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# UNUSUAL NONPROTEIN IMINO ACID AND ITS RELATIONSHIP TO PHENOLIC AND NITROGENOUS COMPOUNDS IN *COPAIFERA*

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**Key Word Index**—*Copaifera* species; Caesalpinioideae; leaf and seed nonprotein imino acid; *N*-methyl-*trans*-4-hydroxy-L-proline; 4-hydroxyhydric acid; 4-hydroxy-1-methylpyrrolidine-2-carboxylic acid; leaf phenolics; Folin–Denis reagent; Kjeldahl analysis.

**Abstract**—The unusual imino acid, *N*-methyl-*trans*-4-hydroxy-L proline has been isolated from leaves of five species of the leguminous tropical tree *Copaifera*, and for the first time characterized by $^1H$ and $^{13}C$ NMR and mass spectrometry. This imino acid can constitute up to 3% of the mature leaf dry weight and 10% of the nitrogen; it also constitutes 2–3% of the dry weight of the seed. Preliminary feeding trials have shown it to be a very effective inhibitor of larval development of the seed-feeding bruchid beetle *Callosobruchus maculatus* and to have significant feeding deterrence of the leaf-feeding lepidopteran *Spodoptera littoralis*. Phenolic compounds, also known to affect herbivores adversely, comprise 6–10% leaf dry weight. However, the imino acid displayed a mean of 50% reactivity compared to standards commonly used in analysis of total phenolics by the Folin–Denis (F–D) assay [gallic acid, tannic acid and (+)catechin], thus resulting in a significant overestimate of phenolics by this assay. It is concluded that assessment of leaf food quality for herbivores of *Copaifera* by assays widely used for this purpose in ecological studies, such as F–D for phenolics and Kjeldahl for total nitrogen content, give some misleading results.

## INTRODUCTION

Secondary chemistry and nitrogen content are among the important factors governing herbivore acceptance and food value of foliage [e.g. 1, 2]. Considerable evidence indicates that plant phenolic compounds can have adverse effects upon plant herbivores, suggesting that they may play an important role in protecting plant tissues [e.g. 3–6]. Because of the great structural diversity of plant phenolics, several different assay procedures have been used for their detection and determination of concentration in plant tissue [e.g. 7–10]. Among these, the Folin-Denis (F–D) assay has been the most widely used for estimation of total phenolic content in plant tissues in both marine [e.g. 11] and terrestrial [e.g. 2, 12–17] ecological studies. Nitrogen is a fundamental element in the diet of herbivores [e.g. 2, 18, 19]. However, nitrogen is measured in most ecological studies by the Kjeldahl method [e.g. 2, 14, 17, 20] which neither reveals differences in the type of nitrogen present nor information about its availability as a dietary source to herbivores.

This study is part of a long-term investigation of the evolution of resin-producing plants in which the leguminous tropical tree *Copaifera* (Caesalpinioideae: Detarieae) has been selected as one of the model genera for detailed study [21–26]. *Copaifera* is an amphi-Atlantic genus with four species in West African rainforests [27] and *ca* 20 other species occurring throughout all the major lowland ecosystems in the Neotropics [22]. The leaf sesquiterpene hydrocarbons have been studied in detail [25, 26, 28–30] and diterpene resin acids have now also been detected in the leaf [31]. Phenolic compounds, analysed by F–D, proanthocyanidins (PA), and vanillin assays and HPLC have also been reported [26, 32, 33]. In this paper we report the isolation and, for the first time, the characterization by $^1H$, $^{13}C$ NMR and mass spectrometry of a nonphenolic nitrogen-containing compound (nonprotein imino acid), which reacts in the F–D assay and also leads to questions regarding nitrogen quality for herbivore diets in *Copaifera* leaves. We have also compared the reactivity of this imino acid with the F–D reagent relative to compounds commonly used as standards in the same assay and to compounds structurally related to the imino acid. As a result, we could quantify the phenol mimicing reaction and gather information on the structure–reactivity relationships. Leaves from adult tree of *C. multijuga* Hayne from the central Amazonian rainforest and *C. langsdorfii* Desf. from woodland in southeastern Brazil were analysed in greatest detail. Comparisons were were made with greenhouse-grown saplings of *C. venezuelana* var. *laxa* Xena and Arroyo, *C. pubiflora* Benth. and *C. officinalis* L. from dry forest of Venezuela. Preliminary analyses were also made of seeds from these plants.

## RESULTS AND DISCUSSION

### *Isolation and identification*

The precipitate formed by treatment of the methanolic extract of the mature leaves of *C. multijuga* and

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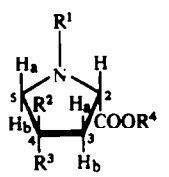
*C. langsdorfii* with diethyl ether has physical and chemical characteristics of a mixture composed only of phenolic compounds. The mixture gives a positive result with F–D and proanthocyanidin (PA) assays, and precipitates BSA from aqueous solution, methodologies used in most ecological studies.

However, a proton decoupled $^{13}C$ NMR spectrum of this mixture revealed that it was composed primarily of a nonphenolic compound containing six distinct carbon atoms. This compound was purified from the water soluble fraction of the precipitated methanolic extract by reverse-phase column chromatography and recrystallized from methanol. Analysis of its $^{1}H$ and $^{13}C$ NMR spectra (see Experimental), including extensive proton decoupling, and one-dimensional $^{13}C$ spin-echo techniques, such as APT[34], DEPT[35, 36] and RECAPT[37], high resolution EI mass spectrometry, IR and optical rotation data allowed the assignment of the structure to *n*-methyl-*trans*-4-hydroxy-L-proline (**1**) (4-hydroxyhygric acid; 4-hydroxy-1-methylpyrrolidine-2-carboxylic acid).

The presence of a carboxyl group was confirmed by methylation of the parent compound to afford the methyl ester (**2**). Definitive confirmation of the structure was obtained by *N*-methylation of *trans*-4-hydroxy-L-proline (**4**). The product was identical in all respects to **1**, which has been previously isolated from the heartwood of another legume, *Afromosia elata* [38] and from the bark of *Croton gubouga* (Euphorbiaceae) [39].

The purified nonprotein imino acid (**1**) showed a strong positive reaction with F–D reagent but neither precipitated BSA from aqueous solution nor gave a positive PA test. A proton decoupled $^{13}C$ NMR spectrum of a sample of the water-insoluble fraction of the precipitated methanolic extract and of the pooled fractions devoid of the imino acid, obtained from the reversed-phase chromatographic column, revealed that the astringency and PA positive reaction were due to mixture of proanthocyanidins (condensed tannins). The imino acid is strongly bound in the precipitated crude methanolic extract to the proanthocyanidins.

The nonprotein imino acid (**1**) constitutes *ca* 3% of the dry weight of mature leaves from two trees of *C. multijuga* and one tree of *C. langsdorfii*. It also constitutes 1–2% of the dry weight of mature leaves of greenhouse-grown saplings of *C. pubiflora*, *C. officinalis* and *C. venezuelana* var. *laxa*, as well as 2–3% of the dry weight of the seeds of all the cited species.




| | $R^1$ | $R^2$ | $R^3$ | $R^4$ |
|---|---|---|---|---|
| **1** | Me | OH | H | H |
| **2** | Me | OH | H | Me |
| **3** | Me | H | OH | H |
| **4** | H | OH | H | H |
| **5** | H | H | OH | H |

*Interference with F–D assay and the structure–reactivity relationship.*

Reactivity of **1** was compared to compounds commonly used as standards in analysis of phenolics by the F–D assay, e.g. gallic acid, tannic acid and (+)catechin. Over a concentration range of 125–1000 ppm the **1** displayed a mean of 50% relative reactivity compared to the standard compounds. Specifically, 1000 ppm of **1** gives an absorbance reading equivalent to that given by 480 ppm of gallic acid, 555 ppm of tannic acid and 505 ppm of (+)catechin. Also, the error over the range of concentrations was very small, indicating that the colour-reaction is persistent even at the lower concentration, and that colour density varies linearly with concentration. Thus, when using the F–D assay, the presence of *N*-methyl-*trans*-4-hydroxy-L-proline (**1**) leads to a significant overestimation (between 13—14%) of phenolics in leaves of *Copaifera* species [33].

The characteristic colour of the F–D assay (which is carried out under basic conditions) with phenolic compounds is caused by partial reduction of the active reagent (a yellow acidic solution containing complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids) from +6 to a mixture of +6 and +5 valence states upon binding to the substrate phenolates, resulting in the production of a complex molybdenum-tungsten blue. The exact nature of these complex blue pigments is still uncertain [40, 41].

*Trans* and *cis*-4-hydroxy-L-proline (**4** and **5**), even at concentrations as high as 30 mg/ml, and the methyl ester of *N*-methyl-*trans*-4-hydroxy-L-proline (**2**), at concentrations in which the free acid **1** is very reactive, do not produce any blue colour in the F–D assay. On the other hand, *N*-methyl-*trans* and *cis*-4-hydroxy-L-prolines (**1** and **3**), at the same concentration, produce *ca* the same colour-density in the F–D assay. Thus, it has been concluded that: (a) the orientation of the hydroxy group at the C-4 position of the imino acids tested does not play any role in the resulting blue-colour reaction; (b) the free carboxyl group in the imino acid **1** and **3** is one of the indispensable sites where the partial reduction of the F–D reagent takes place; and (c) the *N*-methyl group has an important function on the reactivity of the imino acids **1** and **3** in the F–D assay.

Early work by Abraham [42–44] on *trans* and *cis*-4-hydroxyproline (**4** and **5**, respectively), using $^{1}H$ NMR coupling constants, demonstrated that: (a) the cation (pH $< 0.5$) and zwitterion (pH 7) forms of both isomers adopted similar conformation—'$C_S$' or envelope conformation with C-4 projecting out of the plane of the other ring atoms for the *trans* compound and C-5 in the *cis* compounds, (b) the anion (pH 12.9) of both the *cis* and *trans* isomer was undergoing fast inversion about the nitrogen centre.

Comparison of the coupling constants of *N*-methyl-*trans*(**1**) and *cis*-4-hydroxy-L-proline(**3**) with Abraham's values for the *trans* and *cis*-L-hydroxyproline (**4** and **5**) revealed that the *N*-methyl group does not appear to affect the conformation of **1** and **2** relative to **4** and **5** at neutral pH.

Contrary to Abraham's results, when we obtained the $^{1}H$ NMR of *N*-methyl-*trans*-4-hydroxyl-L-proline(**1**) in basic conditions (NaOD in $D_2O$ at pH ~ 12.0), the coupling constants of the *N*-methylated imino acid were almost identical to those obtained at neutral pH. This demonstrates that a predominant conformer exists at

neutral and basic conditions for the *N*-methylated imino acid, and inversion at the nitrogen centre is not occurring on the NMR time scale. Since the methyl group *trans* to the carboxyl group in **1** is preferred based on steric considerations, we suggest that the nitrogen 'lone pair' is *cis* to the carboxyl group long enough to provide a dual site, along with the carboxyl anion, of electron density to be available for binding and reduction of the tungsten-molybdenum complex.

Although this study has dealt specifically with a non-phenolic secondary metabolite of restricted occurrence, there is the possibility of the presence of other non-phenolic secondary compounds, which have molecular characteristics such as adjacent sites of electron density and available electrons as exhibited by **1**, and occur in concentrations which may significantly overestimate the results given by the F–D assay.

*Ecological problems in measurement of total nitrogen content*

All nitrogen in plant tissues may not be available to the herbivores, or the balance between the various nitrogen-containing compounds, which can be effectively used as nutrients, may be unsatisfactory. Therefore, the quality of nitrogen availability for herbivore growth are more limiting than its total quantities [19, 45, 46]. Moreover, nitrogenous compounds, such as some of the nonprotein amino and imino acids, are synthesized and stored in plant tissue in substantial amounts, and are known to be toxic to insects, vertebrate animals and microorganism [e.g. 46–50]. In fact, Bell [47] and Rosenthal and Bell [48] have concluded that nonprotein amino or imino acids may have concomitantly a nitrogen storage function as well as deterrent or toxic roles. Mooney *et al.* [51] also have concluded that, since nitrogenous secondary compounds are readily mobile and metabolized, the major constraint on the use of large amounts of nitrogen in chemical defense probably is its effectiveness and its availability in the habitat.

*Copaifera* species apparently do not fix nitrogen by root nodules, they occur mainly on nutrient-poor soils and therefore nitrogen could be a critical limiting factor. In fact, *C. multijuga* often occurs on extremely nutrient-poor soil in Amazonian rainforest [32]. The mean total leaf nitrogen content in some *Copaifera* species reaches almost 3% dry weight [26, 32] and the nonprotein imino acid also reaches *ca* 3% nitrogen by weight, in mature leaves (before senescence). Since the nonprotein imino acid is 9.65% nitrogen by weight, then it can comprise 0.3% *ca* 10% of the leaf nitrogen. It also comprises 2–3% dry weight of the seed, although we have no measurements of the total nitrogen of the seed.

Preliminary feeding trials have shown methyl-4-*trans*-hydroxy-L-proline to be inhibitory to both seed and leaf-feeding insects. It is a very effective inhibitor of larval development of the seed-eating bruchid beetle *Callosobruchus maculatus* (Upper Volta strain). The compound was mixed with cowpea flour and incorporated into gelatin capsules to give two concentrations (0.05% and 0.01 wt/wt) with five replications for treatment. A mean of $24.7 \pm 3.23$ adults emerged in the control, $1.0 \pm 0.31$ at 0.05% and no adults at 0.1% of the proline compound. With the generalist leaf-feeding lepidopteran, *Spodoptera littoralis*, the compound was incorporated into a disc at concentrations of 100 and 1000 ppm. An index was calculated as $\frac{C-T}{C+T} \times 100$ where $C$ = weight of control disc eaten and $T$ = weight of test disc eaten ($n = 10$). The index for 100 ppm was $11.2 \pm 8.24$ and for 1000 ppm $26.7 \pm 11.41$. Although it is a significant feeding deterrence at 1000 ppm, the index values are not sufficiently great to be a potent compound against the generalist *Spodoptera littoralis* (Fellows, L. and Simmonds, M., Jodrell Labs, Royal Botanic Gardens, Kew U.K., personal communications). However, results might be expected to differ for native herbivores. Also an important aspect of the defensive properties of this proline compound could be the storage of nitrogen in a form that herbivores are unable to use as a dietary nitrogen source (19, 45) but could be mobilized for plant metabolism.

## CONCLUSIONS

The assessment of food quality for herbivores of *Copaifera* leaves simply by assays widely used for this purpose in ecological studies, such as F–D for total phenolics and Kjeldahl for total nitrogen content, will give misleading results. In addition, the presence of the nonprotein imino acid and its possible ecological importance would be obscured. Finally, although we do not know how widespread such problems might be, these results for *Copaifera* provide a warning regarding its possible existence.

## EXPERIMENTAL

*General.* NMR spectra measured at 25 MHz for $^{13}C$ and 100 and 500 MHz for $^{1}H$. Standard pulse parameters for APT, DEPT and RECAPT were used. MS were obtained by direct inlet at 20 eV with temp. prog. of 10° min to 200°. Only strong and medium bands are reported for UV and IR spectra. Mp are uncorr., optical rotations were measured with a quartz microcell (0.1 ml).

*Plant material.* Leaves of *C. multijuga* were collected from the canopy of mature trees RF 4 and RF 4 and seeds from trees RF 12 and RF 13 (trees 46 and 242 for phenological studies at the site) all growing at Ducke Reserve of the Instituto Nacional de Pesquisas da Amazonia, Manaus, Brazil. Leaves of *C. langsdorfii* were collected from a mature tree (JHL 6442) growing in woodland at Campininha Reserve of the São Paulo Forestry Institute, near Moji Guaçu, S. P., Brazil, and seeds from a tree growing at the edge of Rio Pirayu, Paraguari Dept., Paraguay (JHL 6189). Leaves of *C. venezuelana* var. *laxa*. *C. pubiflora* and *C. officinalis* were sampled from saplings grown from seeds under unitform conditions at UCSC greenhouses [26]. Seeds of *C. venezuelana* var. *laxa* (JHL 6134) and *C. officinalis* (JHL 6078) were from trees growing in dry forests in eastern Venezuela and those of *C. pubiflora* (JHL 6115) from a dry forest island in savanna of central Venezuela. Voucher specimens have been deposited in the Herbarium, University of California, Santa Cruz.

*Extraction and isolation procedures for leaves of C. multijuga* and *C. langsdorfii.* Finely ground air-dried leaves were exhaustively and sequentially extd in a Soxhlet apparatus with $CH_2Cl_2$ and MeOH. The MeOH extract, reduced in vol., was fractionated by pptn with $Et_2O$. The ppt. was filtered off on sintered glass, washed several times with $Et_2O$ and dried *in vacuo*. The $H_2O$-sol. fraction of the ppt. was further purified by passing through a

small column packed with octadecyl (C-18) silica previously equilibrated with MeOH. The eluant was evapd to dryness, in a stream of $N_2$, the crystalline residue dissolved in hot MeOH and allowed to recrystallize at room temp. mp 237–241° (decomp.); $[\alpha]_D$ −74.0 (MeOH; *c*0.01); IR $\nu^{KBr}cm^{-1}$: 3240, 2930, 2360, 1625, 1404, 1078, 922, 799, 729, 707; $^1H$ NMR (500 MHz, $D_2O$); $\delta H_2$, 4.05, *dd* (11, 7.5 Hz); $H_{3a}$ *d* 2.44, *dddd* (14, 7.5, 1.8, 1.6 Hz); $H_{3b}$, 2.16, *ddd* (14, 11, 4.5 Hz); $H_4$, 4.55, m; $H_{5a}$, 3.07, *ddd* (12.6, 1.8, 1.4 Hz); $H_{5b}$, 3.78, *dd* (12.6, 4.5 Hz); N-Me $\delta$2.95, *s*. $^{13}C$ NMR (25 MHz, $D_2O$, acetone-$d_6$ as int. std), $\delta$(C-2, 7.09*, *d*; C-3, 40.3, *t*; C-4, 72.0*, *d*; C-5, 64.2, *t*; C-6, 172.6, *s*; N-Me, 44.1, *q* (where * denotes assignments interchangeable). MS *m/z* (rel. int) [calculated for $C_6H_{11}NO_3$: $M_r$, 145, 1578. Found: $M_r$ (HRCIMS) 145.0763] 145.0763(3.4) $[M]^+$, 100.0759(100) $[M-COOH]^+$, 82.0656(30.03) $[M-COOH-H_2O]^+$.

*Extraction, detection and quantitative analyses of* **1** *in leaves and seeds of* C. venezuelana *var* laxa, C. pubiflora, C. officinalis, C. multijuga *and* C. langsdorfii. Leaves were oven-dried at 40° for 3–5 days (*C. venezuelana*, *C. pubiflora* and *C. officinalis*) or air-dried for several weeks (*C. multijuga* and *C. langsdorfii*), ground in a WIG-L- bug and extracted × 3 with $H_2O$-MeOH (9:1) for 2 hr. Small vols of solvent were added to adjust the final conc of the extracts to 1 mg of tissue/0.1 ml of solvent (1% w/v). Seeds were ground in an electric coffee grinder and extracted as above. Quantitative analyses were based on the comparison of the TLC spot areas shown by standard solns of **1** (100, 200, 300 ppm, etc., in $H_2O$-MeOH, 9:1) and those given by the test extracts (mg/0.1 ml). The test extracts and the standard solns were applied (2:1) on 5 × 10 cm HPTLC Silica gel 60 F-254 plates, developed with MeOH–$H_2O$–EtOAc. (2:1:2), dried and the spots visualized by exposure to $I_2$ vapour ($R_f$ 0.35). Tests had also been made to determine if oven or air-drying of leaves increased the amount of imino acid. Using the same type of analyses as inidicated above pairs of leaflets from three trees of *C. multijuga* gave the same results either after being extracted immediately upon collection or by being either air-dried or oven-dried at 40° for 3–5 days.

*Folin–Denis assay.* The assay was developed as given in ref. [40] and described in ref. [53]. Vols were reduced by a factor of 10 for final reaction vols of 11 ml. To 0.1 ml of test solns of known concn, 3 ml of $H_2O$ and 0.5 ml of the Folin–Ciocalteau reagent were added and well mixed; after 30 sec, and before 8 min, 1.5 ml of 20% $Na_2CO_3$ soln were added, mixed and the vol. adjusted to 11 ml. *A* were read 4 hr later at 765 nm against a blank.

*Methylation of* cis *and* trans-4-*hydroxy*-L-*proline.* Each compound (100 mg) (Sigma) was separately dissolved in 3 ml of 80% MeOH and then 1.5 ml 35% HCHO added while stirring. The mixt. was refluxed for 30 min and allowed to cool to room temp. $NaBH_4$ (100 mg) was added, the mixt. stirred for 1 hr, then evapd to dryness *in vacuo*, dissolved in 80% MeOH and filtered on silca gel [54]. $^1H$ NMR (100 MHz, $D_2O$); $\delta H_2$, 3.28, *dd* (11, 5.5); $H_{3a}$–$H_{3b}$, 1.17–1.95, *m*; $H_4$, 4.26, *m*; $H_{5a}$, 3.05–3.28, *m*; $H_{5b}$, 2.55–2.86, *m*; N-Me, 2.48, *s*. $^{13}C$ NMR (25 MHz, $D_2O$, acetone-$d_5$ int. std), $\delta$C-2, 67.7*, *d*; C-3, 39.1, *t*; C-4, 68.1*, *d*; C-5, 63.1, *t*; C-6, 170.9, *s*; N-Me, 40.6, *q*.

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## REFERENCES

1. Bernays, E. A. and Chapman, R. F. (1978) in *Biochemical Aspects of Plant and Animal Coevolution* (Harborne, J. B., ed), pp. 99–141. Academic Press, London.
2. Schultz, J. C. and Baldwin, I. T. (1982) *Science* **217**, 149.
3. Swain, T. (1977) *Annu. Rev. Plant Physiol.* **28**, 479.
4. Swain, T. (1979) in *Biochemistry of Plant Phenolics* (Swain, T., Harborne, J. B. and Sumere, C. F. V., eds) pp. 617–640. Plenum Press, New York.
5. Harborne, J. B. (1980) in *Secondary Plant Products* (Bell, E. A. and Charlwood, B. V., eds), pp. 329–398. Springer, Berlin.
6. Lindroth, R.L. and Batzli, G. O. (1984) *J. Chem. Ecol.* **10**, 229.
7. Hagerman, A. E. and Butler, L. G. (1978) *J. Agric. Food Chem.* **26**, 809.
8. Martin, J. S. and Martin, M. M. (1983) *J. Chem. Ecol.* **9**, 285.
9. Butler, L. G., Price, M. L. and Brotherton, J. E. (1982) *J. Agric. Food Chem.* **30**, 1087.
10. Temple, A. S. (1982) *J. Chem. Ecol.* **8**, 1289.
11. Steinberg, P. D. (1984) *Science* **223**, 405.
12. Gartlan, J. S., McKey, D. B., Waterman, P. G., Mbi, E. N. and Struhsaker, T. T. (1980) *Biochem. Syst. Ecol.* **8**, 401.
13. Coley, P. D. (1983) *Ecol. Monographs* **53**, 209.
14. Tuomi, J., Niemela, P., Haukioja, E., Siren, S. and Neuvonen, S. (1984) *Oecologia* **61**, 208.
15. Schultz, J. C., Nothnagle, P. J. and Baldwin, I. T. (1982) *Am. J. Botany* **69**, 753.
16. Wilson, M. F. (1984) *J. Chem. Ecol.* **10**, 493.
17. Waterman, P. G., Ross, J. A. M. and McKey, D. B. (1984) *J. Chem. Ecol.* **10**, 387.
18. Mattson, W. J. (1980) *Annu. Rev. Ecol. Syst.* **11**, 119.
19. McNeill, S. and Southwood, T. R. E. (1978) in *Biochemical Aspects of Plant and Animal Coevolution* (Harborne, J. B., ed.), pp. 77–98. Academic Press, New York.
20. Capinera, J. L., Renaud, A. R. and Roehrig, N. E. (1983) *J. Chem. Ecol.* **9**, 1425.
21. Langenheim, J. H. (1969) *Science* **168**, 1157.
22. Langenheim, J. H. (1981) in *Advances in Legume Systematics* (Polhill, R. H. and Raven, P. H., eds), pp. 627–655. Kew, U.K., Proc. Int. Legume Congress, Royal Botanical Gard.
23. Langenheim, J. H. (1984) in *Physiological Ecology of Plants of tle Wet Tropics* (Medina, E., Mooney, H. A. and Vasquez-Yanes, C., eds) pp. 189–208. W. Junk, The Hague.
24. Langenheim, J. H., Lincoln, D. E., Stubblebine, W. H. and Gabrielli, A. L. (1982) *Am. J. Botany* **69**, 595.
25. Langenheim, J. H., Convis, C. L., Macedo, C. A. and Stubblebine, W. H. (1986) *Biochem. Syst. Ecol.* **14**, 41.
26. Langenheim, J. H., Macedo, C. A., Ross, M. K. and Stubblebine, W. H. (1986) *Biochem. Syst. Ecol.* **14**, 51.
27. Léonard, J. (1957) *Mem. Cl. Sci. Acad. R. Belgique* **30**, 1.
28. Arrhenius, S. P., Foster, C. E., Edmonds, C. G. and Langenheim, J. H. (1984) *Phytochemistry* **22**, 471.
29. Arrhenius, S. P. and Langenheim, J. H. (1983) *Biochem. Syst. Ecol.* **11**, 361.
30. Langenheim, J. H., Arrhenius, S. P. and Nascimento, J. C. (1981) *Biochem. Syst. Ecol.* **9**, 27.
31. Figliuolo, R., Crews, P. and Langenheim, J. H. (1987) (Unpublished manuscript).
32. Nascimento, J. C. and Langenheim, J. H. (1986) *Biochem. Syst. Ecol.* **14**, 615.
33. McCloskey, L. P. (1984) Ph.D. Dissertation, Univ. of Calif., Santa Cruz.
34. Patt, S. L. and Shoolery, J. N. (1982) *J. Magn. Res.* **46**, 535.
35. Doddrell, D. M., Pegg, D. T. and Bendall, M. R. (1982) *J. Magn. Res.* **48**, 323.
36. Pegg, D. T., Doddrell, D. M. and Bendall, M. R. (1982) *J. Chem. Phys.* **77**, 2745.

37. Crews, P., Naylor, S., Myers, B. L., Loo, J. and Manes, L. V. (1985) *Magn. Reson. Chem.* **23**, 684.
38. Morgan, J. W. W. (1964) *Chem. Ind.* 542.
39. Goodson, J. A. and Clewer, H. W. B. (1919) *J. Chem. Soc.* 923.
40. Singleton, V. L. and Rossi, Jr, J. A. (1965) *Am. J. Enol. Viticutt.* **16**, 144.
41. Bray, H. G. and Thorpe, W. V. (1954) in *Methods of Biochemical Analysis* (Glick, D. ed.), pp. 27–29. Interscience, New York.
42. Abraham, R. J. and McLauchlan, K. A. (1962) *Mol. Phys.* **5**, 195.
43. Abraham, R. J. and McLauchlan, K. A. (1962) *Mol. Phys.* **5**, 513.
44. Abraham, R. J. and Thomas, W. A. (1964) *J. Chem. Soc.* 3739.
45. Wilson, K. G. and Stinner, R. E. (1984) *Oecologia* **61**, 337.
46. Bernays, E. A. (1983) in *Nitrogen as an Ecological Factor* (Lee, J. A., McNeil, S. and Rorison, I. H., eds), pp. 321–344. Blackwell Scientific, Oxford.
47. Bell, E. A. (1977) in *Natural Products and the Protection of Plants* (Marini-Bettolo, G. B., ed.) Scripta Varia 41, pp. 571–595. Vatican City, Pontifical Academy of Science.
48. Rosenthal, G. A. and Bell, E. A. (1979) in *Herbivores: their Interactions with Secondary Plant Metabolites* (Rosenthal, G. A. and Janzen, D. A., eds), pp. 353–385. Academic Press, New York.
49. Rosenthal, G. A. (1982) *Plant Nonprotein Amino and Imino Acids: Biological, Biochemical and Toxicological Properties.* Academic Press, New York.
50. Bell, E. A. (1981) in *The Biochemistry of Plants: a Comprehensive Treatise* (Conn, E. E., ed.), pp. 1–17. Academic Press, New York.
51. Mooney, H. A., Gulmon, S. L. and Johnson, N. D. (1983) in *Plant Resistance to Insects* (Hedin, P. A., ed.), pp. 21–36. American Chemical Society, Washington, D.C.
52. Fowden. L. and Lea, P. J. (1979) in *Herbivores: Their Interactions with Secondary Plant Metabolites* (Rosenthal, G. A. and Janzen, D. A., eds), pp. 135–160. Academic Press, New York.
53. Amerine, M. A. and Ough, C. S. (1980) *Wine and Must Analysis.* Academic Press, New York.
54. Sondergan, B. L., Hemo, J. H. and Charles, G. (1973) *Tetrahedron Letters* **3**, 261.