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Calystegines in wild and cultivated *Erythroxylum* species

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Abstract

Calystegines were identified in the genus *Erythroxylum* for the first time. *Erythroxylum novogranatense var. novogranatense*, a species cultivated for cocaine production, contained 0.2% total calystegines in dry leaves. Forty six *Erythroxylum* herbarium species consisting mostly of leaf tissue were analysed for calystegines, and 38 were found positive. Calystegines were compared qualitatively and quantitatively between individual *Erythroxylum* species. Calystegines A_3 and B_2 were the major calystegines in most species. Total calystegine content reached up to 0.32% dry mass. The simultaneous occurrence of calystegines, cocaine, other alkaloids of a 3α-hydroxy- or 3β-hydroxytropane structure together with nicotine supports the concept of common biosynthetic steps of these alkaloids in *Erythroxylum*. The present results are the basis for further investigations of the phylogenetic origin of tropane alkaloid biosynthesis in the taxonomically remote families Solanaceae and Erythroxylaceae.

Keywords: *Erythroxylum*; Erythroxylaceae; Coca; Tropane alkaloid analysis; Calystegines

1. Introduction

Natural tropane alkaloids of medicinal application, hyoscyamine and scopolamine, are produced in a limited number of species or hybrids, e.g., in the genera *Atropa*, *Datura*, *Hyoscyamus*, and *Duboisia* (Solanaceae). However, alkaloids with the basic tropane bicyclic structure are more widespread in the plant kingdom. In the family Convolvulaceae, being a sister family to Solanaceae within the order Solanales (Stevens, 2001 onward), some *Convolvulus* species contain esterified tropane alkaloids (Jenett-Siems et al., 1998). Some species of the Brassicaceae, Erythroxylaceae, Euphorbiaceae, Proteaceae, and Rhizophoraceae were also reported to accumulate alkaloids with a tropane skeleton and with several structural variations. 4α-Benzylderivatives and pyranotropanes appear in some Proteaceae, and 2-methoxycarbonyl-3β-hydroxytropane benzoylester (cocaine) occur in a few *Erythroxylum* species (Lounasmaa and Tamminen, 1993; Griffin and Lin, 2000). These families belong to various clades in the angiosperms, positioned far away from the Solanales (Stevens, 2001 onward). Recently, a new type of nortropane alkaloids called calystegines was structurally characterised by three to five hydroxyl groups on a nortropane skeleton (Fig. 1). In contrast to most other tropane alkaloids, calystegines are not esterifed. They were initially identified in *Calystegia sepium*, Convolvulaceae (Goldmann et al., 1990), and later detected in numerous other Convolvulaceae (Schimming et al., 1998), and many Solanaceae that produce tropane alkaloids such as *Atropa belladonna* and *Duboisia* species (Draeger, 2004).

The biosynthesis of tropane alkaloids has been intensively investigated in Solanaceae (Robins and Walton, 1993; Hashimoto and Yamada, 1994). The succession

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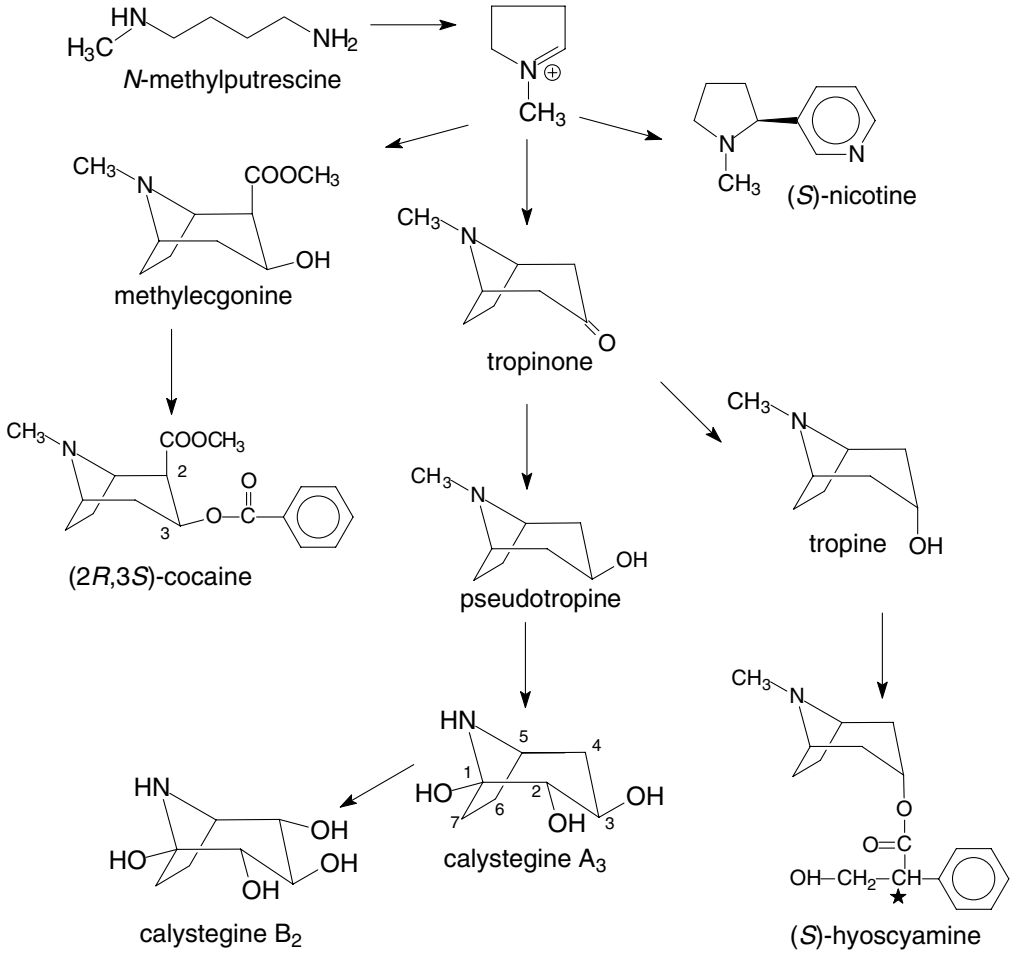

Fig. 1. Current knowledge of nicotine and tropane alkaloid biosynthesis. Arrows may comprise more than one biosynthetic step. Additional alkaloids mentioned in the text are scopolamine = 6,7-epoxyhyoscyamine, calystegine A_5 = 1,3,4-trihydroxynortropane, calystegine B_1 = 1,2,3,6-tetrahydroxynortropane.

of biosynthetic steps was unravelled, and four enzymes of the pathway have been cloned, putrescine *N*-methyltransferase (Suzuki et al., 1999), hyoscyamine 6β-hydroxylase (Matsuda et al., 1991), and two stereospecific tropinone reductases (Nakajima et al., 1993). Calystegines in Solanaceae and Convolvulaceae are formed by the tropane alkaloid biosynthetic pathway (Fig. 1), pseudotropine formation from tropinone being the first calystegine-specific step (Scholl et al., 2001, 2003). The biosynthesis of tropane alkaloids in other plant genera outside the family Solanaceae is less well known, and those investigations that were reported relate mostly to the cultivated *Erythroxylum coca*. Cocaine, the major alkaloid in this species, is a tropane alkaloid with a unique ecgonine base moiety (Fig. 1). Its biosynthesis appears to take place in the leaves of the plant, while in Solanaceae tropane alkaloid biosynthesis is restricted to roots (Leete et al., 1991). Feeding of labelled precursors to *E. coca* indicated biosynthetic steps different from the formation of the tropane bicyclus in Solanaceae (Leete, 1982; Leete and Kim, 1988; Leete et al., 1991). Other tropane alkaloids identified in *Erythroxylum* species, however, do not derive from ecgonine but rather from the stereoisomeric 3α- and 3β-tropine or from the respective tropane-3,6-diols. In addition, nortropane derivatives were also described (Lounasmaa and Tamminen, 1993; Griffin and Lin, 2000; Zuanazzi et al., 2001).

Erythroxylum is the largest genus of four in the family Erythroxylaceae. More than 230 *Erythroxylum* species are distinguished; they are native to subtropical and tropical regions of South America, Africa, South East Asia and Madagascar. The only species of economic interest are cultivated varieties of *E. coca* and *E. novogranatense* because of their cocaine content, which can reach up to 2% of leaf dry mass (Plowman and Rivier, 1983). Cocaine is only accumulated in *E. coca* and *E. novogranatense* and occurs in traces in a few other species; it may not be a distinctive phylogenetic character of the family Erythroxylaceae. The ability of the plants to synthesize the particular ecgonine moiety may be considered to be a singular evolutionary event (autapomorphy) not paralleled in other species and genera. In contrast, the widespread occurrence of other

tropane alkaloids may provide issues for the analysis of the phylogenetic origin of the tropane biosynthetic formation. The pathways in different plant families may be of mono- or polyphyletic origin, i.e., they may have evolved once or several times independently. The question of their phylogenetic origin can only be solved if the distribution of the tropane alkaloids is known beforehand and in detail. In contrast, sequence comparisons of homologous genes from tropane biosynthesis between families and genera alone does not provide evidence for evolution of function. For example, genes with over 50% identity to tropinone reductases termed "putative tropinone reductase" appear almost ubiquitous in the plant kingdom. *Arabidopsis thaliana* possesses16 genes and two pseudogenes annotated as putative tropinone reductase (www.arabidopsis.org), but no tropane alkaloids are formed in the plant (unpublished result). In this study, the distribution of calystegines in 46 different *Erythroxylum* species was analysed. This provides a starting point for further evolutionary and functional analyses using the genes and enzymes involved in the tropane biosynthetic pathway.

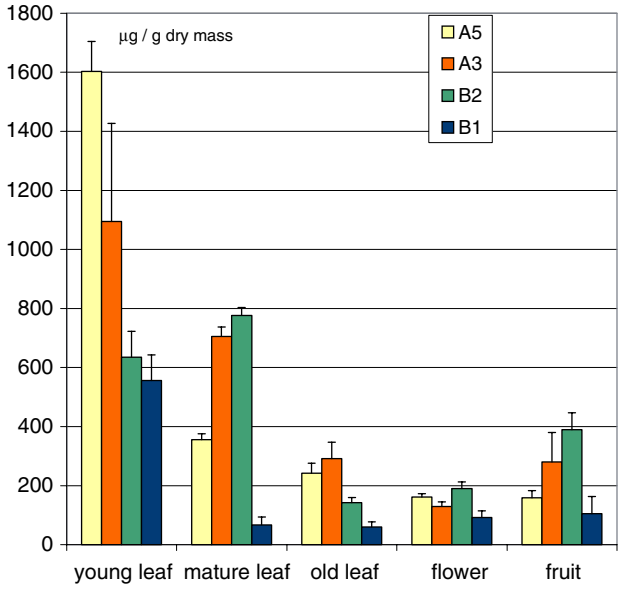

Fig. 2. Concentration of calystegines A5, A3, B2 and B1 in *Erythroxylum novogranatense var. novogranatense* tissues. Error bars indicate standard deviation, $n = 8$.

2. Results

2.1. Calystegines in E. novogranatense

Freshly harvested leaves, fruits and flowers of *E. novogranatense var. novogranatense* were investigated. This allowed to detect calystegines in this genus for the first time. The identification was performed by co-chromatography with reference compounds on thin layer chromatography, by GC with two different columns and detectors (FID, NPD) and by GC–MS. Identification of the major calystegines A_3 and B_2 and further A_5 and B_1 was unambiguous. Additional calystegines, in particular those with a fragmentation pattern of calystegines belonging to the A-group and to the B-group, were observed in minor amounts. However, lack of reference standards precluded their identification. Fourteen different calystegines have been elucidated up to now in Solanaceae and Convolvulaceae, but the composition of each individual plant is, like in *E. novogranatense*, dominated by two compounds, calystegines A_3 or B_2 (Draeger, 2004). Concentration of calystegines in *E. novogranatense* is variable and depends on the plant organ and the developmental stage (Fig. 2). Young leaves contain the highest calystegine concentrations, about 4 mg total calystegines per gram dry mass corresponding to 24 µmol. Similarly, young leaves of *C. sepium* and *A. belladonna* were identified as plant tissues with the maximum amount of calystegines. Concentrations are comparable and reached 14 µmol total calystegines per gram dry young leaves for *C. sepium* (Scholl et al., 2001) and 40 µmol for *A. belladonna* (Draeger et al., 1995). In *A. belladonna*, calystegines in leaves may dominate the total alkaloid composition reaching higher concentration than hyoscyamine and other tropine derivatives. The major alkaloids in leaves of the cultivated species *E. novogranatense* are cocaine and cinnamoylcocaines, together ranging from 1.2% to 2.0% dry mass (Plowman and Rivier, 1983) corresponding to 38–64 µmol per gram dry mass. The same leaf tissue contains 12 µmol per gram dry mass total calystegines, considerably less than cocaine derivatives. For cocaine as well as for calystegines, young leaves are the plant tissues in which the maximum alkaloid concentrations are found (Johnson, 1996).

2.2. Calystegines in other Erythroxylum species

One or several of four different calystegines were found in 38 of the 45 herbarium species examined (Table 1). To the best of our knowledge, for the vast majority of the *Erythroxylum* species, this is the first report on alkaloid identification at all. Most species were never before chemically investigated. Only *E. amazonicum* (Salama et al., 1994), *E. argentinum* (Zuanazzi et al., 2001), *E. campestre* (Aynilian et al., 1974), *E. havanense* (Dominicis and Fernandez, 1991), *E. macrocarpum* (Al-Said et al., 1986), and *E. pulchrum* (Aynilian et al., 1974) were reported to contain alkaloids with a tropane skeleton. The herbarium samples were available in quantities of 150–800 mg dry mass, allowing 3–6 independent extractions. Results were termed as negative, when in none of the extractions calystegines could be quantified. The 50 *Erythroxylum* samples (Table 1) were grouped according to their maximum calystegine

Table 1
Herbarium samples of 45 *Erythroxylum* species

Sample number	Species location and date of collection	Field museum Accession Number	Collector number	Remarks
1	*E. amazonicum* Peyr. vel aff. Venezuela. Estado Falcón. 11°11′30″ N., 69°41′00″ W. Alt. 1200–1400 m. Coll. T. Plowman, P.E. Berry, R. Wingfield; March 1984	F1931666	13413	
2	*E. andrei* sp. nov Brazil. Estado da Bahia. 14°04′ S., 38°58′ W. Sea level. Coll. T. Plowman, A.M. de Carvalho; Feb. 1983	F1916656	12786	
3	*E. argentinum* O.E. Schulz Bolivia. Depto. Tarija. Prov. O'Connor, 21°25′ S., 64°16′ W. Alt. 1700 m. Coll. J.C. Solomon; May 1983	F1929656	10404	
4	*E. bradeanum* O.E. Schulz. Brazil. Município de Santa Maria Madalena 21°56′ S., 41°52′ W. Alt. 450 m. Coll. T. Plowman, H.C. de Lima; Feb. 1983	F1916492	12950	
5	*E. campestre* (Mart.) St. Hil. Paraguay. Coll. Hahn	F1948025	1768	
6	*E. citrifolium* St. Hil. Venezuela. Estado Falcón. 11°11′ N., 69°41′ W. Alt. 1100–1200 m. Coll. T. Plowman, P.E. Berry, R. Wingfield; March 1984	F1931672	13424	
7	*E. coca* var. *coca* Peru, Trujillo desert, collector: unknown			Authenticated by T. Plowman
8	*E. coca* var. *coca* Peru, Tingo Maria, collector: unknown			Authenticated by T. Plowman
9	*E. coca* "*Mate de Coca Boliviana*" Bolivia, La Paz 1998		5 S	Bought on a market in tea bag as "Mate de Coca Boliviana", identified by the alkaloids pattern
10	*E. confusum* Britt. Mexico. Quintana Roo Coll. T. Plowman; May 1982	F1910848	20642	
11	*E. cumanense* H.B.K. Venezuela. Dtto Federal. Alt. 900–950 m. Coll. P.E. Berry; Feb. 1984		4297	
12	*E. cuneifolium* (Mart.) O.E. Schulz Paraguay Parque Nacional Cerro Cora, Coll. Hahn	F1948031	1782	Leaves and twigs
13	*E. cuspidifolium* Mart. Brazil. Estado da Bahia. 13°40′ S., 39°07′ W. Coll. T. Plowman, A.M. de Carvalho; Feb. 1983	F1916662	12810	Leaves and twigs
14	*E. deciduum* St. Hil. Brazilia. Município de Curitiba Coll. T. Plowman, P.E. Berry, F. Juarez; Jan. 1985	F1954563	4457	
15	*E. densum* Rusby Venezuela. Dtto. Federal. Alt. 900–950 m Coll. P.E. Berry; Feb. 1984	F1930165	4298	
16	*E. fimbriatum* Peyritsch vel aff. Coll. T. Plowman	F1899111	11400	
17	*E. foetidum* Plowman ("*nervosum*" Plowman sp. nov. ined.) Venezuela. Ter. Fed. Amazonas. Depto Átures. Puerto Ayacucho, Coll. T. Plowman; April 1984	F1933543	13731	*E.* "*nervosum*" nom. inval., corrected into *E. foetidum*
18	*E. frangulifolium* O.E. Schulz Brazil. Município de Maricá 22°58′ S., 42°54′ W. Sea level, Coll. T. Plowman; Feb. 1983	F1916625	12860	
19	*E. glazioui* O.E. Schulz. Brazil. Município de Cabo Frio. Sea level. Coll. T. Plowman, D. Araujo; Feb. 1985	F1947952	13939	
20	*E. gonocladum* (Mart.) O.E. Schultz Brazil. Município de Macaé. Alt. 1150–1250 m. Coll. T. Plowman, C. Farney, G. Martinelli, S. Pessoa, A. Costa; Nov. 1985	F1950280	585	
21	*E. havanense* Jacq. Venezuela. Dtto Fed.. Alt. 900–950 m. Coll. P.E. Berry; 1979	F1859518	3494	
22	*E. hypoleucum* Plowman Venezuela. Ter. Fed. Amazonas. Depto Rio Negro Coll. T. Plowman; April 1984	F1932963	13700	

23	*E. aff. impressum* O.E. Schulz Peru. Depto Cajamarca. Prov. Jaén. Alt. 520 m. Coll. T. Plowman; July 1986	F1973368	14253	
24	*E. lealcostae* Plowman Brazil. Estado da Bahia. Município de Salvador. 12°55′ S., 38°21′ W. Sea level Coll. T. Plowman; Jan. 1983	F1916645	12770	Leaves and twigs
25	*E. ligustrinum* var. *ligustrinum* DC. (*E. aturense* Plowman sp. nov. ined.) Venezuela. Ter. Fed. Amazonas. Depto Átures. 5°22′ N., 67°33′ W. Coll. T. Plowman; April 1984	F1934043	13773	*E. aturense* Plowman sp. nov. ined. – nom. inval. corrected into *E. ligustrinum var. ligustrinum*
26	*E. macrocarpum* Mauritius Coll. V. Ridges, provided by William C. Evans., Nottingham		5	Endemic plant of Mauritius, leaves and twigs
27	*E. macrophyllum* Cav. (syn. *lucidum E.* H.B.K.) Mexico. Selva alta Perennifolia. 18°18′ N., 94°47′ W. Alt. 300 m. Coll. M. Nee, G. Diggs, F. Ramirez R.; July 1982	F1921436	25112	Former *E. lucidum*, corrected into *E. macrophyllum*
28	*E. macrophyllum* Cav. var. *savannarum* Plowman (*E. savannarum* Plowman sp. nov. ined.) Venezuela. Ter. Fed. Amazonas. Depto Átures Coll. T. Plowman, F. Guánchez; April 1984	F1934047	13757	Independent species *E. savannarum* is not described
29	*E. macrophyllum* Cav. Coll. T. Plowman, H. Kennedy	F1763768	5804	
30	*E. magnoliifolium* St. Hil. Brazil. Município Rio de Janeiro. Mata de Lagôa. Alt. 150–350 m. Coll. T. Plowman; April 1980	F1872652	10101	
31	*E. martii* Peyr. Brazil. Estado da bahia. Município de Ituberá. 13°40′ S., 39°07′ W. Coll. T. Plowman, A.M. de Carvalho; Feb. 1983	F1916659	12805	
32	*E. mattos-silvae* Plowman Brazil. Município de Ilhéus Coll. T. Plowman, L.A.M. Silva, T.S. dos Santos; Feb. 1985	F1944101	13970	
33	*E. mexicanum* H.B.K. Mexico. Município San Andrés Tuxtla. 18°27′30″ N., 95°11′15″ W. Alt. 300 m. Coll. M. Nee, G. Diggs, G. Schatz; July 1982	F1921739	24756	
34	*E. mikanii* Peyr. Brazil. Município de Ilhéus Coll. T. Plowman, L.A.M. Silva, T.S. dos Santos; Feb. 1985	F1944044	13959	
35	*E. mucronatum* Benth. Brazil. Município de Ilhéus Coll. T. Plowman	F1898255	11375	
36	*E. myrsinites* Mart. Brazil. Município de Balsa Nova. Alt. 885 m. Coll. T. Plowman, P.E. Berry; Jan. 1985	F1954578	4490	Leaves and twigs
37	*E. ochranthum* Mart. Brazilia. Município de Ilhéus Coll. T. Plowman, L.A.M. Silva, T.S. dos Santos; Feb. 1985	F1944049	13968	
38	*E. orinocense* H.B.K. Venezuela. Ter. Fed. Amazonas. Depto Átures. Savanna Coll. T. Plowman, F. Guánchez; April 1984	F1934045	13755	
39	*E. ovalifolium* Peyr. Brazil. Município de Maricá. 22°58′ S., 42°54′ W. Sea level Coll. T. Plowman; Feb. 1983	F1916623	12840	
40	*E. passerinum* Mart. Brazil. Município de Cabo Frio. Sea level Coll. T. Plowman, D. Araujo; Feb. 1985	F1947948	13936	
41	*E. pictum* E. Mey. South Africa, Coll. by R.G. Strey, obtained from W.C. Evans.		10277	Stem bark and twigs
42	*E. pulchrum* St Hil. Brazil. Município de Cabo Frio. Sea level Coll. T. Plowman, D. Araujo; Feb. 1985	F1947953	13940	
43	*E. roraimae* (sp. aff. E. roraimae) Klotsch ex O.E. Schulz Brazil. Amapà. Município de Mazagão. 0°10′ N., 51°37′ W. Coll. S. Mori, B. Rabelo, R. Cardoso; Dec. 1984	F1962321	17485	Corrected into "*E. roraimae*"
44	*E. rufum* Cav. Venezuela. Ter. Fed. Amazonas. Depto Átures Coll. T. Plowman, F. Guánchez; April 1984	F1934034	13765	
45	*E. shatona* Macbride Coll. T. Plowman	F1824664	6046	

(*continued on next page*)

Table 1 (*continued*)

Sample number	Species location and date of collection	Field museum Accession Number	Collector number	Remarks
46	*E. splendidum* Plowman sp. nov. Brazil. Município de Valença. Guaibim. 13°18′ S., 39°00′ W. Sea level Coll. T. Plowman, A.M. de Carvalho; Feb. 1983	F1916633	12818	Leaves and twigs
47	*E. suberosum* St. Hil. Coll. Hahn; Jan. 1983	F1948034	1787	
48	*E. suberosum* St. Hil. Brazil. State of Goiás Coll. M.J. Balick et al.; Nov. 1981	F1945882	1308	
49	*E. subrotundum* St Hil. Brazil. Município de Cabo Frio. Armação de Búzios Coll. T. Plowman, D. Araujo; Feb. 1985	F1947945	13942	
50	*E. ulei* O.E. Schulz Coll. T. Plowman, M. Ramirez, P.M. Rury	F1896991	11419	

Voucher specimens of the South American species authenticated by Dr. Timothy Plowman are available in the Field Museum, Chicago, Illinois, and carry an accession number. Species without accession number derive from Mauritius, South Africa or do not have a corresponding voucher specimen in the Field Museum herbarium. They were authenticated by the collectors as indicated. All samples used for analysis were leaves except those indicated otherwise.

concentration (Fig. 3A–C). Leaves of *E. argentinum* (3; numbers relate to Table 1), *E. bradeanum* (4), *E. cuspidifolium* (13), *E. frangulifolium* (18), *E. macrocarpum* (26), *E. roraimae* (43), *E. splendidum* (46), *E. ulei* (50) and one of the leaf samples of *E. macrophyllum* (29) did not show any calystegines. *E. pictum*, of which only stem bark was available, contained calystegine A3 (sample 41, Fig. 3B). Highest calystegine concentrations in *E. ochranthum* reached more than 3.2 mg per gram dry mass (sample 37, Fig. 3C) and were comparable to young leaves of *E. novogranatense* (Fig. 2). Comparison of calystegine contents to other alkaloid concentrations in the respective species is prevented, because the above mentioned reports of alkaloids other than cocaine in *Erythroxylum* do not give quantities. As in many Solanaceae, calystegine B_2 was the major calystegine in most *Erythroxylum* samples; however, some species differed in their calystegine pattern. One *E. coca* (8) sample contained predominantly calystegine B_1. The investigation of more *Erythroxylum* species will be required, before alkaloid pattern may be possibly useful for phylogenetic analysis of the genus and the family Erythroxylaceae.

3. Discussion

Calystegines were identified and measured in the genus *Erythroxylum* for the first time. Most of the *Erythroxylum* species analysed contained at least one calystegine. Nine species either did not show any calystegines or contained only traces that could not be quantified. All analyses were performed to compare calystegines qualitatively and quantitatively between individual samples and species. For quantitative data, the age of the herbarium samples must be taken into consideration. Whether and how much calystegines are degraded during more than 20 years of dry storage cannot be determined. Thus, the data should be considered as minimum calystegine content. However, freshly harvested mature leaves of *E. novogranatense* did not contain more calystegines than average herbarium samples, indicating that the compounds do not seem to degrade easily with time. Preservation of cocaine in *E. coca* leaves was shown for over 44-year-old herbarium samples (Aynilian et al., 1974). Alkaloids in herbarium samples are well preserved provided that the plant tissues were air-dried (Balick et al., 1982). *Erythroxylum* species are known for their cocaine content, but cocaine is found in considerable amounts only in the cultivated *Erythroxylum* varieties of *E. coca* and *E. novogranatense* (Plowman and Rivier, 1983). The specimen of *E. novogranatense* var. *novogranatense* used in this study contained 5.45 mg cocaine, i.e., 18 μmol per gram dry mass in mature leaves confirming published data for cocaine content in this variety (Plowman and Rivier, 1983). Comparison to 12 μmol per gram dry mass total

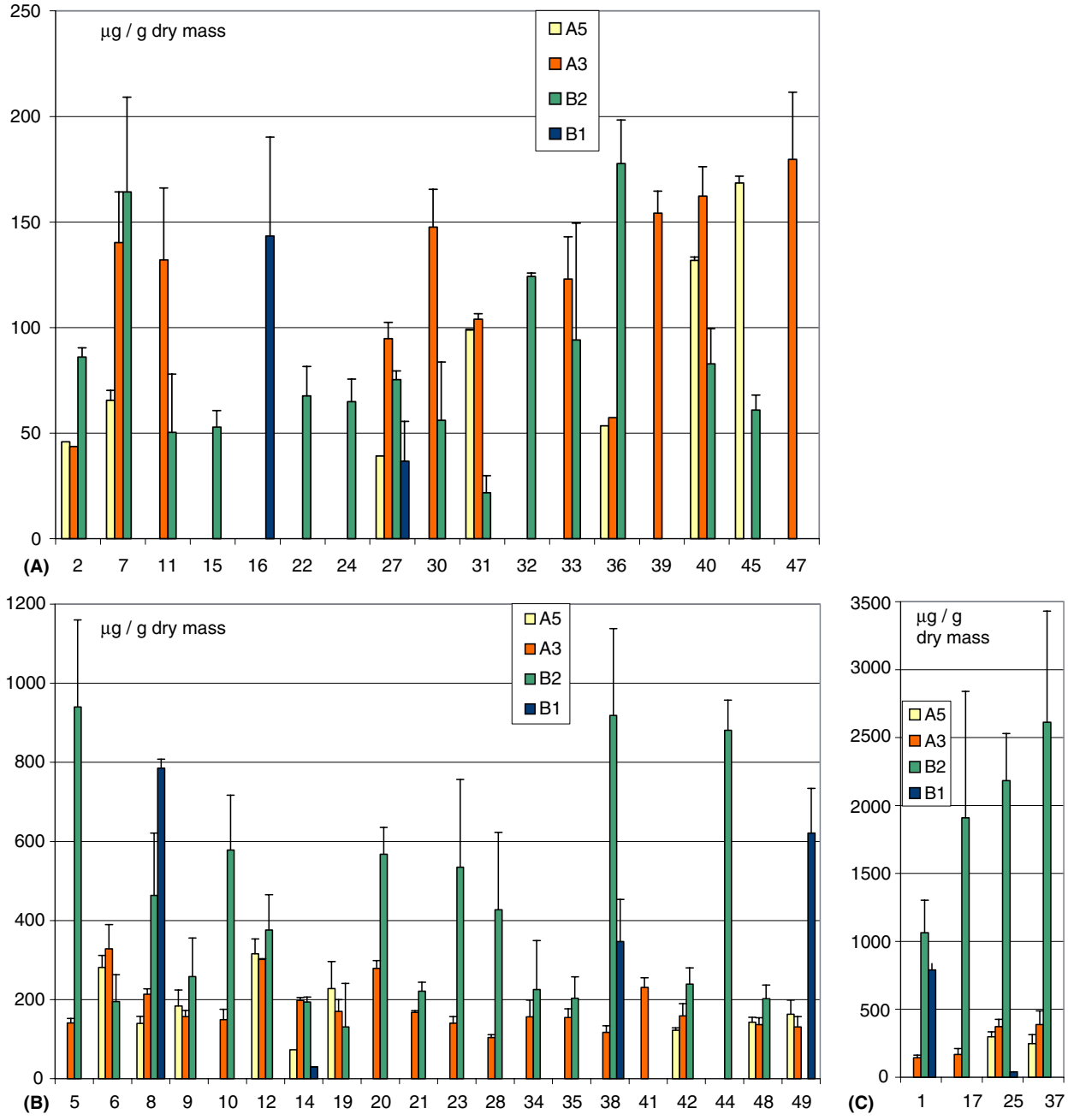

Fig. 3. Calystegines in *Erythroxylum* species. Species numbers relate to Table 1. Error bars indicate standard deviation, $n = 3–6$. (A) Species with calystegines ranging up to 200 µg per gram dry mass. (B) Species with calystegines ranging from 200 to 1000 µg per gram dry mass. (C) Species with calystegines ranging with more than 1000 µg per gram dry mass.

calystegines in the same leaves illustrates that a substantial part of the total tropane alkaloids in leaves are calystegines.

While cocaine is the most well-known alkaloid in the genus *Erythroxylum* due to its strong pharmacological effects, ecgonine derivatives in general are less frequent in *Erythroxylum* species than other alkaloids with a tropane bicyclic skeleton (Lounasmaa and Tamminen, 1993; Bringmann et al., 2000; Griffin and Lin, 2000; Mi et al., 2002; Zanolari et al., 2003). Tropane alkaloids were described with various structures and from different parts of the plants, e.g., stem bark and root bark. 3α- and 3β-hydroxytropane are frequently esterified with various acids. The tropane ring system may carry further hydroxyl groups, predominantly in ring position 6 and 7. The bridge head carbon C1 typically carries a hydroxyl group in calystegines, and equivalent 1-hydroxytropane alkaloids were also reported in *Erythroxylum* species (Moore et al., 1993; Lounasmaa and Tamminen, 1993). *N*-Demethyltropane structures have

been described as well (Christen et al., 1995; Zuanazzi et al., 2001; Khattak et al., 2003). The biosynthetic sequence initially thought specific for ecgonine and cuscohygrine formation in *Erythroxylum* (Leete et al., 1991; Newquist et al., 1993) was later proposed to operate in Solanaceae accordingly, indicating similarities between the biosynthesis of cocaine and hyoscyamine (Abraham and Leete, 1995).

Many species that were reported to possess tropane alkaloids of any structure are currently analysed for calystegines, and many are positive. However, other families remain to be examined, e.g., Brassicaceae, Proteaceae and Rhizophoraceae. Recent taxonomic investigations have related Rhizophoraceae as a sister family to Erythroxylaceae (Setoguchi et al., 1999; Schwarzbach and Ricklefs, 2000). Both belong to the Rosidae and are placed far from the Solanaceae and Convolvulaceae that are in the Lamiidae. Rhizophoraceae were also reported to contain tropane alkaloids, e.g., brugine in *Bruguiera sexangula* and *Bruguiera cylindrica* (Lounasmaa and Tamminen, 1993; Griffin and Lin, 2000). Leaves of *B. cylindrica* and of *Bruguiera gymnorhiza* were found to contain calystegines as well (unpublished results).

The widespread occurrences of calystegines in the American species of *Erythroxylum* and including one South African species point to an ancient origin of this biosynthetic pathway. The genes involved in the calystegine synthesis may, thus, be orthologous and may have evolved at least close to the origin of the genus. For an understanding of the evolutionary development of tropane alkaloid metabolism in taxonomically remote families of angiosperms, biosynthetic steps and enzymes participating in tropane alkaloid formation must now be identified. Co-occurrence of nicotine together with tropane alkaloids in *Erythroxylum* (El-Imam et al., 1988; Christen et al., 1993; Brachet et al., 1997) suggests that the first specific steps of the alkaloid pathway leading to *N*-methyl putrescine and the pyrrolinium cation are common for calystegines, nicotine and cocaine in Solanaceae and Erythroxylaceae. To test this hypothesis, the first specific enzyme of the tropane alkaloids and nicotine formation, putrescine *N*-methyltransferase should be investigated in Erythroxylaceae and compared to those enzymes of the Solanaceae. The amino acid and cDNA sequences of this and of further homologous enzymes with a proved function in tropane alkaloids biosynthesis from taxonomically remote families of angiosperms may then enable conclusions on the evolution of tropane alkaloid metabolism. It is interesting that tropinone reductase-like sequences with an identity of more than 50% to tropinone reductases from *Datura stramonium* (GenBank Accession Nos. L20473 and L20474) are annotated in many plant genome sequencing projects such as Arabidopsis and rice and in EST data banks, e.g., of *Medicago truncatula*. Human and Drosophila genomes also contain such open reading frames, and they contain further typical traits of genes coding for short chain dehydrogenase enzymes. The ubiquitous presence of highly variable ketone reductases may have formed the biochemical background in favour of repeated (or conserved) invention of tropane alkaloid biosynthesis.

4. Experimental

4.1. Plant material

E. novogranatense var. *novogranatense* plants were cultivated for analysis. Fresh plant tissue was freeze-dried. Herbarium samples, which belong to the collection of the late Dr. Timothy Plowman, were obtained from Dr. Laurent Rivier, Lausanne, Switzerland, who cooperated with Plowman during the 1980s. The herbarium collector numbers were compared to the database of the Field Museum Chicago, Illinois (http://www.fieldmuseum.org/). South American species could be related to the equivalent voucher deposited in the Field Museum using the collector number, the collection date and location indicated on the samples as identifier (Table 1). Species originating from Mauritius, South Africa, or those without a correspondent voucher specimen in the Field Museum herbarium were authenticated by the collectors. Voucher specimens of these samples are available at the School of Pharmaceutical Sciences EPGL of Geneva University. All samples used for analysis were leaves if not indicated otherwise.

4.2. Extraction and sample preparation

Dry plant material was ground to a fine homogenous powder by a MM 200 RETSCH ball-mill and finally sieved to an average particle size less than 125 μm. Samples (50–100 mg) were extracted 3 times with 5 ml 50% aqueous methanol. Combined extracts were evaporated and applied to strong cation exchange columns for calystegine purification as described elsewhere (Keiner and Draeger, 2000). Calystegines were eluted with 2 M ammonia and evaporated to 600 μl. These extracts were used for identification of calystegines by thin layer chromatography (Draeger, 2002). Derivatisation before GC separation was done with freeze-dried extract aliquots in pyridine with hexamethyldisilazane and trimethyl chlorosilane for 30 min at 50 °C. Azobenzene was added together with the silylating reagents as internal standard for all measurements. Before injection, samples were filled up to 100 or 500 μl with dry hexane. Crude leaf extracts were also analysed without cation exchange purification. In many cases, several centrifugation steps were necessary before GC analysis to remove particles that formed upon silylation. Some

extracts showed many overlapping signals that made quantification impossible. Therefore these extracts were re-analysed after cation exchange purification as described above.

4.3. Gas chromatography

Calystegines were measured by GC as described elsewhere (Keiner and Draeger, 2000); separation was done twice: (1) gas chromatograph HP 6890 with simultaneous detection by FID and PND, 1 μl split injection with a split ratio of 20:1, column HP1 (30 m × 0.25 mm i.d., 0.25 μm film thickness), carrier gas helium (1 ml min^{-1} constant flow), injection temp. 250 °C, detector temp. 310 °C, temp. programme 160 °C, 5 °C min^{-1} up to 240 °C; (2) gas chromatograph HP 5890, detector HP 5972, 1 μl splitless injection with a splitless period of 1 min, HP5 (30 m × 0.25 mm i.d., 0.25 μm film thickness) carrier gas helium (1 ml min^{-1} constant flow), injection temp. 250 °C, detector temp. 250 °C, EIMS 70 eV, temp. programme 100 °C, 2 min hold, 5 °C min^{-1} up to 240 °C. Quantification was done using the FID signal and the FID/NPD signal ratio for peak purity. Calystegines B2 and A3 were used for calibration, their response by FID and PND, respectively, was almost the same. Calystegine B_1 was measured using the calibration for calystegine B_2, calystegine A3 calibration was used for calystegine A_5 quantification. The quantification limit using 50 mg dry plant material and the aforementioned conditions was 20 μg per gram dry mass. Identification of calystegines was possible, when more than 6 μg per gram dry mass were contained. Cocaine in *E. novogranatense* was measured by GC-FID/NPD in the methanolic extract without cation exchange purification and without silylation. The same temperature programme was used as for calystegine separation. Calibration was done with authentic cocaine.

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