SANDRA SATIE MIZOKAMI

ÁCIDO CAURENÓICO DA *SPHAGNETICOLA TRILOBATA* (L.) PRUSKI INIBE A DOR INFLAMATÓRIA:

EFEITO NA PRODUÇÃO DE CITOCINAS E ATIVAÇÃO DA VIA NO/GMPc/PKG/K+ATP

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Dissertação apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito à obtenção do título de Mestre em Patologia Experimental.

Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior.

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Londrina, 19 de março de 2012.

Dedico este trabalho aos meus pais,
Francisco e Eunice, que estão sempre ao meu
lado me apoiando em tudo que faço e a
minha querida irmã Miriam.

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"A mente que se abre para uma nova idéia
jamais voltará ao seu tamanho original"
Albert Einstein

"O sábio procura a ausência da dor
e não o prazer" Aristóteles

MIZOKAMI, Sandra Satie. **Ácido caurenóico da *Sphagneticola trilobata* (L.) Pruski inibe a dor inflamatória:** efeito na produção de citocinas e ativação da via NO/GMPc/PKG/K+ATP. 2012. 81 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina. Londrina, 2012.

RESUMO

Ácido caurenóico (AC) é um diterpeno presente em muitas plantas, incluindo a *Sphagneticola trilobata* (L.) Pruski. A única evidência do seu efeito antinociceptivo é em inibir as contorções abdominais induzida por ácido acético em camundongos. Portanto, nós aprofundamos o estudo do efeito analgésico do AC e seus mecanismos de ação em diferentes modelos nociceptivos em camundongos. O tratamento com AC via intraperitoneal e via oral, inibiu de maneira dose-dependente a nocicepção inflamatória induzida pela administração de ácido acético. O tratamento oral com AC também inibiu a nocicepção induzida por fenil-*p*-benzoquinona, ambas as fases do teste da formalina e no teste com Adjuvante completo de *Freund's* (CFA). O composto AC também inibiu a hiperalgesia mecânica na inflamação aguda induzida pela carragenina e PGE_2, e inflamação prolongada induzida pelo CFA. Quanto aos mecanismos de ação do AC, houve inibição da produção de citocinas pró-inflamatórias TNF-α e IL-1β. Além disso, o feito analgésico do AC foi inibido pelos pré-tratamentos com L-NAME, ODQ, KT5823 e glibenclamida, demonstrando que o efeito analgésico também é dependente da ativação da via NO/GMPc/PKG/canais de potássio dependentes de ATP, respectivamente. Esses resultados demonstram que AC apresenta efeito analgésico e que seus mecanismos envolvem a inibição na produção de citocinas e ativação da via NO/GMPc/PKG/K+ATP.

Palavras-chave: Ácido caurenóico. Citocina. Óxido nítrico. Dor Inflamatória. K^+ATP.

MIZOKAMI, Sandra Satie. **Kaurenoic acid from *Sphagneticola trilobata* (L.) Pruski inhibits inflammatory pain:** effect on cytokine and activation of NO/cGMP/PKG/K+ATP channels signaling pathway. 2012. 81 f. Dissertation (Master's degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2012.

ABSTRACT

Kaurenoic acid (KA) is a diterpene present in many plants including *Sphagneticola trilobata* (L.) Pruski. The only evidence on its antinociceptive effect is that it inhibits the writhing response induced by acetic acid in mice. Therefore, we further addressed the analgesic effect of KA in different models of pain and its mechanisms in mice. Intraperitoneal and oral treatments with KA dose-dependently inhibited inflammatory nociception induced by acetic acid. The oral treatment with KA also inhibited the nociception induced by phenyl-*p*-benzoquinone, both phases of formalin test and Complete Freund's Adjuvant (CFA). Compound KA also inhibited acute carrageenin- and PGE_2-, and chronic CFA-induced inflammatory mechanical hyperalgesia. Mechanistically, KA inhibited the production of hyperalgesic cytokines TNFα and IL-1β. Furthermore, the analgesic effect of KA was inhibited by L-NAME, ODQ, KT5823 and glybenclamide treatments demonstrating that its analgesic effect also depends on activation of the NO/cGMP/PKG/K+ATP channels signaling pathway, respectively. These results consistently demonstrate that KA presents analgesic effect, and that its mechanisms involve the inhibition of cytokine production and activation of the NO/cGMP/PKG/K+ATP channels signaling pathway.

Keywords: Kaurenoic acid. Cytokine. Nitric oxide. Inflammatory pain. K+ATP.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ALT	Alanina aminotransferase
AMPc	Adenosina monofosfato cíclico
AST	Aspartato aminotransferase
ATP	Adenosina trifosfato
BALF	*Bronchoalveolar lavage fluid*
Cg	Carragenina
CFA	*Complete Freund's Adjuvant*
DMSO	Dimetilsulfóxido
ELISA	Ensaio Imunoenzimático
ERK	*Extracellular-signal-regulated Kinase*
GGT	Gama-glutamiltransferase
GMPc	Guanosina monofosfato cíclico
HPLC	*High-performance liquid chromatography*
IASP	*International Association for the Study of Pain*
Ig E	Imunoglobulina E
IL-1β	Interleucina 1beta
IL1RI	IL-1 *receptor*
IP3	Inositol fosfato
i.p.	Intraperitoneal
i.pl	Intraplantar
JNK	*c- Jun N-terminal Kinase*
KA	*Kaurenoic acid*
KC	queratinocitos
KT5823	inibidor das proteínas quinases dependente de GMPc/PKG
L-NAME	L-nitro-arginina metil ester; inibidor não seletivo da oxido nítrico sintase
LPS	Lipopolissacarídeo
MAPK	*Mitogen-activated protein kinase*
MDA	Malondialdeído
MPO	Mieloperoxidase
NO	*Nitric oxid*
NOS	*Nitric oxid sintase*

ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; inibidor da guanilato ciclase
PBQ	phenil-*p*-benzoquinona
PGE	Prostaglandina
PGE_2	Prostaglandina E2
PKA	*Protein Kinase A*
PKC	*Protein Kinase C*
PKG	*Protein Kinase G*
PLA_2	*phosfolipase* A2
TNF-α	*Tumor necrosis factor alfa*
TPA	12-O-tetradecanoilforbol acetato
VLC	*Vaccum Liquid Chromatography*
v.o.	via oral
δ	Deslocamento químico (ppm)

SUMÁRIO

1 INTRODUÇÃO

1.1 Processo inflamatório

O desenvolvimento da reação inflamatória tem por função destruir ou inativar o agente agressor e reparar o tecido lesionado através dos processos de regeneração ou cicatrização. O processo inflamatório apresenta alterações vasculares e celulares e envolve o desenvolvimento de quatro sinais cardinais: rubor, calor, edema e dor. Dependendo da intensidade e extensão do processo inflamatório pode ocorrer a perda da função (COTRAN, 2000).

Dentre as alterações vasculares que ocorrem durante a inflamação temos a vasodilatação de arteríolas e vênulas e abertura de capilares induzida pela liberação de mediadores químicos. Este processo induz ao aumento do volume sanguíneo no local, resultando no rubor ou eritema, e aumento da temperatura local. O aumento do volume local eleva a pressão hidrostática em conjunto com a vasodilação e abertura de lacunas entre as células endoteliais, contribuindo para o aumento da permeabilidade e extravassamento de líquido rico em proteínas (exsudado) e formação do edema (COTRAN, 2000).

Apesar do volume sanguíneo aumentar no foco inflamatório, há diminuição da sua velocidade de passagem devido a abertura de capilares. Essa redução da velocidade permite a saída dos leucócitos da coluna central do fluxo sanguíneo ou sua marginalização para a periferia do vaso. Essa marginalização contribui para a posterior transmigração dos leucócitos, embora não seja o único fator envolvido. Ademais, ativação de células residentes como macrófagos e mastócitos induz a produção de citocinas como fator de necrose tumoral alfa (TNF-α) e interleucina-1beta (IL-1β), as quais ativam as células endoteliais que passam a expressar moléculas de adesão da família das selectinas. As selectinas são glicoproteínas que apresentam um domínio N-terminal extracelular que se ligam a açúcares. Temos a E-selecitna presente no endotélio, a P-selectina presente no endotélio e em plaquetas e a L-selectina em leucócitos. Essa ligação a carboidratos fornece uma interação fraca entre leucócitos e endotélio vascular resultando no rolamento. A ligação das selectinas aos respectivos carboidratos tem a função de

moléculas de adesão, mas também de receptores resultando na ativação das integrinas. As integrinas interagem com moléculas de adesão da superfamília das imunoglobulinas permitindo a adesão firme dos leucócitos ao endotélio e posterior transmigração (COTRAN, 2000).

Esta emigração dos leucócitos ativados para o foco inflamatório ocorre através de substâncias quimioatratoras. Dentre elas podemos citar os produtos bacterianos, componentes do sistema complemento C3a e C5a, leucotrienos, citocinas e quimiocinas. Os leucócitos ao chegarem ao foco inflamatório fagocitam o agente agressor e podem contribuir para o desenvolvimento da dor. De fato, a inibição do recrutamento celular reduz o desenvolvimento da hiperalgesia. Em modelo de inflamação induzida pela carragenina, os leucócitos recrutados, principalmente neutrófilos, contribuem para o desenvolvimento da hiperalgesia por produzirem a prostaglandina E_2 (CUNHA , 2008).

1.1.1 Dor

> A capacidade de experimentar a dor tem um papel protetor: ela nos adverte de dano tecidual real ou iminente e suscita respostas reflexas coordenadas e comportamentais para manter tais danos ao mínimo (WOOLF, MANNION, 1999).

A dor é um sentimento desagradável porém essencial para o sistema de defesa do corpo. Se não sentissemos dor não teriamos, por exemplo, a ação de retirada do braço em cima da chama quente ou quando nos furamos com algo. Segundo a *International Association from the Study of Pain* (IASP), a dor é considerada uma experiência sensorial e emocional desagradável associada ao dano tecidual potencial ou real (MERSKEY, BODDUK, 1994). Na pratica experimental é utilizado o termo nocicepção para referir-se ao estímulo nocivo através do dano tecidual captados por nociceptores localizados nas terminações das fibras nervosas. Ademais são empregados os termos alodinia e hiperalgesia, sendo o primeiro utilizado para designar dor decorrente de um estímulo não doloroso, como estímulos mecânicos e térmicos inócuos, e hiperalgesia, ao aumento da resposta a estímulos nocivos (CUNHA, 2004; VALERIO, 2009; FERREIRA Jr, 2010).

A percepção do estímulo nocivo ou nociceptivo, seja ele químico, mecânico ou físico, capaz de provocar injúria tecidual é detectado por nociceptores (neurônios nociceptivos). Os nociceptores são terminações nervosas livres de fibras aferentes primárias, e são elas que, quando ativadas, conduzem os estímulos nocivos.

Existem três tipos principais de fibras aferentes primárias que transmitem os estímulos periféricos ao sistema nervoso central: as fibras do tipo Aβ, Aδ e as do tipo C. As fibras Aδ e Aβ são mielinizadas e as do tipo C são amielinizadas. As fibras Aδ conduzem os estímulos mais rapidamente, em torno de 5-30 m/s e respondem a estímulos térmicos e mecânicos, enquanto as fibras do tipo C conduzem os estímulos a uma baixa velocidade (< 1m/s) porem com maior intensidade e respondem a estímulos de origem térmica, mecânica ou química (FERREIRA, 2009). Existem também uma outra classe de nociceptores chamados de “silenciosos” ou “adormecidos” (*silent* ou *sleeping nociceptors*) descritos por Schaible e Schimidt em 1988. Estes nociceptores não respondem a estímulos termicos e mecânicos, mas são ativados durante o processo inflamatório.

Durante a resposta imune contra o agente agressor ou estressor são liberados mediadores inflamatórios intermediarios e finais. Eles ativam receptores que causam a sensibilização neuronal através da modificação do limiar de ativação de canais iônicos, como os de sódio, cálcio e potássio, presente na membrana de neurônios e induzem uma alteração nos potenciais de repouso levando à transmissão do estímulo doloroso da periferia até a medula espinal e, da medula espinal até o sistema nervoso central (tálamo e córtex somatossensorial), onde será interpretada como dor (WILLIS, 1985; RUSSO, BROSE, 1998; WOOLF, COSTIGAN, 1999).

Vários mediadores químicos como endotelinas, substância P, prostaglandina (PG) e aminas simpáticas, sensibilizam de maneira direta os nociceptores (VERRI, 2006). Prostaglandinas e aminas agem sobre as fibras C e nociceptores silenciosos ativando segundos mensageiros como a via da adenosina monofosfato ciclico (AMPc), proteinas quinases (PKA e PKC), fosforilando canais de sódio e levando ao aumento do influxo intracelular de sodio (Na^+). O resultado disto é a despolarização e consequente excitabilidade da membrana neuronal e

transmissão do impulso nervoso (FERREIRA, 1994; CUNHA, 1999; VERRI, 2006).

Tem sido relatada a participação da via das proteinas quinases ativadas por mitógenos (MAPKs) na geração da hiperalgesia. A MAPK é ativada pela fosforilação de resíduos de tirosina e treonina e independente da ativação da PKA e PKC (DINA, 2003). Existem três principais MAPK: quinase ativada por receptores extracelulares (ERK), quinase-N-terminal c-Jun (JNK) e a p38 MAPK. A ERK está envolvidas na proliferação e diferenciação celular e no desenvolvimento da plasticidade neuronal, a p38 e a JNK quinase são proteínas ativadas pelo estresse e participam na resposta à lesão e morte celular. A ativação da ERK no gânglio da raiz dorsal e no corno dorsal de neurônios contribui para o desenvolvimento e manutenção da hipersensibilidade à dor e a p38 tem sido implicada em estados de dor aguda e crônica, neuropática e inflamatória (JIN, 2003).

Existem diferentes modelos experimentais no qual pode se avaliar a eficácia de um fármaco com prováveis propriedades analgésica e anti-inflamatória. Dentre eles podemos citar os modelos de dor manifesta como as contorções abdominais induzidas por ácido acético e fenil-p-benzoquinona, adjuvante completo de Freund (CFA) e teste da formalina. Nesses modelos, o estímulo inflamatório induz a liberação de mediadores que ativam os nociceptores induzindo uma resposta comportamental considerada nociceptiva por ser inibida por fármacos analgésicos. No caso da formalina, este teste apresenta duas fases, sendo os primeiros 5-10 minutos do teste mediados pela liberação de histamina e serotonina, e receptores acoplados a canal iônico. Já a segunda fase é considerada inflamatória, pois depende da produção de citocinas e outros mediadores (VERRI, 2006).

Em outro modelo, o da carrragenina, no qual avaliamos a hiperalgesia mecânica, a carragenina desencadeia a produção de citocinas pró-inflamatórias como o TNF-α, IL-1β e quimicionas como a CXCL1 (KC, quimiocinas derivada de queratinócitos) em camundongos. Essas citocinas induzem a produção de prostaglandina E_2, que por sua vez sensibiliza os nociceptores induzindo a hiperalgesia (dor) (VERRI, 2006). Ademais, a carragenina induz um aumento do estresse oxidativo que é detectado como redução da forma reduzida da glutationa. Esse aumento do estresse oxidativo ou produção de radicais livres pode contribuir para a produção de citocinas pró-inflamatórias. Ao mesmo tempo em que ocorre a

liberação de citocinas pró-inflamatórias também ocorre a liberação de citocinas anti-inflamatórias que contrabalanceiam o desenvolvimento da resposta inflamatória como a IL-10, a qual inibe a produção de TNF-α e lL-1β (BRASILEIRO FILHO, 2006). Dessa forma, existem alguns modelos que são extremamente úteis para a avaliação do efeito e mecanismos de ação de drogas anti-inflamatórias e analgésicas.

1.1.2 Citocinas

As citocinas são um grupo de proteínas que participam ativamente na imunidade inata e adaptativa e também são consideradas mediadores importantes na sensibilização dos nociceptores e no dano neuronal (VERRI, 2006, 2007). As citocinas são produzidas e liberadas em resposta a uma variedade de estímulos inflamatórios, como parasitas, vírus, fungos, bactérias e seus produtos tóxicos (lipopolissacarídeos - LPS), ou em resposta a outras citocinas (VERRI, 2007). Possuem baixo peso molecular, entre 5 kD e 140 kD, e geralmente tem tempo de meia vida curto. Atualmente, já foram descritas mais de 200 citocinas diferentes (BILATE, 2007).

A ação das citocinas ocorre após sua ligação a receptores específicos expressos em células-alvo, no qual desencadeia transdução de sinais no interior da célula (BILATE, 2007). Elas podem agir de maneira autócrina, parácrina ou hormonal, ou seja, as citocinas podem agir em receptores da própria célula produtora, em receptores de células vizinhas e/ou distantes, sendo transportadas pela corrente sanguínea. Há também outro grupo particular de citocinas denominadas quimiocinas (citocinas quimiotáticas) que agem em receptores de membrana acoplados à proteína G (VERRI, 2006) controlando a adesão, quimiotaxia e ativação de vários tipos de leucócitos e o recrutamento de células inflamatórias. As quimiocinas também atuam na hematopoiese, angiogênese, diferenciação celular e metástase de tumores (KRAYCHETE, 2006; BILATE, 2007).

Atualmente é bem aceito que citocinas constituem uma ligação entre injúria celular ou reconhecimento imunológico e os sinais da inflamação local e sistêmicos (BLACKWELL, CRISTMAN, 1996; DINARELLO, 2000; HOPKINS, 2003;

CUNHA, FERREIRA, 2003; CONTI, 2004; VERRI, 2006).

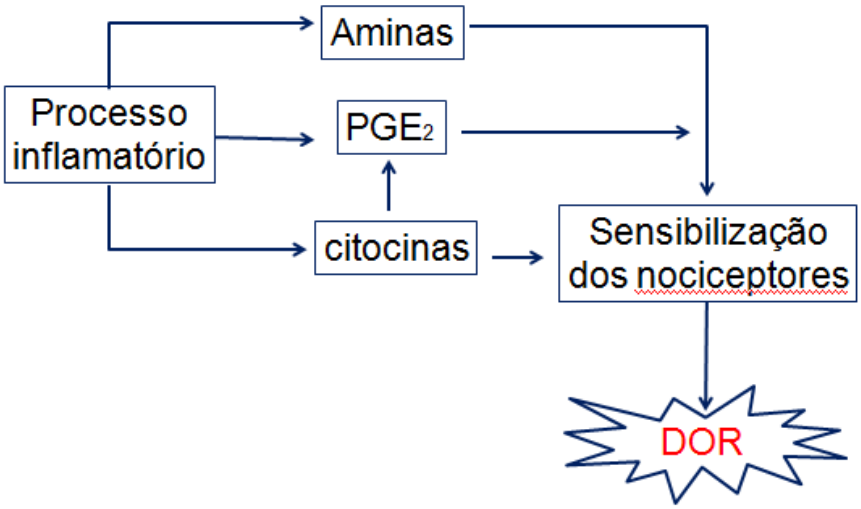
Cunha e colaboradores (1992) demonstraram a participação de citocinas na hiperalgesia mecânica induzida pela administração de carragenina (Cg) em ratos. Uma cascata de citocinas é liberada por células residentes ou que migraram para o foco inflamatório e são responsáveis pela liberação de mediadores finais (prostaglandina, aminas simpáticas e endotelinas) que sensibilizam os nociceptores (CUNHA, 1992, 2005; VERRI, 2004, 2005). Verificou-se também que perifericamente o TNF-α induz hipernocicepção através de duas vias independentes e paralelas: TNF-α ou CXCL1/KC (equivalente a IL-8 em humanos) → IL-1β → PGE_2 e, CXCL1 → aminas simpáticas em camundongos (CUNHA , 2005). Assim, a inibição da produção de citocinas é um mecanismo analgésico importante.

Também existem evidências que o TNF induz a ativação espinal da p38 MAPK na medula espinal nos gânglios da raiz dorsal (JI, 2002; KRAYCHETE, 2006) aumentando a condutividade de canais de sódio dependente de voltagem. A IL-1β ativa o receptor para IL-1β tipo I (IL1RI) induzindo a ativação das proteinas quinases especificas (ex. PKC) que causa a sensibilização neuronal. Há também, expressão desses receptores no gânglio da raiz dorsal, sugerindo a ação autócrina e parácrina da IL-1β no processo sensorial (SOMMER, 2004; KRAYCHETE, 2006). Corroborando a importância da IL-1, em modelos murinos de neuropatia, a administração de antagonistas de receptores IL1RI reduz o comportamento doloroso dos animais (SOMMER, 1999; KRAYCHETE, 2006).

Em resposta à liberação de citocinas pró-inflamatórias há a liberação de citocinas anti-inflamatórias como a IL-10, que inibe a produção de TNF-α e IL-1β (VERRI, 2006; BILATE, 2007).

Uma desregulação ou ativação exagerada de citocinas pró-inflamatórias implica em várias doenças como sepse, artrite reumatóide, doença de Crohn's ou esclerose multipla (SOMMER, KRESS, 2004). Com base no conhecimento das citocinas e sua relação com doenças inflamatórias, tem se utilizado medicamentos que inibem ou controlam sua produção e liberação. Considerando a importância das citocinas em várias doenças e como moléculas imunomoduladoras, são utilizados atualmente várias citocinas recombinantes,

anticorpos, receptores solúveis e antagonistas de receptores de citocinas na prática clínica e vários estão em testes clínicos (BILATE, 2007). Desta forma, drogas que modulem a produção e/ou ação de citocinas são importantes e apresentam perspectivas de desenvolvimento clínico.



1.1.3 Óxido Nítrico

O óxido nítrico (NO) é formado pela óxido nítrico sintase (NOS) a partir da L-arginina tendo como subproduto a L-citrulina. Existem três isoformas de NOS: NOS neuronal (nNOS), NOS endotelial (eNOS) e NOS induzível (iNOS). A nNOS e eNOS são vias constitutivas dependentes de Ca^{+2} e agem em baixas concentrações. A iNOS é independente de Ca^{+2} intracelular e é ativada por macrófagos estimulados por bactérias, toxinas e citocinas e requer algumas horas para ser expressa, mas uma vez sintetizada, libera quantidades maiores de NO. Há relatos da existência de uma quarta isoforma a mNOS presente em mitocondrias (DUSSE, 2003; COELHO, 2009; MICLESCU, GORDH, 2009).

O NO esta envolvido em muitos processos biológicos como vasodilatação do endotelio, relaxamento do músculo liso, inibição da adesão e agregação plaquetária, potencialização e depressão de longo prazo no hipocampo e cerebelo, age como neurotransmisssor com capacidade potencializadora, atuando

na memória e no aprendizado e regulação e liberação de outros neurotransmissores (FLORA FILHO, ZILERSTEIN, 2000; CERQUEIRA, 2002; COELHO, 2009). Desempenha sua função também no sistema imune, no qual ele é o principal mediador citotóxico de células imunes efetoras, sendo uma das mais importantes moléculas reguladora do sistema imune (DUSSE, 2003).

Porém, é importante resaltar que o NO possue papel dubio no qual em altas concentrações o NO pode ser citotóxico não só contra microrganismos mas para o próprio hospedeiro e está relacionado a algumas doenças como a asma, artrite reumatóide, choque séptico, doenças neurodegenerativas (Alzheimer e esclerose multipla), lesões ateroscleróticas, tuberculose e gastrite induzida por *Helicobacter pylori* (BARRETO, 2005; COELHO, 2009).

Varios estudos demonstram que o NO participa na resposta inflamatória podendo ter efeitos tanto pró como anti-inflamatório, tais efeitos dependem da concentração, do local de atuação e do modelo utilizado para se avaliar a dor (MICLESCU, GORDH, 2009).

O oxido nítrico formado pela NOS é rapidamente permeável através de membranas biológicas e ativa a enzima guanilato ciclase solúvel que produz um aumento nos niveis de guanosina monofosfato ciclico (GMPc). O GMPc estimula a proteína quinase dependente de GMPc, a proteina quinase G (PKG), que pode fosforilar diversas proteínas e ativar canais de potássio sensiveis ao ATP (K^+ATP), levando a hiperpolarização ou a saida de Ca^{+2} do citoplasma (MONCADA, 1991; PRADO, 2002; ARUL, KONDURI, 2009; COELHO, 2009), contribuindo para a vasodilatação e modulação da transmissão sináptica (MICLESCU, GORDH, 2009).

A via de sinalização NO/GMPc/PKG/canais K^+ATP contribui para a anti-hiperalgesia em modelos animais de dor e vem sendo alvo de diversos estudos de substancias potencialmente analgésicas. Drogas que bloqueiam de maneira direta a hiperalgesia agem bloqueando a sensibilização dos nociceptores por promover a abertura dos canais de potássio sensíveis a ATP, no qual a saída desse íon da célula contrabalançaria o limiar de ativação aumentado devido à modulação de canais de sódio e calcio durante a sensibilização neuronal (SACHS, CUNHA, FERREIRA, 2004; FERREIRA, 2008).

Para se avaliar o efeito de drogas sobre a via de sinalização do óxido nítrico são utilizados diversos inibidores da via L-arginina/NO/GMPc, uma vez que o uso de inibidores irá inibir a produção de NO e seus efeitos subsequentes (FILHO, ZILBERSTEIN, 2000; FERREIRA, 2008). Todas as isoformas de NOS podem ser inibidas por análogos da arginina N-substituidos como a L^G-monometil-L-arginina (L-NMMA), N-imino-etil-L-ornitina (L-NIO), L^G-amino-L-arginina (L-NAA), L^G-L-arginina (L-NA) e L^G -nitro-L-arginina metil-ester (L-NAME) (DUSSE, 2003).

A figura 1 mostra alguns inibidores da via L-arginina/NO/GMPc utilizados em experimentos.

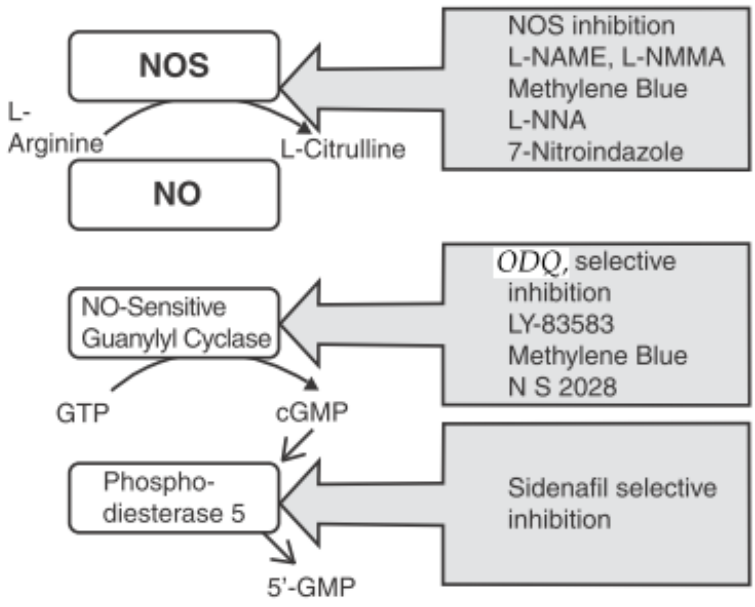


Figura 1: Via do óxido nítrico/guanosina monofosfato ciclico e seus inibidores. Oxido nítrico sintase (NOS) cataliza a formação de óxido nítrico (NO) pela L-arginina. NO ativa a guanilil ciclase solúvel (cGMP), que é catalizada pela guanosina trifosfato (GTP). No final da cascata o cGMP-especifico fosfatidilesterase tipo 5 hidrolase (5'-GMP). Inibidores: L-NAME, N-nitro-L-arginina metil-ester, um bloqueador da NOS; ODQ, 1H-[1, 2, 4] oxadiazolo[4, 3-a]quinoxalin-1-one, um bloqueador seletivo da NO sensível a guanilato ciclase; Sidenafil, um bloqueador seletivo da 5'-cGMP (leva a um aumento dos niveis de cGMP). *Fonte:* MICLESCU, GORDH, 2009.

1.2 Ácido caurenóico

Vários diterpenos vem sendo estudados na literatura por apresentar potencial biológicos e na pratica clinica, dentre eles destaca-se o ácido *ent*-kaur-16-en-19-oic ou ácido caurenóico (AC) (Figura 2), um diterpeno presente em diversas plantas entre as quais destacam-se a *Annona glabra, Aralia continentalis, Copaifera langsdorffii, Mikania glomerata, Mikania laevigata, Sphagneticola trilobata, Vigueira robusta* e *Xylopia frutescen* (CAVALCANTI, 2006; DE CARLI, 2009; LIN, 2009; SANTOS, 2005; SILVA, 2002; TIRAPELLI, 2004;).

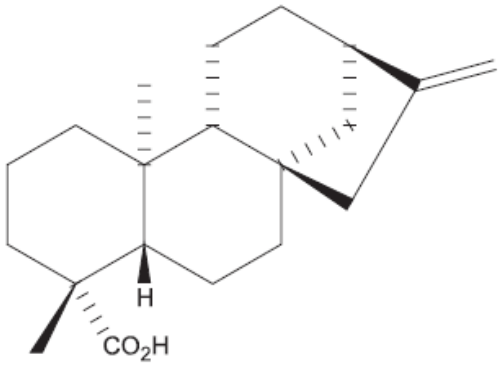


Figura 2 : Estrutura química do ácido caurenóico (*Fonte:* TIRAPELLI, 2004)

O AC apresenta diversas propriedades farmacológicas, dentre elas ação antibacteriana, no qual o extrato de plantas do gênero da *Mikania* contendo frações de AC inibiu o crescimento e a adesão de *Streptococcus mutans* (YATSUDA, 2005), o crescimento de *Staphylococcus aureus, Staphylococcus epidermidis* e *Bacillus cereus,* porem foi ineficaz contra o crescimento de bactérias gram negativas como *Escherichia coli* e *Pseudomonas aeruginosa* (SILVA, 2002). Demonstrou atividade *in vitro*, contra formas tripomastigotas de *Trypanossoma cruzi* (VIEIRA, 2002). Foi observada também atividade antifúngica ao inibir o crescimento de *Candida albicans* (DE CARLI, 2009; SANTOS, 2005).

Possui também efeito anti-tumorogênico no qual o AC apresentou atividade citotóxica contra diferentes linhagens de células tumorais da próstata,

cólon, mama (COSTA-LOTUFO, 2002; HENRY, 2006;) e uma inibição de mais de 95% no crescimento de células obtidas de crianças com leucemia (COSTA-LOTUFO, 2002).

O AC na dose de 10 mg/Kg diminuiu a concentração de glicose no sangue em ratos diabéticos induzido por aloxano, mostrando ser um agente hipoglicemiante (BRESCIANI, 2004).

Tirapelli em 2004 verificou *in vitro*, o efeito do ácido caurenóico em modelo de contração de artérias isoladas de ratos. O endotélio da artéria aorta íntegra ou seus anéis foram conectados a um transdutor de força e as contrações do músculo induzidas por fenilefrina ou cloreto de potássio (KCl). O pré-tratamento com AC reduziu as contrações induzidas por fenilefrina e KCl tanto no endotélio da aorta íntegra quanto nos anéis aórticos e causou o relaxamento do vaso. Isto se deve ao fato do ácido caurenóico bloquear os canais de cálcio pela ativação da via NO/GMPc e abertura dos canais de potássio (TIRAPELLI, 2004).

Cho e colaboradores (2010) avaliaram a atividade anti-espasmótica do diterpeno AC extraído da raiz da *Aralia cordata*. Em seus experimentos foram avaliados a ação do AC na resistência das vias aéreas na fase imediata e tardia da asma induzida por Ig-E em porcos sensibilizados com ovalbumina. Foram avaliados também o recrutamento de neutrófilos e a presença de mediadores químicos no fluido lavado broncoalveolar (BALF). O AC na dose de 50 mg/Kg diminuiu a resistência das vias aéreas na fase tardia da asma (40,52%±3,75%), o recrutamento de neutrófilos para o pulmão (de $8,85\pm0,95x10^5$ células para $3,51\pm0,70x10^5$ células) e a liberação de histamina e a atividade da fosfolipase A2 (PLA_2) analisadas no BALF.

A ação analgésica do ácido caurenóico foi demonstrada por Block em 1998. Extrato composto de ácido caurenóico extraído da *Wedelia paludosa* (ou *Sphagneticola trilobata*) foi administrado intraperitoneal (i.p.) nas doses de 1-60 mg/Kg em camundongos. A dose de 3 mg/Kg do extrato inibiu 55% o número de contorções induzido pela administração de ácido acético 0,6%, inibição maior que com o tratamento com acetominofeno, ácido acetilsalicílico, indometacina e dipirona (35, 38, 38 e 33% de inibição, respectivamente, na dose de 10 mg/Kg).

Ademais, a aplicação tópica do AC inibiu a formação de edema de orelha induzido por óleo de cróton e 12-O-tetradecanoilforbol acetato (TPA) em camundongos (BOLLER, 2010; DE CARLI, 2009). O AC extraído da *Aralia continentalis* também inibiu a formação de edema de pata induzido por Cg, porém não inibiu significativamente a produção de PGE_2 na concentração de 100μM em culturas de células RAW 264.7 ativadas por LPS. Os autores sugerem que esta diferença na atividade anti-inflamatória em modelos *in vivo* e *in vitro* pode estar relacionada ao mecanismo de ação do AC, como a inibição de citocinas pró-inflamatórias, embora, não tenha sido demonstrado que o AC iniba a produção de citocinas (LIN, 2009).

Em modelos de colite induzido pela administração de ácido acético, o AC reduziu significativamente o dano tecidual e o ganho de peso úmido do cólon. Diminuiu de maneira dose-dependente a atividade da mieloperoxidase (MPO), uma enzima presente dentro dos leucócitos e utilizada de forma indireta na análise de migração de leucócitos, especialmente neutrófilos, para o local inflamado, e diminuiu também a concentração de malondialdeido (MDA), um indicador da lipoperoxidação lipídica. Em modelos de colite induzida por ácido acético o aumento da MPO e MDA podem gerar radicais livres e consequente instalação do processo inflamatório e a formação de ulcerações (PAIVA, 2003).

Bürger e colaboradores em 2005 avaliaram a toxicidade aguda e subaguda do AC. Camundongos receberam por via oral (v.o.) extrato hidroalcoolico contendo AC na dose 100-4.000 mg/Kg em uma única dose (toxicidade aguda) ou durante 15 dias (toxicidade subaguda). Os comportamentos dos animais durante o tratamento e amostras de sangue e tecido foram analisados. O tratamento agudo e subagudo com AC não apresentou nenhuma mudança no comportamento em relação a ganho ou perda de peso, alterações no sistema respiratório, nervoso e gastrointestinal, não apresentou também nenhum efeito cutâneo ou aumento da taxa de mortalidade. A análise dos níveis de aspartato animotrasferase (AST), alanina aminotrasferase (ALT) e gama-glutamiltransferase (GGT) não demonstrou lesão hepatocelular e a análise macroscópica do fígado, pulmão, coração, baço e rim não apresentou nenhuma alteração em relação à cor e textura, indicando que o AC não apresenta toxicidade quando administrado em altas doses e por período mais longo.

Baseados nestes dados nós propomos investigar o efeito analgésico do AC em diferentes modelos nociceptivos e determinar seus possíveis mecanismos de ação.

2. OBJETIVOS

2.1 Objetivo geral

Investigar a atividade analgésica do ácido caurenóico, extraído da *Sphagneticola trilobata* (L.) Pruski, em diferentes modelos de dor inflamatória, bem como elucidar seu(s) mecanismo(s) de ação.

2.2 Objetivos específicos

- Avaliar o efeito analgésico do AC em diferentes vias de administração e dose-dependência;

- Avaliar o efeito analgésico do tratamento com AC em modelos experimentais através de diferentes estímulos nociceptivos (ácido acético, modelo da carragenina, formalina, CFA);

- Avaliar o efeito anti-inflamatório do AC em modelos de dor inflamatória aguda e prolongada;

- Avaliar se AC inibe a hiperalgesia induzida por mediadores que sensibilizam os nociceptores como a PGE_2;

- Avaliar se o efeito analgésico do AC estaria relacionado à inibição da produção de citocinas pró-hiperalgésicas como o TNF-α e IL-1β;

- Verificar a participação da via NO/GMPC/PKG/K+ATP no efeito analgésico do AC.

3 ARTIGO PARA PUBLICAÇÃO

Este é um trabalho realizado no Laboratório de Dor, Inflamação, Neuropatia e Câncer, formado pelo artigo científico: Kaurenoic acid from *Sphagneticola trilobata* (L.) Pruski inhibits inflammatory pain: effect on cytokine production and activation of NO/GMPc/PKG/K+ATP channels signaling pathway. Sandra S. Mizokami, Nilton S. Arakawa, Sergio R. Ambrosio, Ana C. Zarpelon, Rubia Casagrande, Thiago H. Ferreira, Fernando Q. Cunha, Waldiceu Aparecido Verri Júnior.

As formatações do artigo seguem as normas da revista *Journal of Natural Products* (Anexo).

Kaurenoic acid from *Sphagneticola trilobata* (L.) Pruski inhibits inflammatory pain: effect on cytokine production and activation of NO/GMPc/PKG/K+ATP channels signaling pathway.

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Abstract

Kaurenoic acid (**1**) is a diterpene present in many plants including *Sphagneticola trilobata* (L.) Pruski. The only evidence on its antinociceptive effect is that it inhibits the writhing response induced by acetic acid in mice. Therefore, we further addressed the analgesic effect of **1** in different models of pain and its mechanisms in mice. Intraperitoneal and oral treatments with **1** dose-dependently inhibited inflammatory nociception induced by acetic acid. The oral treatment with **1** also inhibited the nociception induced by phenyl-*p*-benzoquinone, both phases of formalin test and Complete Freund's Adjuvant (CFA). Compound **1** also inhibited acute carrageenin- and PGE_2-, and chronic CFA-induced inflammatory mechanical hyperalgesia. Mechanistically, **1** inhibited the production of hyperalgesic cytokines TNFα and IL-1β. Furthermore, the analgesic effect of **1** was inhibited by L-NAME, ODQ, KT5823 and glybenclamide treatments demonstrating that its analgesic effect also depends on activation of the NO/cGMP/PKG/K+ATP channels signaling pathway, respectively. These results consistently demonstrate that **1** presents analgesic effect, and that its mechanisms involve the inhibition of cytokine production and activation of the NO/cGMP/PKG/K+ATP channels signaling pathway.

Key words: Kaurenoic acid, cytokine, nitric oxide, inflammatory pain, K+ATP.

Introduction

Kaurenoic acid or *ent*-kaur-16-*en*-19-oic acid (**1**) is a diterpene obtained from a great number of plants, and is a major compound in *Sphagneticola trilobata* (*Wedelia paludosa*, *Acmella brasiliensis*, Asteraceae)[1], which is popularly known in Brazil as "*Arnica-do-mato", "pseudo-arnica", "picão-da-praia" and "vedélia".* There is evidence that **1** presents biological effects by inhibiting the inflammatory process such as carrageenin-induced paw edema[2,3] and TPA-induced ear edema.[4] Furthermore, in a model of asthma in guinea pigs, **1** inhibited the ovalbumin challenge-induced airway resistance in immunized animals as well as the production of histamine and activity of phospholipase A_2.[5] *In vitro* experiments also demonstrated that **1** inhibits LPS-induced production of nitric oxide[2,3] and prostaglandin E_2 (PGE_2) as well as the expression of cyclooxygenase-2 and inducible NO synthase (iNOS) in RAW 264.7 macrophages.[3] This inhibition of cyclooxygenase-2 and iNOS expression was probably related to inhibition of NFκB activation.[3] On the other hand, there is also evidence that **1** does not inhibit LPS-induced NFκB activation, nitrite production or mRNA expression of pro-inflammatory cytokines such as TNF-α and IL-1β, and cyclooxygenase-2.[6] Thus, the possible effect of **1** in these inflammatory pathways is still controversial and was only addressed *in vitro*.

Additional relevant biological effects of **1** are the vasorelaxant effect via NO/cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG)/ ATP sensitive potassium channels (K+ATP) and calcium channels depending on the experimental model;[7] and anti-microbial effects against *Bacillus cereus* and *Mycobacterium tuberculosis*.[8,9]

Sphagneticola trilobata is popularly used for rheumatic inflammatory diseases and fever.[1,11] Of note, rheumatic diseases such as rheumatoid arthritis are accompanied by pain.[12] In this sense, it was demonstrated that **1** inhibits acetic acid-induced writhing response in mice.[11,13] However, it remains to be determined if **1** inhibits inflammatory pain in other models and its analgesic mechanisms.

In terms of inflammatory pain, the sensitization of primary nociceptive neurons (nociceptors) occurs during inflammation, producing an increase in pain sensation (hyperalgesia). This sensitization is caused by direct action of inflammatory

mediators such as prostaglandins (e.g., PGE_2, PGI_2) and sympathetic amines (e.g., dopamine, epinephrine) on their receptors present in the membrane of nociceptors. It is also accepted that the release of these direct-acting hyperalgesic mediators is preceded by the release of a cascade of cytokines.[14,15] In the carrageenin model of paw inflammation, this cascade is initiated with the release of TNF-α.[16] Additionally, increasing attention has been drawn to the activation of the via NO/cGMP/PKG/K+ATP channels (nitric oxide/cyclic guanosine monophosphate/protein kinase G/ATP sensitive potassium channels) as an important analgesic signaling pathway since it is activated by morphine and other drugs.[17]

In view of the information presented above, it was investigated as to whether the antinociceptive effect of **1** depends on counteracting cytokines production/release and activation of the NO/cGMP/PKG/K+ATP channels analgesic signaling pathway in inflammatory pain.

Results and Discussion

Kaurenoic acid (1) Inhibits Writhing response Induced by Acetic Acid and Phenyl-*p*-Benzoquinone (PBQ). In the first series of experiments, the antinociceptive effect of **1** was evaluated in acetic acid and PBQ induced pain-like behavior. Mice were treated with **1** (3-30 mg/kg; DMSO 2% diluted in saline) ip (Figure 1A) or po (Figure 1B) 30 min before injection of acetic acid 0.8% or with 10 mg/kg of **1** po 30 min before injection of phenyl-*p*-benzoquinone (1890 µg/kg, Figure 1C), or vehicle (saline or DMSO 2% diluted in saline, respectively). All doses of **1** inhibited acetic acid-induced writhing response without differences between doses when it was administrated ip (Figure 1A). On the other hand, the po treatment with **1** produced a dose-dependent inhibition of acetic acid-induced writhing with significant differences between the doses of 10 and 30 compared to 3 mg/kg of **1**, and there was no difference between the doses of 10 and 30 mg/kg of **1** (Figure 1B). This difference might be related to **1** pharmacokinetics since ip absorption is expected to be faster than po absorption, and there were no differences in the effect of **1** at the doses of 10 and 30 mg/kg irrespectively to the route of administration. Therefore, it is unlikely that **1** is susceptible to gastrointestinal environment. In this sense, the dose of 10 mg/kg po of **1** was selected for next experiments regarding overt pain-like

behavior. Previous data also detected that **1** inhibits acetic acid-induced writhing response.[11,13] The po treatment with **1** also inhibited PBQ-induced writhing. Acetic acid and PBQ models of writhing response share some mechanisms as well as some mechanisms are model specific. For instance, these models share the participation of prostanoids.[18] In fact, the treatment with indomethacin (cyclooxygenase inhibitor, 5 mg/kg, ip, 40 min) inhibited acetic acid (Figure 1A)- and PBQ (Figure 1C)-induced writhing response.

Kaurenoic acid (1) Inhibits Paw Flinch and Time Spent Licking the Paw Induced by Formalin and Complete Freund's Adjuvant (CFA). Mice were treated with **1** as described above and received 25 µL of formalin 1.5% intraplantarly (i.pl.). Compound **1** inhibited both phases of formalin test regarding paw flinching (Figure 2A) and time spent licking the paw (Figure 2B). The first phase of formalin test is considered to be a direct effect of formalin in TRPA1 receptors present in primary nociceptive neurons[19] together with the involvement of histamine and serotonin release/action on nociceptors.[20] Considering that **1** inhibits histamine release in a model of asthma,[5] it is possible that this mechanism contributes to the inhibition of the first phase of formalin test. The second phase of the formalin test depends on indirect mechanisms such as prostaglandin and cytokine production.[21] Therefore, **1** could be inhibiting the action of direct and indirect acting inflammatory mediators. The CFA-induced inflammation is also dependent on cytokine production.[8] In Figures 2C and 2D, it was detected that **1** inhibited both paw flinching and licking behaviors induced by CFA (10 µl/paw), respectively.

Treatment with Kaurenoic Acid (1) inhibits Carrageenin- and CFA-induced Mechanical Hyperalgesia. Next, the antinociceptive effect of **1** was tested in the carrageenin (100 µg/paw)- and CFA (10 µL/paw)-induced mechanical hyperalgesia models. The po treatment with **1** dose-dependently (1-10 mg/kg) inhibited carrageenin-induced mechanical hyperalgesia (Figure 3A). No significant effects were observed with 1 mg/kg. On the other hand, 3 mg/kg of **1** significantly inhibited carrageenin-induced hyperalgesia at 1 and 3 h after stimulus, and 10 mg/kg inhibited the carrageenin-induced mechanical hyperalgesia 1-5 h after stimulus with significant differences compared to the dose of 1 mg/kg of **1** (Figure 3A). Therefore,

10 mg/kg of **1** was selected for mechanical hyperalgesia-related experiments. Indomethacin treatment significantly inhibited carrageenin-induced mechanical hyperalgesia (Figure 3A). To further address the possible therapeutic usefulness of the compound, mice were daily treated with **1** starting 1h after CFA i.pl. injection during 7 days (Figure 3B). There was significant inhibition of mechanical hyperalgesia at all time points evaluated except in the first day after CFA injection for both **1** and control drug indomethacin (Figure 3B).

In addition to inflammatory hyperalgesia, **1** also inhibits carrageenin-induced paw edema,[2,3] supporting a wider potential therapeutic applicability of **1** in inflammatory conditions.

Kaurenoic Acid (1) Inhibited TNF-α and IL-1β Production Induced by Carrageenin. Carrageenin-induced inflammatory hyperalgesia is mediated by a cascade of cytokines.[16] In turn, the hyperalgesic role of cytokines is mediated by the production of final sensitizing mediators such as PGE_2 and sympathetic amines.[16,22,23] Three h after injection of carrageenin mice were terminally anesthetized and the cutaneous plantar tissue was collected for cytokine (TNF-α and IL-1β) measurement (Figure 4). Compound **1** inhibited carrageenin-induced production of TNF-α (Figure 4A) and IL-1β (Figure 4B) in the paw tissue. This inhibition of cytokine production by **1** might account for the inhibition of cyclooxygenase-2 expression[3] since both cytokines induce it.[14,16] Furthermore, the present data demonstrating the inhibition of cytokine production by **1** sheds light in a controversy on whether it inhibits or not NFκB activation and mRNA expression of TNF-α and IL-1β as determined in vitro,[3,6] indicating that these mechanisms might be of importance in vivo and the inhibition of cytokine production is probably related to the inhibition of NFκB activity as previously described.[3] It is noteworthy to mention that, to our knowledge, this is the first study to demonstrate that **1** inhibits cytokine production. Besides being important cytokines in carrageenin hyperalgesia, TNF-α and IL-1β are considered important targets to control a great number of inflammatory diseases.[24]

Kaurenoic Acid (1) Reduced PGE_2-induced Mechanical Hyperalgesia. Mice were treated with **1** (10 mg/kg, po) or vehicle 30 min before i.pl. injection of

PGE_2 (100 ng/paw) (Figure 5). There was significant inhibition of PGE_2-induced mechanical hyperalgesia at 3 h by **1** treatment. Since PGE_2 acts directly on nociceptive neurons to produce mechanical hyperalgesia, it is likely that the analgesic effect of **1** might be through activation of neuronal events.[14,16]

Kaurenoic Acid (1) Inhibits Inflammatory Pain by Activating the NO/cGMP/PKG/K+ATP Channels Signaling Pathway. Mice were treated with L-NAME (NOS inhibitor, 10-90 mg/kg, ip) 60 min before **1** (10 mg/kg, po) treatment (figure 6A). After additional 30 min, mice received i.pl. injection of carrageenin (100 µg/paw), and mechanical hyperalgesia was evaluated 1, 3 and 5 h after injection of carrageenin. Corroborating the data presented above, **1** inhibited the carrageenin-induced mechanical hyperalgesia. In turn, L-NAME dose-dependently inhibited **1** analgesic effect. The dose of 10 mg/kg of L-NAME did not affect **1** antinociception, and the doses of 30 and 90 mg/kg of L-NAME significantly inhibited **1** effect. The dose of 90 was also statistically different of the 10 mg/kg dose of L-NAME. There was no difference between the doses of 30 and 90 mg/kg of **1** (Figure 6A). Similar results were obtained at 1 and 5 h after carrageenin injection (data not shown), but we presented only the 3rd h to increase the clarity of data. Therefore, in addition to inhibition of cytokine production, the antinociceptive effect of **1** depends on induction of NO production. Although we did not determine the isoform of NOS responsible for **1**-induced NO production, it has been shown that selective inhibitors of nNOS and eNOS, but not iNOS, reduced the vasorelaxing effects of **1**.[7] This information[7] also explains the apparent contradiction with previous data demonstrating that **1** inhibits LPS-induced NO production and iNOS expression.[3,6] Therefore, it is likely that **1** presents a dual effect on NO production since it can increase NO production by activating nNOS and eNOS,[7] and reduce NO production by inhibiting the expression of iNOS.[3] In the present experimental conditions, it is very likely that **1** is activating nNOS, because a series of studies demonstrated that nNOS-derived NO is a prominent analgesic mechanism shared by morphine, diclofenac and other clinically available drugs.[17] NO also inhibits the hyperalgesia induced by PGE_2 and other direct acting hyperalgesic mediators.[17] Thus, the present result on the role of NO in the analgesic effect of **1** explains the inhibition of PGE_2-induced hyperalgesia (Figure 5). NO activates the cGMP/PKG/K+ATP channels signaling pathway to induce

analgesia, therefore, the next experiments assessed whether **1** would also activate similar mechanisms to reduce inflammatory mechanical hyperalgesia. Mice were treated with ODQ (Figure 6B, soluble cGMP inhibitor; 0.3 mg/kg, ip, diluted in 2% DMSO in saline, 30 min), KT5823 (Figure 6C, PKG inhibitor; 0.5 µg/mice, ip, diluted in 2% DMSO in saline, 5 min), glybenclamide (Figure 6D, K+ATP channels blocker; 0.3 mg/kg, ip, diluted in 5% of Tween 80 in saline, 45 min) or the respective vehicle before **1** (10 mg/kg, po). After additional 30 min mice received i.pl. injection of carrageenin (100 µg/paw). Again, **1** significantly inhibited the carrageenin-induced mechanical hyperalgesia at 1, 3 and 5 h after stimulus, and the treatment with ODQ (Figure 6B), KT5823 (Figure 6C) or glybenclamide (Figure 6D) significantly inhibited **1** analgesic effect. Thus, the analgesic effect of **1** depends on the activation of the NO/cGMP/PKG/K+ATP channels signaling pathway, which diminishes the nociceptive transmission resulting in diminished inflammatory mechanical hyperalgesia.[17]

Kaurenoic Acid (1) Showed no Muscle-relaxing or Sedative Effects. Per oral treatment with 10 mg/kg of **1** did not alter the motor response of the test animals 1.5, 3.5 and 5.5 h after treatment (n = 6). These time points were based on the 30 min of pretreament plus 1, 3 and 5 h until mechanical hyperalgesia measurement in the carrageenin model. The vehicle control responses and **1**-treated animals in the rota-rod test were: 120 s vs 120 s (1.5 h); 120 s vs 120 s (3.5 h); and 120 s vs 120 s (5.5 h), respectively (data not shown). These results support the notion that **1** is diminishing the nociceptive threshold induced by inflammation, and not that the mice are incapable of responding because of muscle-relaxing or sedative effects.[25] Previous data showed that **1** reduces methanphetamine-induced locomotor enhancement suggesting sedative effects.[13] However, this effect was achieved with 300 mg/kg of **1**, a 30 fold higher dose compared to the present experimental condition.

Kaurenoic Acid (1) did not Exhibit Any Effect in the Hot Plate Test in Naive Mice. Mice were treated with **1** (10 mg/kg, po route) or morphine hydrochloride (8 mg/kg, ip route) and the thermal nociception was evaluated before, and 1.5, 3.5 and 5.5 h after treatment (data not shown) in the same manner as for rota-rod test.

Morphine hydrochloride treatment increased the thermal nociceptive threshold as expected because of its central analgesic effects. On the other hand, **1** did not alter the thermal threshold of mice (data not shown). This result further supports a peripheral neuronal effect of **1** upon inflammatory pain, since the hot plate test is considered to be modulated by supraspinal mechanisms.[26]

In conclusion, the present study has further analyzed the antinociceptive activity of **1** upon inflammatory pain and has provided novel evidence on its effect in a variety of inflammatory pain models and for its mechanism of action. The antinociceptive mechanisms of action of **1** may depend on: (a) inhibition of pro-hyperalgesic cytokine production, TNFα and IL-1β; (b) inhibition of cyclooxygenase-2, and consequently PGE_2 production;[2,3] (c) reduction of hyperalgesia produced by a directly acting mediator such as PGE_2; (d) activation of the NO/cGMP/PKG/K+ATP channels signaling pathway. As a consequence, there is reduced sensitization of nociceptors due to reduced cytokine and PGE_2[2,3] production and reduced nociceptive transmission related to activation of the NO/cGMP/PKG/K+ATP channels signaling pathway. Therefore, the presence in high concentrations of **1** it presents promising antinociceptive activity, it merits further pre-clinical and clinical investigation.

Experimental Section

General Experimental Procedures. Nuclear Magnetic Resonance (NMR) spectra were run on a Bruker DPX 400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). Samples were dissolved in $CDCl_3$, and the spectra were calibrated with the solvents signals at δ 7.26 (^{1}H) and δ 77.0 (^{13}C). Vaccum Liquid Chromatography (VLC) was carried out using silica gel 60H (Merck, art. 7736) in glass columns with 5-10 cm i.d.[27] High Performance Liquid Chromatography (HPLC) analyses were accomplished using a Shimadzu CBM-20A liquid chromatography controller, operating with the LCsolution software, equipped with a Shimadzu UV-DAD detector SPD-M20A and a Shimadzu ODS column (4.6 x 250 mm, 5 µm, 100 Å).

In vivo experiments, mice received per oral (1, 3, 10 and 30 mg/kg) or intraperitoneal (3, 10 and 30 mg/kg) treatment with kaurenoic acid (**1**) or vehicle (2% DMSO in saline) 30 min before inflammatory stimulus. The doses of inflammatory

stimuli were determined previously in our laboratory in pilot studies and based on previous work.[16,18,25,28-30] Mechanical hyperalgesia was evaluated 1-5 h after carrageenin (100 μg/paw), 3 h after PGE_2 (100 ng/paw), or 1-7 days after CFA (10 μL/paw) stimulus. All inflammatory stimuli induced only ipsilateral (in the paw the stimulus was injected) mechanical hyperalgesia. IL-1β and TNF-α levels were evaluated 3 h after carrageenin (100 μg/paw) injection. The writhing response was evaluated for 20 min after po injection of acetic acid or phenyl-p-benzoquinone. The paw flinching and licking nociceptive responses were quantified for 30 min after formalin 1.5% (25 μL/paw) or CFA (10 μL/paw) injection.

Plant Material. The air-dried roots of *Sphagneticola trilobata* were collected in September 2011 at Horto de Plantas Medicinais (23°19'41"S/51°12'14"W) of Centro de Ciências Agrárias at Universidade Estadual de Londrina (UEL). A voucher specimen was identified by Profa. Dra. Vieira, A.O.S. of the Departamento de Biologia Animal e Vegetal (Centro de Ciências Biológicas, UEL) and was deposited at Herbário da Universidade Estadual de Londrina (FUEL) no. 49306, collected by N.S. Arakawa-001.

Isolation and Identification of kaurenoic acid (1). The air-dried roots was pulverized and then exhaustively extracted with dichloromethane (900 mL) at room temperature, to give 1,2 g crude extract, which was suspended in 300 mL methanol/H_2O (9:1) and filtered. The soluble fraction was partitioned using n-hexane (300 mL, four times), which resulted in 0.7 g n-hexane-soluble fraction after solvent evaporation under reduced pressure. The n-hexane-soluble fraction was chromatographed over Silica gel 60 (0.063 – 0.200 mm) using vaccum chromatography with n-hexane and increasing amounts of ethyl acetate as eluents (250 mL each fraction). The second fraction (0.41 g) was washed with cold methanol, to afford kaurenoic acid (KA, *ent*-kaur-16-en-19-oic acid; 800 mg, purity 96%, as determined by HPLC), which was identified by spectroscopic analysis, compared by HPLC with authentic standard and comparison with literature data.[31,32]

Animals. Male Swiss mice (25-30 g) from the Universidade Estadual de Londrina, Londrina, Parana, Brazil were used in this study. Mice were housed in standard clear plastic cages with free access to food and water, a light/dark cycle of 12:12 h, and kept at 21 ^{o}C. All behavioural testing was performed between 9 a.m.

and 5 p.m. in a temperature-controlled room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 1440.2011.09). All efforts were made to minimize the number of animals used and their suffering.

Nociception Tests. Electronic Pressure-meter Test. Mechanical hyperalgesia was tested in mice as previously reported.[33] In a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors, 15-30 min before the start of testing. The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic anesthesiometer; Insight, Ribeirao Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were tested before and after treatment. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements 3 h after stimulus. Withdrawal threshold was 9.1 ± 0.4 g (mean ± SEM; n = 30) before injection of the hyperalgesic agents (e.g., PGE_2, CFA or carrageenin).

Writhing Response Tests. The phenyl-*p*-benzoquinone (PBQ) and acetic acid-induced writhing models were performed as previously described.[18] PBQ (diluted in DMSO 2%/saline, 1890 µg/kg), acetic acid (0.8% v/v, diluted in saline, 10 mL/kg), or vehicle was injected into the peritoneal cavities of mice pre-treated with kaurenoic acid (**1**) (3-30 mg/kg, ip route). Each mouse was placed in a large glass cylinder and the intensity of nociceptive behaviour was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection. The writhing response consisted of a contraction of the abdominal muscle together with a stretching of hind limbs. The intensity of the writhing response was expressed as the cumulative writhing score over 20 min. Different individuals administered each test, prepared solutions to be injected, and performed the injections.

Formalin Test. The number of paw flinches and time spent licking the paw were determined between 0-30 min after intraplantar injection of 25 µL of formalin 1.5%, as previously described.[25,34] The period was divided in intervals of 5 min, and clearly demonstrated the presence of the first and second phases, which are characteristic of the method.[25,34] Results were presented as first (0-15 min) and second phase (20-30 min).

Hot Plate Test. Mice were placed in a 10 cm-wide glass cylinder on a hot plate (IITC Life Science Inc. Woodland Hills, CA) maintained at 55 °C. Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 5–9 s. The latency was also evaluated 1.5, 3.5 and 5.5 h after test compound administration. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cut-off) was set at 30 s to avoid tissue damage.[23]

Measurement of Motor Performance. In order to discard possible non-specific muscle relaxant or sedative effects of kaurenoic acid **(1)**, mice motor performance was evaluated on the rota-rod test.[28] The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (Ugo Basile, Model 7600). The bar rotated at a constant speed of 22 rotations per min. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 180 s. Animals were treated with vehicle (Tween 80 20% in saline) or 1 (10 mg/kg, po), and testing was performed 1.5, 3.5 and 5.5 h after treatement. The cut-off time used was 180 s.

Cytokine Measurement. Mice were treated with vehicle or kaurenoic acid (**1**) (10 mg/kg, po) 30 min before carrageenin (100 µg/paw) stimulus. Three h after the injection of carrageenin, mice were terminally anesthetized, and the skin tissues were removed from the injected and control paws (saline and naive). The samples were homogenized in 500 µL of buffer containing protease inhibitors, and IL-1β and TNF-α levels were determined as described previously[29,30] by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. The results are expressed as picograms (pg) of cytokine/paw. As a control, the concentrations of these cytokines

were determined in animals injected with saline.

Statistical Analysis. Results are presented as mean ± S.E.M. of experiments made on 5 (Figures 3A, 4-6) or 6 (Figures 1-2, 3B) animals per group, and are representative of two separate experiments. Differences between groups were evaluated by analyses of variance (one-way ANOVA) followed by the Tukey's test. Statistical differences were considered to be significant at $p < 0.05$.

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Legends to Figures

Figure 1. Kaurenoic acid (**1**) inhibits writhing response induced by acetic acid and phenyl-*p*-benzoquinone (PBQ). Mice were treated ip (Panel A) or po (Panel B) with **1** (3-30 mg/kg) or vehicle (DMSO 2% diluted in saline) 30 min before an acetic acid (Panel A) injection. In another set, mice were treated with **1** (10 mg/kg, po, 30 min) vehicle 30 min before a phenyl-*p*-benzoquinone (PBQ, Panel C) injection. Control groups were treated with indomethacin (5 mg/kg, ip) 40 min before acetic acid or PBQ injection. The cumulative number of writhings (writhing score) was evaluated for 20 min. *n* = 6 mice per group per experiment, representative of two separate experiments. [* $p < 0.05$ compared with the saline group, $^{\#}$ $p < 0.05$ compared to the vehicle group and ** $p < 0.05$ compared to the dose of 3 mg/kg of **1** (one-way ANOVA followed by Tukey test)].

Figure 2. Kaurenoic acid (**1**) inhibits paw flinch and time spent licking the paw induced by formalin and complete Freund's adjuvant (CFA). Mice were treated with **1** (10 mg/kg, po, 30 min) or vehicle before the injection of formalin (25 µL of 1.5% formalin in saline, Panels A and B) or CFA (10 µL/paw, Panels C and D). The total number of flinches (Panels A and C) and time spent licking the paw (Panels B and D) were evaluated for 30 min and expressed between 0-15 and 20-30 min (Panels A and B) or the cumulative number (Panels C and D). *n* = 6 mice per group per experiment, representative of two separate experiments. [* $p < 0.05$ compared with the saline group, $^{\#}$ $p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey test)].

Figure 3. Treatment with kaurenoic acid (**1**) inhibits carrageenin- and CFA-induced mechanical hyperalgesia. Mice were treated with **1** (1-10 mg/kg, po, 30 min) or vehicle before the carrageenin (100 µg/paw) injection (Panel A). In another set, mice were treated daily **1** (10 mg/kg, po) starting 1 h after CFA (10 µL/paw) injection (Panel B). Control groups were treated with indomethacin 40 min (5 mg/kg, ip) before carrageenin or every 24 h starting 1 h after CFA (2 mg/kg, po). The intensity of hyperalgesia was measured 1-5 h (Panel A) or every 24 h (Panel B) by the electronic pressure-meter test. *n* = 5 mice per group per experiment, representative of two

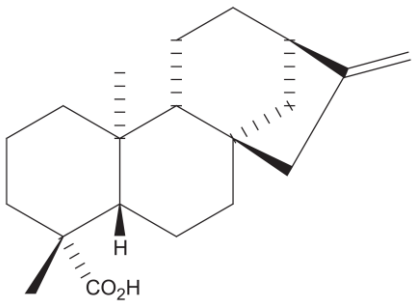
separate experiments. [* $p < 0.05$ compared with the saline group, $^{\#}$ $p < 0.05$ compared to the vehicle group and ** $p < 0.05$ compared to the dose of 1 mg/kg of **1** (one-way ANOVA followed by Tukey test)].

Figure 4. Kaurenoic acid (**1**) inhibited TNF-α and IL-1β production induced by carrageenin. Mice were treated with **1** (10 mg/kg, po, 30 min) or vehicle before the i.pl. injection of carrageenin. Three hours after carrageenin injection, mice were sacrificed and paw skin samples were collected for the determination of TNF-α and IL-1β production. n = 5 mice per group per experiment, representative of two separate experiments. [* $p < 0.05$ compared with the saline group, and $^{\#}$ $p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey test)].

Figure 5. Kaurenoic acid (**1**) reduced PGE_2-induced mechanical hyperalgesia. Mice were treated with **1** (10 mg/kg, po, 30 min) or vehicle before PGE_2 (100 ng/paw) injection. The intensity of mechanical hyperalgesia was measured 3 h after stimulus injection by the electronic pressure-meter test. n = 5 mice per group per experiment, representative of two separate experiments. [* $p < 0.05$ compared with the saline group, $^{\#}$ $p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey test)].

Figure 6. Kaurenoic acid (**1**) inhibits inflammatory pain by activating the NO/cGMP/PKG/K+ATP channels signaling pathway. Mice were treated with L-NAME (NOS inhibitor, 10-90 mg/kg, ip) 60 min before **1** (10 mg/kg, po) treatment (Panel A). After additional 30 min, mice received i.pl. injection of carrageenin (100 µg/paw), and mechanical hyperalgesia was evaluated after 3 h. Mice were treated with ODQ (Panel B, soluble cGMP inhibitor; 0.3 mg/kg, ip, diluted in 2% DMSO in saline), KT5823 (Panel C, PKG inhibitor; 0.5 µg/mice, ip, diluted in 2% DMSO in saline, 5 min), glybenclamide (Panel D, K+ ATP channels blocker; 0.3 mg/kg, ip, diluted in 5% of Tween 80 in saline, 45 min) or the respective vehicle before **1** (10 mg/kg, po). After additional 30 min mice received i.pl. injection of carrageenin (100 µg/paw). Mechanical hyperalgesia was measured 1, 3 and 5h after stimulus injection. n = 5 mice per group per experiment, representative of two separate experiments. [* $p < 0.05$ compared with the saline group, $^{\#}$ $p < 0.05$ compared to the vehicle(s) group, ** $p < 0.05$ compared to the groups treated only with **1**, and ^{f} $p < 0.05$ compared to the dose of 10 mg/kg of L-NAME (Panel A) (one-way ANOVA followed by Tukey test)].

Structure Sheet



1

Figure 1

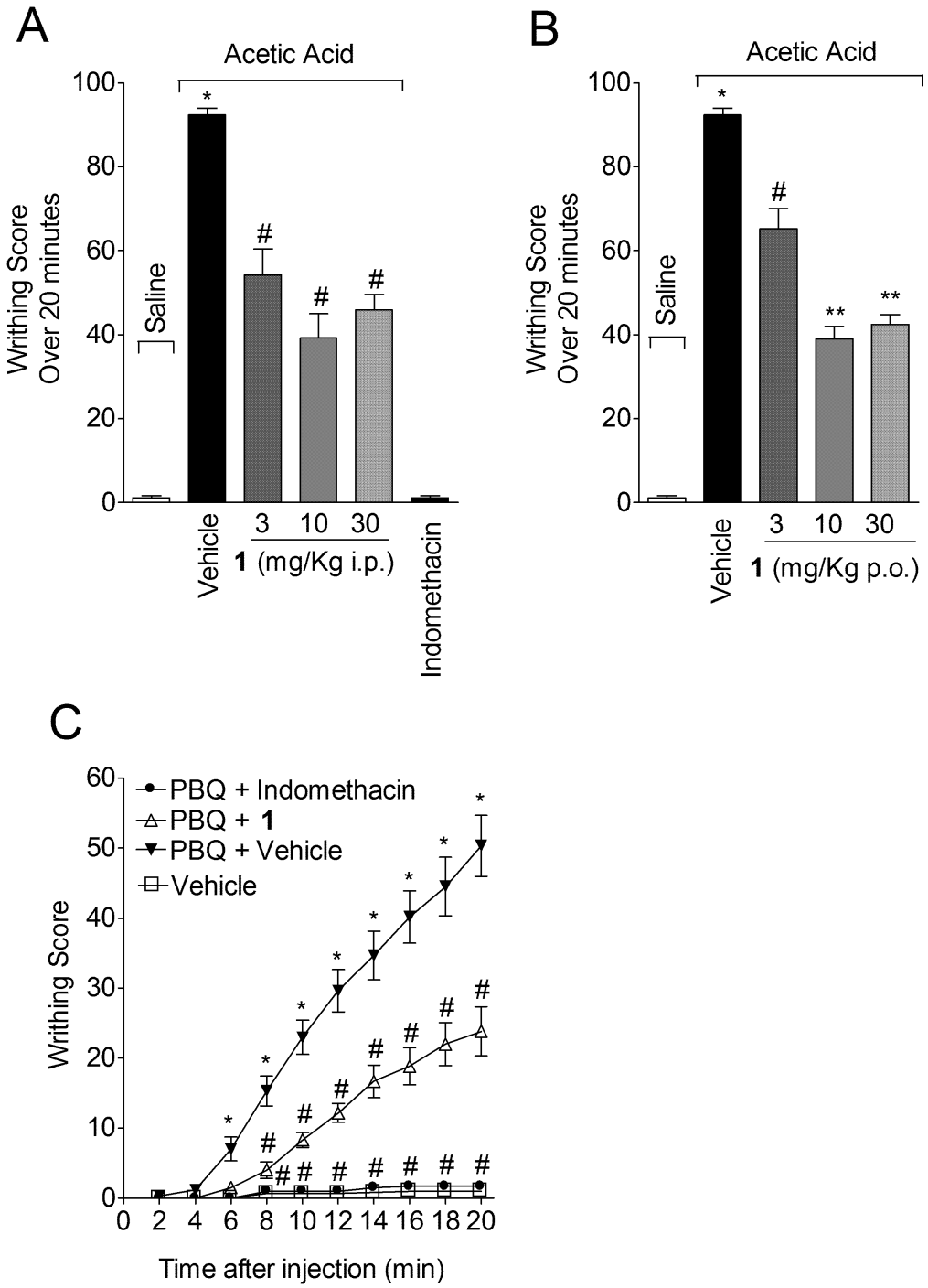

A
Acetic Acid
Writhing Score
Over 20 minutes
Saline
Vehicle
3
10
30
1 (mg/Kg i.p.)
Indomethacin
B
Acetic Acid
Writhing Score
Over 20 minutes
Saline
Vehicle
3
10
30
1 (mg/Kg p.o.)
C
PBQ + Indomethacin
PBQ + 1
PBQ + Vehicle
Vehicle
Writhing Score
Time after injection (min)

Figure 2

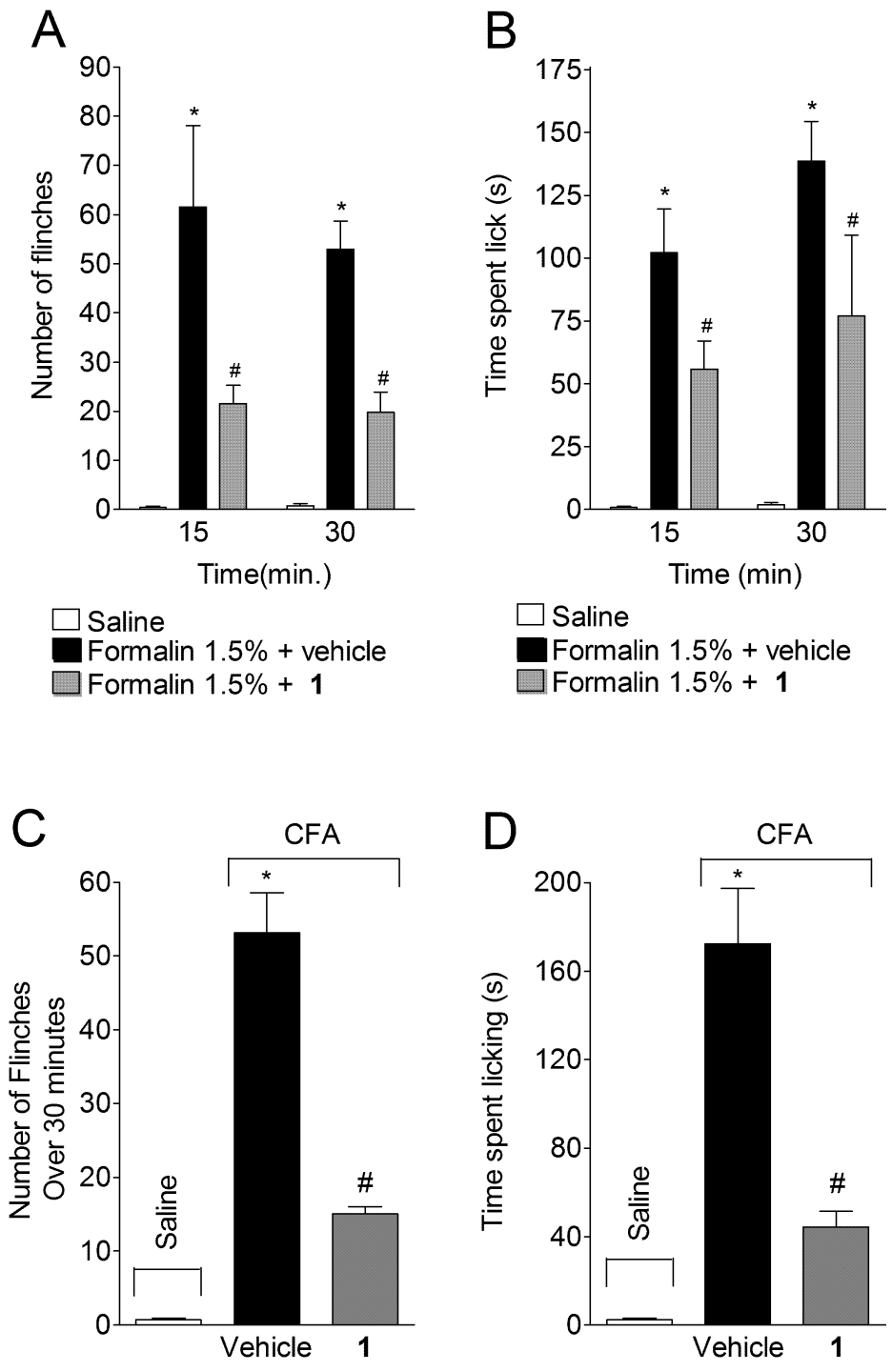

A
Number of flinches
90
80
70
60
50
40
30
20
10
0
15
30
Time(min.)
Saline
Formalin 1.5% + vehicle
Formalin 1.5% + 1
B
Time spent lick (s)
175
150
125
100
75
50
25
0
15
30
Time (min)
Saline
Formalin 1.5% + vehicle
Formalin 1.5% + 1
C
CFA
Number of Flinches
Over 30 minutes
60
50
40
30
20
10
0
Saline
Vehicle
1
D
CFA
Time spent licking (s)
200
160
120
80
40
0
Saline
Vehicle
1

Figure 3

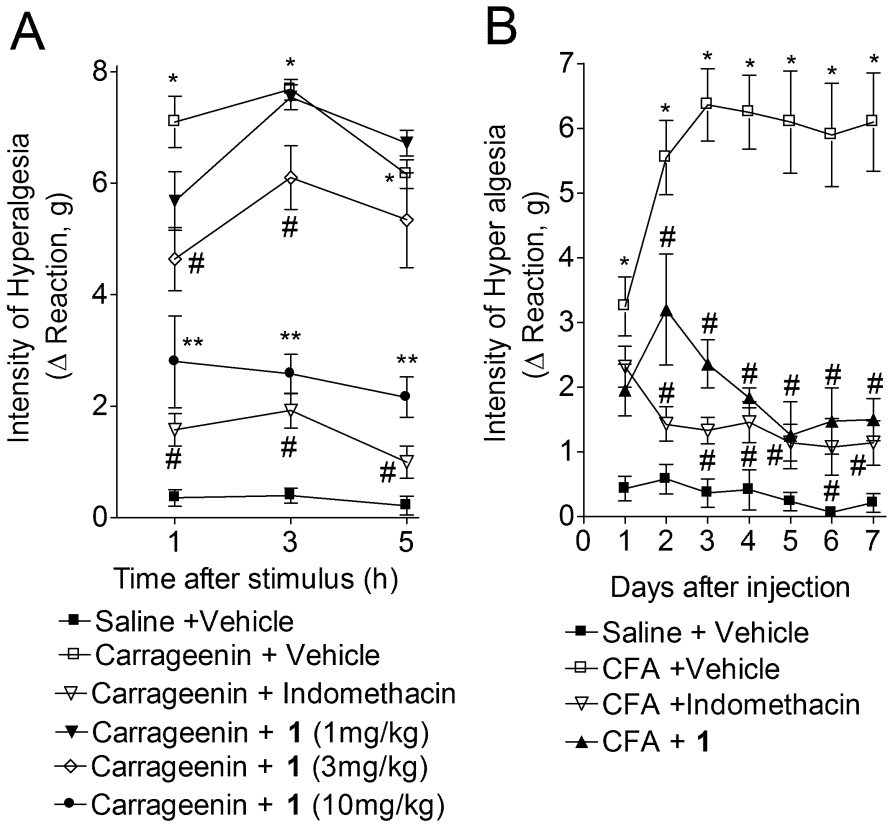

A
Intensity of Hyperalgesia
(Δ Reaction, g)
Time after stimulus (h)
Saline +Vehicle
Carrageenin + Vehicle
Carrageenin + Indomethacin
Carrageenin + 1 (1mg/kg)
Carrageenin + 1 (3mg/kg)
Carrageenin + 1 (10mg/kg)
B
Intensity of Hyper algesia
(Δ Reaction, g)
Days after injection
Saline + Vehicle
CFA +Vehicle
CFA +Indomethacin
CFA + 1

Figure 4

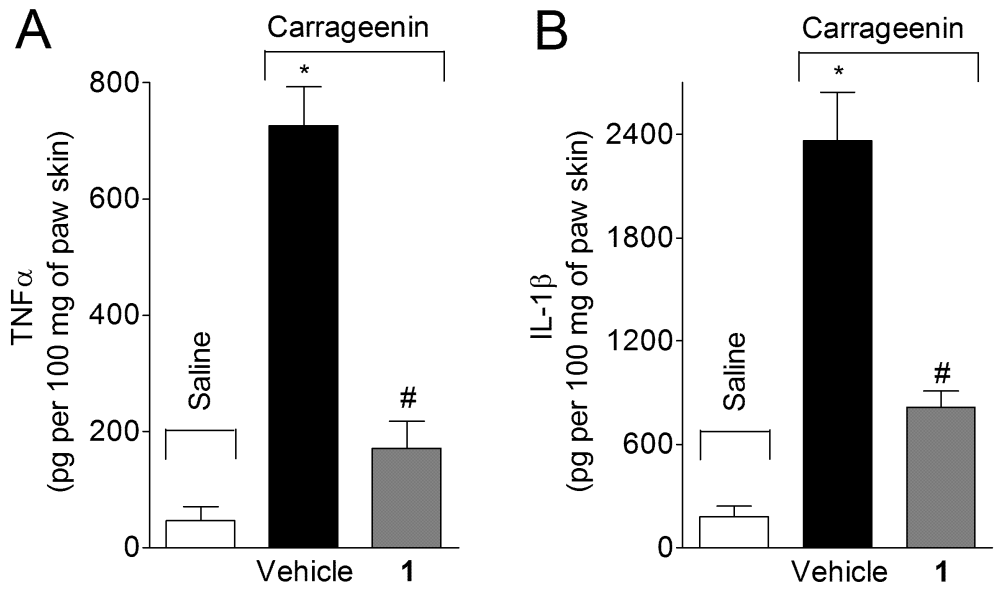

A
Carrageenin
*
800
600
400
200
0
TNFα
(pg per 100 mg of paw skin)
Saline
#
Vehicle
1
B
Carrageenin
*
2400
1800
1200
600
0
IL-1β
(pg per 100 mg of paw skin)
Saline
#
Vehicle
1

Figure 5

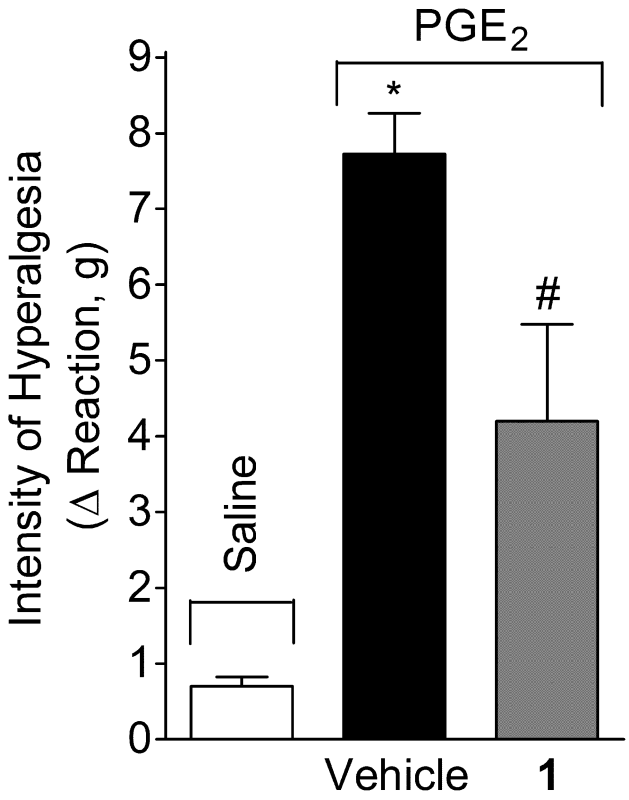
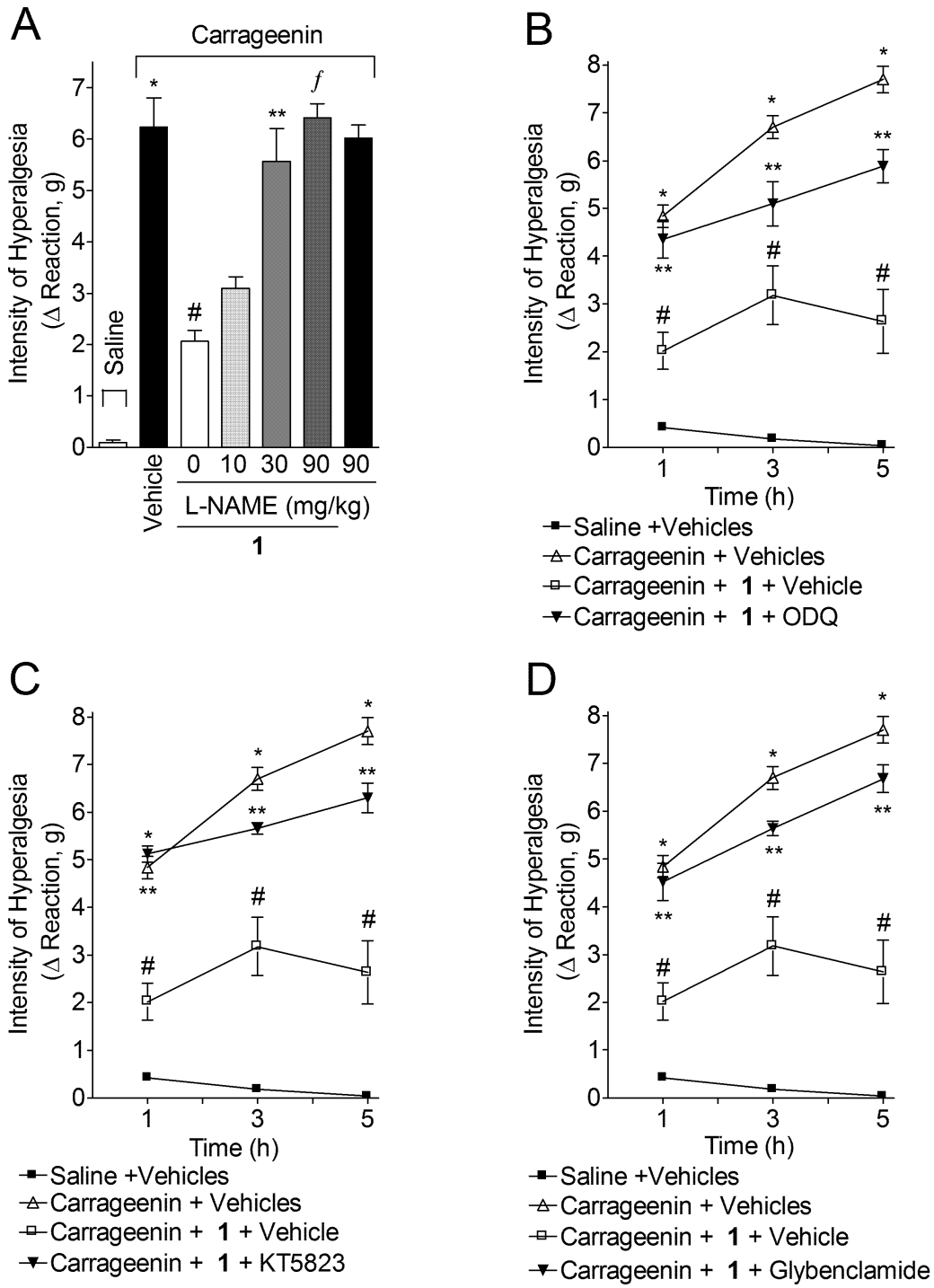

PGE2
*
#
Intensity of Hyperalgesia
(Δ Reaction, g)
Saline
0
1
2
3
4
5
6
7
8
9
Vehicle
1

Figure 6


A
Carrageenin
Intensity of Hyperalgesia (Δ Reaction, g)
Saline
Vehicle
0 10 30 90 90
L-NAME (mg/kg)
1
B
Time (h)
Saline +Vehicles
Carrageenin + Vehicles
Carrageenin + 1 + Vehicle
Carrageenin + 1 + ODQ
C
Carrageenin + 1 + KT5823
D
Carrageenin + 1 + Glybenclamide

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ANEXOS

ANEXO A

Preparation and Submission of Manuscripts

Title Page

The title should appear on a separate page and should be followed by the author names and the institution name and address. The title, author name(s), and affiliations should all appear on their own respective line of text. Place an asterisk after the name of the author to whom enquiries regarding the paper should be directed and include that author's telephone and fax numbers and e-mail address. Author affiliations must be footnoted using the following symbols in order (which should be used as superscripts): †, ‡, §, ⊥, ‖, ∇, O. In article titles, it is not necessary to use words like "new" or "novel" (with the latter referring specifically to a compound based on an unprecedented carbon skeleton) or to specify the number of new substances obtained.

Abstract

The abstract, detailing, in one paragraph, the problem, experimental approach, major findings, and conclusions, should appear on the second page. It should be double spaced and should not exceed 200 words for Full Papers and Reviews or 100 words for Notes and Rapid Communications. Compounds mentioned in the abstract, and given as specific Arabic numerals that are bolded in the text, should also be accompanied in the abstract by the same bolded numerals. The abstract should be on a separate page and should be untitled.

Introduction

The manuscript should include an untitled introduction stating the purpose of the investigation and relating the manuscript to similar research.

Results and Discussion

The results should be presented concisely. Tables and figures should be designed to maximize the presentation and comprehension of the experimental data. The

discussion should interpret the results and relate them to existing knowledge in the field in as clear and brief a fashion as possible. Authors submitting a manuscript as a Note should omit the heading “Results and Discussion”. For Full Papers of unusual length, subheadings may be included within the Results and Discussion section.

Experimental Section

The presentation of specific details about instruments used, sources of specialized chemicals, and related experimental details should be incorporated into the text of the Experimental Section as a paragraph headed General Experimental Procedures. The general order for inclusion should be as follows: melting points; optical rotations; UV spectra; CD spectra; IR spectra; NMR spectra; mass spectra; and chromatographic and other techniques.

In a separate paragraph, experimental biological material should be reported as authenticated if cultivated or from a natural habitat, and the herbarium deposit site and voucher number should be recorded. The month and year when the organisms were collected should be stated, and it is recommended that the exact collection location be provided using a GPS navigation tool. All microorganisms used experimentally should bear a strain designation number and the culture collection in which they are deposited. The scientific name (genus, species, authority citation, and family) should be presented when first mentioned in the body of the manuscript. Thereafter, the authority should be eliminated, and the generic name should be reduced (except in tables and figure legends) to the first capital letter of the name (but avoid ambiguity, if two or more generic names have the same first letter).

If the biological material has not been identified as to species, the manuscript will not be considered for publication unless a special protocol has been followed. Thus, a voucher specimen of the organism should be deposited with a recognized taxonomist for the particular group of organisms in question. The taxonomist should then assign to the specimen an identifying number unique to the organism so that any additional collections of the same organism would bear this same number. The number will be retained until the organism is completely identified. The taxonomist should write a brief taxonomic description to be included in the manuscript, which should state how the organism in question relates morphologically to known species. Contributors are

encouraged to use DNA sequence analysis to assist with the taxonomic identification of unknown microorganisms, and to deposit these data in GenBank (http://www.ncbi.nlm.nih.gov/). Photographs of incompletely identified organisms may be included as Supporting Information. Authors should be aware of the fact that large-scale collection of marine or terrestrial organisms may have ecological effects. Authors describing an investigation derived from large-scale collections should thus include a statement in their manuscript (in the “Biological Material” paragraph of the Experimental Section) explaining why the collection had no significant adverse ecological effect or justifying such effect in terms of the benefit from the resulting work.

Authors who purchase dried “herbal remedies” or other materials from companies must mAKe provision for their proper deposit in a herbarium, for access by future workers. When a commercially available extract is obtained, the extraction procedure from the organism of origin must be specified. The identification of the extract should be supported by an HPLC trace of known secondary metabolite constituents of the organism, which should be included in the manuscript as Supporting Information.

When physical and spectroscopic data are presented in the body of the manuscript, the following general style must be used (with the various commonly used techniques presented in this same order):

> **Romucosine (1):** colorless needles (CHC3); mp 152–153 °C; [α]25D –110 (*c* 0.4, CHCl3); UV (EtOH) *λ*max(log ε) 235 (4.23), 275 (4.18), 292 (sh) (3.52), 325 (3.41) nm; IR (Nujol) vmax 1680, 1040, 920 cm–1; 1H NMR (CDC3, 400 MHz) *δ* 8.11 (1H, d, *J* = 7.6 Hz, H-11), 7.54–7.28 (2H, m, H-9, H-10), 7.27 (1H, m, H-8), 6.59 (1H, s, H-3), 6.10, 5.97 (each 1H, d, *J* = 1.5 Hz, OC*H*2O), 4.86 (1H, dd, *J* = 13.7, 4.4 Hz, H-6a), 4.44 (1H, m, H-5a), 3.77 (3H, s, NCOOC*H*3), 3.06 (1H, m, H-7a), 2.99 (1H, m, H-5b), 2.91 (1H, m, H-7b), 2.82 (1H, m, H-4a), 2.61 (1H, m, H-4b); 13C NMR (CDCl3, 100 MHz) *δ* 155.8 (C, N*C*OOCH3), 146.8 (C, C-2), 143.0 (C, C-1), 135.8 (C, C-7a), 130.7 (C, C-11a), 128.7 (CH, C-8), 127.79 (C, C-3a), 127.78 (CH, C-9), 127.2 (CH, C-10), 127.0 (CH, C-11), 125.6 (C, C-3b), 117.3 (C, C-1a), 107.6 (CH, C-3), 100.9 (CH2, OCH2O), 52.7 (CH3, NCOO*C*H3), 51.7 (CH, C-6a), 39.2 (CH2, C-5),

34.5 (CH2, C-7), 30.4 (CH2, C-4); EIMS *m*/*z* 323 [M]+ (98), 308 (28), 292 (5), 262 (20), 248 (21), 236 (81), 235 (100), 206 (17), 178 (27), 88 (17); HREIMS *m*/*z* 323.1152 (calcd for C19H17O4N, 323.1158).

The correct presentation of NMR spectroscopic data is shown in the table below.

Table 1. NMR Spectroscopic Data (400 MHz, C_6D_6) for Aurilides B (**1**) and C (**2**)

	aurilide B (**1**)			aurilide C (**2**)	
position	δ_C, type	δ_H (J in Hz)	HMBC[a]	δ_C	δ_H (J in Hz)
1	170.0, C			170.2	
2	58.9, CH	3.23, m	1, 3, 4, 5	59.6	3.08, m
3	13.8, CH_3	1.21, d (7.1)	1, 2	14.0	1.25, d (7.1)
4	36.1, CH_3	2.63, s	2, 5	36.8	2.55, s
5	172.1, C			172.1	
6	54.3, CH	5.12, dd (9.0, 7.4)	5, 7, 9	54.4	5.15, dd (9.0, 5.0)
7	31.0, CH	1.97, m		32.0	1.98, m
8	20.1, CH_3	1.15, d (7.0)	6, 7, 9	20.4	1.17, d (7.0)
9	17.3, CH_3	1.25, d (7.0)	6, 7, 8	17.5	1.28, d (7.0)
10	169.9, C			170.11	
11	51.8, CH_2	4.40, d (18.0) 3.80, d (18.0)	10, 12, 13	51.9	4.39, d (18.0) 3.80, d (18.0)
12	36.8, CH_3	3.23, s	11, 13	37.1	3.22, s
13	170.0, C			170.14	
14	58.6, CH	5.24, d (10.0)	13, 18, 19, 20	58.7	5.26, d (10.0)
15	33.9, CH	2.48, m	14, 16, 18	34.1	2.49, m
16	27.4, CH_2	1.86, 1.30, m	14, 15, 17	27.6	1.89, 1.30, m
17	12.1, CH_3	1.03, t (7.1)		12.2	1.03, t (6.9)
18	14.8, CH_3	0.85, d (7.0)	15, 16	15.1	0.86, d (7.0)
19	30.7, CH_3	2.88, s	20	30.6	2.85, s
20	173.1, C			173.2	
21	54.7, CH	4.78, dd (8.8, 8.8)	20, 22	54.9	4.75, dd (8.6, 7.5)
22	31.7, CH	1.98, m		31.0	1.95, m
23	18.1, CH_3	0.89, d (6.0)	21, 22, 24	18.9	0.88, d (6.0)
24	20.2, CH_3	0.90, d (6.0)	23	20.3	0.90, d (6.0)
25	170.3, C			170.3	
26	78.5, CH	4.90, d (6.1)	25, 27, 31	80.4	4.54, d (7.5)
27	37.2, CH	2.17, m	26, 30	30.5	2.36, m
28	26.1, CH_2	1.50, 1.14, m	29	18.7	1.00, d (7.0)
29	11.8, CH_3	0.83, t (7.7)	27, 28	18.4	0.88, d (7.0)
30	14.9, CH_3	1.03, d (6.0)	26, 27, 28	169.7	
31	169.3, C			128.3	
32	128.0, C			146.0	7.75, t (9.0)
33	145.3, CH	7.74, t (9.0)	31, 42	30.9	2.14, m
34	30.9, CH_2	2.19, m	32, 33, 42	71.2	3.98, m
35	71.0, CH	3.97, m	34	41.2	2.02, m
36	41.1, CH	2.07, m	43	82.6	5.17, d (11.2)
37	82.5, CH	5.18, d (11.2)	1, 36, 38, 44	132.1	
38	131.4, C			134.6	5.62, t (7.7)
39	134.2, CH	5.61, t (7.7)	37, 44	21.4	1.95, 1.92, m
40	21.4, CH_2	1.95, 1.92, m	38, 39, 41	14.3	0.89, t[b]
41	14.1, CH_3	0.89, t[b]	39, 40	12.8	1.95, s
42	12.7, CH_3	1.95, s	31, 32, 33	10.1	0.66, (7.0)
43	10.2, CH_3	0.64, d (7.0)	35, 36, 37	11.4	1.54, s
44	11.3, CH_3	1.54, s	37, 38, 39		
NH (1)		7.69 br, d (9.1)	10		7.66 br, d (9.1)
NH (2)		6.75 br, d (8.8)	25		6.70 br, d (8.8)

[a]HMBC correlations, optimized for 6 Hz, are from proton(s) stated to the indicated carbon.
[b]Signal partially obscured.

The correct format to present elemental analysis data is: anal. C 72.87, H 11.13%, calcd for C37H68O6, C 73.02, H 11.18%. The structures of compounds are expected to be supported by high-resolution mass spectrometry or elemental analysis. Melting point determinations should not be provided for compounds described as “amorphous solids”. The unit of concentration to be used for optical rotation

measurements is grams per 100 mL. UV extinction coefficient data should be provided as log ϵ values, to two places of decimals. In reporting 1H NMR data of diastereotopic methylene protons, the one at lower field should be listed as the “a” proton and that at the higher field as the “b” proton, as in “H-10a” and “H-10b”, respectively. If two proton or carbon signals in an NMR spectrum appear at the same chemical shift but are still distinguishable, an additional decimal place (three for 1H NMR data and two for 13C NMR data) may be used to designate the resonance in question. Carbon-13 NMR data should be reported to the nearest 0.1 ppm with the number of attached protons designated using the C, CH, CH2, and CH3 notation.

Acknowledgment

The Acknowledgment section should include credits [initial(s) and last name] for technical assistance, financial support, and other appropriate recognition.

References and Notes

References to the literature and all notes, regardless of their nature, should be numbered in order of appearance in the manuscript and cited in the text with superscript numbers. Each reference may have its own citation number, or alternatively, references referring to the same topic may be grouped under a common number using alphabetical subdesignations (e.g., 1a, 1b, 1c, etc.). Each note should be assigned its own number. References and notes should follow the format shown:

(1) Dumdei, E.; Andersen, R. J. *J. Nat. Prod.* **1993**, *56*, 792–794.

(2) Cordell, G. A. *Introduction to Alkaloids: A Biogenetic Approach*; John Wiley & Sons: New York, 1981; p 43.

(3) Pelletier, S. W.; Mody, N. V. In *The Alkaloids*; Rodrigo, R. G. A., Ed.; Academic Press: New York, 1981; Vol. 18, Chapter 2, pp 100–216.

(4) Zheng, G.; KAKisawa, H. *Chin. Sci. Bull.* **1990**, *35*, 1406–1407; *Chem. Abstr.* **1991**, *114*, 43213*m*.

(5) Meyer, B. N. Brine Shrimp Toxicity: Certain Components of *Stapelia, Coryphantha, Lupinus,* and *Quinoa*. Ph.D. Thesis, Purdue University, West Lafayette, IN, 1983, p 35.

(6) Davis, R. U.S. Patent 5,708,591, 1998.

(7) The biogeographic zone comprising Madiera, the Canary Islands, the Cape Verde Islands, and the Azores.

For additional information on the reference and note format to use, see *The ACS Style Guide*, 3rd ed. (2006) (http://pubs.acs.org/books), available from Oxford University Press, Order Department, 2001 Evans Road, Cary, NC 27513 (http://www.oup.com).

The author is responsible for the accuracy and completeness of all references. In particular, authors must cite all of the references from their own work on a particular topic, such as all papers published or submitted on the constituents of a given organism under consideration. Because subscribers to the Web edition are now able to click on the “CAS” tag following each reference to retrieve the corresponding CAS abstract, reference accuracy is critical. Journal abbreviations should be those used by *Chemical Abstracts* [see *Chemical Abstracts Service Source Index (CASSI) 1907–2004*].

The author should supply the Editor with copies of related manuscripts that are cited as “in press” or “submitted” for use by the editors and the reviewers in evaluating the manuscript under consideration.

Nomenclature

It is the responsibility of the authors to provide correct nomenclature. All nomenclature must be consistent and unambiguous and should conform with current American usage. Insofar as possible, authors should use systematic names similar to those used by Chemical Abstracts Service, the International Union of Pure and Applied Chemistry, and the International Union of Biochemistry and Molecular Biology.

Chemical Abstracts (*CA*) nomenclature rules are described in Appendix IV of the *Chemical Abstracts Index Guide*. A list of ring systems, including names and numbering systems, is found in the *Ring Systems Handbook*, American Chemical Society, Columbus, OH, 2003, and its latest cumulative supplement. For CA nomenclature advice, consult the Manager of Nomenclature Services, Chemical Abstracts Service, PO Box 3012, Columbus, OH 43210-0012. A name generation service is available for a fee through CAS Client Services, 2540 Olentangy River Road, PO Box 3343, Columbus, OH 43210-0334; tel: (614) 447-3870; fax: (614) 447-3747; or e-mail: answers@cas.org.

For IUPAC rules, see:

- *Nomenclature of Inorganic Chemistry, Recommendations, 1990*; Blackwell Scientific Publications: Oxford, England, 1990.
- *A Guide to IUPAC Nomenclature of Organic Compounds, Recommendations, 1993*; Blackwell Scientific Publications: Oxford, England, 1993.
- *Nomenclature of Organic Chemistry, Sections A–F and H*; Pergamon Press: Elmsford, NY, 1979.
- *Compendium of Macromolecular Nomenclature*; Blackwell Scientific Publications: Oxford, England, 1991.
- *Biochemical Nomenclature and Related Documents*, 2nd ed.; Portland Press, Ltd.: London, England, 1992.
- Selected IUPAC recommendations can be found on the Web at http://www.chem.qmw.ac.uk/iupac/iupac.html.
- The ACS Web site has links to nomenclature recommendations: http://chemistry.org.

Abbreviations

Abbreviations are used without periods. Standard abbreviations should be used throughout the manuscript. All nonstandard abbreviations should be kept to a

minimum and must be defined in the text following their first use. The preferred forms of some of the more commonly used abbreviations are mp, bp, °C, K, s, min, h, mL, *μ*L, kg, g, mg, *μ*g, cm, mm, nm, mol, mmol, *μ*mol, ppm, TLC, GC, NMR, MS, UV, CD, and IR. For further information, refer to *The ACS Style Guide* (2006).

Graphics

The quality of the illustrations depends on the quality of the originals provided. Figures cannot be modified or enhanced by the journal production staff. The graphics must be submitted as part of the manuscript file and are used in the production of the Journal (material deposited as Supporting Information will not be published in the print edition). Contrast is important.

Remove all color from graphics, except for those graphics that you would like to have considered for publication in color (see Color section below for details).

(1) Layout. In preparing structures for publication, layout is critical. Equations, schemes, and blocks of structures are presented in the Journal either in one-column or two-column format.

For efficient use of journal space, single-column illustrations are preferred.	**single (preferred)**	**double**
width		
minimum		10.5 cm (4.13 in.)
maximum	8.25 cm (3.25 in.)	17.78 cm (7 in.)
maximum depth	24 cm (9.5 in.)	24 cm (9.5 in.)

Authors are advised that structural material labeled as a “Figure” is placed at the top or bottom of a page, as is all two-column material. All structural material that should immediately follow certain text must be designed to fit the one-column format, and its location in the text must be indicated on the manuscript. Structures, arrows, and compound designators should be arranged so as to make maximum use of the width afforded by the one-column or two-column format.

(2) Content. Abbreviations such as Me for CH3, Et for C2H5, and Ph (but not φ) for C6H5 are acceptable. Make liberal use of “R and X groups” in equations, schemes, and structure blocks to avoid the repetition of similar structures. Do not repeat a structure; the number alone of an earlier structure can be used if a compound occurs several times. Schemes are numbered with Arabic numerals. Within schemes, structures should be numbered with boldface Arabic numerals, consecutively from left to right, top to bottom, regardless of the order in which the compounds are discussed in the text. Schemes should be footnoted in the manner described below for Tables. It is not necessary to give reagents and conditions in complete detail, since this detail is contained in the Experimental Section. Where needed, numbers such as NMR chemical shifts may be included directly on structural formulas.

(3) Dimensions. For best results, illustrations should be submitted in the actual size at which they should appear in the Journal. Original illustrations that do not need to be reduced to fit a single or double column will yield the best quality. Lettering should be no smaller than 4.5 points. (Helvetica or Arial type works well for lettering.) Lines should be no thinner than 0.5 point. Lettering and lines should be of uniform density.

If artwork that should be reduced must be submitted, larger lettering and thicker lines should be used so that, when reduced, the artwork meets the above-mentioned parameters.

Complex textures and shading to achieve a three-dimensional effect should be avoided. To show a pattern, a simple cross-hatch design should be used.

Digital graphics should be submitted as TIFF images with the following minimum resolution requirements: Black and white line art	1200 dpi
Grayscale art	600 dpi
Color art	300 dpi

Chemical Structures

Structures should be produced with the use of a drawing program such as ChemDraw. Structure drawing preferences (preset in the ACS Stylesheet in ChemDraw) are as follows:

1. As drawing settings select:

chain angle	120º
bond spacing	18% of width
fixed length	14.4 pt (0.508 cm, 0.2 in.)
bold width	2.0 pt (0.071 cm, 0.0278 in.)
line width	0.6 pt (0.021 cm, 0.0084 in.)
margin width	1.6 pt (0.056 cm, 0.0222 in.)
hash spacing	2.5 pt (0.088 cm, 0.0347 in.)

2. As text settings select:

font	Arial/Helvetica
size	10 pt

3. Under the preferences choose:

units	points
tolerances	5 pixels

4. Under page setup choose:

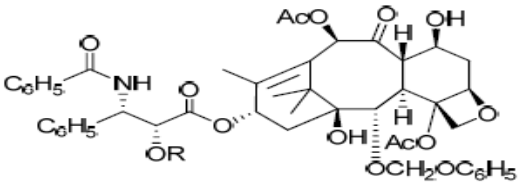
Paper	US Letter
Scale	100%

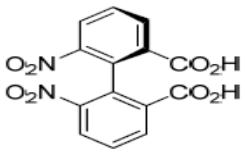
5. Using the ChemDraw ruler or appropriate margin settings, create structure blocks, schemes, and equations having maximum widths of 11.3 cm (one-column format) or

23.6 cm (two-column format). Note: if the foregoing preferences are selected as cm values, the ChemDraw ruler is calibrated in cm. ChemDraw graphics will be reduced to 75% during production.

6. Embolden compound numbers, but not atom labels or captions.

7. Authors are urged to use only a single configurational descriptor (heavy line or dashed line, but not both) when defining a stereocenter in a chemical structure. Atoms should be kept outside of rings wherever possible. Rather than rectangular solid and dashed lines, authors should use solid and dashed wedges to indicate configurations, as shown below. Dots at ring junctions intended to represent hydrogen atoms should not be used. Structures should be drawn in a neat manner ready for direct reproduction, and should not be cluttered or overlapping. Any arrows and numbering used for atoms in figures should not come into contact with bonds or ring systems. See an example of a prepared structure using ChemDraw with the specified preferences below. In molecules containing a chiral biphenyl axis, it is recommended that one of the aromatic rings be drawn in the plane of the paper and the second one be rotated out of the plane of the paper, to reflect the *P* or *M* conformation about the biphenyl bond (see below for example).





Authors using other drawing packages should, in as far as possible, modify their program's parameters so that they reflect the above guidelines.

Tables

These should be numbered consecutively with Arabic numerals and should be placed as they should appear in the paper. Footnotes in tables should be given

lowercase letter designations and be cited in the table by italic superscript letters. The sequence of letters should proceed by line rather than by column. If a footnote is cited both in the text and in a table, insert a lettered footnote in the table to refer to the numbered footnote in the text. Each table should be provided with a descriptive heading, which, together with the individual column headings, should make the table, as nearly as possible, self-explanatory. In setting up tabulations, authors are requested to keep in mind the type area of the journal page (17.8 × 25.4 cm) and the column width (8.5 cm), and to make tables conform to the limitations of these dimensions. Arrangements that leave many columns partially filled or that contain much blank space should be avoided.

Figures

Figures should be constructed in keeping with the column width, line width, and font size specified above (see Structural Drawings). All illustrations should be numbered as “Figures”, with Arabic numerals. Blocks of chemical structures should not be designated as “Figures”. Each figure must be identified outside the frame of the figure.

(1) Photographs. Digital photographs are accepted. Photographs that are single or double column width so that they will not have to be reduced work best.

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Table of Contents Graphic (called “synopsis graphic” in the manuscript template instructions)

A graphic must be included with each manuscript for the Table of Contents (TOC) of the Web edition of the Journal issue in which the Communication, Review, Full Paper, or Note will appear. This graphic should capture the reader’s attention and, in conjunction with the manuscript’s title, should give the reader a quick visual impression of the type of chemistry described and/or the biological results obtained.

Structures in the TOC graphic should be constructed as specified in the ‘Chemical Structures’ section above. The TOC graphic may be up to 4.7 in. (12.0 cm) wide and 1.8 in. (4.6 cm) tall. (See detailed instructions at the Paragon Plus Web site.) Text should be limited to labels for compounds, reaction arrows, and figures. The use of color to enhance the scientific value is highly encouraged. The TOC graphic should be inserted on a separate page at the end of the manuscript file. The title and author list will be added during production.

Recommendations for Crystal Structure Papers

Although the results of crystal structure determinations are frequently of interest to readers of the Journal, details of crystal structure experiments are generally not. Results appropriate for the Journal are not, however, sufficient to allow referees to assess the quality of an X-ray structure determination. Thus, it is recommended that manuscripts involving such determinations be accompanied by material provided for the benefit of the reviewers only. Authors should submit the following minimum materials, in tabular form where possible, for each compound for which X-ray crystallographic supplementary data are available.

Published Manuscript:

(1) Crystal data, including chemical formula, formula weight, crystal system and space group, cell dimensions (with uncertainties), number of formulas per unit cell, calculated density, radiation used, and wavelength.

(2) Final fractional atomic coordinates. Hydrogen atom coordinates should be included only if they have been experimentally determined or refined. Calculated coordinates should be provided as reviewer’s material.

(3) A *brief* outline of procedures used for data collection and refinement, including the method used for intensity measurement, 0 limits, portion of the full sphere collected, handling of absorption (if applicable), method of refinement, number of reflections used in the refinement and criteria for their choice, treatment of hydrogen atoms, and final *R* factor.

(4) A perspective diagram (perhaps prepared by ORTEP, PLUTO, or similar programs) that gives the atom-numbering scheme if it is not unambiguous

from the remainder of the paper. If the figure is a stereoview, it should be provided reduced to correct size, about 55–60 mm between images.

Besides a description of the structure, other information (i.e., important distances, torsion angles, results of best plane calculations, etc.) may be included if appropriate. A note should be cited at an appropriate place in the manuscript and included in the References and Notes Section: “Crystallographic data for the structure(s) reported in this paper have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).”

Reviewer’s Material:

(1) Any calculated coordinate (e.g., hydrogen atoms).

(2) A full list of bond distances (and their uncertainties).

(3) A full list of bond angles (and their uncertainties).

All tables should be clearly legible, the contents nonredundant, and their interpretation immediately obvious. It is recommended that authors provide this information in the form of a Crystallographic Information File (CIF) for each compound for which X-ray crystallographic data are determined, with each CIF being separated from any other Supporting Information files.

Authors will deposit the tables of final fractional atomic coordinates and the full list of bond lengths and angles at the Cambridge Crystallographic Data Centre on notification of the acceptance of their paper. A checklist of data items for deposition is available at http://www.ccdc.cam.ac.uk.

Supporting Information

Authors are encouraged to provide Supporting Information in order to keep their manuscripts to a reasonable length. The Web edition of this journal can accommodate almost any type of supplementary data (e.g., reproductions of spectra, experimental procedures, tabulated spectroscopic/spectrometric data, expanded

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