Lichen Metabolites. 1. Inhibitory Action Against Leukotriene B_4 Biosynthesis by a Non-Redox Mechanism

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Of several lichen metabolites isolated from *Parmelia nepalensis* and *Parmelia tinctorum*, the didepsides atranorin (**4**) and diffractaic acid (**5**), as well as (+)-protolichesterinic acid (**7**), inhibited LTB_4 biosynthesis in polymorphonuclear leukocytes. Ethyl hematommate (**3**) and (+)-usnic acid (**1**) were only weak inhibitors, while methyl β-orcinolcarboxylate (**2**) and gyrophoric acid (**6**) were inactive at concentrations up to 60 μM. Redox properties of the compounds were evaluated in terms of inhibition of nonenzymatic lipid peroxidation in model membranes, reactivity against the stable free radical 2,2-diphenyl-1-picrylhydrazyl, and deoxyribose degradation as a measure of hydroxyl-radical generation. The results revealed that lichen metabolites neither acted as antioxidants against the peroxidation process in model membranes nor did they scavenge or produce free radicals, suggesting that the inhibitory effects on LTB_4 biosynthesis was due to specific enzyme interaction rather than a nonspecific redox mechanism.

Lichens are complex plants consisting of a symbiotic association of a fungus and an alga. They produce characteristic secondary metabolites that are unique with respect to those of higher plants. Several lichen extracts have been used for various remedies in folk medicine, and screening tests with lichens have indicated the frequent occurrence of metabolites with antibiotic,[1] antimycobacterial,[2] antiviral,[3−5] analgesic,[6] and antipyretic properties.[6] Other literature reports of biological activities of these naturally occurring compounds are scarce. This may partly be due to the difficulties encountered with collecting substantial amounts of plant material, as most of the lichen species grow as scattered patches, mainly on stones or on tree trunks.

In addition to their reputed effectiveness in the treatment of many diseases, some lichens have also been used traditionally as medicinal plants to treat bronchitis and inflammatory disorders.[7] Because leukotrienes (LT), the products of 5-lipoxygenase (5-LO) metabolism of arachidonic acid, play an important role in a variety of pathophysiological states in humans,[8−10] particularly those involving inflammation, we speculate that these mediators might be influenced by lichen-derived compounds.

Furthermore, a distinct class of lichen metabolites is the depsides. These types of compounds are formed by condensation of two or more hydroxybenzoic acids whereby the carboxyl group of one molecule is esterified with a phenolic hydroxyl group of a second molecule. Owing to the phenolic nature of their chemical structures, these molecules are interesting candidates for evaluating their effects on leukotriene biosynthesis, as a major class of inhibitors often contains a hydroxylated aromatic ring.[11] Moreover, two small-molecule lichen-derived metabolites, protolichesterinic acid and lobaric acid, have been reported to inhibit 5-LO from porcine leukocytes.[12] The latter has also been shown to inhibit peptide leukotriene formation.[13] Lichen depsides have also been described to inhibit prostaglandin biosynthesis.[14]

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Table 1. Inhibition of LTB_4 Biosynthesis in Bovine PMNL by Lichen Metabolites

lichen metabolites	LTB_4 biosynthesis IC_{50} (μM)
(+)-usnic acid (**1**)	42 ± 2.2[a]
methyl β-orcinolcarboxylate (**2**)	>60
ethyl haematommate (**3**)	40 ± 6.4[a]
atranorin (**4**)	6 ± 0.4[a]
diffractaic acid (**5**)	8 ± 2.3[a]
gyrophoric acid (**6**)	>60
(+)-protolichesterinic acid (**7**)	9 ± 1.3[a]

[a] Indicated values are mean ± SD. Inhibition was significantly different with respect to that of the control; $n = 3$ or more, $p < 0.05$. Nordihydroguaiaretic acid and anthralin were used as standard inhibitors (IC_{50} = 0.4 ± 0.21 and 37 ± 4.6 μM, respectively).[15]

As part of our continuing search for plant-derived potent antiinflammatory and antiproliferative agents, the in vitro inhibitory action against leukotriene biosynthesis of some well-known lichen metabolites has been studied in bovine polymorphonuclear leukocytes (PMNL).[15,16] These compounds, whose structures are members of the most common classes of lichen metabolites,[17] were isolated from either *Parmelia nepalensis* Tayl. or *Parmelia tinctorum* Nyl. (Parmeliaceae):[18] the dibenzofuran derivative, (+)-usnic acid (**1**); the 2,4-dihydroxybenzoates, methyl β-orcinolcarboxylate (**2**) and ethyl hematommate (**3**); the didepsides, atranorin (**4**) and diffractaic acid (**5**); the tridepside, gyrophoric acid (**6**); and (+)-protolichesterinic acid (**7**), an aliphatic α-methylene butyrolactone. In addition to the 5-LO assay, tests for prooxidant and antioxidant properties of the compounds were performed to ascertain their mode of action against the 5-LO enzyme. Redox properties were evaluated in terms of inhibition of nonenzymatic lipid peroxidation in model membranes, reactivity against the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), and deoxyribose degradation as a measure of hydroxyl-radical formation.[15,19]

Results and Discussion

Lichen metabolites were evaluated for inhibition of LTB_4 biosynthesis in vitro using Ca-ionophore A23187-stimulated bovine PMNL. Table 1 summarizes the inhibitory

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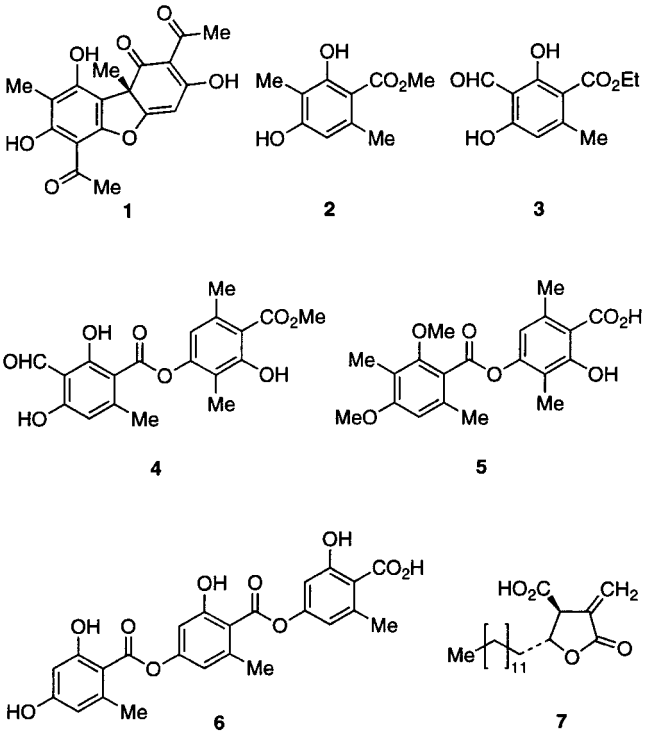

activities of the compounds as expressed by their IC_{50} values. Several compounds of this study had IC_{50} values in the micromolar range. The didepsides **4** and **5** were the most potent representatives, with IC_{50} values of 6 and 8 μM, respectively. Also, the structurally different compound **7** afforded a similar IC_{50} value. Even though the monomeric 2,4-dihydroxybenzoate **3** shares the structural pattern with the orcinol moiety of **4**, it was markedly less active. The tricyclic compound **1** was also a weaker inhibitor of LTB_4 biosynthesis. The tridepside **6** and compound **2** were not active at concentrations up to 60 μM.

Many natural extracts and compounds isolated therefrom that inhibit 5-LO have been reported in the literature.[11] Their structures often bear little resemblance to each other; however, there is one feature common to many of these compounds. They behave as antioxidants by being preferentially oxidized themselves or by generating a reactive species that can be oxidized. In this fashion, they can act as alternative substrates for 5-LO either by 5-LO-induced oxidation of the inhibitor or by oxidation of their metabolic species. Many 5-LO inhibitors also inhibit nonenzymatic lipid peroxidation,[20,21] and their inhibitory action is believed to be due to scavenging of intermediate radicals that are formed within the active site of the enzyme. This is not surprising considering that conversion of arachidonic acid into LTB_4 via 5-LO is a radical-based oxidation. To further characterize the nature of the inhibitory action of the lichen metabolites, several other experiments were performed.

We used bovine brain phospholipid liposomes as model membranes[22] to evaluate the inhibitory effects of the compounds on nonenzymatic lipid peroxidation. Lipid peroxidation was stimulated with the azo initiator 2,2′-azo-bis-(2-amidinopropane) hydrochloride (AAPH).[23] Furthermore, radical scavenging antioxidants react rapidly with the stable free radical DPPH to give the reduced 2,2-diphenyl-1-picrylhydrazine. Accordingly, we determined the ability of the lichen metabolites to scavenge radical intermediates by measuring spectrophotometrically the decrease of DPPH concentration. However, according to our studies, lichen metabolites were neither inhibitors of the peroxidation process in model membranes nor did they scavenge free radicals. No inhibitory effects against lipid peroxidation were observed for any of the compounds at concentrations as high as 200 μM (Table 2). Also, except for **4**, which showed moderate reactivity, no appreciable amounts of reduced hydrazine were formed by the most potent inhibitors, documenting their lack of ability to react with a free radical (Table 2). By contrast, the standard 5-LO inhibitor nordihydroguaiaretic acid was also an effective inhibitor of lipid peroxidation and highly reactive against DPPH.

A further aspect associated with inhibition of lipoxygenase enzymes results from observations that several inhibitors can give rise to one-electron oxidation species, which play a key role in enzyme inactivation.[24,25] Moreover, direct effects of reactive oxygen species on lipoxygenases have been demonstrated.[26,27] Therefore, we defined prooxidant properties of the compounds by the deoxyribose assay, which is a sensitive test for the production of hydroxyl radicals.[28] The release of 2-thiobarbituric acid reactive material is expressed as malondialdehyde (MDA) and reflects a measure for hydroxyl-radical formation. However, with the exception of the less active 5-LO inhibitor **1** and the inactive compound **2**, which generated approximately half the amount of hydroxyl radicals as did the standard agent anthralin,[15,19] we did not observe any deoxyribose degradation from lichen metabolites (Table 2). Overall, prooxidant actions of lichen metabolites can be ruled out by the result of this experiment, suggesting that hydroxyl radicals are not involved in enzyme inhibition by this structurally diverse class of compounds. Also, the lack of inhibitory effects against free radical-induced, nonenzymatic lipid peroxidation and the lack of appreciable reactivity against stable free radicals suggest that a simple

Table 2. Redox Properties of Lichen Metabolites

lichen metabolites and standard compounds	LPO IC_{50} (μM)[a]	k_{DPPH} (M^{-1} s^{-1})[b]	$^{\bullet}OH$[c]
(+)-usnic acid (**1**)	>200	<1	1.47 ± 0.35[d]
methyl β-orcinolcarboxylate (**2**)	>200	5.95 ± 0.38[d]	1.43 ± 0[d]
ethyl hematommate (**3**)	>200	12.24 ± 1.36[d]	0.30 ± 0.07[d]
atranorin (**4**)	>200	11.46 ± 0.49[d]	0
diffractaic acid (**5**)	>200	<1	0.12 ± 0
gyrophoric acid (**6**)	>200	3.63 ± 1.11[d]	0.12 ± 0.03
(+)-protolichesterinic acid (**7**)	>200	<1	0.08 ± 0.01
nordihydroguaiaretic acid[e]	2 ± 0.2	>100[d]	0.23 ± 0.01[d]
anthralin[e]	79 ± 8.6	24.2 ± 4.2[d]	2.89 ± 0.14[d]

[a] Inhibition of AAPH-induced lipid peroxidation in bovine brain phospholipid liposomes; $n = 3$ or more. [b] Reducing activity against 2,2-diphenyl-1-picrylhydrazyl with equimolar amount of test compound (mean values ± SD, $n = 3$; control 0.8).[15] [c] Deoxyribose degradation as a measure of hydroxyl-radical formation. Indicated values are μmol of malondialdehyde/mmol of deoxyribose released by 75 μM test compound (mean values ± SD, $n = 3$; controls < 0.1).[15] [d] Values are significantly different with respect to control; $p < 0.01$). [e] Positive controls.[15]

redox effect does not explain the activity of lichen metabolites. Accordingly, the inhibitory effects against the 5-LO enzyme appear to be due to enzyme interaction rather than to a nonspecific redox mechanism. Thus, lichen metabolites compare favorably in these tests with the frequently used antipsoriatic agent anthralin (Table 1), which is a well-known generator of hydroxyl radicals, and these species are responsible for the undesired proinflammatory reaction of the skin associated with anthralin therapy.[29]

In this context, it is interesting to note that none of the lichen metabolites of this study has a catechol, hydroquinone, or pyrogallol subunit, which may play a role in redox-based 5-LO inhibition. These phenolic subunits may inhibit the enzyme by virtue of their iron chelating properties or by reductive inactivation.[30] Compounds containing these structural features have been characterized as redox inhibitors of 5-LO.[15,31]

In conclusion, the present study shows that several lichen metabolites are inhibitors of 5-LO. In contrast to many other inhibitors that may exhibit more potent inhibition of 5-LO than the lichen metabolites under consideration, atranorin (**4**), diffractaic acid (**5**), and (+)-protolichesterinic acid (**7**) have been identified as nonredox inhibitors of LTB_4 biosynthesis. As such, they should not be attended with a variety of adverse effects, particularly those due to the formation of reactive radical species, which have frequently been observed as byproducts of 5-LO inhibition.

Experimental Section

General Experimental Procedures. For analytical instruments and methods, see Müller et al.[15]

Isolation and Identification of Lichen Metabolites. Lichen metabolites **2**−**4** and **7** were isolated from *P. nepalensis* as described and identified by comparison with authentic samples.[18]

Lichen metabolites **1**, **5**, and **6** were isolated from *P. tinctorum*, collected from Dhulikhel (1700 m), Nepal, and authenticated by comparison with a herbarium specimen (no. 80-24) preserved at National Herbarium and Plant Laboratory, Godawari, Nepal. The shade-dried, powdered plant material (225 g) was extracted in a Soxhlet extractor with hexane followed by C_6H_6 and EtOAc to afford hexane (3.29 g), C_6H_6 (3.35 g), and EtOAc (29 g) extracts, respectively. The hexane and C_6H_6 extracts were pooled and chromatographed on Si gel (4.5 × 11 cm, 60−120 mesh) using a hexane−EtOAc gradient and then a EtOAc−MeOH gradient with an elution rate of 0.5 mL/min, collecting 25 mL in each fraction. The fractions were monitored on TLC (detection by 5% H_2SO_4) and pooled. The residue obtained from fractions 64−67 (hexane−EtOAc, 4:1) was recrystallized to afford **1** (18 mg); mp 199 °C (lit.[32] mp 201.5−202.5 °C). Fractions 156−163 (EtOAc−MeOH, 19:1) afforded **5** (31 mg); mp 189 °C (lit.[33] mp 192−193 °C). Evaporation of the EtOAc extract afforded a white powder that was further purified by repeated recrystallization (EtOAc−MeOH) to give **6** (73 mg); mp 214 °C (lit.[34] mp 223 °C). Spectroscopic (^{1}H NMR, FTIR, MS) data of the isolated compounds were consistent with those of literature reports or authentic samples.

Inhibition of LTB_4 Biosynthesis. Inhibition of 5-LO was determined using Ca-ionophore-stimulated bovine PMNL (10^7 cells/mL), as previously described.[15,16] Test compounds were preincubated for 15 min at 37 °C, and the concentrations of LTB_4 and 5-hydroxyeicosatetraenoic acid released after 10 min were measured by reversed-phase HPLC analysis. Inhibition was calculated by the comparison of the mean values of test compound ($n = 3$) with control ($n = 6$−8) activity: (1 − test compound/control) × 100. Inhibition was statistically significant compared to that of the control (Student's *t*-test; $p < 0.05$). Each IC_{50} value was derived by interpolation of a log inhibitor concentration versus response plot using four or more concentrations of the compound, spanning the 50% inhibition point.

Determination of the Reducing Activity Against 2,2-Diphenyl-1-picrylhydrazyl.[15,19] To 1 mL of the test compound solution (100 mM) was added 1 mL of DPPH solution (100 mM), each in Me_2CO−phosphate buffered saline (1/1 v/v), and the reduction of DPPH was followed spectrophotometrically at 516 nm. Plots of the reciprocal of DPPH concentrations against time gave straight lines, and the second-order rate constants were obtained from the slopes and are expressed as mean values ($n = 3$−6).

Degradation of 2-Deoxy-D-ribose. The deoxyribose assay was conducted as described.[15,19] The reaction mixtures contained the following reagents at the final concentrations stated: 0.3 mL of KH_2PO_4−KOH buffer, pH 7.4 (30 mM), 0.2 mL of H_2O (double distilled), 0.2 mL of 2-deoxy-D-ribose (2 mM), 0.2 mL of $FeCl_3 \cdot 6H_2O$ (0.1 mM), and 0.1 mL of the test compound solution (75 μM). Appropriate blanks and controls with the vehicle (MeCN) were conducted. The reaction mixtures were incubated for 2 h at 37 °C in a shaking H_2O bath. Trichloroacetic acid (1.0 mL, 2.8% w/v) and 1.0 mL of 1% (w/v in 0.05 N NaOH) 2-thiobarbituric acid (TBA) were added, and the samples were heated at 100 °C for 15 min and then cooled in an ice bath (5 min). Then, 2.0 mL of the reaction mixtures were treated with 0.05 mL of 36% (w/v) HCl and 2.0 mL of 1-butanol, and the mixtures were vigorously shaken in a vortex mixer (Heidolph) for 15 s. The organic layer was separated by centrifugation at 1500 *g* (15 min), and the absorbance at 532 nm was measured against butanol. Calibration was performed using a MDA standard prepared by hydrolysis of 1,1,3,3,-tetraethoxypropane.[35] TBA reactive material is expressed in terms of μmol MDA per mmol deoxyribose.

Assay of Lipid Peroxidation in Bovine Brain Phospholipid Liposomes. Bovine brain phospholipids were prepared as described by Gutteridge.[22] Inhibition of lipid peroxidation was performed essentially as described.[15,19] The following reagents were added to glass tubes in the order and at the final concentrations stated: 0.3 mL KH_2PO_4−KOH buffer, pH 7.4 (30 mM), 0.39 mL H_2O (double distilled), 0.2 mL liposomes (1 mg/mL), 0.1 mL of AAPH (10 mM), 0.01 mL test compound solution (variable concentrations). Appropriate blanks and controls with the vehicles (Me_2CO) were conducted. The reaction mixtures were incubated for 1 h at 37 °C in a shaking H_2O bath. 2,6-Di-*tert*-butyl-4-methylphenol (10 μL, 20% w/v), 0.5 mL of 25% (w/v) HCl, and 0.5 mL of 1% TBA were added, and TBA-reactive material was measured as described above. AAPH (10 mM) as stimulator resulted in the formation of 3.52 ± 0.20 nmoles MDA/mg phospholipid.

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