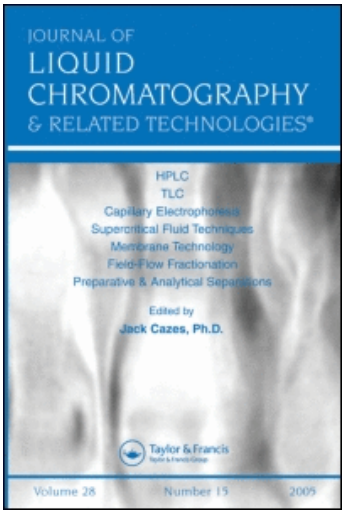

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SEPARATION OF DITERPENOID ALKALOIDS BY HIGH PERFORMANCE CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT

Complex diterpenoid alkaloids of close R_f values have been separated by High Performance Centrifugal Partition Chromatography (HPCPC). Model LLB-M (Series 1000) by Sanki Laboratories Inc. was used for these separations. Especially highly polar (on Al_2O_3) alkaloids which could not be isolated by conventional methods using a solid support, owing to irreversible retention, could be isolated easily as this separation method can be used in the reverse phase mode by careful selection of the biphasic solvent system. The HPCPC instrument was found easy to handle, dependable, applicable to the entire range of polarity of diterpenoid alkaloids, and gave reproducible results. When some of the fractions furnished a mixture of minor alkaloids, the mixture was resolved by a quick separation technique such as separation on a Chromatotron and preparative TLC. In all, eight different mixtures of known as well as crude alkaloidal mixtures were separated and the results are reported here.

Isolated pure known compounds were identified through their spectroscopic (IR., ^{1}H, ^{13}C, DEPT NMR) and physical data, as well as comparison of their TLC behavior with that of authentic samples. A new diterpenoid alkaloid, 13-acetylvakhmatine (**20**), was also isolated in one of the separations.

INTRODUCTION

Natural products have been a key source for the discovery of new drugs.[1] Isolation of the active components from a natural product has always been associated with complex separation problems due to the enormous chemical complexity of the extracts, but recent advances in separation sciences have facilitated the isolation of these active components from natural products.

Crude extracts of natural products which show desirable biological activity are subjected to activity-guided fractionation until an active component is isolated and identified. This exploratory process of fractionation typically involves sub-optimal chromatographic conditions; hence, in order to avoid destruction of potentially labile components, utmost care must be taken throughout the entire process of isolation.

At present, most of the chromatographic separations of natural products are being carried out on solid supports. However, SiO_2, Al_2O_3 or reverse phase adsorbents are not chemically inert.[2] Separation of a natural product on alumina or silica gel sometimes results in recovery of only 70-90%. Sometimes severe losses of valuable materials result because of irreversible adsorption on a solid support. In addition, isolation of artifacts have also been reported due to chemical reactions of the substrates with solid phase adsorbents.

A High Performance Centrifugal Partition Chromatograph (HPCPC), which utilizes centrifugal force to enhance phase separation, provides a new dimension in the area of separation science. HPCPC is based on liquid-liquid partitioning and is an excellent alternative to circumvent the problems associated with solid phase adsorbents and to preserve the chemical integrity of mixtures subjected to fractionation.[1] The HPCPC instruments from Sanki Laboratories are designed entirely with inert materials compatible with virtually all solvents. In model LLB-M (Series 1000) No. 3216, used by the authors, the actual liquid-liquid partitioning takes place in a single-piece integrated cartridge (rotor). This design solves the problem of leakage associated with earlier models. Depending on the model, they are suitable for work with any quantity ranging from 100 mg to 1500 g (for the largest model). With these advantages, HPCPC is gaining popularity as a separation method for natural products, and especially in the bioassay-guided fractionation of extracts.

Diterpenoid alkaloids have attracted attention because of their potent CNS, cardiovascular, smooth muscle, and skeletal muscle effects, extreme toxicity towards mammalian organisms, and reduction of blood pressure and heart rate.[3-6] A separation of alkaloids of *Aconitum nagarum* var. *lasiandum* by high-speed counter current chromatography using $CHCl_3$-MeOH-citric acid / mono sodium phosphate buffer (pH 4.3) (4:3:2) has been reported.[7] In order to test the usefulness of HPCPC in the fractionation of diterpenoid alkaloids, we have carried out several sets of separations of complex diterpenoid alkaloid mixtures which are summarized below.

EXPERIMENTAL

General Procedures

Isolation of alkaloids was carried out on a High Performance Centrifugal Partition Chromatograph (HPCPC), Sanki Laboratories Inc., USA, model LLB-M (Series 1000), No. 3216, equipped with a HPLC pump model SSI 222C. Melting points are corrected and were determined on a Thomas-Kofler hot stage equipped with a microscope and a polarizer. ^1H (300.13 MHz) and ^{13}C (75.47 MHz) NMR spectra were recorded on a Bruker AC-300 spectrometer in $CDCl_3$. The ^{13}C NMR chemical shift multiplicities were determined from DEPT spectra. Chromatographic separations on a Chromatotron[8] were carried out on rotors coated with a 1 mm thick layer of Al_2O_3, 60 PF-254, 365 (EM 1104) or SiO_2 60 GF 254 (EM 7749).

General procedure for the separation of an alkaloidal mixture on HPCPC

Selection of a bi-phasic solvent system for each set of separation experiment was made on the basis of the partition coefficient (P) of the mixture to be separated. Counter current fractionation has been found most successful for those components of a sample which have P mobile/stationary phase within the range of 5>P>0.1. Partition coefficients of the alkaloidal mixtures were determined by dissolving 100 mg of the mixture in 4 mL each of upper and lower phases and then determining the amount of the mixture distributed in each phase. Selection of a stationary and a mobile phase was made on the basis of distribution of alkaloids in each phase.

The phase containing most of the alkaloid mixture was selected as the stationary phase, while the phase containing the least alkaloids was the mobile phase. We used two bi-phasic solvent systems: **A**-hexane:CH_2C_{l2}:MeOH:H_2O (7.5:7.5:12:4) and **B** - C_6H_6:$CHCl_3$:MeOH:H_2O (5:5:7:2). After degassing, the stationary phase was pumped in ascending mode with a rotor speed of 300 rpm at a flow rate of 10 mL / min. The degassed mobile phase was pumped in

descending or ascending mode depending on the density of the mobile phase. The rotor speed was kept between 1000 - 1300 rpm (determined by observing the displacement of the stationary phase) and flow rate at 3 mL / min. As soon as the displacement of the stationary phase stopped, the alkaloidal mixture was dissolved in 4 mL each of the stationary and the mobile phase and injected through a syringe into the sample loop (10 mL capacity). Fractions of 4 to 10 mL were collected with a fraction collector (Gilson, model:FC-220K Race Track fractionator).

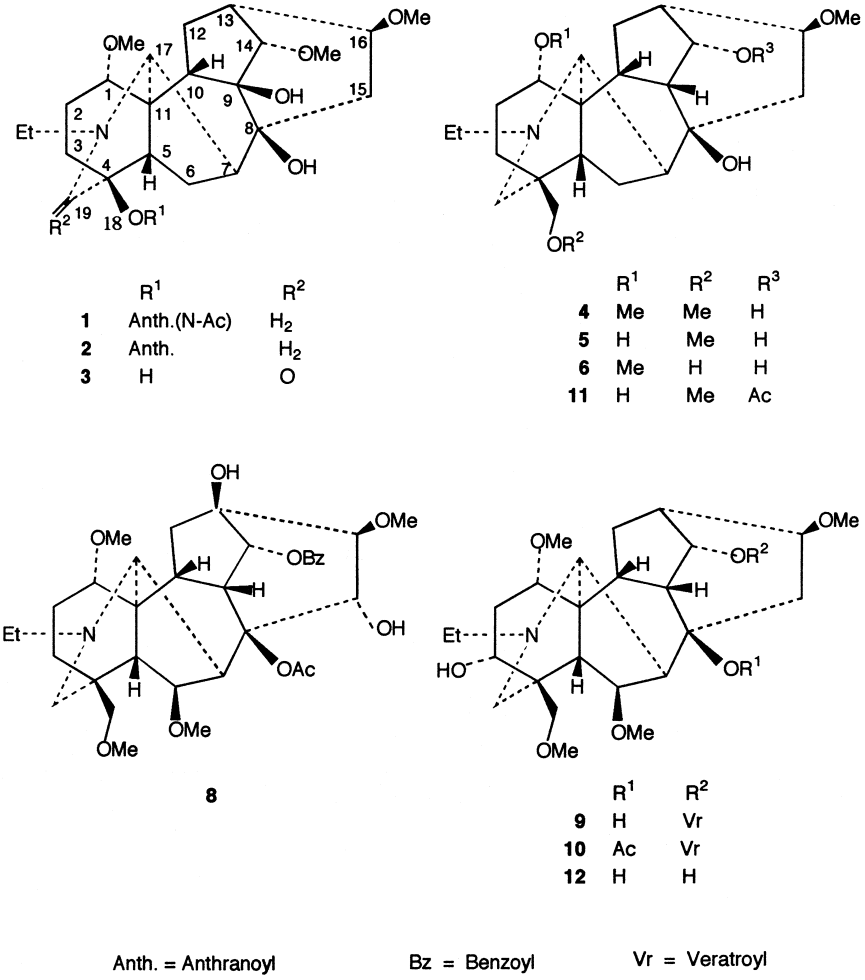
After completion of the separation, the solvent remaining in the partition chambers was displaced with compressed air and washed with MeOH in the ascending mode. Fractions were concentrated using a speed vac concentrator (Savant Speed Vac Concentrator, model:SVC 200 H). The total recovery of the compounds was between 95 - 98% in each experiment. The identity of each alkaloid obtained was established by TLC, Co-TLC behavior, and comparison of the ^{1}H, ^{13}C and DEPT NMR spectra with those of an authentic sample. Many of these alkaloids undergo methanolysis, hydrolysis, and some stereochemical changes when heated with MeOH or H_2O.[2]

Separation #1. A known mixture containing Lappaconitine(1), N-Deacetyllappaconitine (2) and Oxolappaconine (3)

Before carrying out separation of the crude diterpenoid alkaloid mixtures we decided to evaluate the resolving efficiency of the instrument using a mixture of known diterpenoid alkaloids having similar structures and close R_f values. The R_f values of lappaconitine, *N*-deacetyllappaconitine and oxolappaconine on a silica gel TLC plate with $CHCl_3$: MeOH (95:05) are 0.50, 0.34 and, 0.24, respectively. For this purpose a mixture of lappaconitine (1, 99 mg), *N*-deacetyllappaconitine (**2**, 80 mg) and oxolappaconine (**3**, 87 mg) was prepared and resolved on a HPCPC using solvent system **A**. The upper layer was used as a stationary phase and the lower layer as a mobile phase; 9 mL fractions were collected.

Separation was achieved in reverse phase (judged by TLC on SiO_2 plates), i.e. the polar compounds were separated first followed by the non-polar compounds.

Fractions 8-13 afforded oxolappaconine (**3**, 70 mg, 80.5%), $C_{23}H_{35}NO_7$, mp 181-182°.[9] Fractions 20-35 gave *N*-deacetyllappaconitine (**2**, 48 mg, 60.0%), $C_{30}H_{42}N_2O_7$, mp 213-214°.[10] Fractions 41-77 gave lappaconitine (**1**, 88 mg 88.9%), $C_{32}H_{44}N_2O_8$, mp 229-230°.[10] Other fractions (46 mg) consisted of a mixture of the above alkaloids (**1-3).** This separation resulted into a total recovery of 95%.



	R^1	R^2
1	Anth.(N-Ac)	H_2
2	Anth.	H_2
3	H	O

	R^1	R^2	R^3
4	Me	Me	H
5	H	Me	H
6	Me	H	H
11	H	Me	Ac

8

	R^1	R^2
9	H	Vr
10	Ac	Vr
12	H	H

Scheme 1

Separation #2. A known mixture containing Talatizamine (4), Isotalatizidine (5), and Cammaconine (6)

A mixture (140 mg) was prepared by combining talatizamine (**4**, 45 mg), isotalatizidine (**5**, 45 mg) and cammaconine (**6**, 50 mg). The R_f values of talatizamine, isotalatizidine and cammaconine on an alumina plate with $CHCl_3$: MeOH (97:03) are 0.69, 0.48, and 0.24, respectively. The separation was carried out on a HPCPC using solvent system **B**. The upper layer was used as a

stationary phase and the lower layer as a mobile phase. Sixty fractions (9 mL each) were collected. The separation was achieved in the normal phase (judged by TLC on Al_2O_3). This separation resulted in a total recovery of 97%.

Fractions 9-12 afforded talatizamine (**4**, 42 mg, 93.3%), $C_{24}H_{39}NO_5$, mp 145-146°;[10] fractions 13-15 yielded isotalatizidine (**5**, 38 mg, 84.4%), $C_{23}H_{37}NO_5$, mp 116-117°;[10] and fractions 17-60 gave cammaconine (**6**, 39 mg, 78.0%), $C_{23}H_{37}NO_5$, mp 135-137°.[10] Other fractions (17 mg) consisted of a mixture of alkaloids (**4-6**). A total recovery of 97% was achieved.

Separation #3. "Merck Potent Aconitine"

"Merck Potent Aconitine" once available commercially,[11] contains 92-95% of aconitine along with small amounts of mesaconitine and 3-deoxyaconitine. Purification of "Merck aconitine" by column chromatography or by vacuum liquid chromatography[12] with hexane:ether has always resulted in the formation of some oxidised and *N*-oxide products.

In order to avoid the oxidation and *N*-oxidation, we carried out separation of three grams of "Merck Potent Aconitine" (loaded 1 g at a time) on a HPCPC. Solvent system A was used for the separation of "Merck Potent Aconitine." Eighty fractions (7 mL each) were collected and the total recovery was 99%.

The HPCPC fractions 1-36 afforded polar alkaloids (214 mg). Isolation and identification work of these polar alkaloids is in progress. Fractions 37-67 gave aconitine (**7**, 2.63 g), $C_{34}H_{47}NO_{11}$, mp 202-205°.[10] After completion of the separation, the stationary phase was pumped out and HPCPC columns were washed with MeOH which afforded 3-deoxyaconitine (**8**, 142 mg), $C_{34}H_{47}NO_{10}$, mp 177-180°[10] the least polar (Al_2O_3 TLC) fraction of the mixture. Hence, the separation in this case resulted in a reverse phase mode.

Separation #4. Alkaloids of *Aconitum falconeri*

An alkaloidal mixture from *A. falconeri* (319 mg, pH-4.5) was resolved on HPCPC with solvent system A. The upper layer was used as a stationary phase and the lower layer as a mobile phase. The alkaloids were isolated in the reverse phase mode.

The HPCPC fractions 18-22 afforded falconerine (**9**, 26 mg), $C_{34}H_{49}NO_{10}$, amorphous.[13] The HPCPC fractions 34-39 afforded 8-acetylfalconerine (**10**, 18 mg), $C_{36}H_{51}NO_{11}$, mp 162-163°.[14] Other fractions (261 mg) consisted of a mixture of alkaloids.

Separation # 5. Other alkaloids of *Aconitum falconeri*

The ^{1}H and ^{13}C NMR spectra of fractions 11-12 (16 mg) and fractions 27 (5 mg) from the above separation (#4) showed the presence of some interesting polar alkaloids which were not earlier isolated from *A. falconeri*. Hence, the polar alkaloidal mixture of *A. falconeri* (1.048 g) was resolved on HPCPC using the same solvent system and conditions as for the separation # 4 .

A combined fraction (14-23) (172 mg) of HPCPC separation # 5 was quickly purified on a Chromatotron with an Al_2O_3 rotor with gradient elution in increasing polarity with hexane, C_6H_6, EtOAc and MeOH. Elution with hexane: C_6H_6:EtOAc (1:1:1.5) afforded condelphine (**11**, 14 mg), $C_{34}H_{39}NO_{10}$, amorphous,[15] while elution with hexane:C_6H_6:EtOAc:MeOH (1:1:2:0.03) gave falconerine (**9**, 20 mg), $C_{34}H_{49}NO_{10}$, amorphous,[13] and ezochasmanine (**12**, 16 mg), $C_{25}H_{41}NO_7$, mp 115-118°.[16] Elution with hexane:C_6H_6:EtOAc:MeOH (1:1:2:0.06) gave neoline (**13**, 20 mg), $C_{24}H_{39}NO_6$, mp 159-161°.[15,17] Finally elution of the rotor with hexane:C_6H_6:EtOAc:MeOH (1:1:2:0.1) gave isotalatizidine (**5**, 19 mg), $C_{23}H_{37}NO_5$, mp 116-117°.[10]

A combined fraction (24-43) (301 mg) of HPCPC # 5 was resolved on a Chromatotron with an Al_2O_3 rotor with gradient elution with hexane:toluene: EtOAc (1:1:1) to afford falconerine (**9**, 40 mg)[13] while elution with hexane: toluene:EtOAc:MeOH (1:1:1.25 to 1:1:1.5 :0.02) gave a mixture of two compounds which on further purification on an Al_2O_3 rotor with gradient elution with hexane:toluene:EtOAc (1:1:1.25) gave pseudaconitine (**14**, 28 mg), $C_{36}H_{51}NO_{12}$, mp 205-208°.[10]

A combined fraction (44-74) (305 mg) of HPCPC # 5 was resolved on a Chromatotron with an Al_2O_3 rotor. Gradient elution of the rotor with hexane: C_6H_6: EtOAc (1:1:0.8) afforded 8-acetylfalconerine (**10**, 33 mg), $C_{36}H_{51}NO_{11}$, mp 162-163°.[14] Elution with hexane:C_6H_6:EtOAc:MeOH (1:1:1:0.01) gave indaconitine (**15**, 18 mg), $C_{34}H_{47}NO_{10}$, mp 203-204°,[10] while elution with hexane :C_6H_6:EtOAc:MeOH (1:1:1:0.02) gave yunaconitine (**16**, 15 mg), $C_{35}H_{49}NO_{11}$, mp 141-143°.[10]

Separation # 6. Polar and non-polar alkaloids of *Aconitum falconeri*

During the separation of an alkaloidal mixture from *A. falconeri* on HPCPC we were able to isolate a very polar mixture of alkaloids. These polar alkaloids are very minor and could not be eluted even with MeOH (100%), either on a column or on a Chromatotron. This prompted an attempt to isolate these polar alkaloids on a HPCPC.

With the solvent system **A**, we resolved 12 g of mixture of alkaloids from *A. falconeri* (pH-4.5) on HPCPC (1 g at a time) which gave 1.2 g of polar and 10.3 g of a non polar alkaloidal mixture. Further purification of these polar alkaloids is in progress.

Separation # 7. Polar alkaloids of *Consolida ambigua*

The aerial parts and the seeds of *Consolida ambigua* L. P. W. Ball and V. Heywood (Syn. *Delphinium ajacis* L) (Ranunculacea)[18] are rich in alkaloids, most of which are of the norditerpenoid-type.[10,13] Thirty alkaloids have been isolated from the seeds and leaves of this plant. The alkaloidal mixture ($CHCl_3$ soluble) of *C. ambigua* (9.76 g) was resolved over a VLC[12] alumina column with gradient elution. Elution with $CHCl_3$:MeOH (9:1) gave a mixture of polar alkaloids (1.34 g). Solvent system **B** was used to resolve the alkaloidal mixture on a HPCPC. The upper phase was used as a stationary phase and the lower phase as a mobile phase.

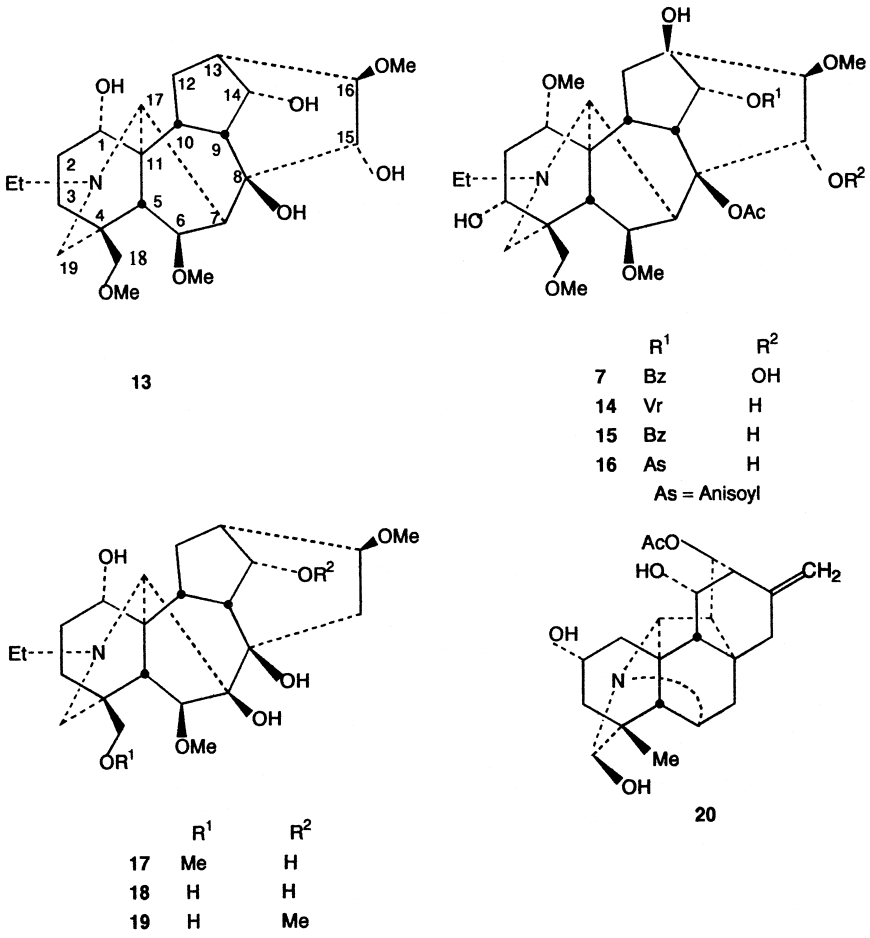
HPCPC fraction 14 afforded delcosine (**17**, 91 mg), $C_{24}H_{39}NO_7$, mp 203-204°,[11] while fractions 21-23 gave takaosamine (**18**, 87 mg), $C_{23}H_{37}NO_7$, mp 174-175°.[13]

HPCPC fractions 15-16 (284 mg) consisted of a mixture of two compounds and was subjected to further resolution on a HPCPC using solvent system **B** . Fractions 24-28 afforded gigactonine (**19**, 19 mg), $C_{24}H_{39}NO_7$, mp 168-169°;[10] fraction 34 gave some more delcosine (**17**, 18 mg), $C_{24}H_{39}NO_7$, mp. 203-204°.[10] Other fractions of HPCPC separation #7 consisted of a mixture of alkaloids.

Separation # 8. Two polar C_{20}- diterpenoid alkaloids from *Consolida ambigua*

The $CHCl_3$ soluble alkaloids of *C. ambigua* (separation #7) was resolved over a VLC column with gradient elution. Final elution of the column with MeOH gave a mixture of polar alkaloids (1.05 g). A 910 mg sample of this polar alkaloid mixture was subjected to resolution on HPCPC using solvent system **B**.

Fractions 56-58 afforded a new C_{20} diterpenoid alkaloid 13-acetyl-vakhmatine (**20**, 46 mg), $C_{22}H_{29}NO_5$.[19] Fractions 33-34 also afforded a homogeneous C_{20}-diterpenoid alkaloid (20 mg). Structure elucidation work on this alkaloid is in progress. Other fractions (823 mg) consisted of a mixture of alkaloids.



Scheme 2

CONCLUSION

We have found HPCPC to be a very useful instrument for the isolation of polar as well as non polar complex diterpenoid alkaloids. With HPCPC we were able to isolate the known polar compounds isotalatizidine,[5] condelphine,[11] ezochasmanine,[12] neoline,[13] and yunaconitine[16] for the first time from *A. falconeri*, and 13-acetylvakhmatine,[20] a new alkaloid from *Consolida ambigua*. The solvent systems used in these separations consist of all liquids without any buffer solutions. Solids used in buffer solutions may clog the system.

With a combination of HPCPC and some quick separation techniques of VLC, Chromatotron and preparative TLC, where contact time of the compounds with the solid adsorbents is reduced, diterpenoid alkaloids can be isolated in a pure state. Since many of the *Aconitum* and *Delphinium* plant species are used as herbal medicines, HPCPC will be the most appropriate instrument for the bioassay-guided fractionation of these herbs. The new model (Series 1000) affords fast separations without any problems of leakage or clogging.

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