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Conjugation of L-NAME to prenyloxycinnamic acids improves its inhibitory effects on nitric oxide production

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ABSTRACT

A series of **10** compounds resulting from the conjugation of O-prenylated naturally occurring benzoic and cinnamic acids to L-NAME were synthesized and tested together with the corresponding unprenylated parent molecule as anti-inflammatory agents for their inhibitory effects on NO production in LPS-stimulated RAW 264.7 macrophages. Results indicated that the coupling between O-geranyl and O-isopentenylcinnamic acids and L-NAME led to products with an enhanced activity when compared to the parent compounds.

The nitric oxide (NO) radical, synthesized in vivo by the oxidation of L-arginine catalyzed by either the inducible (*i*-) or constitutive (*c*-) isoforms of NO synthase (NOS), is involved in several physiological and pathological processes in mammals, most of which have been largely reviewed in the last decade.[1] It is also well known how an excessive production of NO by *i*NOS in macrophages is involved in various acute and chronic inflammatory diseases.[2–4] High levels of NO are implicated in carcinogenesis,[5,6] either by causing mutagenesis, deamination of DNA bases[7–9] or promoting the formation of carcinogenic *N*-nitroso compounds in vivo.[10] This latter biotransformation implies the rapid and spontaneous reaction of NO with triplet O_2 to form stable anions, like nitrite and nitrate,[11,12] that in turn non-enzymatically *N*-nitrosylate the primary and secondary amines to produce carcinogenic nitrosamines.[10] Moreover under inflammatory conditions, macrophages can greatly increase their production of both NO and superoxide anion simultaneously, which rapidly react with each other to form peroxynitrite anion, thus playing an additional damaging role in inflammation and also possibly in the multistage process of carcinogenesis.[13] The peroxynitrite anion is then able to activate the constitutive and inducible forms of cyclooxygenase (COX-1 and COX-2, respectively), which are rate determining enzymes for prostaglandin biosynthesis during the inflammatory process.[14,15]

Oxyprenylated natural products (isopentenyloxy-, geranyloxy-, and the less spread farnesyloxy-compounds) represent a family of secondary metabolites that were previously considered as biosynthetic intermediates of the most widespread C-prenylated derivatives. However, recent evidence suggests that these natural products are important biologically active phytochemicals. More than 350 related molecules have been isolated from plants primarily belonging to the families of Rutaceae, Apiaceae, and Compositae, comprising common edible vegetables and fruits, as well as from fungi and bacteria. A wide variety of compounds containing a prenyloxy side chain were isolated and these comprise alkaloids, coumarins, flavonoids, cinnamic and benzoic acids, phenols, alcohols, aldehydes, anthraquinones, chalcones, lignans, xanthones, aceto- and benzophenones. Many of the isolated oxyprenylated natural products were shown to have remarkable anti-cancer, anti-inflammatory, anti-microbial, and anti-fungal effects using in vitro and in vivo models. The chemistry and pharmacology of this class of natural products has been recently reviewed.[16] In this context, one of these oxyprenylated secondary metabolites, namely 4′-geranyloxyferulic acid (**1**), was seen to exert in vivo beneficial effects both as an anti-inflammatory agent, being an inhibitor of COX-2[17] expression and *i*-NOS activity,[18] and as a dietary feeding colon cancer chemopreventer.[19] The pharmacological properties of compound (**1**) has been recently reviewed.[20]

Literature reports indicate that inflammation represents the basis of severe acute and chronic syndromes like cancer, cardiovascular diseases, neurological disorders, and several others.[21] Thus

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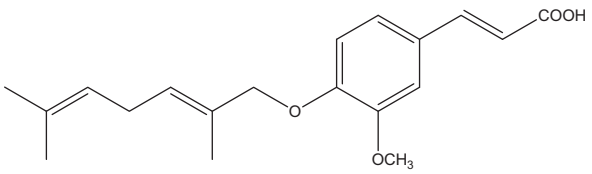
the search for novel, alternative, and safer anti-inflammatory agents as well as the better description of the mechanism of action underlying the observed effects, is a field of research of current and growing interest.



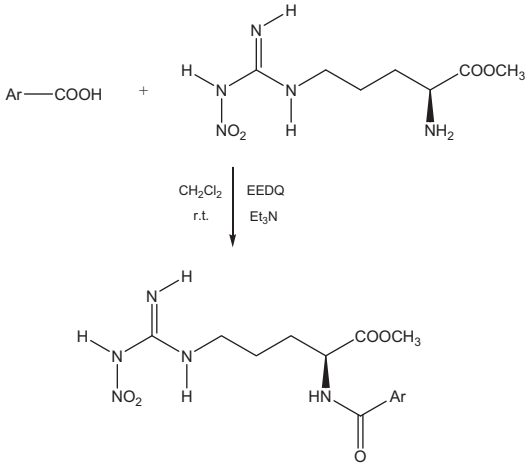
(**1**)

As a continuation of our ongoing studies aimed to better depict the phytochemical and pharmacological properties of natural and semisynthetic oxyprenylated phenylpropanoids and polyketides, also in this work we synthesized and investigated the effects of **10** compounds resulting from the conjugation via an amide bond of selected oxyprenylated benzoic and cinnamic acids to L-nitroarginine methyl ester (L-NAME) with the aim to analyze a potential synergism of action between the well known anti-NOS activity of L-NAME and the anti-inflammatory effects of O-prenyl derivatives,[16,17] and of their unbound counterparts on NO production in bacterial lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages.

The chemical structures of the compounds we studied are illustrated in Figure 1.

Chemical synthesis and natural sources of compounds (**1–4**), (**9–11**), and (**17 and 18**) have been already reported,[22–24] while compound **9**, recently isolated from *Piper crassinervum* H.B.K. (Piperaceae),[25] was synthesized from commercially available methyl *p*-hydroxybenzoate and geranyl bromide in the presence of K_2CO_3 as the base in acetone following the already reported scheme for the geranylation of phenolic acids.[22] Conjugated adducts were synthesized by condensation between L-NAME hydrochloride and the prenyloxy acid promoted by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in the presence of Et_3N (Scheme 1).

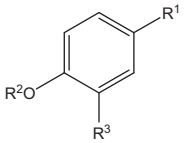
This brief and simple reaction provided the desired products after crystallization (*n*-hexane) with the following yields: 55% (**5**), 61% (**6**), 62% (**7**), 54% (**8**), 56% (**13**), 56% (**14**), 62% (**15**), 60% (**16**), 58% (**19**), and 61% (**20**).



Scheme 1. Synthesis of L-NAME adducts

All compounds were then assessed for their potential to inhibit the LPS-induced production of NO in murine macrophage RAW 264.7 cells pre-stimulated by LPS (1 μg/mL). Pro-inflammatory agents, like LPS, can significantly increase NO production in macrophages through activation of *i*-NOS.[26] The treatment of RAW 264.7 macrophages for 24 h induced NO production, which was then quantified by the chromogenic Griess reaction, measuring the accumulation of nitrite, a stable metabolite of NO. Table 1 reports the level of inhibition of NO production expressed as IC_{50} (μM). L-NAME and indomethacin were used as controls. For all compounds we performed the MTT test using the same cell line. Results obtained from this assay indicated that every adduct had virtually no effects on cell viability (data not shown).

From results obtained and reported in the Table 1 all L-NAME conjugated products, with the exception of compound (**13**), are



1 R^1 = CH=CH-COOH, R^2 = geranyl, R^3 = OCH_3
2 R^1 = CH=CH-COOH, R^2 = isopentenyl, R^3 = OCH_3
3 R^1 = CH=CH-COOH, R^2 = isopentenyl, R^3 = H
4 R^1 = CH=CH-COOH, R^2 = geranyl, R^3 = H
5 R^1 = CH=CH-CO-LNAME, R^2 = isopentenyl, R^3 = OCH_3
6 R^1 = CH=CH-CO-LNAME, R^2 = geranyl, R^3 = OCH_3
7 R^1 = CH=CH-CO-LNAME, R^2 = isopentenyl, R^3 = H
8 R^1 = CH=CH-CO-LNAME, R^2 = geranyl, R^3 = H
9 R^1 = COOH, R^2 = isopentenyl, R^3 = OCH_3
10 R^1 = COOH, R^2 = geranyl, R^3 = OCH_3
11 R^1 = COOH, R^2 = isopentenyl, R^3 = H
12 R^1 = COOH, R^2 = geranyl, R^3 = H
13 R^1 = CO-LNAME, R^2 = isopentenyl, R^3 = OCH_3
14 R^1 = CO-LNAME, R^2 = geranyl, R^3 = OCH_3
15 R^1 = CO-LNAME, R^2 = isopentenyl, R^3 = H
16 R^1 = CO-LNAME, R^2 = geranyl, R^3 = H
17 R^1 = CH_2CH_2COOH, R^2 = isopentenyl, R^3 = H
18 R^1 = CH_2CH_2COOH, R^2 = geranyl, R^3 = H
19 R^1 = $CH_2CH_2COLNAME$, R^2 = isopentenyl, R^3 = H
20 R^1 = $CH_2CH_2COLNAME$, R^2 = geranyl, R^3 = H

Figure 1.

Table 1
Inhibitory effects of the synthetized compounds on the LPS-induced NO production in RAW 264.7 cells

Compound	IC_{50} (μM)
1	100.24 ± 0.12[g]
2	>200
3	>200
4	>200
5	64.19 ± 0.13[d]
6	67.19 ± 0.09[e]
7	62.61 ± 0.04[c]
8	41.65 ± 0.06[a]
9	>200
10	>200
11	>200
12	>200
13	>200
14	53.12 ± 0.21[b]
15	110.50 ± 0.04[i]
16	81.18 ± 0.05[f]
17	>210
18	>200
19	103.10 ± 0.03[h]
20	125.00 ± 0.17[j]
Indomethacine[a]	148.00 ± 0.47[k]
L-NAME[a]	170.04 ± 0.37[l]

Data are expressed as mean ± S.E.M. (n = 4). Different letters along column indicate statistically significant differences at P <0.05 (Tukey's test).
[a] Positive controls.

able to decrease to different extents more efficiently than L-NAME and indomethacine, the over-production of NO in LPS-stimulated RAW 264.7 macrophages. The more significant result to this concern was obtained with the conjugated compound (**8**) resulting from the coupling of *p*-coumaric acid with L-NAME (IC_{50} = 41.65 ± 0.06 μM). Other prenyloxy cinnamic acid derivatives performed slightly worse but recording an homogeneity in obtained data, being the values of inhibition comprised in the range 62.61–67.19 μM. This pattern of results was different for oxyprenylated benzoic acid derived products (**13–16**) for which only one compound, namely (**14**), provide an excellent decrease in NO production by macrophages, with an IC_{50} value of 53.12 ± 0.21 μM. Compounds (**16**) and (**15**) showed activity lower, but still significantly higher than that of the reference compounds (Tukey's test). On the contrary, molecule (**13**) showed an IC_{50} value by far higher. Among active compounds, the derivative of oxyprenylated dihydrocinnamic acid (**20**) induced the lowest inhibition of NO production (IC_{50} = 125.00 ± 0.17 μM.).

Trying to depict a reasonable mechanism of action underlying the observed effects, structural considerations led to hypothesize that these chemicals might act as prodrugs able, once inside the cell, to deliver two biologically active portions, (e.g., L-NAME and the free acid), that in turn may sinergically act on different pro-inflammatory targets, like NOS,[1] COX,[17] and others, thus resulting in a decrease of NO release by RAW 264.7 cells. This kind of approach, namely coupling of L-NAME to other biologically active chemical entities via a peptidic linkage, is described herein for the first time to the best of our knowledge.

In terms of structure activity relationships it is evident that the α,β-unsaturated double bond is a key structural requirement for activity, being clear that cinnamic acid derivative conjugates are more efficient than the benzoic and dihydrocinnamic acid derivative ones. Moreover the presence of a geranyl side chain, like in compounds (**6**), (**8**), (**14**), and (**16**) render the molecule more active than their O-isopentenylated counterparts. This last observation is in accordance with what has been already reported for such oxyprenylated secondary metabolites in terms of anti-inflammatory activity.[17] Finally the OCH_3 group in position 3 of the aromatic ring seem not to play a definitive role in this context.

In conclusion, exploiting peptide-like derivatives resulting from the coupling of L-NAME and oxyprenylated naturally occurring acids, we have disclosed herein that the NO production inhibitory activity of L-NAME can be greatly improved by its linkage to other known anti-inflammatory agents like prenyloxycinnamic acids. Stating the well known importance of inflammation in the pathogenesis of several severe diseases, the pivotal role played by COX and NOS in several types of cancer,[27] and the efficacy of L-NAME and oxyprenylated secondary metabolites against severe syndromes, for example colon cancer,[28] the findings described herein will be of certain interest in the next future to the development of a novel class of prodrugs able to generate two or more biologically sinergically active portions able to efficiently reduce the inflammatory process acting on different endocellular targets.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.050.

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