SUPPLEMENTAL DATA

RESULTS

Ingenuity Pathways Analysis – To gain a further insight into the potential mechanisms of the effects of n-Pen c 13 in mice, the identified proteins were mapped to networks available in the Ingenuity database. Three networks were identified and were ranked by the score in the *p*-value calculation of the IPA assay, which ranged from 11 to 53. The highest-scoring network revealed a significant link with hematological disease, immunological disease, and respiratory disease. Furthermore, cancer, cellular growth, proliferation, and cellular assembly and organization might also be influenced in the other two networks. The components of these networks are shown in Supplemental Table 4. The scores take into account the number of focus proteins and the size of the network to approximate the relevance of the network to the original list of focus proteins. We then focused on the network with the highest score, which consisted of 21 focus molecules and 14 interconnecting molecules ("non-focused" proteins that were not present in our list). We then carried out a Function & Disease analysis and Canonical Pathway analysis of the 35 identified proteins in the highest-scorings network using Ingenuity software. The Function & Disease analysis results showed a significant correlation with acute allergic pulmonary eosinophilia (7 proteins) and cell movement (10 proteins), while the Canonical Pathway analysis revealed that actin cytoskeleton signaling, leukocyte extravasation signaling, integrin signaling, NRF2-mediated oxidative stress response, FAK signaling, tight junction signaling, and acute phase response signaling were the most significant signaling pathways modulated by n-Pen c 13 (Supplemental Fig.S3). We also carried out data mining on the highest-scoring network to explore two proteins (galectin-3, laminin-γ1) associated with allergic airway inflammation, but not in our proteomics data (Supplemental Table 3).

EXPERIMENTAL PROCEDURES

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) – Total mRNA of lung tissue was isolated with QuickPrep Micro mRNA Purification Kit (GE Healthcare) according to the manufacturer's instructions. Lastly, the mRNA samples were added 40 U RNase out (Invitrogen), which is a potent inhibitor of ribonucleases. The mRNA from experimental samples was used to synthesize cDNA using MMLV reverse transcriptase (EPICENTRE® Biotechnologies) and oligo (dT) as a primer. cDNAs were amplified using specific sets of primers listed in Supplemental Table 1. Levels of mRNA expression of cytokines and chemokines were determined by PCR in a 20 µl reaction volume using Taq ReadyMix (Kapa Biosystems) on the ABI Prism 2720 (Applied Biosystems, Foster City, Calif., USA). For each of the samples, PCR products were separated in a 2% agarose gel visualized under ultraviolet light. Densitometric analysis of PCR products was carried out using the UVP image analysis system and normalized by β-actin.

Signaling pathway analysis – Proteins found to be differentially expressed by 2-D DIGE analysis were subjected to Ingenuity Pathways Analysis (IPA) (Ingenuity Systems) and used as a starting point for building biological networks. This analysis uses computational algorithms to identify networks consisting of focus proteins (proteins that were present in our list) and their interactions with other proteins ("non-focused") in the knowledge base. Scores were calculated for each network according to the fit of the network to the set of focus proteins and used to rank networks on the Ingenuity analysis. IPA uses the proteins from the highest-scoring network to extract a connectivity pathway that relates candidate proteins to each other based on their interactions. The involved Function & Disease and Canonical Pathways were shown to be significantly associated with these candidates. In addition, we also searched for other proteins involved in this network using the build tools of IPA.

Supplemental Table 1.

Primer sets for reverse transcriptase–polymerase chain reaction analysis

Targets	Sequences		Product size (bp)
	Forward	Reverse	
IL-4	5'-GAAGAACACCACAGAGAGTGAGCT -3'	5'-GACTCATTCATGGTGCAGCTTATCG-3'	180
IL-5	5'-GTGAAAGAGACCTTGACACAGCTG-3'	5'-CACACCAAGGAACTCTTGCAGGTA-3'	290
IL-13	5'-AAACTGCAGCAAGACCGTGAGTCC-3'	5'-TGGCAGACAGGAGTGTTGCTCTGG-3'	311
CCL17	5'-CAGGGATGCCATCGTGTTTCT-3'	5'-GGTCACAGGCCGCTTTATGTT-3'	179
CCL22	5'-CTGGGTGAAGAAGCTACTCCAT-3'	5'-TAGAAACTTGGGAGGTGTGTGG-3'	493
TNF-α	5'-GGCAGGTCTACTTTGGAGTCATTGC-3'	5'-ACATTCGAGGCTCCAGTGAATTCGG-3'	307
IL-10	5'-ACCTGGTAGAAGTGATGCCCCAGGCA-3'	5'-CTATGCAGTTGATGAAGATGTCAAA-3'	237

Supplemental Table 2.

Experimental design for 2-D DIGE comparison of lung lysates from n-Pen c 13-sensitized and PBS-treated mice.

Gel No	Cy2	Cy3	Cy5
1	Pooled std	n-Pen c 13	PBS
2	Pooled std	n-Pen c 13	PBS
3	Pooled std	PBS	n-Pen c 13
4	Pooled std	PBS	n-Pen c 13

Supplemental Table 3.

Identification of proteins with altered expression levels in the n-Pen c 13-treated versus control mouse lung by nano-LC-MS/MS analysis.

Protein name	Spot No.	Theoret. Mass (pI/kDa)	Sequence coverage[a]	NCBI Accession no.	Average ratio (2-D DIGE)[b]
Cytoskeleton and related proteins					
F-actin-capping protein subunit alpha-1	4	5.34/32.919	34%	gi|595917	1.23
Actin, cytoplasmic 1	5	5.29/41.71	27%	gi|74213524	1.89
Coronin-1A	6	6.05/50.957	19%	gi|74192405	1.76
Macrophage-capping protein	11	6.73/39.216	25%	gi|74138419	1.66
Rho GDP-dissociation inhibitor 2	15	4.97/22.836	51%	gi|33563236	1.61
Cofilin-1	21	8.22/18.548	66%	gi|6680924	1.37
Vinculin	24	5.54/123.781	46%	gi|148669535	-1.39
Annexin A2	36	7.55/38.652	68%	gi|6996913	-1.22
Coactosin-like protein	34	5.13/16.507	53%	gi|159163110	1.74
Actin related protein 2/3 complex, subunit 5	43	5.47/16.218	39%	gi|74185013	1.23
Moesin	45	6.22/67.725	50%	gi|70778915	-1.29
Protein folding					
Protein disulfide-isomerase A3	3	5.88/56.643	44%	gi|26353794	1.21
78 kDa glucose-regulated protein	37	5.07/72.377	54%	gi|2506545	1.22
Heat shock protein 90 kDa beta (Grp94), member 1	38	4.74/92.418	52%	gi|6755863	1.21
Oxidoreduction					
Alcohol dehydrogenase ($NADP^+$)	13	6.9/36.564	50%	gi|10946870	1.28
Ferritin light chain 1	17	5.66/20.788	83%	gi|148690909	3.89
Superoxide dismutase [Mn]	19	8.8/24.662	41%	gi|53450	1.2
Peroxiredoxin 1	22	8.26/22.162	53%	gi|6754976	1.22
Aldehyde dehydrogenase, mitochondrial	28, 29	7.53/56.49	38%/58%	gi|74226796	-1.32/-1.33
Electron transfer flavoprotein subunit alpha, mitochondrial	30	8.62/34.988	21%	gi|146345417	-1.46
Carbonyl reductase (NADPH) 2	32, 33	9.1/25.942	43%/50%	gi|6671688	-1.37/-1.47
Aldehyde dehydrogenase 1 family, member A1	42	7.89/54.447	51%	gi|191804	-1.32
Immune response					
Annexin A1	12	6.97/38.71	55%	gi|70912321	1.26
Advanced glycosylation end product-specific receptor	9, 10	5.78/42.642	15%/17%	gi|110225341	-1.34/-1.41
Cellular metabolism					
Alpha-enolase	7, 8	6.37/47.111	29%/39%	gi|70794816	1.45/1.53
Phosphoglycerate mutase 1	14	6.67/28.685	63%	gi|12844989	1.34
Fatty acid-binding protein, epidermal	18	6.14/15.127	31%	gi|6754450	1.86

Transaldolase	39	6.57/37.363	24%	gi|33859640	1.21
Transketolase	40	7.23/67.588	47%	gi|6678359	1.23
Pyruvate kinase isozymes M1/M2	41	7.18/57.878	61%	gi|1405933	1.24
Propionyl CoA-carboxylase alpha-subunit	44	7.02/79.61	40%	gi|15667251	-1.22
Others					
Serum deprivation response protein	1, 2	5.15/46.74	31%/17%	gi|20270267	-1.69/-1.78
Translationally-controlled tumor protein	16	4.76/19.45	49%	gi|6678437	1.33
Glutathione S-transferase Mu 1	20	7.71/25.953	52%	gi|6754084	-1.47
Heterogeneous nuclear ribonucleoprotein A3	23	8.2/31.862	32%	gi|82918359	1.41
Serum albumin	25, 26	5.78/68.688	24%/21%	gi|74137565	-1.43/-1.63
Dihydropyrimidinase-related protein 2	27	5.95/62.239	24%	gi|40254595	-2.04
Carbonic anhydrase 2	31	6.49/29.015	44%	gi|33243954	-1.37
Chloride intracellular channel protein 3	35	5.98/26.829	29%	gi|46395972	1.38

a. Sequencing coverage is defined as the percentage of the whole length of the protein sequence which is covered by matched peptides identified by the nano-LC-MS/MS analysis.

b. Ratio of protein expression levels were calculated using DeCyder software as the -fold change between normalized spot volume between n-Pen c 13-sensitized mice and PBS-treated mice tissue lysates (Student's *t* test was based on the log of the ratio between n-Pen c 13-sensitized mice and PBS-treated mice; $p < 0.05$).

Supplemental Table 4.

Physical function analysis using IPA generated three networks which are ordered by a score denoting significance. The highest-scoring network, which comprises 21 proteins in our list, revealed significant changes in hematological disease, immunological disease, and respiratory disease. In addition, the other networks revealed changes in cancer, cellular growth, proliferation, cellular assembly, and organization.

Top Network	Score	Focus Protein	Molecules
Hematological disease, immunological disease, respiratory disease	53	21	**ACTB**, Actin, Actin-Actn-Ptk2-Pxn-Vcl, **AGER**, **ALB**, **ALDH2**, Alpha actin, **ANXA1**, **ANXA2**, Calpain, **CAPZA1**, **CFL1**, **CORO1A**, **COTL1**, **DPYSL2**, **ENO1**, ERK, F Actin, **FABP5L2**, FSH, Hsp90, **HSP90B1**, **HSPA5**, IL1, Mapk, MHC Class I (complex), **MSN**, NFkB (complex), **PDIA3**, PHACTR1, **PKM2**, PLS1, **SOD2**, **TPT1** (includes EG:22070), **VCL**
Cancer, cellular growth and proliferation, hematological disease	25	12	ACO1, ALDH16A1, **ALDH1A1**, **ARPC5**, **CA2**, **CAPG**, Cbp/p300-Maf-Nfe2l2, **CBR2**, CTNNB1, **ETFA**, FABP, FTH1, **FTL**, GADD45A, glutathione, **GSTM5**, **HNRNPA3**, HNRPDL, HRAS, IFI6, IFNG, IREB2, NAMPT, NR3C1, **PCCA**, PCCB, PLOD1, PPARG, RPS10, **SDPR**, **TALDO1**, TGFB1, TNF, TRAF6, VDAC2
Cellular assembly and organization, cancer, hematological disease	11	6	**AKR1A1**, **ANXA1**, APP, **ARHGDIB**, ATP5B, BAX, BBC3, BCL2L11, BIK, Cofilin, CTSL1, EPB41L3, FPR2, GABBR1, GAPDH (includes EG:2597), HBA2, KLC1, KLC2, LIMK1, NUMB, P2RX7, PFN1, **PGAM1**, PPIA (includes EG:5478), **PRDX1**, PRDX2, retinoic acid, S100B, SERPINF1, SMPD1, **TKT**, TPI1, TRA@, TUBB, YWHAZ

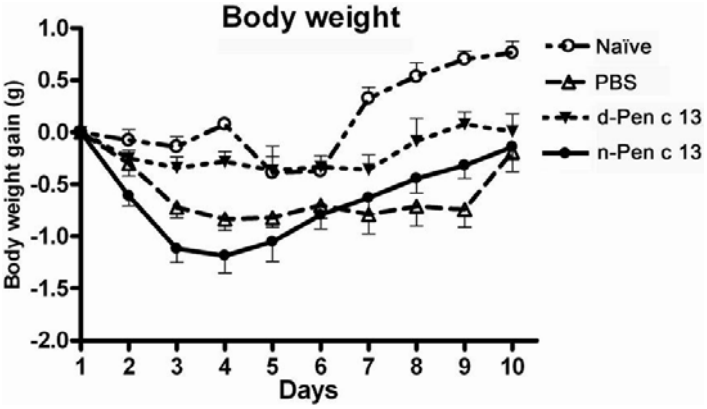

Fig. S1. Average body weight changes in the mice during administration of PBS, d-Pen c 13, or n-Pen c 13 and in naïve mice. The body weight in the different groups was monitored throughout intratracheal administration.

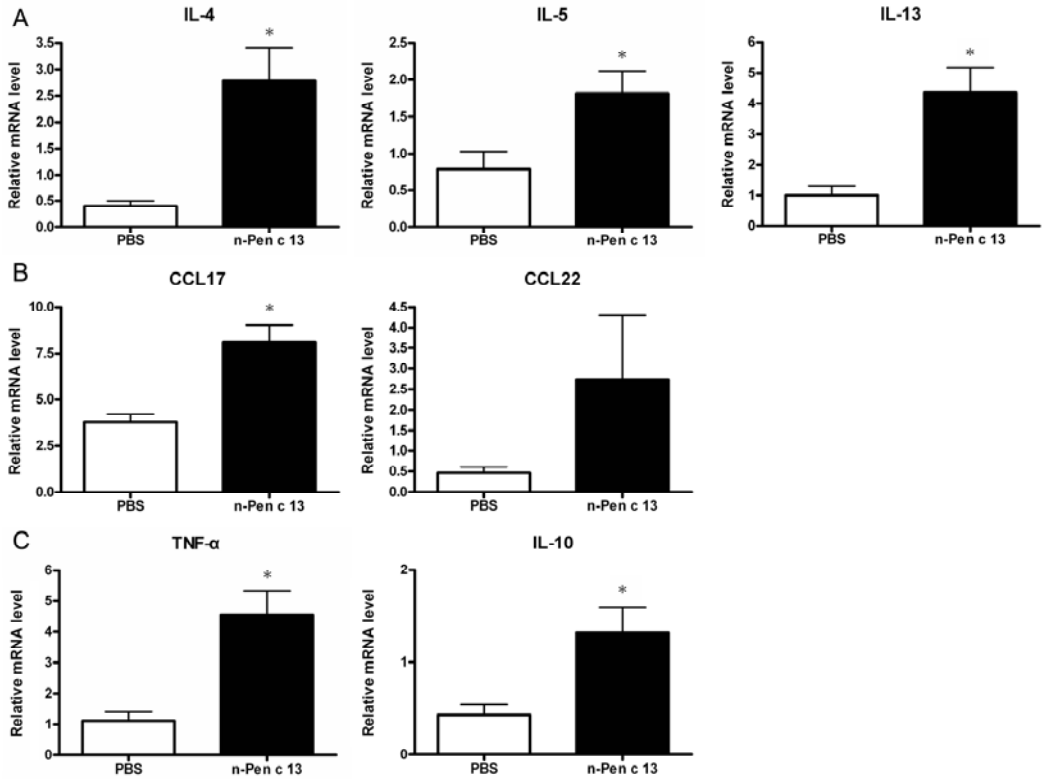

Fig. S2. The cytokine, chemokine, and pro-inflammatory mediator mRNA expression in the lungs after exposure to active Pen c 13 or PBS for 10 consecutive days.
A, Th2 cytokines (IL-4, IL-5, and IL-13). B, Th2 chemokines (CCL17 and CCL22). C, Pro-inflammatory mediators (TNF-α and IL-10). In the lungs from n-Pen c 13 sensitized mice, significant increases in the mRNA expression of IL-4 (7.0-fold), IL-5 (2.3-fold), IL-13 (4.3-fold), CCL17 (2.1-fold), TNF-α (4.1-fold) and IL-10 (3.1-fold) were observed compared with the control animals. Although not statistically significant, the levels of CCL22 in repeated n-Pen c 13-exposed animals showed a tendency to increase (5.9-fold). The results were analyzed using the paired Student's *t* test (n = 4-5 per group). *$p < 0.05$ compared to PBS-treated mice. Values for all measurements were expressed as the mean ± SEM.

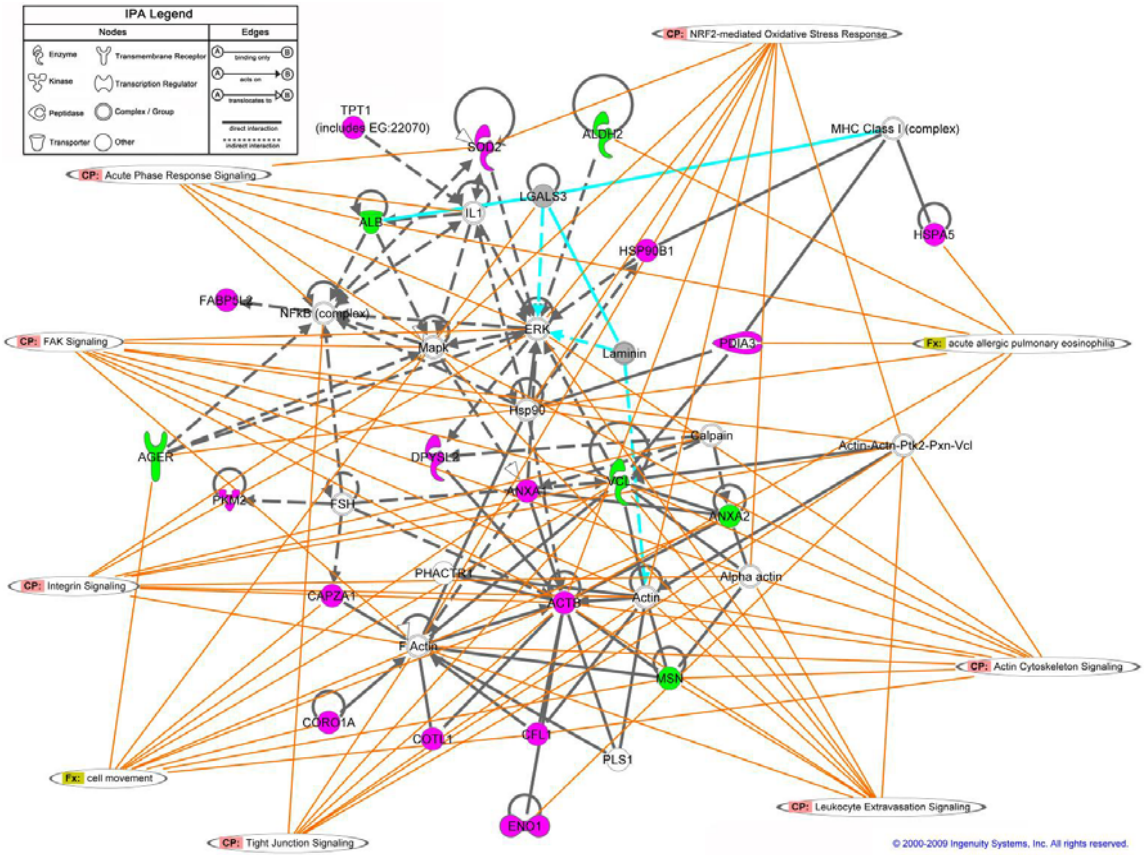

Fig. S3. Molecular pathway analysis by Ingenuity Pathways Analysis (IPA). The significant connections of the most highly-scoring network, consisting of 21 proteins showing markedly altered expression in the 2-D DIGE analysis (Supplemental Table 3), plus additional interacting molecules, are indicated. Upregulated proteins are shown in red and downregulated proteins in green. Two molecules found by the data mining tools of IPA (build tools) are shown in gray. The highlight tool of IPA (overlay tools) revealed significant changes in Function & Disease (acute allergic pulmonary eosinophilia and cell movement) and Canonical Pathway (actin cytoskeleton signaling, leukocyte extravasation signaling, integrin signaling, NRF2-mediated oxidative stress response, FAK signaling, tight junction signaling, and acute phase response signaling), which are shown as orange lines. Fx, Function & Disease; CP, Canonical Pathway. Nodes are displayed using various shapes that represent the functional class of the proteins. Edges are displayed with various labels that describe the nature of the relationship between the nodes. HSP90B1 (Grp94), HSPA5 (Grp78), MSN (Moesin).

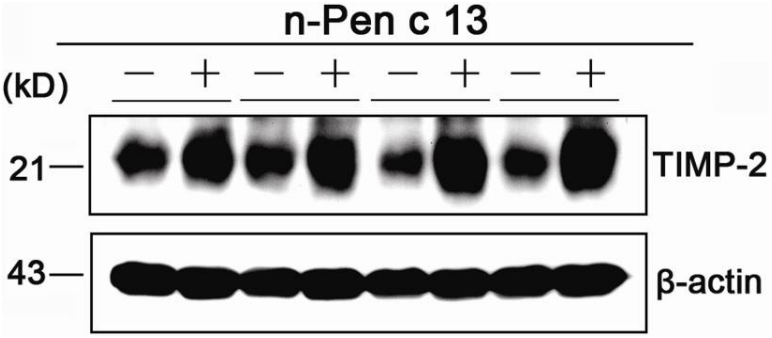

Fig. S4. TIMP-2 expression is increased in lung tissue after n-Pen c 13 inoculation.
Western blotting was performed on lung lysates from n-Pen c 13-sensitized (+) and PBS-treated (-) mice to determine levels of TIMP-2. The results shown are representative of those from 4 separate experiments. β-actin was used as the loading control.

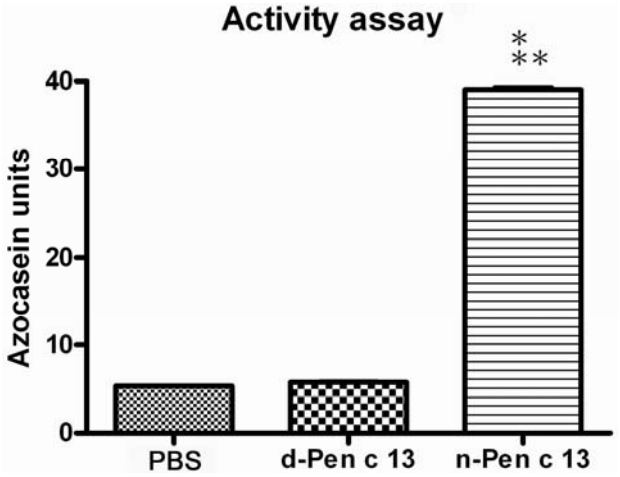

Fig. S5. Evaluation of Pen c 13 activity in the azocasein assay.
The n-Pen c 13 and d-Pen c 13 were assayed for proteolytic activity using azocasein as a substrate. Azocasein (Sigma) was dissolved at a concentration of 5 mg/ml in 100 mM Tris/HCl, pH 8.0. The azocasein solution (90 μl) was mixed with aliquots (10 μl) of proteins (100 μg/ml) and the mixture incubated for 1 h at 37℃, then nondigested azocasein was precipitated by adding 4 μl of 100% trichloroacetic acid (Merck, Darmstadt, Germany) and leaving the reaction mixture on ice for 30 min. The tubes were then centrifuged for 5 min at 8,000 × *g* at 4℃ and 50 μl of each supernatant added to 50 μl of 1 M NaOH and the absorbance at 436 nm of the released azo dye measured on a spectrophotometer as an indicator of proteolytic activity. n = 4/group; data are expressed as the mean ± SEM. $p < 0.001$ compared to *PBS or **d-Pen c 13.

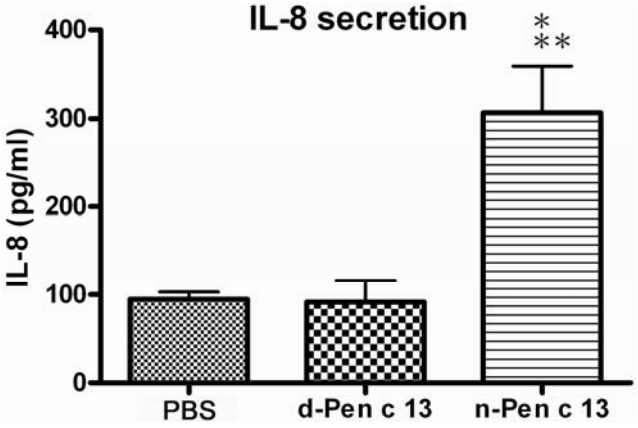

Fig. S6. Effect of Pen c 13 on IL-8 secretion by A549 cells.
A549 cells was plated in 24-well plates (2×10^4 cells/well), then, at 85% confluence, were washed twice with PBS, then 3.0 nM d-Pen c 13 or n-Pen c 13 in PBS or PBS alone was added. After 18 h, the culture supernatants were harvested and IL-8 levels determined by ELISA. n = 4/group; data are expressed as the mean ± SEM. $p < 0.01$ compared to *PBS-treated or **d-Pen c 13-treated cells, respectively.

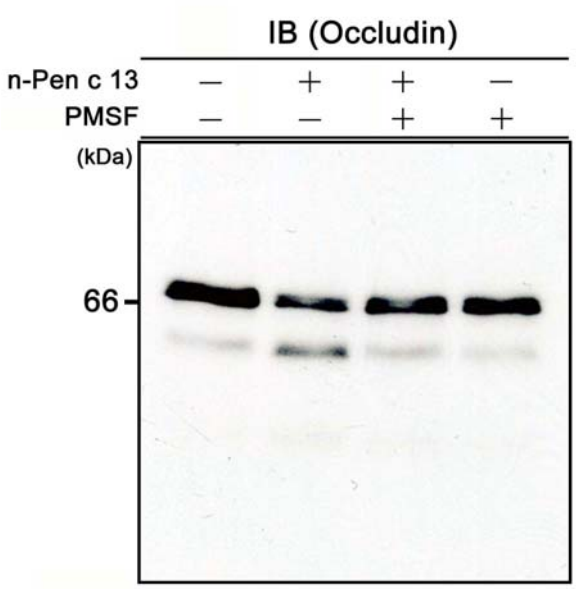

Fig. S7. Effect of protease inhibitor on n-Pen c 13-induced proteolysis of occludin.
Immunoblots of occludin from NCI-H441 cell monolayers after incubation for 2 h at 37℃ in the presence or absence n-Pen c 13 (30 nM) and 20 μM PMSF (a serine class protease inhibitor). Protease activity of Pen c 13 was inhibited by addition of PMSF for 2 h on ice prior to experimentation.