Scheme 1). When **4** was treated with boiling DMF in the presence of CH_3COOK for 1 h, simultaneous inversion at the C-3 position and elimination at the C-6 position took place to give methyl 3-hydroxy-5-cholen-24-oate (**5**),[26,27] which in turn was hydrogenated to give the

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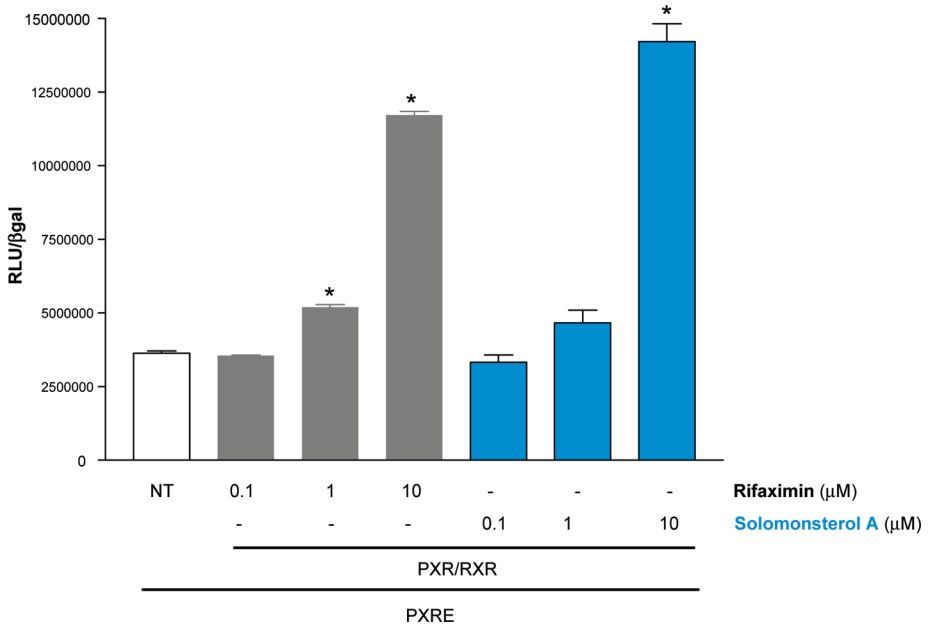

Figure 2. Luciferase reporter assay performed in HepG2 transiently transfected with pSG5-PXR, pSG5-RXR, pCMV-βgal, and p(cyp3a4)TKLUC vectors and stimulated for 18 h with rifaximin or solomonsterol A (0.1, 1, and 10 μM). *P < 0.05 versus not treated (NT) (n = 4).

required A/B *trans* ring junction in **6**.[28] The simultaneous introduction of the 2β,3α-dihydroxy functionality was achieved by the following three-step sequence:[29,30] (a) elimination at the C3-position and consequent introduction of the Δ-2 double bond; (b) epoxidation with *m*-CPBA; and (c) acid catalyzed ring-opening of the epoxide to afford diol **10**. β-Elimination and epoxidation were found to proceed with excellent regioselectivity and stereoselectivity, respectively, as determined by an analysis of NMR spectra and comparison of the NMR data of **8** and **9** with related compounds.[29,30] According to the Fürst−Plattner rule,[31] epoxide ring-opening with sulphuric acid in THF provided the desired 2β,3α-diol **10**, exclusively.[30] The ^{1}H NMR signals of H-2 and H-3 (broad singlet at 3.89 ppm and broad singlet at 3.85 ppm, respectively) also confirmed the *trans*-diaxial disposition of the two hydroxy groups in **10**.

Reduction of the methyl ester at C24 with $LiBH_4$ afforded triol **11** in 92% yield. Treatment of **11** with 10 equivalents of the triethylammonium−sulfur trioxide complex at 95 °C afforded the ammonium sulfate salt of solomosterol A, which was transformed via ion exchange into the desired target trisodium salt **1** (Scheme 1). The complete match of optical rotation, NMR, and HRMS data of solomonsterol A with that of the natural product secured the identity of the synthetic derivative.

This synthesis was completed in a total of 10 steps starting from commercially available hyodeoxycholic acid (**2**) and had an overall yield of 31%. This route enabled us to prepare sufficient quantities of solomonsterol A (**1**) to be further characterized in pharmacological tests.

PHARMACOLOGICAL EVALUATION

Anti-Inflammatory Activity of Solomonsterol A in a Model of Colitis Induced by TNBS Administration to PXR Transgenic Mice. We have first investigated whether the synthetic solomonsterol A (**1**) transactivates hPXR in PXR transactivation assay. As illustrated in Figure 2, solomonsterol A (**1**) was equally effective as rifaximin in transactivating the hPXR in HepG2 cells. The relative EC_{50} was 2.2 ± 0.3 μM for rifaximin and 5.2 ± 0.4 μM for solomonsterol A (n = 3).

Colon inflammation that develops in mice administered TNBS (trinitrobenzenesulfonic acid) is a model of a Th1-mediated disease with dense infiltrations of lymphocytes/macrophages in the *lamina propria* and thickening of the colon wall.[32,33] In order to assess whether solomonsterol A would exert immune-modulatory activity, TNBS was administered to C57Bl/6 transgenic mice expressing the human PXR. The hPXR mice express the human PXR in the mice PXR-null background.[10] Previous studies have shown that the intestinal inflammation that develops in these mice in response to TNBS and other chemical agents could be rescued by using agonists for hPXR, and therefore, this mouse strain represents a validated model to study the effects of hPXR activation. A previous report has shown that treating hPXR transgenic mice with rifaximin, a human PXR agonist, rescues the animals form colitis induced by TNBS.[10] In these experiments, mice were treated with solomonsterol A (**1**) and rifaximin for 7 days starting 3 days before intrarectal administration of TNBS (Figure 3). As shown, administering hPXR transgenic mice with solomonsterol A (**1**) effectively attenuated colitis development as measured by assessing local and systemic signs of inflammation. Thus, similarly to rifaximin, treatment with **1** at the dose of 10 mg/kg protected against the development colitis, as measured by the diarrhea score and weight loss (Figure 3A and B, n = 6−7; *p < 0.05 versus naïve; **p < 0.05 versus TNBS group), and reduced the macroscopic score of colitis as well as the microscopic score (Figure 3C−D, n = 6−7; *p < 0.05 versus naïve; **p < 0.05 versus TNBS group). As shown in Figure 3E, histopathological samples of colon removed from hPXR transgenic mice administered TNBS had an extensive cellular infiltrate, submucosal edema, and large areas of epithelial erosions. These changes were robustly attenuated by pretreatment with solomonsterol A (**1**) or rifaximin.

These effects were supported by an attenuation of signs of inflammation-driven immune dysfunction induced by TNBS administration. Thus, similarly to rifaximin, solomonsterol A

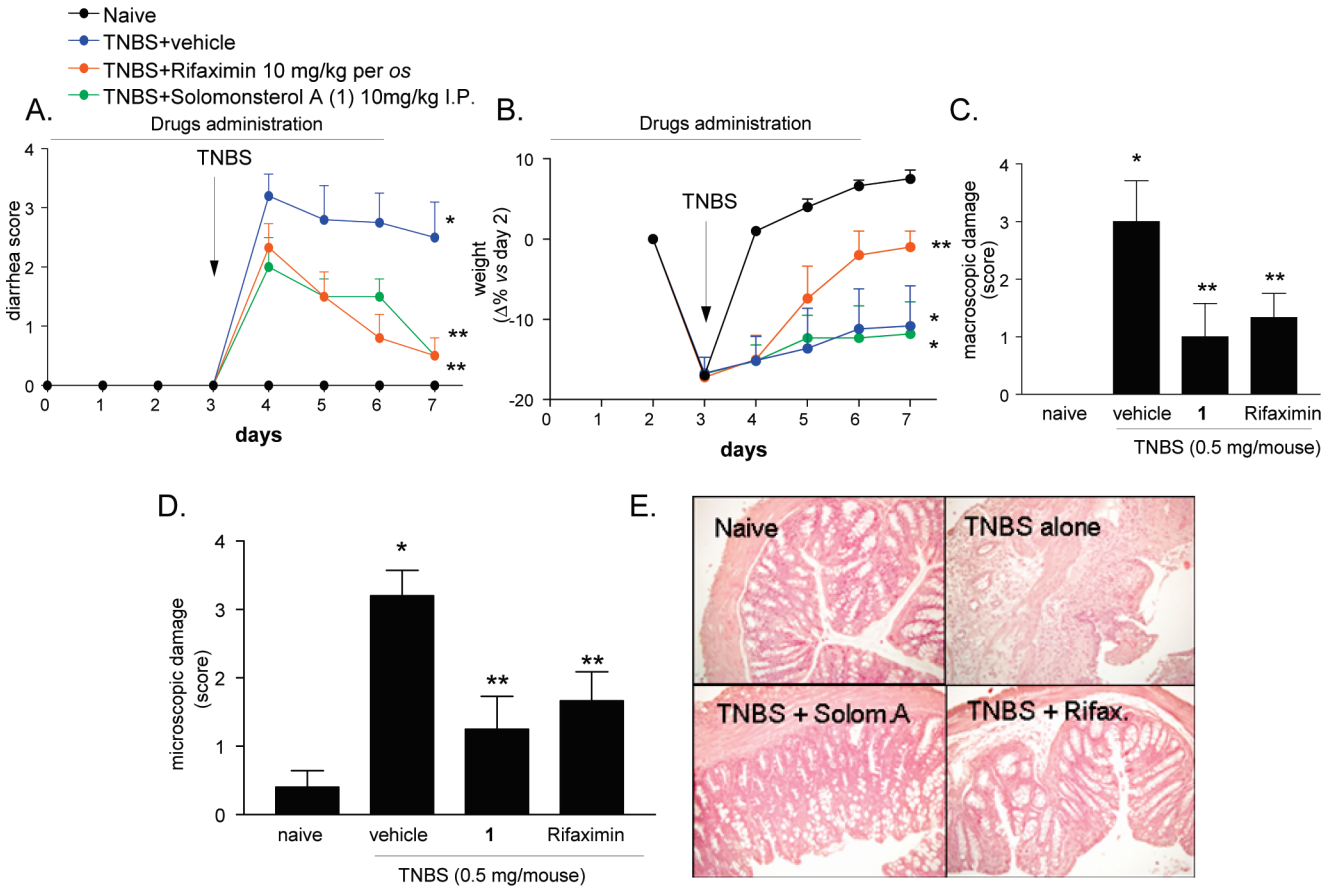

Figure 3. Colitis was induced by intrarectal administration of 0.5 mg of TNBS per hPXR mouse, and animals were sacrificed 4 days after TNBS administration. Solomonsterol A (**1**), 10 mg/kg, and rifaximin, 10 mg/kg, were administered once daily intraperitoneally (I.P.) or orally (per *os*), respectively, for 3 days before TNBS. The severity of TNBS-induced inflammation (A, diarrhea score; B, weight loss; and C, macroscopic colon damage) is modulated by rifaximin and solomonsterol A (**1**) administration. (D) Microscopic colon damage. (E) Histological analysis of colon samples (original magnification 40×, H&E staining). TNBS administration causes colon wall thickening and massive inflammatory infiltration in the *lamina propria*.

(**1**) reduced neutrophil accumulation in the colonic mucosa as assessed by measuring MPO (myeloperoxidase) activity, as well as the expression of a number of signature cytokines and chemokines including TNFα (tumor necrosis factor alfa), IFNγ (interferon gamma), IL-12p70 (interleukin-12 p70 subunit), and MIP-1α (macrophage inflammatory protein-1α) (Figure 4). Of interest, both rifaximin and solomonsterol A (**1**) effectively increased the colon expression of IL-10 (interleukin-10), a key counter-regulatory cytokine. A similar pattern, thought nonsignificant for solomonsterol A (**1**), was observed for TGFβ (transforming growth factor beta) mRNA, a growth factor whose colon expression is linked to the generation of a subset of regulatory T cells (Treg, regulatory T cells)[34] (Figure 4F and G, $n = 6-7$; $^{*}p < 0.05$ versus naïve; $^{**}p < 0.05$ versus TNBS).

Finally we found that administering hPXR mice with solomonsterol A (**1**) effectively triggered PXR activation in vivo. Indeed, as shown in Figure 4H, both solomonsterol A (**1**) and rifaximin caused a potent induction in the expression of Cyp3A11. In the mice, Cyp3A11 is the orthologue of the human CYP3A4 gene, and it is a PXR regulated gene highly expressed in the intestine. These data strongly indicated that solomonsterol A and rifaximin are PXR agonists in vivo (Figure 4H, $n = 6-7$; $^{*}p < 0.05$ versus naïve; $^{**}p < 0.05$ versus TNBS).

Solomonsterol A Inhibits LPS-Induced NF-κB DNA Binding Activity. In addition, we have evaluated the effect of solomonsterol A on NF-κB (nuclear factor-kappaB) DNA binding activity in LPS (lipopolysaccharide)-activated monocyte cell line. As shown in Figure 5, compared to LPS alone, cotreatment with solomonsterol A resulted in robust reduction of LPS induced NF-κB DNA binding activity as measured in EMSA assay. These results suggest that solomonsterol A inhibits the LPS-induced cytokines and chemokine expression by suppressing NF-κB DNA-binding activity.

Therapeutic Effect of Solomonsterol A. Because these data demonstrated that prophylactic treatment with solomonsterol A (**1**) effectively protects against colitis development, we have investigated whether this agent is effective in driving the healing of an established active colitis.

For this purpose, solomonsterol A was administered in a therapeutic manner in mice rendered colitic by TNBS administration. As illustrated in Figure 6, when administered to mice on day 1 after TNBS administration, solomonsterol A (**1**) effectively attenuated clinical signs of colitis (Figure 6A and B), including the diarrhea score and wasting disease. In addition, solomonsterol A (**1**) attenuated the macroscopic and microscopic scores as well as the MPO activity, a measure of neutrophil infiltration into the colonic mucosa (Figure 6C−E).

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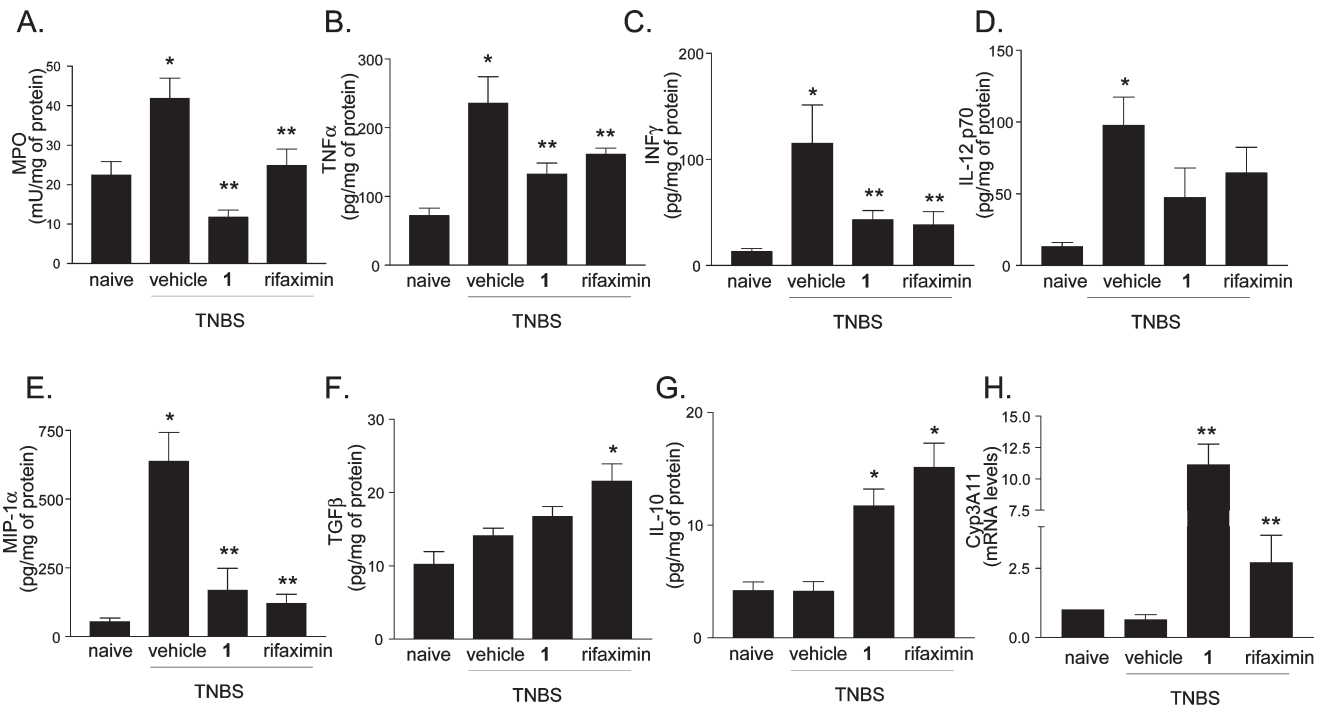

Figure 4. MPO (A), TNFα (B), INFγ (C), IL-12 p70 (D), MIP-1α (E), TGF-β1 (F), and IL-10 (G) protein levels in colons obtained 5 days after the administration of TNBS alone or in combination with rifaximin and **1**. Treatments inhibit the increase of neutrophil infiltration (MPO levels) and proinflammatory factors induced by intrarectal administration of TNBS. Data represent the mean ± SE of 6−7 mice per group. (*$p < 0.05$ vs naïve; **$p < 0.05$ vs TNBS). (H) Effects of rifaximin and solomonsterol A in colon mRNA levels of a PXR target gene such as Cyp3A11 (data are the mean ± SE of 5 mice per group; *$p < 0.05$ solomonsterol A vs rifaximin; **$p < 0.05$ vs TNBS).

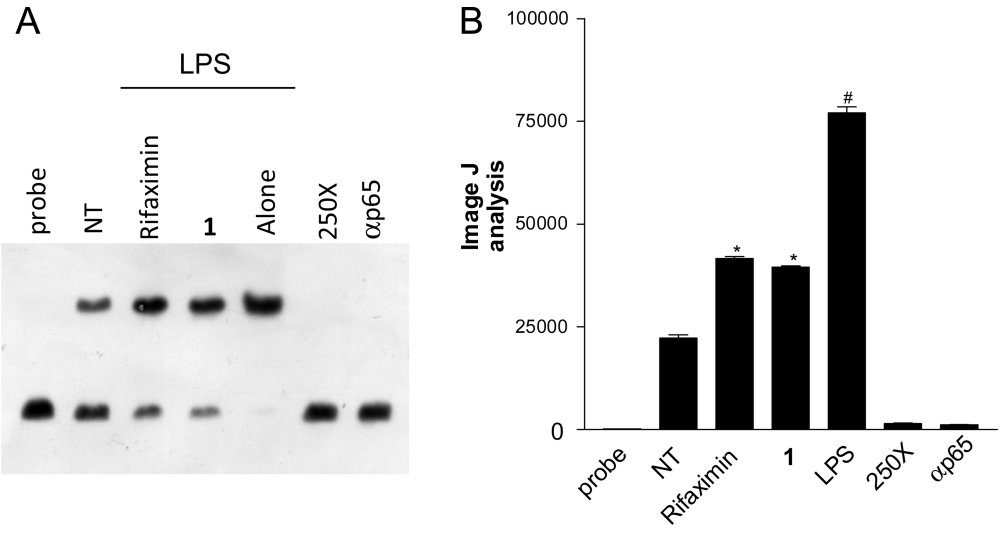

Figure 5. Rifaximin and solomonsterol A (**1**) suppress LPS-induced NF-κB DNA binding activity in THP-1 (human acute monocytic leukemia) cells, a monocyte-like cell line. The cells were pretreated with rifaximin (10 μM), **1** (10 μM), or vehicle for 3 h and then stimulated with LPS (1 μg/mL) for 20 h. (A) DNA binding activity of the NF-κB p65 subunit was analyzed by EMSA. For the competition assay, a 250-fold excess of unlabeled probe was added together with the labeled probe. For the supershift assay, 1 μg of antibody against NF-κB p65 was added together with the nuclear extract. Similar results were observed in three independent experiments. (B) Densitometric analysis of p65 binding to NF-κB responsive elements.

CONCLUSIONS

In the present study, we have reported the first total synthesis of solomonsterol A, a marine natural product endowed with potent anti-inflammatory activity, mediated by its ability to act as an hPXR agonist as demonstrated by the induction of a canonical target gene of PXR in colon, such as Cyp3A11 (Figure 4 H). The synthetic route, short and amenable to scale-up, allowed us to prepare sufficient quantities of solomonsterol A and to evaluate it in animal models of colitis. In this study, we have shown that activation of PXR receptor by solomonsterol A (**1**) exerts

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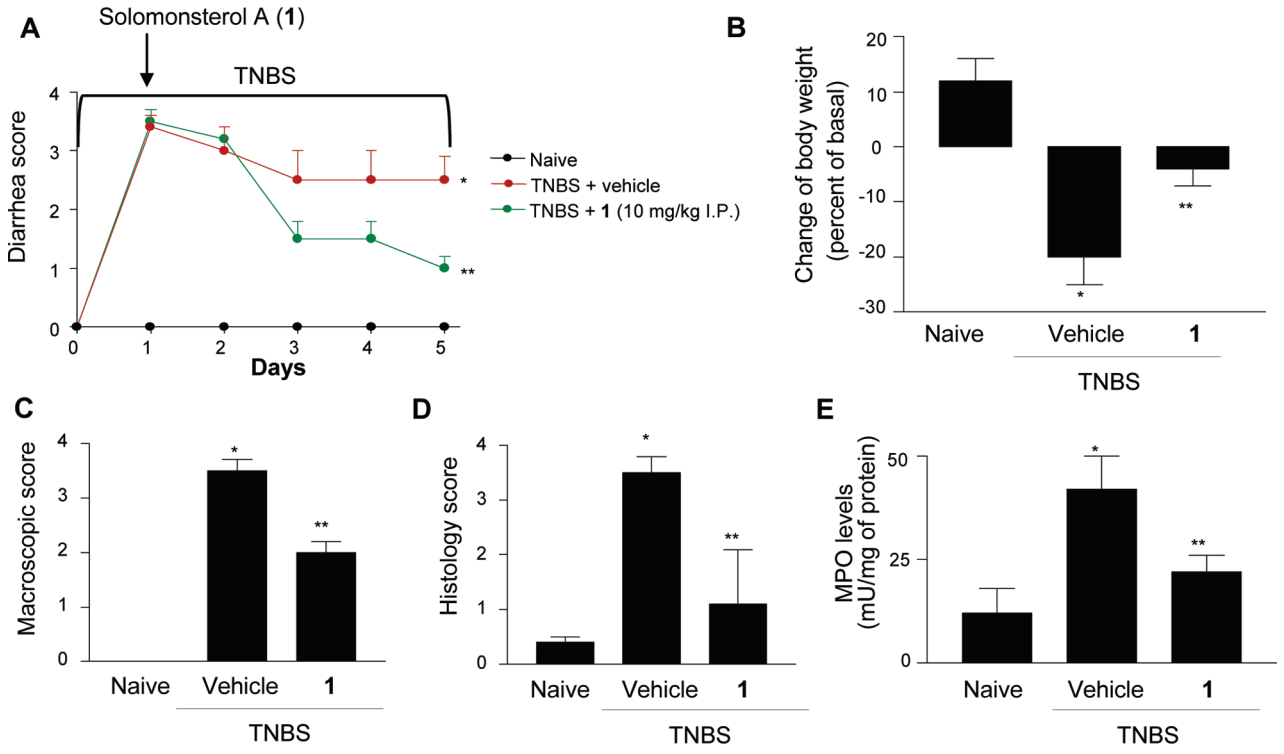

Figure 6. Colitis was induced by intrarectal administration of 0.5 mg of TNBS per mouse, and animals were sacrificed 5 days after TNBS administration. Solomonsterol A (**1**), 10 mg/kg, was administered once daily starting on day 1 after TNBS administration. The severity of TNBS-induced inflammation (A, diarrhea score; B, weight loss; C, macroscopic colon damage; D, microscopic score damage; and E, MPO activity) was reduced by solomonsterol A (**1**) administration. Body weight is expressed as delta percentage versus the weight of mice on the day before TNBS administration. Data represent the mean ± SE of 4−6 mice per group (*p < 0.05 vs naïve; **p < 0.05 vs TNBS).

beneficial effects on the development of colitis induced by TNBS in transgenic mice harboring human PXR. PXR is a master gene regulating the activity of a variety of genes involved in xeno- and endobiotic metabolism in the liver and gastrointestinal tract.[4−7] However, in recent years it has been demonstrated that PXR activation regulates important effector functions in the immune system, preventing the activation of NF-κB in epithelial and immune cells.[7,35]

TLR4 responds to LPS by regulating the expression/activity of NF-κB, a master regulator of inflammation, whose hyper-activation in the intestinal mucosa is mechanistically linked to the development of inflammation and dysregulated immunity in patients with Crohn's disease (CD) and ulcerative colitis (UC).[36] Because NF-κB is essential in regulating inflammatory signals in IBD, we investigated whether the beneficial effects exerted by solomonsterol A could be linked to the inhibition of this signaling mechanism. Indeed, supporting previous observations made with rifaximin, our results demonstrate that hPXR activation by solomonsterol A (**1**) prevents the binding of the p65 subunit of NF-κB to its responsive element in target genes. Reduction of p65 binding in the EMSA could be the result of several biochemical interactions of NF-κB with hPXR. One possible explanation is the formation of a protein−protein complex between p65 and PXR, thereby reducing the amount of p65 that translocates into the nucleus.[35]

In addition, we have demonstrated that treating TNBS mice with solomonsterol A (**1**) attenuated the release of TNFα in the inflamed tissue. TNFα is a signature cytokine in IBD and a validated therapeutic target in these disorders. Therefore, inhibition of TNFα might have mechanistic relevance in the beneficial effects of solomonsterol A (**1**) in TNBS-induced colitis. Another important observation we made is that both rifaximin and solomonsterol A induced the expression of IL-10 and TGFβ in the colon of colitic mice. Because IL-10 and TGFβ are key counter-regulatory cytokines instrumental in the generation of regulatory T cells, i.e., a subset of T cells that attenuates adaptive dysregulated immunity; these data, seem to support a novel concept, i.e., that PXR activation is endowed with the potential to induce regulatory T cells in inflammation.

In conclusion, we have demonstrated that solomonsterol A (**1**) is a potent agonist of hPXR. Using transgenic mice expressing the hPXR, we have provided evidence that the novel compound attenuates colitis development and modulates the expression of pro-inflammatory cytokines by a NF-κB dependent mechanism. In addition, we have provided the first evidence that solomonsterol A (**1**) might trigger the expression of counter-regulatory cytokines (IL-10, TGFβ).

EXPERIMENTAL SECTION

Chemistry. Specific rotations were measured on a Jasco P-2000 polarimeter. High-resolution ESI-MS spectra were performed with a Micromass Q-TOF mass spectrometer. NMR spectra were obtained on Varian Inova 400 and Varian Inova 500 NMR spectrometers (^{1}H at 400 and 500 MHz, ^{13}C at 100 and 125 MHz, respectively) equipped with a SUN microsystem ultra5 hardware and recorded in $CDCl_3$ (δ_H =7.26 and δ_C =77.0 ppm) and CD_3OD (δ_H = 3.30 and δ_C = 49.0 ppm). All of the detected signals were in accordance with the proposed structures. Coupling constants (J values) are given in Hertz (Hz), and chemical shifts (δ) are reported in ppm and referred to $CHCl_3$ and CHD_2OD as

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internal standards. Spin multiplicities are given as s (singlet), br s (broad singlet), d (doublet), or m (multiplet).

HPLC was performed with a Waters Model 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401. The purity of all of the intermediates, checked by ^{1}H NMR and HPLC, was greater than 95%. The purity of tested solomonsterol A was determined to be always greater than 95% by analytical HPLC analysis on a Macherey-Nagel Nucleodur 100-5 C18 (5 μm; 10 mm i.d. × 250 mm) column using 32% MeOH/H_2O (isocratic mode) at a flow rate of 1 mL/min.

Reaction progress was monitored via thin-layer chromatography (TLC) on Alugram silica gel G/UV254 plates. Silica gel MN Kieselgel 60 (70−230 mesh) from Macherey-Nagel Company was used for column chromatography. All chemicals were obtained from Sigma-Aldrich, Inc. Solvents and reagents were used as supplied from commercial sources with the following exceptions. Tetrahydrofuran, toluene, dichloromethane, ether, and triethylamine were distilled from calcium hydride immediately prior to use. All reactions were carried out under argon atmosphere using flame-dried glassware. Compounds **3**−**5** were prepared as described previously.[26]

*Methyl 3β-Hydroxy-5α-cholan-24-oate (**6**).* An oven-dried 250 mL flask was charged with 10% palladium on carbon (100 mg) and compound **5** (2.00 g, 5.15 mmol), and the flask was evacuated and flushed with argon. Absolute methanol (50 mL) and dry THF (50 mL) were added, and the flask was flushed with hydrogen. The reaction was stirred at room temperature under H_2 (1 atm) for 4 h. The mixture was filtered through Celite, and the recovered filtrate was concentrated to give 1.70 g of crude product. The residue was subjected to column chromatography on silica gel eluting with hexane−EtOAc (9:1, 0.5% TEA) to give 1.60 g of pure **6** (80%). $[\alpha]_{24}^{D}$ = +3.4 (*c* 0.54, $CHCl_3$); selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.64 (3H, s), 3.56 (1H, m), 2.33 (1H, m), 2.19 (1H, m), 0.89 (3H, d, *J* = 6.3 Hz), 0.78 (3H, s), 0.63 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.8, 71.3, 56.4, 55.8, 54.3, 51.5, 44.8, 42.6, 41.1, 38.0, 36.9, 35.4, 35.3 (2C), 32.0, 31.3, 31.0, 30.9, 28.6, 28.1, 24.1, 21.2, 18.2, 12.5, 12.3; HRMS-ESI *m*/*z* 391.3227 ($[M + H]^+$, $C_{25}H_{43}O_3$ requires 391.3212.

*Methyl 3β-Tosyloxy-5α-cholan-24-oate (**7**).* To a solution of **6** (1.50 g, 3.84 mmol) in dry pyridine (30 mL), *p*-toluenesulfonyl chloride (2.20 g, 11.5 mmol) was added. The solution was stirred at room temperature for 24 h and then poured into cold water (20 mL) and extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layer was washed with saturated $NaHCO_3$ solution (30 mL) and water (30 mL), and then dried over anhydrous $MgSO_4$ and evaporated in vacuo to give 2.09 g of **7**, which was subjected to the next step without any purification. Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.79 (2H, d, *J* = 8.2 Hz), 7.33 (2H, d, *J* = 8.2 Hz), 4.41 (1H, m), 4.23 (1H, m), 3.66 (3H, s), 2.45 (3H, s), 2.34 (1H, m), 2.22 (1H, m), 0.89 (3H, d, *J* = 5.9 Hz), 0.77 (3H, s), 0.63 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.8, 148.8, 129.8 (2C), 127.7 (2C), 124.3, 82.7, 56.6, 56.1, 54.2, 51.7, 45.0, 41.6, 40.1, 37.0, 35.5 (2C), 35.2, 35.0, 32.1, 31.3, 31.2, 29.9, 28.6, 28.3, 24.4, 21.9, 21.4, 18.5, 14.4, 12.3; HRMS-ESI *m*/*z* 545.3311 ($[M + H]^+$, $C_{32}H_{49}O_5S$ requires 545.3301.

*Methyl 5α-Chol-2-en-24-oate (**8**).* Lithium bromide (3.30 g, 38.4 mmol) and lithium carbonate (2.8 g, 38.4 mmol) were added to a solution of **7** (2.08 g, 3.84 mmol) in dry DMF (150 mL), and the mixture was refluxed for 1.5 h. After cooling to room temperature, the mixture was slowly poured into 10% HCl solution (150 mL) and extracted with CH_2Cl_2 (3 × 150 mL). The combined organic layer was washed successively with water, saturated $NaHCO_3$ solution, and water, and then dried over anhydrous $MgSO_4$ and evaporated to dryness to give a white solid residue that was purified on a silica gel column by eluting with hexane−EtOAc (9:1, 0.5% TEA) to obtain pure **8** (1.19 g, 83% over two steps). $[\alpha]_{24}^{D}$ = +18.6 (*c* 2.07, $CHCl_3$). Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.48 (2H, br s), 2.25 (1H, m), 2.11 (1H, m), 0.83 (3H, d, *J* = 6.2 Hz), 0.65 (3H, s), 0.56 (3H, s). ^{13}C NMR (100 MHz $CDCl_3$): δ 175.2, 126.2 (2C), 56.7, 56.0, 54.2, 51.7, 42.8, 41.6, 40.2, 39.9, 35.8, 35.5, 34.3, 32.0, 31.3, 31.1, 30.5, 28.9, 28.3, 24.4, 21.1, 18.5, 12.2 (2C); HRMS-ESI *m*/*z* 373.3117 ($[M + H]^+$, $C_{25}H_{41}O_2$ requires 373.3107.

*Methyl 2α,3α-Epoxy-5α-cholan-24-oate (**9**).* To a solution of **8** (1.09 g, 2.93 mmol) in CH_2Cl_2 (66 mL) were added water (46.2 mL) and Na_2CO_3 (1.15 g, 10.8 mmol). The reaction mixture was stirred vigorously, and *m*-chloroperbenzoic acid (708 mg, 4.10 mmol) was added slowly. The mixture was stirred for 4 h at room temperature, and then the aqueous layer was extracted with CH_2Cl_2 (3 × 35 mL). The combined CH_2Cl_2 extracts were washed successively with 5% Na_2SO_3 solution (100 mL), saturated $NaHCO_3$ solution (100 mL), and water (100 mL), dried over anhydrous $MgSO_4$, and evaporated to dryness to give 1.13 g of crude epoxide **9**, which was subjected to the next step without any purification. Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.65 (3H, s), 3.14 (1H, br s), 3.09 (1H, br s), 2.34 (1H, m), 2.20 (1H, m), 0.90 (3H, d, *J* = 6.7 Hz), 0.74 (3H, s), 0.63 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.2, 56.5, 56.0, 53.8, 52.6, 51.7, 51.3, 42.6, 40.2, 40.7, 38.5, 36.4, 35.8, 35.6, 31.9, 31.3, 31.2, 30.9, 28.6, 28.3, 24.4, 21.1, 18.5, 13.2, 12.2; HRMS-ESI *m*/*z* 389.3036 ($[M + H]^+$, $C_{25}H_{41}O_3$ requires 389.3056.

*Methyl 2β,3α-Dihydroxy-5α-cholan-24-oate (**10**).* A solution of epoxide **9** (1.13 g, 2.93 mmol) in THF (70 mL) was treated with 1 N H_2SO_4 (7.32 mL, 7.32 mmol) solution and stirred for 24 h at room temperature. After neutralization with saturated $NaHCO_3$ solution, the mixture was evaporated to a fifth of the initial volume, diluted with water (50 mL), and extracted with ethyl acetate (3 × 50 mL). The combined organic extracts were washed with water, dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness. Purification on a silica gel column by eluting with CH_2Cl_2−MeOH (9:1) afforded pure **10** (870 mg, 73% over two steps). $[\alpha]_{24}^{D}$ = +0.67 (*c* 0.46, $CHCl_3$). Selected ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.89 (1H, br s), 3.85 (1H, br s), 3.64 (3H, s), 2.34 (1H, m), 2.20 (1H, m), 0.97 (3H, s), 0.89 (3H, d, *J* = 6.7 Hz), 0.63 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.4, 72.1, 70.9, 56.6, 56.0, 53.7, 51.7, 42.6, 40.7, 40.2, 39.2, 36.3, 35.6, 35.1, 32.1, 31.9, 31.3, 31.2, 28.4, 28.3, 24.4, 21.1, 18.5, 14.9, 12.4; HRMS-ESI *m*/*z* 407.3181 ($[M + H]^+$, $C_{25}H_{43}O_4$ requires 407.3161.

*5α-Cholan-2β,3α,24-triol (**11**).* Dry methanol (320 μL, 7.88 mmol) and $LiBH_4$ (3.94 mL, 2 M in THF, 7.88 mmol) were added to a solution of the methyl ester **10** (800 mg, 1.97 mmol) in dry THF (10 mL) at 0 °C under argon, and the resulting mixture was stirred for 4 h at 0 °C. The mixture was quenched by the addition of NaOH (1 M, 4 mL) and then allowed to warm to room temperature. Ethyl acetate was added, and the separated aqueous phase was extracted with ethyl acetate (3 × 1 5 mL). The combined organic phases were washed with water, dried (Na_2SO_4), and concentrated. Purification by silica gel eluting with CH_2Cl_2−MeOH (9:1) gave alcohol **11** as a white solid (685 mg, 92%). $[\alpha]_{24}^{D}$ = −1.2 (*c* 0.12, $CHCl_3$). Selected ^{1}H NMR (400 MHz, CD_3OD): δ 3.81 (1H, br s), 3.77 (1H, m), 3.67 (1H, br s), 3.52 (1H, m), 1.01 (3H, s), 0.96 (3H, d, *J* = 6.0 Hz), 0.70 (3H, s). ^{13}C NMR (100, CD_3OD): δ 73.3, 70.5, 63.1, 57.0, 56.6, 55.6, 43.0, 41.6, 40.5, 40.2, 36.0, 35.3, 35.2, 32.3, 31.7, 29.6, 29.5, 28.7, 28.6, 24.6, 21.1, 18.9, 14.2, 12.3; HRMS-ESI *m*/*z* 379.3154 ($[M + H]^+$, $C_{24}H_{43}O_3$ requires 378.3134.

*5α-Cholan-2β,3α,24-tryl-2,3,24-sodium trisulfate (**1**).* The triethylamine−sulfur trioxide complex (2.80 g, 15.8 mmol) was added to a solution of triol **11** (600 mg, 1.58 mmol) in dry DMF (2 mL) under an argon atmosphere, and the mixture was stirred at 95 °C over the weekend. Then, the reaction mixture was quenched with water (1.6 mL). The solution was poured over a silica gel column to remove excess $SO_3 \cdot NEt_3$. The product was eluted by MeOH and followed by evaporation of the solvent to yield a yellow solid [tris-(triethylammonium sulfate) salt]. To the solution of the solid in methanol (15 mL) was added Amberlite CG 120 sodium form (20 g). The mixture was stirred for 5 h at room temperature. The resin was removed by filtration, and the filtrate was concentrated to obtain compound **1** as a white solid (972 mg, 90%). $[\alpha]_{24}^{D}$ = +4.6 (*c* 0.8, CH_3OH). Selected

^{1}H NMR (400 MHz, CD_3OD): δ 4.73 (1H, br s), 4.70 (1H, br s), 3.69 (2H, m), 1.00 (3H, s), 0.95 (3H, d, J = 6.3 Hz), 0.69 (3H, s). ^{13}C NMR (400 MHz, CD_3OD): δ 76.2, 75.6, 69.3, 57.5, 57.3, 56.4, 43.2, 41.3, 39.7, 38.5, 36.4, 36.2 (2C), 32.8, 32.6, 30.3, 28.8, 28.7, 26.9, 24.7, 21.5, 18.6, 14.4, 12.3. HRMS-ESI m/z 661.1415 $[M - Na]^-$, $C_{24}H_{39}Na_2O_{12}S_3$ requires 661.1399.

Biological Assays. *Plasmids, Cell Culture, Transfection, and Luciferase Assays.* All transfections were made using Fugene HD transfection reagent (Roche). For PXR mediated transactivation, HepG2 cells, plated in a 6-well plate at 5×10^5 cells/well, were transfected with 100 ng of pSG5-PXR, 100 ng of pSG5-RXR, 200 ng of pCMV-β-galactosidase, and with 500 ng of the reporter vector containing the PXR target gene promoter (CYP3A4 gene promoter) cloned upstream of the luciferase gene (pCYP3A4promoter-TKLuc). At 48 h post-transfection, cells were stimulated for 18 h with rifaximin or solomonsterol A (0.1, 1, and 10 μM). Cells were lysed in 100 μL of diluted reporter lysis buffer (Promega), and 0.2 μL of cellular lysate was assayed for luciferase activity using the Luciferase Assay System (Promega). Luminescence was measured using an automated luminometer. Luciferase activities were normalized for transfection efficiencies by dividing the relative light units by β-galactosidase activity expressed from cells cotransfected with pCMV-βgal.

Quantitative Real-Time PCR. A 50 ng template was added to the PCR mixture (final volume 25 μL) containing the following reagents: 0.2 μM of each primer and 12.5 μL of 2X SYBR Green qPCR master mix (Invitrogen, Milan, Italy). All reactions were performed in triplicate, and the thermal cycling conditions were 2 min at 95 °C, followed by 40 cycles at 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s in a iCycler iQ instrument (Biorad, Hercules, CA). The mean value of the replicates for each sample was calculated and expressed as cycle threshold (CT: cycle number at which each PCR reaction reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔCT) between the CT value of the sample for the target gene and the mean CT value of that sample for the endogenous control (GAPDH). Relative expression was calculated as the difference ($\Delta\Delta CT$) between the ΔCT values of the test sample and of the control sample (not treated) for each target gene. The relative quantitation value was expressed and shown as $2^{-\Delta\Delta CT}$. All PCR primers were designed with PRIMER3-OUTPUT software using published sequence data from the NCBI database. The primers' sequences were as follows: mGAPDH, CTGAGTATGTCGTGGAGTCTAC and GTTGGTGGTGCAGGATGCATTG; mCyp3A11, TGAAACCACC-AGTAGCACAC and CCATATCCAGGTATTCCATCTCC.

Electrophoretic Mobility Shift Assay (EMSA). The NF-κB DNA-binding activity was determined by EMSA. After treatment, nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Inc.). EMSA probes were created by biotinylating the 3′ end of the single stranded oligonucleotides using a biotin 3′ end DNA labeling kit (Pierce Biotechnology, Inc.) according to the manufacturer's protocol. The biotinylated oligonucleotides were annealed by boiling for 1 min and then allowing them to slowly cool to room temperature. The consensus nucleotide sequence for NF-κB was 5′-AGA GAT TGC CTG ACG TCA GAC AGC TAG-3′. The EMSA binding reaction was performed by utilizing a LightShift chemiluminescent EMSA kit (Pierce Biotechnology, Inc.). A nuclear extract was incubated in 1× binding reaction buffer containing 50 mM KCl, 10 mM EDTA, 25 ng/mL poly dI-dC, 5 mM $MgCl_2$, and the biotinylated probe. After a 20 min incubation at room temperature, the reaction mixture was electrophoresed on a nondenaturing 6% polyacrylamide gel and then transferred to a nylon membrane. The transferred mixture was UV-cross-linked to the membrane and detected by chemioluminescent reagents (Pierce Biotechnology, Inc.). For the competition assay, a 200-fold excess of unlabeled probe was added together with the labeled probe. For the supershift assay, 1 μg of antibody against NF-κB p65 was added together with the nuclear extract.

Animals. For the TNBS studies, humanized (h)PXR mice, 8−10 weeks of age, were provided by Frank J. Gonzalez (Laboratory of Metabolism, Centre for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland). hPXR male mice were housed in temperature- and light-controlled rooms and were given tap water and pelleted standars mouse chow ad libitum. Mice were housed under controlled temperatures (22 °C) and photoperiods (12:12-h light/dark cycle), and allowed unrestricted access to standard mouse chow and tap water. The present protocol was approved by the Italian Minister of Health and conforms to national guidelines. The ID for this project is #11/2010-B and authorization was released to Professor Stefano Fiorucci, as principal investigator, last on January 25, 2010.

Induction of Colitis. Mice were fasted for 16 h and lightly anesthetized by intraperitoneal injection of 100 μL of ketamine/xylazine solution[32] for 10 g body weight and then administered intrarectally (i.r.) with the haptenating agent TNBS (0.5 mg/mouse) dissolved in 50% ethanol, via a 3.5 French (F) catheter equipped with a 1 mL syringe. The catheter was advanced into the rectum for 4 cm, and then the haptenating agent was administered in a total volume of 150 μL. To ensure the distribution of the agent within the entire colon and cecum, mice were held in a vertical position for 30 s.

Solomonsterol A (10 mg/kg) and rifaximin (10 mg/kg) were dissolved in DMSO (10 mg/100 μL), diluted in methylcellulose 1%, and administered intraperitoneally (i.p.) or orally, once daily, respectively, at the final volume of 100 μL/mouse, 3 days before the induction of colitis. At this time, the TNBS-alone group received the vehicle alone (methylcellulose 1% in a final volume of 100 μL/mouse) every day.

In another experiment, a therapeutic model, solomonsterol A (10 mg/kg) was administrated once daily starting on day 1 after colitis induction. Mice were analyzed for the presence of diarrhea, body weight, and survival. The body weight was expressed as delta percentage versus the weight of mice on the day before TNBS administration.

Four days after TNBS administration, surviving mice were sacrificed, colons were removed and either immediately snap-frozen in liquid nitrogen and stored at −80 °C until use or formalin fixed. The macroscopic appearance was analyzed considering the presence of indurations, edema, thickness, and evidence of mucosal hemorrhage. Grading was performed in a blind fashion. For histological examination, tissues were fixed in 10% buffered formalin phosphate, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Histology images were captured by a digital camera (SPOT-2; Diagnostic Instruments Inc., Burroughs, MI) and analyzed by specific software (Delta Sistemi, Rome, Italy). The degree of colon inflammation was examined microscopically in transversal sections[33] and graded semiquantitatively from 0 to 4 (0, no signs of inflammation; 1, very low level; 2, low level of leukocyte infiltration; 3, high level of leukocyte infiltration, high vascular density, and thickening of the colon wall; 4, transmural infiltration, loss of goblet cells, high vascular density, and thickening of the colon wall). Grading was performed blind by observers.

Colon Myeloperoxidase (MPO) Activity and Cytokine Levels. Colon samples were lysed in 1 mL of lysis buffer T-PER (Pierce, Rockford, USA) and finely minced. Afterward, tissues were centrifuged at 10,000g for 15 min at 4 °C. Colon homogenates were used to determine MPO activity, after two freeze/thaw cycles, using a spectrophotometric assay with trimethylbenzidine (TMB) as a substrate and normalized for the protein levels. Colon TNFα and IL-10 levels in tissue homogenates were quantified by ELISA (SABioscences) according to the manufacturer's instructions and normalized for the protein levels.

Statistical Analysis. All values are expressed as the mean ± SE. The number (n) of experiments or mice used in the experiments is shown.

Comparisons of more than 2 groups were made with a one-way analysis of variance with post-hoc Tukey tests. Differences were considered statistically significant if p was <0.05.

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ABBREVIATIONS USED

APCs, antigen presenting cells; CD, Crohn's disease; CYP3A4, cytochrome P450 3A4; HepG2, human hepatocyte; IBD, inflammatory bowel diseases; IFNγ, interferon gamma; IL-10, interleukin-10; IL-12p70, (interleukin-12 p70 subunit); LPS, lipopolysaccharide; MDR1, multidrug resistance 1; MIP-1α, macrophage inflammatory protein-1α; MPO, myeloperoxidase; NF-κB, nuclear factor-kappaB; NRs, nuclear receptors; PXR, pregnane-X-receptor; PXREs, PXR response elements; RXR, retinoid-X-receptor; TGFβ, transforming growth factor beta; ;TNBS, trinitrobenzenesulfonic acid; THP-1, human acute monocytic leukemia; TNFα, tumor necrosis factor alpha; UC, ulcerative colitis

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