1-Peptidyl-2-arachidonoyl-3-stearoyl-sn-glyceride: An Immunologically Active Lipopeptide from Goat Serum (Capra hircus) Is an Endogenous Damage-Associated Molecular Pattern

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Experiments were undertaken to isolate a component of the serum of goat (Capra hircus) that is effective at mediating an innate immune response. This report describes the isolation and structure elucidation of 1-(N-acetyl-ALYD-KGYTSKEQKDVG)-2-arachidonoyl-3-stearoyl glyceride (I) and its immunomodulatory activity. A dose–response relationship for inflammatory cytokine and chemokine production and release from human fibroblasts incubated with nanomolar concentrations of I was shown. Moreover, the membrane transport role of the diacylglycerol moiety in I is demonstrated with nanomolar quantities of the transfected N-acetyl peptide moiety of I also inducing inflammatory cytokine and chemokine production and release. The apparent EC₅₀ for I was 3 ng/mL (1 nM). The likely biological role for naturally occurring I as a damage-associated molecular pattern is postulated.

The search for new medically useful natural products is a well-accepted process that frequently results in lead molecules that are further developed as pharmaceutical agents. Despite the fact that these new agents rarely become marketable drug products, a large number of medicinal agents are now derived from natural sources. In addition, many traditional places to look for these unique natural products. Rarely, however, has mammalian serum been used as a biological source. hamm and associates reported a serum fraction derived from the goat (Capra hircus) that was effective as an adjunctive therapy with standard antibiotics for treatment of suppurative lower respiratory disease in horses. They reported that 86% of horses treated with the serum fraction, in addition to standard antibiotics, recovered within three weeks, whereas only 10% of horses treated with antibiotic alone recovered. To the best of our knowledge, there have been no further reports in the literature that describe the active principle(s) from goat serum responsible for this effect. Therefore, a series of experiments were carried out to isolate the agent or agents responsible for the effect described by Hamm and associates. This report describes the isolation and structure elucidation of 1-peptidyl-2-arachidonoyl-3-stearoyl glyceride (I) as an active chemical entity in the caprine serum fraction and the innate immunomodulating activity of I as an endogenous damage-associated molecular pattern.

Results and Discussion

Through a series of dialysis and chromatographic separations, native compound I was isolated from normal goat serum as a tryptophan complex, and the initial concentration of I in the serum was estimated to be 50 ng/mL. No compound I was detectable in normal goat, macaque, or human plasma. Fractions generated during the purification procedures were chromatographically analyzed and assayed for their ability to induce IL-8 mRNA expression and IL-8 secretion by human fibroblasts. A preparative C₄ reversed-phase (RP) HPLC fraction containing the biologically active compound I (tryptophan complex (see Figure S1, Supporting Information) was used for mass spectrometric analysis.

Functional group characterization of I using two-dimensional NMR spectroscopy (500 MHz) showed multiple amide ¹H NMR signals (4–5 ppm) indicating a peptide and an aliphatic lipid prosthetic group (0.8–1.2 ppm), and signals between 8 and 9 ppm indicated aromaticity (data not shown). There was no signal in the ³¹P NMR spectrum indicating the absence of a phosphorus atom in the natural product (data not shown). Therefore we subjected a sample of the RP-HPLC-purified natural compound I (tryptophan complex to Edman degradation. The amino acid sequence identified was XLYDKGYTSKEQKD(CVGI...), where the N-terminal amino acid indicated by X either was a nontraditional amino acid or was masked by a prosthetic group. The sequence of the C-terminal amino acids that were identified had a degree of uncertainty due to the low molar abundance of peptide remaining after the previous 14 analysis cycles.

Compound I (as a tryptophan complex) was collected by preparative-scale RP-HPLC and analyzed by liquid chromatography–mass spectrometry (LC/ms) and LC/tandem mass spectrometry. The major component in the LC/ms analysis eluted at 1.4 min, exhibited strong absorbance at 218 and 280 nm, and had a MW of 204 (Supporting Information, Figure S2, including the positive-ion electrospray ESIMS of the major component). LC-ms/ms analysis of the m/z 205 ion ([M + H]⁺) was consistent with the structure for tryptophan. Further evidence was obtained by comparing the LC-MS/MS spectrum of the unknown MW 204 component with an authentic standard of tryptophan and confirmed the presence of tryptophan at an estimated molar ratio of 200:1 relative to I.

Additional automated LC-MS/MS experiments were performed to investigate the presence of low-level peptides. The ion trap mass spectrometer was configured to acquire MS/MS spectra of all components above a relatively low signal threshold. One of the peptides was found to have a MW of 1689 and was comprised of a series of fragment ions (m/z: 261.09, 340.27, 564.00, and 845.45) that were consistent with the partial sequence identified by Edman degradation. The ion at m/z 845.45 was determined to be a M⁺⁺ ion after deconvolution and observing a 0.5 amu separation of the...
neutral NH$_3$ from the fragment (detected in the positive-ion spectrum without added matrix (tryp- 

cation, Figure S4). No molecular ion corresponding to the intact parent molecule was not observed. The 

yl isopentanolamine (X1 in the Edman degradation sequence analysis) esterified to the C-terminal isoleucine, and N-acetyllalanine is the derivatized amino acid (X$_1$) that could not be identified by Edman degradation sequence analysis.

Synthesis of I and the peptide moiety were undertaken to establish the chemical structure of I and to begin to elucidate its biological function. The chemical equivalent of synthetic compound I and natural compound I was established by ESIMS analysis. The ESIMS data are in the Supporting Information (Figures S7 and S8), as is a representative HPLC chromatogram of purified synthetic I (Figure S9).

To determine the bioequivalence between the natural product and synthetic I, normal human fibroblasts were exposed to I, and IL-6, IL-8, MCP-1, and MIP-1α expression was measured. IL-6 has both pro-inflammatory and anti-inflammatory actions and is secreted by a wide variety of cell types in response to pathogen-associated molecular patterns (PAMPs).3,4 and IL-6 is one of the most important mediators of the acute phase response.5,6 IL-8 (CXCL8) is a chemotactin and activator of the CXC chemokine family and is also an important mediator of the inflammatory response.7 Secreted by a wide variety of cell types, IL-8 functions to recruit neutrophils to phagocytose pathogens and other foreign antigens. MCP-1 (CCL2) is a member of the CC chemokine family that is secreted in response to cell injury or pathogen infection, thus recruiting monocytes, memory T cells, and dendritic cells to the site of injury or infection.8,9 MIP-1α (CCL3), or macrophage inflammatory protein, activates neutrophils and stimulates the production and release of other pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α.9

Fibroblasts were treated with various concentrations of synthetic I for 24 h: the media were reserved for ELISA assays of IL-6 and IL-8, and mRNA was extracted from the cells to measure all four cytokines. IL-6 mRNA was elevated 20% in response to synthetic I and 40% with natural I relative to a β-actin control (p < 0.05). It was found that 5 ng/mL of synthetic I was approximately equivalent to the natural product diluted 1:100 (Figure 1). Compound I induced IL-8 expression by about 5-fold compared to untreated fibroblasts, in dose–response studies (Figure 2). The negative dose–response relationship with respect to mRNA expression suggests possible cell toxicity, substrate inhibition, or a negative feedback mechanism at the higher concentrations of I. Secreted IL-8 measured by ELISA showed a positive linear dose–response with increasing concentrations of synthetic I from 0.3 to 3 ng/mL, where the latter concentration appears to give a maximal effect (EC$_{50}$). Both synthetic I (3 ng/mL) and the natural product (1:100 dilution) induced a 3-fold increase in MCP-1 mRNA relative to the β-actin control and increased MIP-1α mRNA expression relative to β-actin by 15% (Figure 3).

The foregoing experimental results were used to establish the bioequivalence of the natural and the synthetic products. Further, a possible maximum effective concentration (EC$_{99}$) for I at 3 ng/
mL (nominally 1 nM) was established. These results indicate that compound 1 is effective at a “cytokine-like” concentration.

Given the amino acid sequence for the peptide in 1, the nonredundant sequence database for homologous sequences was searched using the NCBI BLAST search tool BLASTP at http://blast.ncbi.nlm.nih.gov/Blast.cgi on January 19, 2009.10 The peptide showed identical sequence homology to amino acids 558–574 in the transient receptor potential channel-related protein 1 (TRPC-1). No significant homology with other known proteins or peptides was observed. A comparison of the amino acid sequence at positions 557–574 from bovine, mouse, and human TRPC-1 with the peptide sequence in 1 is presented in Figure 2, where X represents the N- and C-terminal prosthetic groups of 1.

The TRPC family of proteins belongs to the TRP superfamily of nonvoltage-gated cation channel proteins, and seven TRPC family members have been described in mammals. TRPC-1 is the human homologue to Drosophila TRP first discovered by Wes and associates.11 Members of the TRPC family, including TRPC-1, appear to be conserved within the animal kingdom, and the trp gene family is expressed in a wide variety of tissues and cell types including immune cells.12 The membrane topology of TRPC-1 suggests that the amino acids comprising the peptide are extracellular, in the region between the sixth membrane spanning unit and the pore-forming seventh membrane spanning unit.13 Sequence homology of this region of TRPC-1 with the other members of the TRPC family is relatively low (~30%) as compared to ~80% for the N-terminal sequence.11

The trpc1 gene is located on human chromosome 3 at position q22–q24. Interestingly, trpc1 is expressed as multiple unique transcripts and splice variants14 and has multiple loci for NFκB binding.15 This raises the possibility that the region coding for the peptide is a separate reading frame for transcription. Examination of the trpc1 gene and its various splice variants revealed that no stop codon corresponding to the C-terminal isoleucine was present. Therefore, it is unlikely that the peptide in 1 is transcribed from trpc1 as a separate reading frame. TRPC-1 is assembled in the endoplasmic reticulum (ER), and we hypothesize that ER-embedded TRPC-1 is the source of this peptide.

The peptide moiety in compound 1 did not elicit comparable responses in fibroblast stimulation until it was 1000-fold more concentrated (3 μM). Therefore, we speculated that the diacylglycerol moiety enabled the peptide to cross the cell membrane to reach an intracellular site of action. To test this hypothesis and to ascertain the biological role of the diacylglycerol moiety in compound 1, if any, the peptide moiety transfected into human fibroblasts or diacylglycerol alone were evaluated by measuring the expression of mRNA (IL-1β, IL-6, IL-8, IL-18, and IL-33). The peptide/lipofectamine complex enabled the peptide (~5 nM) to cross the cell membrane and substantially increase the mRNA expression for the inflammasome-mediated proteins IL-1β, IL-18, and IL-33, as compared to the untreated fibroblasts or fibroblasts treated with lipofectamine alone (Supporting Information, Figure S10). A 6-fold higher concentration (~30 nM) of diacylglycerol was required to stimulate mRNA expression of IL-33 and IL-18, but this could not stimulate IL-1β mRNA in fibroblasts relative to lipofectamine alone (Supporting Information, Figure S10, lower right panel). Whereas the 6-fold higher concentration of diacylglycerol induced an increase in IL-33 mRNA expression that is comparable to the transfected peptide, the increase in IL-18 mRNA expression was significantly less in the diacylglycerol-treated fibroblasts (~20% versus ~350% for the transfected peptide).

The transfected peptide induced a significant increase in IL-6 and IL-8 mRNA expression relative to the untreated fibroblasts (p < 0.05) but not when compared to the cells treated with lipofectamine alone (p > 0.05). On the other hand, diacylglycerol-treated cells had no effect on IL-6 and IL-8 mRNA expression relative to untreated cells (data not shown). These data suggest that the role of the diacylglycerol is to facilitate transportation of the peptide.
across the cell membrane, and the cytokine/chemokine expression that is mediated by 1 arises from the peptide alone. Further, these data suggest that the intracellular peptide signaling from 1 is mediated through the inflammasome and that IL-6 and IL-8 production may arise as a downstream event from IL-1β secretion and feedback signaling through the IL-1β receptor. This hypothesis for signaling is currently being investigated in our laboratories.

Accordingly, a new immunomodulatory compound originally isolated from caprine serum has been described as 1-peptidyl-2-arachidonoyl-3-stearoylglyceride 1, the peptide portion from which was found to be 100% homologous to a unique region of TRPC-1. After synthesis of 1, we demonstrated that both 1 and the transfected peptide moiety of 1 can modulate the signaling in human fibroblasts with an EC₉₀ in the range 1–10 nM.

IL-6 and IL-8 are important innate immune cytokines that are not only expressed by fibroblasts and other nonimmune cells but are also expressed by activated immune cells. IL-6 and TGFβ are required for the differentiation of naïve mouse CD4+ T-lymphocytes into Th17 cells. Recently it has been shown that human Th17 cell differentiation is mediated by IL-1β, IL-6, and IL-23. The in vitro data suggest that the peptide in 1 may activate the host innate immune response against pathogen infection or other cellular injury and may induce Th17 cell differentiation in vivo. Furthermore, 1 and the transfected peptide moiety of 1 stimulated the increased expression of the chemokines MCP-1 and MIP-1α in fibroblasts, and the function of these proteins are to recruit leukocytes and macrophages. These data suggest a role for 1 and the peptide moiety of 1 as damage-associated molecular patterns in the host response to pathogen infection or other cell injury. This hypothesis is currently under investigation using fibroblasts derived from specific knockout mice and in vivo models of Gram-positive bacteria, Gram-negative bacteria, and viral infections that are relevant to serious human diseases.

Finally, it is noteworthy that, whereas 1 was isolated from normal caprine serum, we have been unable to detect 1 in normal caprine, equine, nonhuman primate (cynomolgous monkey), or human plasma, but have been able to detect 1 in the serum of each at the estimated concentration of 50 ng/mL. We postulate that 1 is derived from platelets in the clotting process and is not present in normal plasma. However, 1 may be present in the plasma of sepsis patients or patients with certain chronic idiopathic diseases. We are currently evaluating this hypothesis as well.

Figure 2. IL-8 dose–response to synthetic 1. IL-8 mRNA expression (top) and secreted IL-8 protein (bottom) from human fibroblasts after treatment with increasing concentrations of synthetic 1 show a negative dose–response to mRNA expression and a positive dose–response to secreted protein. Statistical significance relative to the control is indicated as * (p < 0.05) or ** (p < 0.001).
Stearoyl-2-arachidonoyl-

The internal serine is substituted with proline in bovine TRPC-1. An amino acid sequence from bovine, human, and murine TRPC-1.

Clara, CA).

analysis of the synthetic product (Sigma peptide synthesizer (Advanced Automated Peptide Protein Technologies, Inc., Louisville, KY). ESIMS/MS spectra of the purified natural product were acquired in the positive-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Varian Associates, Inc., Palo Alto, CA). MS/MS analysis was performed using a Procise Protein Sequencer (Manchester, UK). The instrument was equipped with a 337 nm nitrogen laser. Automated Edman degradation at 280 nm (ABI 759A with a 2.4 µmol microbore flow cell). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra of the purified natural product were acquired in the positive-reflection mode using a Kratos AXIMA-CFR mass spectrometer (Manchester, UK). The instrument was equipped with a 337 nm nitrogen laser, a 20 kV extraction voltage, and time-delayed extraction. Saturated dihydroxybenzoic acid in 50% acetonitrile and 10% tribasic ammonium citrate (9:1) served as the matrix. Automated Edman degradation sequence analysis was performed using a Procise Protein Sequencer (Applied Biosystems, Inc.). Peptide synthesis was performed on an AAPPTEC 348 Sigma peptide synthesizer (Advanced Automated Peptide Protein Technologies, Inc., Louisville, KY). ESIMS/MS analysis of the synthetic product (1) was performed using a Thermo-Finnigan Surveyor HPLC-LQ DECA ion trap LC-MS/MS in the +ve mode (ThermoFisher Scientific, Pittsburgh, PA). Chromatography of the serum fractions was performed on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA), with a Zorbax C8 1 × 150 mm column (Agilent, Santa Clara, CA).

**Figure 3.** Comparison of natural and synthetic 1-induced MCP-1 and MIP-1α mRNA expression by human fibroblasts. (Top) MCP-1 mRNA expression in human fibroblasts is approximately equivalent (3-fold increase) for natural 1 (1:100 dilution) and synthetic 1 (3 ng/mL). Data are normalized to β-actin expression. (Bottom) MIP-1α mRNA expression by natural 1 (1:100 dilution) and synthetic 1 (3 ng/mL) are equivalently increased (~60%) in human fibroblasts relative to the β-actin control. Statistical significance relative to the control is indicated as * (p = 0.01), ** (p = 0.001), and *** (p = 0.003).

<table>
<thead>
<tr>
<th>Bovine</th>
<th>Human</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>QLYDKGYPKEKDCVGI</td>
<td>-S-</td>
<td>-S-</td>
</tr>
<tr>
<td>1</td>
<td>XLYDKGYSKEKDCVGI</td>
<td>X</td>
</tr>
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</table>

**Figure 4.** Sequence homology of the peptide in 1 with the internal amino acid sequence from bovine, human, and murine TRPC-1. The internal serine is substituted with proline in bovine TRPC-1. The N-terminal X in 1 is N-acetyllalanine and the C-terminal X is 1-earoaryl-2-arachidonoyl-sn-glycerol.

**Experimental Section**

**General Experimental Procedures.** 1H NMR and 31P NMR of the natural product 1 were performed on an Inova (500 MHz) spectrometer (Varian Associates, Inc., Palo Alto, CA). Mass spectrometry of the natural product 1 was performed on a LTQ linear ion trap mass spectrometer (ThermoFisher Scientific, Pittsburgh, PA). Separation was achieved using a Paradigm MS4 HPLC (Michrom Bioresearches) and HTS-PAL autosampler (LEAP Technologies) with a 20 µL sample loop and a 1 × 50 mm MAGIC C18 reversed-phase column (Michrom Bioresearches) and an in-line UV detector monitoring the column effluent at 280 nm (ABI 759A with a 2.4 µL microbore flow cell). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra of the purified natural product were acquired in the positive-reflection mode using a Kratos AXIMA-CFR mass spectrometer (Manchester, UK). The instrument was equipped with a 337 nm nitrogen laser, a 20 kV extraction voltage, and time-delayed extraction. Saturated dihydroxybenzoic acid in 50% acetonitrile and 10% tribasic ammonium citrate (9:1) served as the matrix. Automated Edman degradation sequence analysis was performed using a Procise Protein Sequencer (Applied Biosystems, Inc.). Peptide synthesis was performed on an AAPPTEC 348 Sigma peptide synthesizer (Advanced Automated Peptide Protein Technologies, Inc., Louisville, KY). ESIMS/MS analysis of the synthetic product (1) was performed using a Thermo-Finnigan Surveyor HPLC-LQ DECA ion trap LC-MS/MS in the +ve mode (ThermoFisher Scientific, Pittsburgh, PA). Chromatography of the serum fractions was performed on an Ultimate 3000 HPLC (Dionex, Sunnyvale, CA), with a Zorbax C8 1 × 150 mm column (Agilent, Santa Clara, CA).

**Table 4.** IL-8 ELISA Bioassay Results of Serum Fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>untreated control</th>
<th>1 mg/mL</th>
<th>100 µg/mL</th>
<th>10 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>dialysate</td>
<td>0.999</td>
<td>3.50</td>
<td>3.49</td>
<td>0.469</td>
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<tr>
<td>MeOH/CHCl3 soluble</td>
<td>0.084</td>
<td>0.111</td>
<td>0.147</td>
<td>0.126</td>
</tr>
<tr>
<td>MeOH/CHCl3 insoluble</td>
<td>0.114</td>
<td>3.80</td>
<td>3.75</td>
<td>2.14</td>
</tr>
</tbody>
</table>

Data are presented as the means of duplicate absorbance (450 nm) measurements.

**Animal Material.** Normal, sterile, endotoxin-free, goat serum (lot #SG30-2045) was collected from a closed, disease-free herd (Equitech-Bio, Inc., Kerrville, TX) and shipped frozen in 500 mL bottles for extraction.

**Extraction and Chromatographic Isolation.** Serum (500 mL) was thawed at room temperature and dialyzed in 10 kDa molecular weight cutoff tubing (SnakeSkin, ThermoFisher Scientific) against deionized water for 24 h with stirring in a cold room. The dialysate was frozen and lyophilized, and the solid residue extracted with 0.1 mL of methanol/chloroform (2:1, v/v) for each 1 mg of solid residue. The undissolved residue was collected by centrifugation, and the methanol/chloroform-soluble material was recovered after evaporation in vacuo. Bioassays indicated that the methanol/chloroform-insoluble residue contained 1 (Table 4). This fraction was dissolved in 10 mL of deionized water and dialyzed in a 7 KDa molecular weight cutoff dialysis cassette (Slide-a-Lyzer, Thermo Scientific) against deionized water at 4 °C with stirring (200 rpm) for 24 h. This second dialysate was frozen and lyophilized. The solid residue was extracted with 0.1 mL of methanol/chloroform (2:1) for each 1.0 mg of solid. The methanol/chloroform-soluble fraction was separated from the undissolved solids by centrifugation and evaporated in vacuo. Biological assay showed that compound 1 was again in the methanol/chloroform-insoluble fraction (Table 5).

A sample (200 µL) in water was chromatographically analyzed. The solvent gradient was 5–65% solvent A (5% acetonitrile + 0.1% trifluoroacetic acid) to solvent B (90% acetonitrile + 0.1% trifluoroacetic acid) over 60 min, followed by a 5 min wash with 90% solvent B, at a flow rate of 50 µL/min. Detection was at 214 and 280 nm. Fractions were collected manually every minute for bioassay.

**N-Terminal Sequence Analysis of the Natural Product 1.** Briefly, phenylisothiocyanate was reacted with the terminal amino group of the peptide to form a phenylthiocarbamoyl derivative. Then, under
Table 5. Serum Fractions-Induced mRNA Expression in Human Fibroblasts

<table>
<thead>
<tr>
<th>mRNA</th>
<th>untreated control</th>
<th>retentate</th>
<th>methanol/ formic acid soluble</th>
<th>methanol/ formic acid insoluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>52.3</td>
<td>271.1</td>
<td>66.4</td>
<td>423.5</td>
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<tr>
<td>IL-8</td>
<td>4.4</td>
<td>166.5</td>
<td>6.9</td>
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<tr>
<td>MCP1</td>
<td>56.9</td>
<td>197.1</td>
<td>86.1</td>
<td>254.3</td>
</tr>
<tr>
<td>MIP-1/β</td>
<td>82.4</td>
<td>404.6</td>
<td>91.9</td>
<td>414.2</td>
</tr>
</tbody>
</table>

* Data are the means of duplicate absorbance (450 nm) measurements.

mildly acidic conditions, the terminal amino acid was removed to produce a phenylalanylhydroxantoin (PTH) derivative of the amino acid and generate a free amino group on the next residue in the peptide sequence. The PTH-AA was identified quantitatively by HPLC by comparison to the retention time of the known standard reference PTH-AA. This process was then repeated to identify the next amino acid. At the end of each cycle during sequencing, results of the chromatography were automatically collected in data files. The amount (in pmol) of each amino acid detected in each cycle and the difference in yield for each amino acid in each cycle compared to the previous cycle were calculated.

ESIMS/MS of the Natural Product 1. HPLC-purified 1 (0.7 mg) was dissolved in 0.5 mL of 0.1% formic acid in water, and 10 μL was loaded onto the column through the sample loop. The sample was eluted using a binary gradient where solvent A was 98% H2O and 2% trifluoroacetic acid and solvent B was 10% H2O and 90% acetone with 0.1% formic acid and 0.01% trifluoroacetic acid. The gradient was formed from the initial conditions of 2% B to 50% B over 20 min and then washed with 100% B for 5 min.

MALDITOFMS of the Natural Product 1. The sample of 1 was dissolved in chloroform/methanol (4:1), and 0.8 μL was deposited onto the same planar surface of the sample disk. The sample mixtures were dried at room temperature prior to mass spectrometric analysis. Hexa-acetylated lipid A 1,4,6-bisphosphate from wild-type E. coli (Sigma Aldrich, St. Louis, MO) served as an external standard for calibration.

Hydrolysis and Derivationization of the Natural Product 1. Native 1 has been found to be labile to acid hydrolysis. Therefore, 100 μg of native 1 was subjected to mild acid hydrolysis conditions (1 N HCl in phosphate-buffered saline) for 18 h at 25 °C. The postreaction salts were removed using a C18 spin column (ThermoFisher Scientific), and the hydrolysis products were eluted from the column using 50% CH3CN/0.1% trifluoroacetic acid. The final product was brought to dryness by evaporation in vacuo at 4 °C. The hydrolysis products were derivatized using the N-terminal sulfonation reagent 4-sulfophenylationthiocyanate and analyzed in situ by MALDI-TOFMS in an α-cyano-4-hydroxycinnamic acid matrix using the method described by Wang et al. 4-Sulfophenylisothiocyanate was dissolved in 20 mM sodium bicarbonate (pH 9.5) to a final concentration of 10 μg/mL. A 50-fold excess of the 4-sulfophenylisothiocyanate solution (80 μL) was added to 4 μL of 1 (4 μg/μL). The sulfonation reaction was incubated at 55 °C for 45 min, and the reaction was quenched by addition of 5% trifluoroacetic acid. The sample was loaded onto a micro pipet tip and dried. The peptide-conjugated resin was acetylated at the N-terminal amine of the peptide by the addition of 10% acetic anhydride in N,N-diisopropylethylamine (20%) and N,N-dimethylacetamide (70%). After 2 h at room temperature the resin was filtered, washed successively with N,N-dimethylacetamide and dichloromethane, and lyophilized. The acetylated and fully protected peptide was cleaved from the resin using 20 mL of a 1:4 solution of 1,1,3,3,3-hexafluor-2-propanol in dichloromethane. After 2 h at room temperature, the resin was filtered and washed with 2 mL of the cleavage solution. The filtrate was evaporated in vacuo using a rotary evaporator. A sample was analyzed by mass spectrometry to confirm the expected mass at m/z 3286 (data not shown).

1-Stearoyl-2-arachidonoyl-sn-glycerol (Sigma Aldrich, St. Louis, MO) was acetylated to the C-terminal isoleucine carboxyl of the fully protected acetylated peptide using the dicyclohexylcarbodiimide/dimethylaminopyridine (DCC/DMAP) coupling reaction. 1-Stearoyl-2-arachidonoyl-sn-glycerol (5 mg) was dissolved in 2 mL of dichloromethane and mixed with 2 equiv of the fully protected acetylated peptide dissolved in 2 mL of dichloromethane and 1 equiv of DCC/DMAP, also dissolved in 2 mL of dichloromethane. The reaction was allowed to proceed overnight at room temperature. The reaction mixture was dried in vacuo, and the protecting groups were removed in situ by the addition of 8 mL of the deprotection solution (2.5% 1,2-ethanold, 94% dichloromethane, and 2.5% trifluoroacetic acid). After a 2 h incubation period at room temperature in the presence of the deprotection solution the reaction mixture was filtered and the filtrate was evaporated in vacuo.

Purification of the crude product was accomplished by reversed-phase preparative scale HPLC using a Jupiter Proteo column (Phenomenex, Inc., Torrance, CA). A binary mobile phase gradient (solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.1% trifluoroacetic acid in acetonitrile) was formed from 5% to 95% solvent B over 20 min (4.5% per min) at a flow rate of 1 mL/min, and the effluent was collected by monitored by monitored absorbance at 220 nm. The synthetic product 1 eluted at 22.9 min under these conditions. The crude product (63 mg) was dissolved in 4 mL of acetonitrile and 2 mL of water and loaded onto the preparative scale column. The synthetic product was collected in 22.5 mL of the mobile phase (95% acetonitrile/0.1% trifluoroacetic acid). The effluent was evaporated in vacuo. The final weight of the synthetic product 1 was 2.5 mg (4% yield) with a purity determined by HPLC analysis of 98.9%.

ESIMS/MS of Synthetic 1. In one experiment, the mobile phase was A (1% 2-propanol, 0.03% trifluoroacetic acid in H2O) and B (1% 2-propanol, 0.01% trifluoroacetic acid in acetonitrile, 0.03% trifluoroacetic acid in CH3CN). In a second experiment, the mobile phase was A (0.1% formic acid in H2O) and B (CH3OH). The column was post-treated with 0.1 M LiOH by syringe pump at 5 μL/min. The mobile phase gradient was formed from 5% B to 80% B (10 min), 80% B to 95% B (13.0–27.0 min), 95% B to 5% B (35.0–36.0 min), and 5% B (36.0–41.0 min). The column was a Zorbax XDB-C8, 3.5 μm, 1 × 50 mm (see Supporting Information, Figures S7 and S8).

mRNA Expression Experiments. Normal primary human fibroblasts obtained from the Coriell Institute (Camden, NJ) were cultured in 70 mm dishes in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO2. Human fibroblasts were incubated in the presence of the serum fractions, compound 1, the peptide moiety of 1, or diacylglycerol. In the initial experiments, the differences were examined between fibroblasts treated with the various serum fractions, synthetic 1 (the peptide moiety from 1 or diacylglycerol), fibroblasts treated with LPS as a positive control, and fibroblasts without treatment. The culture medium was stored at −80 °C for ELISA of the secreted cytokine proteins (see below). Total RNA from fibroblasts was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA), according to the manufacturer’s protocol. To verify the expression of transcripts, 2 μg of total RNA was reverse-transcribed using Superscript-III reverse transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. Transcripts were quantified using SYBR Green PCR amplification (Perkin-Elmer, Waltham, MA). Primers employed in these studies are outlined in Table 6. All transcripts were normalized to β-actin. Briefly, 0.4 ng/μL of cDNA was tested with 10 μM of each specific primer and 1× SYBR Green in a total volume of 20 μL.

ELISA for IL-8 Protein. Human IL-8 was measured by ELISA according to the manufacturer’s specifications (eBioscience, San Diego, CA) in the supernatants of fibroblasts cultured in complete DMEM with varying amounts of compound 1. Briefly, high-affinity binding plates were coated with antihuman IL-8 and incubated overnight at 4 °C. Wells were washed with wash buffer five times for 1 min at each wash and then blocked with assay diluent for 60 min at room temperature. Samples were added to the wells along with diluted standards and incubated for 2 h. Wells were washed and detection antibody was applied for 30 min, wells were washed again, an Avidin-HRP antibody was applied for 30 min, unbound antibody was removed by washing as described, and tetramethylbenzidine substrate was added...
Table 6. Primer Sequences for Real-Time PCR

<table>
<thead>
<tr>
<th>gene</th>
<th>forward primer</th>
<th>reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>5′-GGCCCTAAACGATGAGTGGCTCC-3′</td>
<td>5′-GGCCCTGCGTGAAGCC-3′</td>
</tr>
<tr>
<td>IL-6</td>
<td>5′-TCTCTTCCACACGCGTC-3′</td>
<td>5′-AAGGCCAGAGCAACAAC-3′</td>
</tr>
<tr>
<td>IL-8</td>
<td>5′-CCGCGTCAGCTGGTAAAGGGTGC-3′</td>
<td>5′-GGTGAGGAGTGGTGGTATTCCTGATG-3′</td>
</tr>
<tr>
<td>IL-18</td>
<td>5′-CTGCTAGAGATAATGGCACCACCGGACC-3′</td>
<td>5′-GTTCTCAGAGAGTGGTAGAATATTTCACAC-3′</td>
</tr>
<tr>
<td>IL-33</td>
<td>5′-GGACCTATGCTGAGGCTTCAAAATG-3′</td>
<td>5′-CACTAAATACACTCGAGATCGTCTGC-3′</td>
</tr>
<tr>
<td>MCT-1</td>
<td>5′-GGTCACTGCAGCGATGACATC-3′</td>
<td>5′-GGACACCTGCTGATTGAGTCTGCTATC-3′</td>
</tr>
<tr>
<td>MIP1α</td>
<td>5′-GAGAATCTTCAAGGGCTCGGACAATC-3′</td>
<td>5′-CTGCCCTGCTGAGTCTCTACAC-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>5′-GTGCGGCAAGGATGGCAAAA-3′</td>
<td>5′-GGCAATCCACAGGAGTAGTCTTAT-3′</td>
</tr>
</tbody>
</table>

Acknowledgment. The authors thank Dr. E. Blankenhorn, of Drexel University College of Medicine (Philadelphia, PA), for her valuable insights into the transcriptional regulation of the trpc1 gene. The authors also thank Dr. H. Guo, of Drexel University College of Medicine (Doylespon, PA), for his suggestions regarding the lipofectamine transfection experiment. The authors also acknowledge the assistance of T. Crabtree of Commonwealth Biotechnology Laboratories, Inc. (Richmond, VA) for the Edman degradation sequence analysis, A. Beaasley in the laboratory of Dr. R. J. Cotter, at the Mid-Atlantic Mass Spectrometry Laboratory, Department of Pharmacology, Johns Hopkins University School of Medicine (Baltimore, MD), for the MALDITOF analysis of the natural product, Dr. M. D. Hail and Dr. D. Detlefsen of Novartis, LLC (Monmouth Junction, NJ), for the ESIMS-MS, 1H NMR, and 13P NMR of the natural product, and B. Rariden of Young’s Service Company (Lancaster, PA), for the lyophilization of the peptide fractions. This work was funded by Drexel University College of Medicine (Philadelphia, PA), for the Institute of Hepatitis and Virus Research (Doylespon, PA), and TherimmuneX Pharmaceuticals, Inc. (Doylespon, PA).

Supporting Information Available: HPLC chromatograms of the natural product isolated from goat serum, MS/MS of the product ion (M+2) for the N-terminal peptide fragment of natural product, MALDITOFMS of the tryptophan complex, PS MALDITOFMS of the peptide fragments generated after mild acid hydrolysis of natural product, deconvoluted ESIMS of synthetic compound 1, ESIMS of synthetic compound 1, HPLC chromatogram of the purified synthetic compound 1, and the cytokine mRNA induced by 10 ng/mL (~5 nM) of the transfected peptide from 1 or diacylglycerol alone. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

1-Peptidyl-2-arachidonoyl-3-stearoyl-sn-glyceride: An Immunologically Active Lipopeptide from Goat Serum (Capra hircus) is an Endogenous Damage-Associated Molecular Pattern

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Figure 1. C₈ reversed-phase HPLC chromatograms of natural 1. The parent molecule was determined to comprise the 10.42 min peak in the top preparative scale chromatogram. The peaks eluting between 4.83 min and 9.36 min comprised peptide degradation products of the 10.42 min parent molecule 1 as determined by MALDITOFMS and ESIMS. The peaks eluting between 2.96 min and 4.0 min comprised oligosaccharides and small peptides and were not identified. The component eluting at 22.9 min is an artifact and was present in solvent blanks (data not shown). The bottom chromatogram is the analytical chromatogram of the peak eluting at 10.42 min that was collected from preparative scale chromatography. Purified 1 was determined to be a tryptophan complex and was used in the bioassays.
Figure 2. LC/MS of natural 1. (panel A) LC/UV 280 nm, (panel B) LC/MS total ion current chromatogram, (panel C) extracted ion chromatogram (XIC) of the major component, m/z 205, (panel D) XIC of the minor component with MW 1689 Da, and (panel E) the ESIMS of the minor component at retention time = 3.95 min, MW 1689 Da. From a comparison of the intensities of the XIC’s, the ratio of the abundance of compound 1-to-tryptophan is approximately 1-to-200.
Figure 3. Product ion mass spectrum of \( m/z \) 845.5 from peak at retention time 3.95 min.

The ms/ms spectrum is consistent with the sequence acetylALYDGYTSKEKD, MW = 1689 Da. Specific ion fragments detected in the mass spectrum are bolded.
**Figure 4.** MALDITOFMS of the Tryptophan complex of 1 without added matrix. The region from m/z 800 to m/z 2600 was scanned and 4 major ion fragments from the N-terminal and the C-terminal regions of 1 were observed. The high mass ion fragment at m/z 1282.71 (unlabeled) is consistent with [acetylALYDKGYTSKE-NH₃]⁺. The low mass ion at m/z 1133.30 is the y-ion fragment [DCVGI(DAG)]⁺ where DAG is diacylglycerol (1-stearoyl-2-arachidonoyl-sn-glycerol). The major ion at m/z 1208.47 and its daughter ion at m/z 1223.61 are the z-ion and y-ion fragments (respectively) of the Tryptophan adduct of [KEQKDCVGI]⁺. The y-ion at 1207.53 is [TSKEQKDCVGI]⁺. The mass spectrum is consistent with the sequence shown.
Figure 5. PSD MALDI TOFMS of the Tryptophan complex of 1 in a dihydroxybenzoic acid matrix. The region from m/z 600 to m/z 4000 was scanned and the ion fragments comprise the complete structure of 1. The high mass fragment at m/z 2016.70 is the a-ion of the complete linear peptide sequence of 1. The low mass y-ion at m/z 645.76 is the diacylglycerol C-terminal prosthetic group. The mass spectrum is consistent with the sequence shown.
Figure 6. PSD MALDITOFMS of the peptide fragments generated after mild acid hydrolysis of native 1. The hydrolysis products were sulfonated as SPITC derivatives on the $N$-terminal amino acid to suppress b-ion fragmentation. The assignments for the ion fragments are given in Table 3 of the main text.
Figure 7. Deconvoluted ESIMS of synthetic 1. The experiment was performed on a Thermo-Finnegan Surveyor HPLC-LCQ Classic Ion Trap LC/MS system in the +ve mode. The mobile phase was A [1% 2-propanol, 0.03% TFA in H₂O] and B [1% 2-propanol (containing 2 mmol NH₄OAc in 9:1 2-propanol:H₂O) 0.03% trifluoroacetic acid in CH₃CN]. The raw data generated for the peak eluting at a retention time of 6.23-6.47 min was deconvoluted using ProMass for Xcalibur Version 2.5 SR1. The deconvoluted spectrum shows a pattern consistent with the mass expected for the peptide moiety in 1 minus diacylglycerol with a single charged ion at 2059, a double charged ion at 1030.2 and a triple charged ion at 687.5. The isotopomers for the single charge had a spacing of 1 amu, the double charge a spacing of 0.5 amu, and the triple charge a spacing of 1/3 amu in agreement with expectations.
Figure 8. ESIMS of synthetic 1. The experiment was run on a Thermo-Finnegan Surveyor HPLC-LCQ DECA Ion Trap LC/MS system in the +ve mode. The mobile phase was A [0.1% formic acid in H₂O] and B [CH₃OH]. The column was post-treated with 0.1M LiOH by syringe pump at 5μL/min. The gradient ran from 5%B-80%B (10 min), 80%B-95%B (13.0-27.0 min), 95%B(27.0-35.0 min), 95%B-5%B (35.0-36.0 min) and 5%B (36.0-41.0 min). The major ion mass of 1045.4 was attributed to the x-ion of CVGI(DAG) where DAG is diacylglycerol.
Figure 9. Reversed-phase HPLC chromatogram of purified synthetic 1. 25 μl of purified synthetic 1 was analyzed by reversed-phase HPLC using a Jupiter® Proteo (Phenomenex, Inc., Torrance, CA) column (90 Å, 4 μ, 4.6 x 250 mm) and a binary mobile phase gradient (Solvent A: 0.1% trifluoroacetic acid in water Solvent B: 0.1% trifluoroacetic acid in acetonitrile) formed from 5% to 95% Solvent B over 20 minutes (4.5% per min) at a flow rate of 1 mL/min. The effluent was continuously monitored at 220 nm. The area % integration of 1 eluting at 22.849 min was 99.45%.
**Figure 10.** Cytokine mRNA induced by 10 ng/mL (~ 5 nM) of the transfected peptide from 1 or diacylglycerol. Inflammasome-mediated protein mRNA expression for IL-1β, IL-18, and IL-33 was substantially increased over untreated control fibroblasts or fibroblasts treated with lipofectamine alone. The panel in the lower right corner shows the effect on mRNA expression for IL-1β, IL-33, and IL-18 in fibroblasts incubated with 12.5 ng/mL (~ 30 nM) diacylglycerol alone as compared to untreated control fibroblasts. Data are normalized to β-actin mRNA expression.