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Short communication

Separation of *E–Z* isomeric macrocyclic spermine alkaloids of *Verbascum pseudonobile* and *Verbascum phoeniceum* and of their derivatives using thin-layer chromatography

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Abstract

Twelve *E–Z* isomeric pairs of *Verbascum pseudonobile* Stoj. et Stef. and *Verbascum phoeniceum* L. (Scrophulariaceae) macrocyclic spermine alkaloids and of related compounds and six related dihydro derivatives were studied by TLC on silica in nine systems and on alumina in four systems. In most cases the *Z* isomers exhibited higher retention than their *E* counterparts. Four *E–Z* isomeric pairs of cinnamic acid amides were studied for comparison.

Keywords: Verbascum; Alkaloids; Spermine alkaloids

1. Introduction

Paskov et al. [1] established in the 1960s that the total alkaloid-containing extract of *Verbascum pseudonobile* Stoj. et Stef. (Scrophulariaceae), a species endemic in Bulgaria, exhibited a hypotensive and spasmolytic effect. Following an extended preclinical [1–4] and clinical study, this extract has been released in Bulgaria for the production and distribution of tablets and suppositories in spasmolytic indication under the name Verbascan.

A crystalline substance was isolated from this extract in 1971 and named verbaskine [5]. Its structure was elucidated later [6]. It has been found to represent a macrocyclic compound containing spermine and amidically bound (*E*)-cinnamic and phenylpropionic acid (see **21** in Table 1).

The number and the structures of the main alkaloids present in the natural mixture have been established recently [7]. Major components (more than 90% of the mixture) are the following four macrocyclic lactam spermine alkaloids, derivatives of the *E* and *Z* isomers of cinnamic and 3,4-dimethoxycinnamic acid: verbacine (**1**, about 50%), verballocine (**2**, about 40%), isoverbasitrine (**4**, about 10%) and verbasitrine (**3**, about 1%). It has also been found [7] that verbaskine (**21**) is not a natural substance, but an

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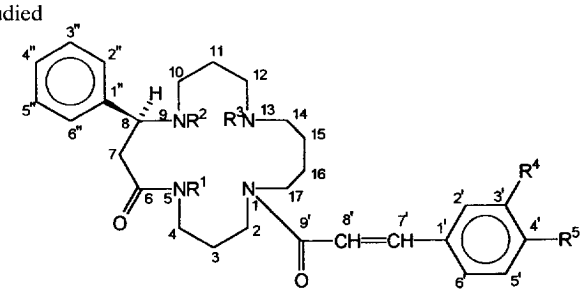
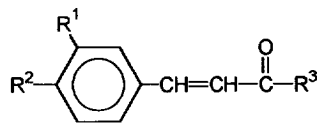
Table 1
Structures of the compounds studied



Compound	R^1	R^2	R^3	R^4	R^5	$C^{7'}$–$C^{8'}$ bond
1 (verbacine)	H	H	H	H	H	*trans*
2 (verballocine)	H	H	H	H	H	*cis*
3 (verbasitrine)	H	H	H	$-OCH_3$	$-OCH_3$	*trans*
4 (isoverbasitrine)	H	H	H	$-OCH_3$	$-OCH_3$	*cis*
5	H	H	H	H	H	Dihydro
6	H	H	H	$-OCH_3$	$-OCH_3$	Dihydro
7 (verbascenine)	H	H	$-COCH_3$	H	H	*trans*
8 (verballoscenine)	H	H	$-COCH_3$	H	H	*cis*
9	H	H	$-COCH_3$	$-OCH_3$	$-OCH_3$	*trans*
10	H	H	$-COCH_3$	$-OCH_3$	$-OCH_3$	*cis*
11	H	$-COCH_3$	$-COCH_3$	H	H	*trans*
12	H	$-COCH_3$	$-COCH_3$	H	H	*cis*
13	H	$-COCH_3$	$-COCH_3$	$-OCH_3$	$-OCH_3$	*trans*
14	H	$-COCH_3$	$-COCH_3$	$-OCH_3$	$-OCH_3$	*cis*

Compound	R^1	$R^2 + R^3$	R^4	R^5	$C^{7'}$–$C^{8'}$ bond
15	H	$-CH_2-$	H	H	*trans*
16	H	$-CH_2-$	H	H	*cis*
17	H	$-CH_2-$	$-OCH_3$	$-OCH_3$	*trans*
18	H	$-CH_2-$	$-OCH_3$	$-OCH_3$	*cis*
19	H	$-CH_2-$	H	H	Dihydro
20	H	$-CH_2-$	$-OCH_3$	$-OCH_3$	Dihydro
21 (verbaskine)	H	–CO–	H	H	*trans*
22	H	–CO–	H	H	*cis*
23	H	–CO–	$-OCH_3$	$-OCH_3$	*trans*
24	H	–CO–	$-OCH_3$	$-OCH_3$	*cis*
25	H	–CO–	H	H	Dihydro
26	H	–CO–	$-OCH_3$	$-OCH_3$	Dihydro
27	$-CH_3$	–CO–	H	H	*trans*
28	$-CH_3$	–CO–	H	H	*cis*
29	$-CH_3$	–CO–	$-OCH_3$	$-OCH_3$	*trans*
30	$-CH_3$	–CO–	$-OCH_3$	$-OCH_3$	*cis*
31	$-CH_3$	–CO–	H	H	Dihydro
32	$-CH_3$	–CO–	$-OCH_3$	$-OCH_3$	Dihydro

Table 1 (*continued*)



Compound	R^1	R^2	R^3	C^7–C^8 bond
33	$-OCH_3$	$-OCH_3$	$-NH_2$	*trans*
34	$-OCH_3$	$-OCH_3$	$-NH_2$	*cis*
35	H	H	$-NH_2$	*trans*
36	H	H	$-NH_2$	*cis*
37	H	H	$-N(CH_3)_2$	*trans*
38	H	H	$-N(CH_3)_2$	*cis*
39	H	H	$-N(CH_2CH_3)_2$	*trans*
40	H	H	$-N(CH_2CH_3)_2$	*cis*

artifact produced, like its analogues (**22–24**), from the native alkaloid (**1–4**) by the attack of phosgene, which often contaminates chloroform used as an extractant [7,8]. In addition, N(9),N(13)-methylene derivatives (cyclic aminals) of **1–4** have also been isolated (**15–18**) from plant extracts as artificial condensation products of **1–4** with formaldehyde [7,8].

In the course of preparative and structural studies, a number of derivatives of the main alkaloids **1–4**, including their N(13)-monoacetylated (**7-10**) and N(9),N(13)-diacetylated (**11–14**) analogues have been produced [7,8]. Substance **7**, N(13)-acetylverbacine, is identical with the alkaloid verbascenine [8] which has been isolated from *Verbascum phoeniceum* L. and *Verbascum nigrum* L. (Scrophulariaceae) and structurally characterized [9]. Its N(9)-acetylated derivative prepared semi-synthetically [9] is identical with N(9),N(13)-diacetylverbacine (**11**) [7,8]. Later, from *Verbascum phoeniceum* L. collected in western Bulgaria, along with **1**, **2** and verbascenine (**7**), the (7′,8′)-*Z* isomer of the latter was isolated, which is identical with N(13)-acetylverballocine (**8**) prepared earlier [8]. This natural substance was named verballoscenine [8]. Its additional acetylation yields a derivative identical with N(9),N(13)-diacetylverballocine (**12**) [8].

This paper is devoted to the study of the thin-layer chromatographic (TLC) behaviour of the *Z*–*E* isomeric pairs of alkaloids, their derivatives and the respective (7′,8′)-dihydro analogues in mobile phases differing in principle. For the sake of comparison with the retention sequence, some simpler isomeric pairs, namely the amides of (*E*)- and (*Z*)-cinnamic (**35–40**) {cinnamic acid amide (**35**) has also been isolated from the extracts of *Verbascum pseudonobile* [10]} and 3,4-dimethoxycinnamic (**33** and **34**) acids were chromatographed.

2. Experimental

2.1. Analytes

Natural alkaloids **1–4** were prepared from plant source using preparative TLC [7,8].

The dihydro derivatives **5**, **6**, **25** and **26** were prepared by catalytic hydrogenation of **1** (or **2**), **3** (or **4**), **21** (or **22**) and **27** (or **28**), respectively [7,8].

Compounds **15–20** were prepared by treatment of **1–6** with formaldehyde and **21–26** by treatment of **1–6** with phosgene [7,8].

Compounds **21–26** were converted into **27–32** by partial methylation according to Ref. [6].

The *Z* isomers **34**, **36**, **38** and **40** were prepared by photoisomerization of their respective *E* isomers by a 3-h exposure of their 0.2% ethanolic solutions to UV radiation from a Camag 254-nm UV lamp placed 3 cm above the liquid level (no filter) [11].

Substances **1–14** and **21–32** are C(8) -*S*-(−)- and substances **15–20** are C(8) -*S*-(+)- [7,9].

2.2. Solvents

Solvents were of analytical-reagent grade unless indicated otherwise: acetic acid (Merck, Darmstadt, Germany); 1-butanol (Merck), not redistilled; chloroform, stabilized with 1% ethanol (Merck), not redistilled; diethylamine (Fluka, Buchs, Switzerland), not redistilled; ethanol (96%, v/v), chemically pure, redistilled; ethyl acetate (Merck), redistilled; methanol (Merck), not redistilled; and toluene (Merck), not redistilled.

2.3. Chromatographic techniques

Kieselgel 60 F_{254} and Aluminiumoxid 60 F_{254} glass plates were obtained from Merck.

A Camag 20/20 chromatographic tank was used, with equilibration for 30 min before chromatography. Volumes of 3 μl of 2% chloroform solutions of the compounds tested were spotted. The distance between the origin and the solvent front was 15 cm.

Detection was effected by quenching of background fluorescence or with Dragendorff's reagent (D144a [12]). The amidic derivatives of (*E*)-3,4-dimethoxycinnamic acid (**3**, **9**, **13**, **17**, **23**, **29** and **33**) showed a bluish fluorescence when excited at 365 nm.

TLC systems

The following solvent systems were used: S1 = butanol–acetic acid–water (40:10:50, v/v/v), the organic phase being used; S2 = toluene–96% ethanol (90:10, v/v); S3 = toluene–96% (v/v) ethanol (90:10, v/v), diethylamine in the gas phase; S4 = toluene–tetrahydrofuran (40:60, v/v); S5 = toluene–tetrahydrofuran (40:60, v/v), diethylamine in the gas phase; S6 = ethyl acetate–methanol (80:20, v/v); S7 = ethyl acetate; S8 = toluene–tetrahydrofuran (70:30, v/v); and S9 = toluene–ethyl acetate (70:30, v/v).

2.4. Selection of solvent systems

In the first stage of the study, solvents conventionally used for analytes of similar polarity and especially for *Verbascum* alkaloids were successfully tested. For instance, Partridge's [12,13] long-known mixture (S1) gave good separations of most of the substances in our set, but with slow running, necessitating subsequent removal of acetic acid, and hydrolysed compounds **15**–**20** to **1**–**6**. Benzene–96% ethanol (80:20) [5] was less suitable and was modified by replacing benzene with toluene (S2) and by combining it with diethylamine (S3). S6, used for verbascenine by Seifert et al. [9], separated **7** well from its isomer verballoscenine **8**.

The later stage of our study was inspired by Snyder et al.'s [14] conclusion that closely related compounds (*threo–erythro* isomers in his case) were separated better when "less strongly localizing" (lower *m*) solvents were used. In addition to the protic (hydrogen donor plus acceptor) constituents of solvents S1–3 and S6, we therefore tested aprotic (hydrogen acceptor) solvents (S4 and S7–S9).

3. Results and discussion

The results for all the compounds under study (Table 1) are presented in Table 2. The *E*–*Z* isomerism applies to positions 7′–8′ of the alkaloids and their derivatives (**1**–**32**) and α–β in the cinnamic acid amides **33**–**40**.

In the *E* isomers the benzene ring–C=C bond–amide group sequence may assume a planar shape in which olefinic and carbonylic double bonds are (in solution) mainly in s-*cis* conformation [15] (Fig. 1a). In the unsubstituted amides **33** and **35**, both NH_2 and carbonyl oxygen may interact with suitably placed active sites of the adsorbent. In the *Z* isomers **34** and **36** (Fig. 1b) one can assume repulsion between the systems of the carbonyl and the benzene ring, thus carbonyl oxygen is pointing in a direction opposite to that of the benzene ring, which may form a weak hydrogen bond with the amidic NH_2 group. This was mentioned long ago for analogous substances [16]. The amidic NH_2 group of **34** and **36** would therefore "localize" less easily on the adsorbent. This might explain the prevalence of the higher retention of the unsubstituted *E* isomers of amides (**33**, **35**) than that of their *Z*

Table 2
R_F values of the compounds studied[a]

Compound[b]	$R_F \times 100$[c]												
	S1	S2	S3		S4	S5	S6	S7		S8		S9	
	Si	Si	Si	Al	Si	Si	Si	Si	Al	Si	Al	Si	Al
1 (*E*)	36	2	13	42	·	3	3[d]	·	·	·	·	·	·
2 (*Z*)	32	2	13	38	·	3	3[d]	·	·	·	·	·	·
3	28	2	13	33	·	3	3[d]	·	·	·	·	·	·
4	27	2	13	33	·	3	3[d]	·	·	·	·	·	·
5 (H)	33	2	17	44	·	3	3[d]	·	·	·	·	·	·
6 (H)	29	2	13	37	·	3	3[d]	·	·	·	·	·	·
7	44	8	29	39	3	9	42	·	4	·	·	·	·
8	39	6	26	35	3	9	33	·	4	·	·	·	·
9	34	5	25	32	3	9	32	·	4	·	·	·	·
10	34	4	22	28	3	9	27	·	4	·	·	·	·
11	68	12	35	46	5	11	57	·	5	·	·	·	·
12	61	10	32	40	5	11	42	·	5	·	·	·	·
13	55	9	31	40	5	11	42	·	5	·	·	·	·
14	55	7	28	35	5	11	34	·	5	·	·	·	·
15	36[e]	16	48	58	24	51	72	4	32	·	·	·	·
16	32[e]	14	45	54	20	45	61	4	25	·	·	·	·
17	28[e]	13	45	54	14	41	61	4	20	·	·	·	·
18	27[e]	12	42	50	12	39	52	4	17	·	·	·	·
19 (H)	33[e]	16	49	59	25	51	71	4	31	·	·	·	·
20 (H)	29[e]	13	45	54	15	40	55	4	21	·	·	·	·
21	79	17	44	56	28	44	85	17	24	·	·	·	·
22	74	15	40	52	22	39	77	12	17	·	·	·	·
23	71	14	40	51	16	33	77	8	15	·	·	·	·
24	68	13	36	46	14	31	69	6	12	·	·	·	·
25 (H)	77	16	45	57	25	46	82	16	23	·	·	·	·
26 (H)	70	14	41	50	15	33	76	8	15	·	·	·	·
27	78	18	50	61	32	58	83	17	35	·	·	·	·
28	73	16	47	57	27	53	74	12	26	·	·	·	·
29	72	16	47	58	23	50	74	10	23	·	·	·	·
30	67	14	44	54	20	46	62	7	19	·	·	·	·
31 (H)	76	18	52	62	31	58	81	16	33	·	·	·	·
32 (H)	69	16	49	58	20	50	66	9	22	·	·	·	·
33	71	13	18	24	23	15	81	32	24	·	·	·	·
34	71	15	25	33	37	12	81	42	38	·	·	·	·
35	77	16	23	30	34	23	86	50	35	·	·	·	·
36	77	18	30	38	46	39	86	57	52	·	·	·	·
37	83	27	65	76	53	64	83	51	82	33	49	14	49
38	83	27	65	76	53	64	83	51	77	33	44	14	43
39	90	35	76	84	72	78	89	73	90	53	64	29	65
40	90	30	71	84	69	74	89	68	87	48	57	25	57

[a] Si = on silica; Al = on alumina.
[b] Dihydro derivatives are marked (H). Otherwise odd numbers refer to *E* and even numbers to *Z* forms.
[c] For easier reading of the table, zeros were replaced by dots (dots do *not* indicate the absence of data.
[d] Unsatisfactory shape of the spot near the origin.
[e] In the acidic system S-1 the cyclic aminals **15–20** hydrolyse and migrate in the form of compounds **1–6**. Similarly, hydroxylaminolysis was observed when the silica plate was impregnated with $NH_2OH \cdot HCl$ [0.2% (w/v) methanolic solution], followed by the S3 basic system.

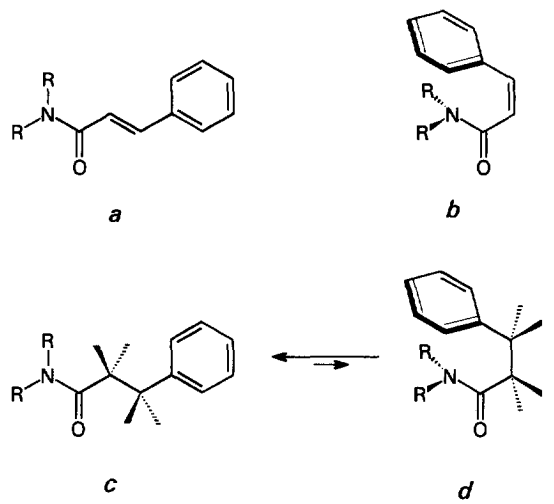


Fig. 1. Structure differences between (a) (*E*)-cinnamamides, (b) (*Z*)-cinnamamides and (c, d) dihydrocinnamamides [(c) antiperiplanar and (d) synperiplanar conformations].

counterparts **34** and **36**. There was no separation in S1, S6, S8 and S9.

The introduction of alkyl substituents on the N atom decreases the polarity of the amidic group and its participation in the localization. Model building shows that in the *Z* isomer the amidic group is at an angle to the vinylbenzene plane. The out-of-plane positioning of the carbonyl oxygen in the *Z* isomer **40** is likely to increase the localization (and retention) in comparison with the planar counterpart **39**. The exceptions (no separation) in S-1, S-3 (alumina) and S-6 might be attributed to the high polarity of the solvent (R_F too high).

N,N-Dimethylcinnamamides **37** and **38** were not separated in any system on silica. This might be attributed to the relatively small size of the methyl groups, which only moderately screen off the carbonyl oxygen accessible to acidic silanols of the amorphous silanol surface. In the case of the crystalline planar surface of alumina, the out-of-plane positioning of the carbonyl oxygen in **38** might be sufficient for the higher retention of this isomer in S7–S9.

If the diethylamides **39** and **40** were considered as valid models of the alkaloids, higher retention of the *Z* alkaloid forms, as observed in systems in which they do separate, would be explained by the better accessibility of the carbonyl oxygen. This explanation, although attractive at first sight, may become less plausible if it is taken into consideration that the close proximity of the macrocycle may obstruct the amide part of the molecule. It is possible that in the *E* isomers (cf., Fig. 1a) the vinylbenzene moiety would stick out and obstruct more adsorption sites (with which the localizing solvent molecules might otherwise interact [17]) than this is the case in the compact *Z* isomer (Fig. 1b). This "displacement" mechanism might be an alternative explanation of the higher retention of the *Z* isomers of the alkaloids and their derivatives.

Isomeric pairs **1–2** and **3–4** are not separated in the systems S2–S9 on silica, but S3 on non-acidic alumina was able to separate **1** and **2**. It is possible that the electrostatic interaction between N(13) (secondary amino group, basic) and the acidic silanols minimizes the difference in the retention of the isomers. The situation is different for the non-acidic alumina. For **3–4**, see later (secondary effects?). One can also speculate that the NH in the N(13) position influences the conformation of the alkaloid itself or of the analyte–adsorbent complex (and thus the availability of the cinnamamide carbonyl or the positioning of the vinylbenzene moiety) in such a way as to minimize the retention difference of the *E–Z* pairs.

Both **1–2** and **3–4** are separated by the acidic S1 system.

The resolution of *E–Z* isomers was not improved in systems containing less strongly localizing, aprotic solvents (cf., [14,18,19]). According to Refs. [20] and [21], exceptions to the improvement of separation of closely related isomers by less strongly localizing (lower *m*) solvents are known.

In the case of the dihydro derivatives of the alkaloids, as in that of the unsaturated isomer pairs, both factors already discussed may be considered, namely the difference in the accessibility of the carbonyl oxygen and the "displacement" mechanism.

Assuming that the antiperiplanar conformation (Fig. 1c) is preferred, one would expect a lower retention for the dihydro derivative than

for the corresponding *Z* isomer (Fig. 1b). This was in fact borne out (Table 2), but the order of the respective dihydro and *E* compounds is variable. Among other reasons, this may be because other conformations, in addition to the antiperiplanar conformation, may also participate in the dissolved or adsorbed form of the alkaloid.

Solvents S2–S3 and S4–S5 show the influence of diethylamine (a proton acceptor). In the case of the alkaloids **1–32** it seems to displace the analytes very strongly. This also applies to S-3 in the amides **37–40**. In the case of S4–S5, the diethylamine effect is reversed for the unsubstituted amides **33–36**. Amidic NH_2 seems to localize more strongly than diethylamine.

Substitution of the benzene ring by two methoxy groups in the 3′,4′ position increases the retention (lowers the R_F value) in most cases.

The *ortho*-dimethoxy grouping seems to be a localizing centre. This effect varies; in some cases there is little or no difference. One can speculate on the secondary effects such as H-bonding with the polar solvent localized on the adsorbent surface or in the mobile phase [17].

Methylene derivatives **15–20** (products of condensation of **1–6** with formaldehyde) can be compared with **1–6** and the urea derivatives **27–32** (products of condensation of **1–6** with phosgene). Owing to their hydrolysis to **1–6**, **15–20** cannot be studied in the acidic system S1. In other systems one observes a markedly weaker retention of **15–20** [with tertiary N(9) and N(13)] when compared with more strongly basic and more strongly localizing secondary amines **1–6**.

In neutral systems on silica, **15–20** are mostly more retained than their respective analogues **21–26** (the difference is especially striking in the case of S-7). In systems with diethylamine (S3 and S5) on silica and in S3 and S7 on the non-acidic alumina, **21–26** are more strongly retained than **15–20**. This suggests that there exists at least a weak electrostatic interaction between **15–20** and the acidic silanols.

The N(5)-methylated derivatives **27–32** are less retained than their respective N(5)-unmethylated (more strongly basic) analogues **21–26**.

Acetylation in positions N(9) and N(13) impairs retention (compare **1–4** with **7–10** and **7–10** with **11–14**). Dinitrophenylation of **7–10** in the N(13) position (data not shown) acts even more strongly in the same direction.

4. Conclusions

In primary cinnamamides the amide NH_2 or carbonyl oxygen can localize. The localization of the former probably prevails in the unscreened *E* isomer, yet in the *Z* isomer (**34**, **36**) it may be blocked by the weak hydrogen bond with the benzene ring.

Introduction of N-alkyl substituents eliminates the amidic NH_2 and the dimethyl- and diethylaminocinnamamides mainly localize by the carbonyl, which, especially in the diethyl *Z* isomer, is shifted outside the plane of the benzene ring and made more accessible for interactions with the adsorbent surface.

In the macrocyclic compounds, either a situation similar to that with N,N-diethylcinnamamides occurs or, alternatively, displacement of the adsorbed molecules of the polar component of the eluent by the vinylbenzene moiety of the analyte plays a role. Both mechanisms may explain the tendency of higher retention for *Z* isomers. Replacement of protic components of the solvent systems by aprotic ones did not improve the *E*–*Z* resolution.

The prevalence of the antiperiplanar conformation in the dihydro derivatives (cf., Fig. 1c and d) may explain why they are less retained than the respective unsaturated *Z* analogues.

Substitution of the benzene ring by *meta*- and *para*-methoxy groups increased the retention in most cases. Methylation in the N(5) position and acetylation in the N(9) and N(13) positions impaired retention. The influence of CH_2 and CO bridging between N(9) and N(13) was studied.

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