Phytochemistry, Vol. 22, No. 7, pp. 1615–1617, 1983.
Printed in Great Britain.

0031–9422/83 $3.00 + 0.00

CIS-4-HYDROXYPIPECOLIC ACID AND 2,4-*CIS*-4,5-*TRANS*-4,5-DIHYDROXYPIPECOLIC ACID FROM *CALLIANDRA*

JOHN T. ROMEO, LEE A. SWAIN and ANTHONY B. BLEECKER

Department of Biology, University of South Florida, Tampa, FL 33620, U.S.A.

(*Revised received* 20 *December* 1982)

Key Word Index—*Calliandra pittieri*; Mimosoideae; *cis*-4-hydroxypipecolic acid; 2,4-*cis*-4,5-*trans*-4,5-dihydroxypipecolic acid; imino acid; pipecolic acid derivatives; stereoisomers.

Abstract—*cis*-4-Hydroxypipecolic acid and 2,4-*cis*-4,5-*trans*-4,5-dihydroxypipecolic acid were isolated from leaves of *Calliandra pittieri*. A system for resolving the eight imino acids isolated from *Calliandra* is described.

INTRODUCTION

Rare pipecolic acid derivatives are relatively common in *Calliandra* species. Previous chemical work has established the presence of: three monohydroxylated pipecolic acids (*trans*-4-, *trans*-5- and *cis*-5-hydroxypipecolic acids); three dihydroxylated pipecolic acids (*trans–cis*, *trans–trans* and *cis–cis*-4,5-dihydroxypipecolic acids); and 2,4-*trans*-acetylaminopipecolic acid in various species [1–4]. The *cis–cis*-4,5-dihydroxypipecolic acid and the *trans*-4-acetylaminopipecolic acid are known only from *Calliandra*. In this paper we report the isolation of two additional rare imino compounds from this genus: *cis*-4-hydroxypipecolic acid, a compound previously reported only from *Strophanthus scandens* [5], and 2,4-*cis*-4,5-*trans*-4,5-dihydroxypipecolic acid (2*S*-carboxy-4*S*,5*S*-dihydroxypiperidine), known only from *Derris elliptica* and two species of *Isoberlina* [6, 7].

RESULTS AND DISCUSSION

During the isolation of the *cis–cis*-4,5-dihydroxypipecolic acid from *C. pittieri* [4], a second blue-gray ninhydrin positive, brick red UV fluorescent, isatin positive compound soluble in 50% ethanol was detected. With high voltage paper electrophoresis (pH 1.9) the substance comigrated with a standard of *trans–cis*-4,5-dihydroxypipecolic acid. On the amino acid analyser, however, the substance was eluted as an early peak at 11.5 min, 1.5 min prior to the other three dihydroxyisomers which co-elute at 13 min. The compound was also distinguished from the other isomers on the basis of 1D-PC in three solvent systems (Table 1). The compound was tentatively identified as 2,4-*cis*-4,5-*trans*-4,5-dihydroxypipecolic acid, the fourth dihydroxy stereoisomer to be detected in the genus. After isolation, identity was confirmed by synthesis and by comparison of IR and NMR spectra with those of an authentic standard.

In the same ethanolic extract, a green ninhydrin positive, brick red UV fluorescent, isatin positive substance was detected. Amino acid analyser analysis and chromatographic data were sufficiently different from those of the *trans*-4-hydroxypipecolic acid to suggest the *cis* form. The compound was isolated, and identity confirmed by synthesis and by comparison of an IR spectrum with that of an authentic standard [5].

With the isolation of these two additional hydroxylated pipecolic acid derivatives, all possible stereoisomeric forms of the 4-, 5- and 4 and 5-hydroxy compounds have been detected in various species of the genus *Calliandra*. The occurrence of both isomers of hydroxylated pipecolic acids in a single plant species has been previously reported. Schenk and Schutte [5] found both *cis* and *trans* forms of 4-hydroxypipecolic acid in *Strophanthus scandens* and Despontin *et al.* [8] found both isomers of 5-hydroxypipecolic acid in *Gymnocladus dioicus*. The widespread occurrence of this phenomenon in *Calliandra* is noteworthy. Species may have both forms of the 4-, 5-, 4 and 5-

Table 1. Amino acid analyser (AAA) elution times and R_{ala} values in chromatographic and electrophoretic systems for *cis*-4-, *trans*-4-, *cis*-5- and *trans*-5-hydroxypipecolic acids and all four dihydroxypipecolic acid isomers

	AAA (min)	*n*-BuOH–HCO_2H–H_2O	*t*-AmOH–lutidine–H_2O	80% PhOH–H_2O	HVE, pH 1.9
cis-4	15.3	0.76	0.97	1.22	0.67
trans-4	16.8	0.91	1.20	1.25	0.67
cis-5	18.5	0.82	0.98	1.27	0.74
trans-5	19.6	0.91	1.22	1.20	0.68
cis–cis	13	0.40	0.49	0.90	0.66
cis–trans	11.5	0.42	0.63	0.70	0.61
trans–trans	12.9	0.63	1.02	0.89	0.66
trans–cis	13	0.50	0.71	0.78	0.61

Table 2. Distribution of mono- and dihydroxypipecolic acid isomers in *Calliandra* species

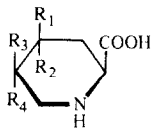
	cis-4	*trans*-4	*cis*-5	*trans*-5	*cis–cis*	*cis–trans*	*trans–trans*	*trans–cis*
C. angustifolia	—	M	M	—	—	—	S	—
C. densifolia	S	—	—	M	—	S	—	—
C. eriophylla 1	—	S	—	M	—	—	—	M
C. eriophylla 2	M	S	—	M	—	—	—	M
C. carbonaria	S	S	S	—	M	—	M	—
C. mexicana	M	S	—	M	—	M	—	M
C. pittieri	S	—	S	M	M	S	—	—
C. speciosa	M	S	M	M	—	—	—	—

S, Strong concentration (> 1 mg/g dry wt); M, moderate (0.1–1 mg/g dry wt); W, weak (< 0.1 mg/g dry wt).

and a variety of dihydroxy combinations (Table 2). An interesting trend which is emerging from the study of the distribution of these compounds within the genus is the tendency for the dihydroxy isomer(s) which is present in a particular species to contain the same absolute configuration about the number 4- and 5-carbons as the monohydroxy isomers with which it co-occurs. For example, *C. angustifolia* which contains the *trans–trans*-dihydroxy isomer has the *trans*-4- and *cis*-5-monohydroxy isomers, the potential precursors of the compound (Table 2, Fig. 1). *C. pittieri* which contains both the *cis–cis*-dihydroxy and the *cis–trans*-dihydroxy isomers contains the *cis*-4-, *cis*-5- and the *trans*-5-monohydroxy compounds as expected, while *trans*-4-hydroxypipecolic acid is not detected. This correlation of stereospecificity at carbons 4 and 5 between the mono and dihydroxy isomers is quite consistent in the several species which have been examined. Data for a few of these are shown in Table 2. Occasionally, a monohydroxy compound with a hydroxyl at a position not occupied in the dihydroxy compound is also detected (e.g. *cis*-4 in *C. eriophylla*-2). While a number of *Calliandra* species do not contain dihydroxypipecolic acid isomers, various combinations of monohydroxypipecolic acids as well as pipecolic acid and proline are found in all species (e.g. *C. speciosa*, Table 2).

Because of similarity in electrophoretic and chromatographic R_fs of: (1) *cis*-4 and *trans*-4; (2) *cis*-5 and *trans*-5; and (3) the *cis–cis*, *cis–trans*, *trans–trans* and *trans–cis* isomers in the common solvent and electrophoretic buffers used most often by chemotaxonomists, one must use extreme caution in interpreting results from such reports. Resolution of all compounds is obtained only by using the combination of techniques described in the Experimental. All compounds are unequivocally resolved with three chromatographic systems, HVE and amino acid analyser analysis (Table 1). We believe that *cis*-5-hydroxypipecolic acid (and possibly *cis*-4 as well) may be much more common in legumes than was previously thought [Romeo, J. T., unpublished results].

In an attempt to determine the stability of our compounds under varying environmental conditions and, hence, their usefulness as potential chemical taxonomic markers, a number of experiments were performed. Tests with three species indicate that while proline and pipecolic acid levels in leaves vary in response to short-term water deficit stress, the other imino acids maintain remarkably constant levels [Romeo, J. T., unpublished results]. Post collection changes in concentrations also are minimal. Several of the compounds show high insecticidal activity suggesting a functional basis for their evolutionary selection [Romeo, J. T., unpublished results]. The distribution data of the nine pipecolic acid derivatives in *Calliandra* species appear to have taxonomic significance. A large number of leaf and seed samples are currently being chemically surveyed in conjunction with a systematic revision of the genus.



pipecolic acid	$R_1 = R_2 = R_3 = R_4 = H$
cis—4—OH pip.	$R_1 = OH$, $R_2 = R_3 = R_4 = H$
trans—4—OH pip.	$R_2 = OH$, $R_1 = R_3 = R_4 = H$
cis—5—OH pip.	$R_3 = OH$, $R_1 = R_2 = R_4 = H$
trans—5—OH pip.	$R_4 = OH$, $R_1 = R_2 = R_3 = H$
trans–trans—4,5—OH pip.	$R_2 = R_3 = OH$, $R_1 = R_4 = H$
trans–cis—4,5—OH pip.	$R_2 = R_4 = OH$, $R_1 = R_3 = H$
cis–cis—4,5—OH pip.	$R_1 = R_3 = OH$, $R_2 = R_4 = H$
cis–trans—4,5—OH pip.	$R_1 = R_4 = OH$, $R_2 = R_3 = H$

Fig. 1. Structure of stereoisomeric forms of various pipecolic acid derivatives.

EXPERIMENTAL

Chromatographic methods. Solvents used for PC were: (1) *n*-BuOH–HCO_2H–H_2O (15:3:2), (2) *t*-AmOH–lutidine–H_2O (89:89:57), (3) 80% PhOH–H_2O (w/v) in the presence of NH_3 vapor. HVE was performed on paper in buffer (pH 1.9, 60 V/cm, 35 min). Automated amino acid analysis was performed on a modified Dionex amino acid analyser adapted for fluorometric detection [9]. This instrument interfaced via an A/D converter with a Hewlett-Packard desk top computer (model 9825A) which stored fluorometer readings at 0.5 sec intervals during the run, and used this information to calculate the integrals of each fluorescence peak [9].

Extraction of leaf samples. Leaf samples were extracted by the method of ref. [10]. Dried leaf material (75 mg) was extracted × 3 with MCW (MeOH–$CHCl_3$–H_2O, 12:5:1). The combined supernatant was separated into an upper aq. and a lower $CHCl_3$ layer by the addition of 1.5 ml H_2O and 1 ml $CHCl_3$. The aq. layer was removed and evaporated completely under a stream of air. The residue was redissolved in 0.5 ml 25% EtOH. This soln was used directly for chromatographic and electrophoretic analysis. Samples for the amino acid analyser were prepared by utilizing

100 μl of the above soln, drying it under a stream of air and redissolving in 0.5 ml 0.2 M Na buffer (pH 2).

Resolution of mono- and dihydroxypipecolic acid isomers. A system for the resolution of all isomeric forms of the mono- and dihydroxypipecolic acid isomers was developed. HVE on paper, pH 1.9, in one direction was followed by 1D-PC in either solvent 1 or 3. A combination of this procedure together with the amino acid analyser analysis distinguished all compounds (Table 1).

Collection and documentation of material. Leaves of *Calliandra pittieri* were collected in July 1982 from a tree growing in Medellin, Colombia. Small samples of other leaves for distribution analysis were taken from herbarium sheets at Universidad Nacional de Colombia. Voucher samples of all specimens are deposited at the Herbarium in the Instituto de Ciencias Naturales in Bogotá.

Isolation of 2,4-cis-4,5-trans-4,5-*dihydroxypipecolic acid.* The neutral amino acid fraction of an ethanolic extract of oven dried *C. pittieri* leaves (800 g) was prepared as reported elsewhere [4]. The thick yellow syrup obtained was diluted with a small vol. of Na citrate buffer (0.2 M citrate, pH 3.1), and placed on a column (2 × 100 cm) of Dowex 50 × 8, cation exchange resin (Na^+), 200–400 mesh. Amino acids were eluted initially with citrate buffer, pH 3.1. After 250 ml of buffer had passed through the column, the pH was increased to 3.3, and after an additional 250 ml had eluted, the pH was increased another 0.2 pH units to 3.5. Fractions (10 ml) were collected at a flow rate of 1 ml/min. Fraction 38, containing only the *cis–trans*-dihydroxypipecolic acid isomer, was desalted on a small column of CG 120 (H^+) resin. The imino acid was eluted from the column with 2 M NH_4OH and evaporated under vacuum. The *cis–trans* isomer was recrystallized in the hydrochloride form from aq. EtOH (yield 75 mg). Fractions 39–80, containing the *cis-trans*- and *cis–cis*-dihydroxy isomers as well as *cis*-4- and *cis*-5-hydroxypipecolic acids, were combined and desalted as above. The concentrate was diluted with a small vol. of 1.5 M HCl and passed through a column (2 × 100 cm) of Dowex 50 × 8, (H^+) resin, 200–400 mesh. Amino acids were eluted with 1.5 M HCl at a flow rate of 1 ml/min in 10 ml fractions. Fractions 50–60 contained only the two dihydroxy isomers, while fractions 65 through 90 contained *cis*-4- and *cis*-5-hydroxypipecolic acids as well as two protein amino acids. Fractions 50–60 were evaporated under vacuum and desalted. The mixture was concd, and the two compounds were separated by ascending PC on Whatman 3MM paper using solvent system 3. The *cis–trans* isomer migrates as the lower band. This was cut from the paper, extracted with a large vol. of H_2O, and placed on a small CG-120 (H^+) column to remove residual PhOH. The *cis–trans* isomer was recrystallized in aq. EtOH as the hydrochloride and combined with the isolate obtained from the citrate fractionation (total yield 580 mg).

Isolation of cis-4-*hydroxypipecolic acid.* Fractions 65–91 obtained from the HCl elution described above were combined and desalted. The concd mixture was subjected to ascending PC on Whatman 3MM paper using solvent system 1. *cis*-4-Hydroxypipecolic acid, which migrates behind cis-5 and ahead of the two unidentified ninhydrin reacting compounds, was cut from the paper, extracted with a large vol. of H_2O, filtered and evaporated under vacuum. Remaining traces of *cis*-5-hydroxypipecolic acid were removed by subjecting this material to HVE (pH 1.9) and eluting the *cis*-4 band. The uncontaminated *cis*-4 isomer was evaporated to a yellow syrup, placed in the freezer at −10° for 24 hr, and crystallized by the addition of 95% EtOH to the frozen material (total yield 145 mg).

Synthesis of 2,4-cis-4,5-trans-4,5-*dihydroxypipecolic acid and cis-4-hydroxypipecolic acid.* The *cis–trans*-dihydroxy isomer was synthesized along with the *trans–trans* isomer from L-Baikiain (Calbiochem) as previously described [3]. The weaker of the two ninhydrin blue bands obtained co-migrated with the isolated *cis–trans* isomer using HVE (pH 1.9).

cis-4-Hydroxypipecolic acid was synthesized from *trans*-4-hydroxypipecolic acid via an epimerization [11]. A small amount of the *trans*-4 isomer (5 mg), isolated from *C. haematocephala*, was heated in satd aq. $Ba(OH)_2$ in a sealed tube at 155° for 12 hr. After desalting on a small CG-120 (H^+) column, the product was analysed chromatographically. Two ninhydrin green spots were visualized; the first co-chromatographed with the *trans*-4 isomer; the second showed R_f mobilities in all solvent systems identical with the natural *cis*-4 isomer. HVE (pH 1.9) mobility and analyser R_f were also identical to those of the natural compound.

Spectral analysis. IR spectral data for *cis*-4-hydroxypipecolic acid matched those provided in the lit. [5]. IR and NMR spectral data for the *cis–trans* isomer corresponded to spectra provided by G. Dardenne (Faculte des Sciences Agronomiques, Gembloux, Belgium) for an authentic standard.

Acknowledgements—We thank G. Dardenne for providing IR and NMR spectra and E. Forero for assistance in obtaining leaf material. This material is based upon work supported by the National Science Foundation under Grant No. DEB 8005567.

REFERENCES

1. Marlier, M., Dardenne, G. and Cassimir, J. (1972) *Phytochemistry* **11**, 2597.
2. Marlier, M., Dardenne, G. and Cassimir, J. (1979) *Phytochemistry* **18**, 479.
3. Bleecker, A. B. and J. T. Romeo (1981) *Phytochemistry* **20**, 1845.
4. Bleecker, A. B. and J. T. Romeo (1983) Phytochemistry **22**, 1025.
5. Schenk, V. W. and H. R. Schutte (1963) *Flora* **153**, 426.
6. Marlier, M., Dardenne, G. and Cassimir, J. (1976) *Phytochemistry* **15**, 183.
7. Shewry, P. R. and L. Fowden (1976) *Phytochemistry* **15**, 1981.
8. Despontin, J., Marlier, M. and Dardenne, G. A. (1977) *Phytochemistry* **16**, 387.
9. Bleecker, A. B. and Romeo, J. T. (1982) *Analyt. Biochem.* **121**, 295.
10. Singh, T. N., Aspinall, D., Paleg, L. G. and Boggess, S. F. (1973) Aust. J. Biol. Sic. **26**, 45.
11. Clark-Lewis, J. W. and P. I. Mortimer (1961) *J. Chem. Soc.* 189.