Journal of Chromatography, 423 (1987) 23-31
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3900

CHARACTERIZATION AND QUANTIFICATION OF PAF-ACETHER (PLATELET-ACTIVATING FACTOR) AS A HEPTAFLUOROBUTYRATE DERIVATIVE OF 1-O-ALKYL-2-ACETYL-*sn*-GLYCEROL BY CAPILLARY COLUMN GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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(First received October 15th, 1986; revised manuscript received August 18th, 1987)

SUMMARY

A gas chromatographic method with a glass capillary column and electron-capture detection is proposed for the characterization and quantification of 1-O-hexadecyl-2-O-acetyl-*sn*-glycero-3-phosphocholine (PAF C_{16}) using the corresponding 1-O-hexadecyl-2-O-acetyl-*sn*-glycero-3-heptafluorobutyrate derivative and, as an internal standard, 1-O-octadecyl-2-O-methyl-*sn*-glycero-3-phosphocholine. The reproducibility was approximately 5% and amounts as low as 20 pg could be measured. The method was specific and allowed the quantification of PAF C_{16} in supernatants from stimulated human polymorphonuclear neutrophils.

INTRODUCTION

Paf-acether is a phospholipid mediator implicated in anaphylactic and inflammatory reactions [1,2]. It is a potent platelet and neutrophil activating agent

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produced by basophils, macrophages, polymorphonuclear neutrophils (PMN), platelets [3], eosinophils, mast cells and endothelial cells [4,5].

The structure of paf-acether from animal sources was shown to be 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine [6–10]. Paf-acether from human PMN has been reported to be a mixture of 1-O-hexadecyl- and 1-O-octadecylalkyl ethers [7,9,11–13]. The presence of various other molecular species including 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine has also been described [13,14]. The analysis of these compounds requires quantification of small amounts in mixtures of lipids containing much larger amounts of potentially interfering compounds.

The actual method for the quantification of paf-acether is based on the monitoring of platelet activation either using the assessment of platelet aggregation in an aggregometer [1,3,4,15,16] or monitoring the release of radiolabelled serotonin previously incorporated into platelets [10,17,18]. These bioassays are very sensitive [10] but give results in terms of the equivalent weights of synthetic compounds and do not allow one to discriminate between the various molecular species of paf-acether that exhibit different specific biological activities [16].

More recently, physico-chemical methods based on mass spectrometric (MS) detection have been described. They include selected-ion monitoring after electronic-impact ionization [11,12], fast atom bombardement (FAB) [19] and gas chromatography (GC)–negative-ion chemical ionization mass spectrometry [20].

In this paper, we propose a simple, rapid and reliable method for the quantification and characterization of 1-O-octadecyl- and 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine. Phospholipids were extracted from physiological fluid, then hydrolysed with phospholipase C and rapidly converted into heptafluorobutyrate (HFB) derivatives prior to GC with electron-capture detection (GC–ECD). The GC–ECD method using a high-resolution capillary column is highly sensitive and specific, and it may become a useful tool for the quantification and characterization of the various molecular species of paf-acether.

EXPERIMENTAL

Reagents

Analytical-reagent grade solvents were redistilled before use. 1-O-Hexadecyl-2-O-acetyl-*sn*-glycero-3-phosphocholine (PAF C_{16}), 1-O-octadecyl-2-O-acetyl-*sn*-glycero-3-phosphocholine (PAF C_{18}), 1-O-octadecyl-2-O-methyl-*sn*-glycero-3-phosphocholine (OMe PAF C_{18}) and 1-O-hexadecyl-2-O-acetyl-*sn*-glycerol (HAG) were purchased from Novabiochem (Laüfelchingen, Switzerland). 1-O-Octadecyl-2-O-propionyl-*sn*-glycero-3-phosphocholine (propio PAF C_{18}) was purchased from Seratec (Epinay/Seine, France). Phospholipase C (PLC) from *Clostridium welchii* and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, U.S.A.); ionophore A 23187 (Calbiochem, La Jolla, CA, U.S.A.) was stored at $-70°C$ as a 1 m*M* solution in dimethyl sulphoxide (DMSO); heptafluorobutyric anhydride was obtained from Pierce (Rockford, IL, U.S.A.) and 1-O-[^{3}H]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine from Amersham (Amersham, U.K.). A solution of tris(hydroxymethyl)aminomethane (10 m*M*), sodium chloride (0.137 *M*), magnesium chloride (1 m*M*), calcium chloride (10

m*M*) and potassium chloride (2.6 m*M*) (Tris buffer) was prepared and the pH was adjusted to 7.4 with hydrochloric acid. Hanks balanced salt solutions (HBSS) without or with Ca^{2+} and Mg^{2+} (Eurobio, Paris, France) were used for neutrophil purification and stimulation, respectively.

Preparation of neutrophils

Human neutrophils were purified according to previously described procedures [21]. Replicate tubes with $5 \cdot 10^7$ neutrophils in 1 ml of HBSS containing 0.25% lipid-free BSA were stimulated or not for 40 min at 37°C with 10 μM ionophore A 23187 (final concentration). The samples were centrifuged at 400 *g* for 10 min and the supernatants were collected and pooled prior to storage at −20°C.

Extraction of paf-acether and analogues

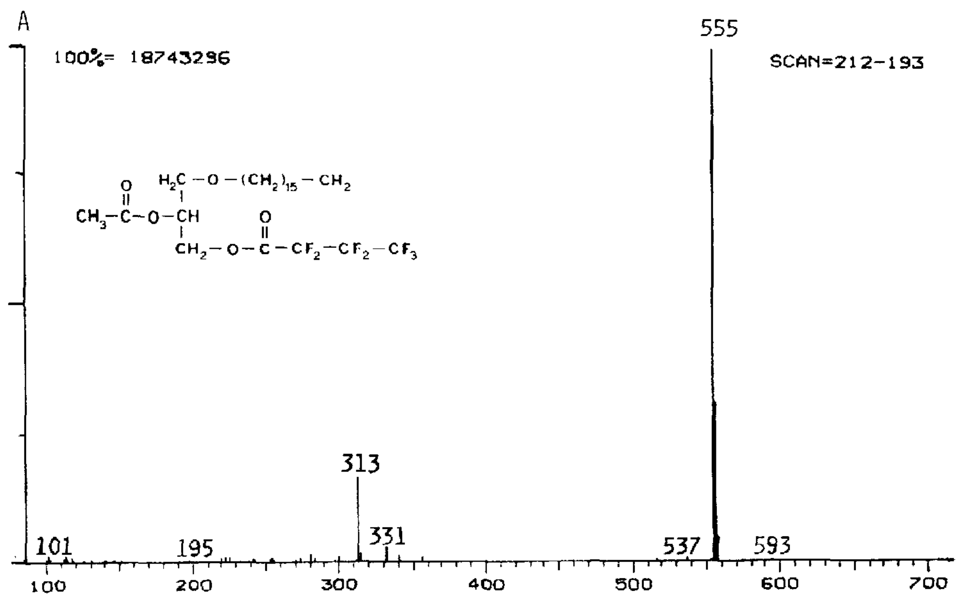
Extraction was carried out using the Bligh and Dyer technique [22]. Briefly, chloroform and methanol were added to the aqueous solutions containing PAF C_{16} or C_{18}, propio PAF C_{18}, OMe PAF C_{18} or cell-free supernatants from human neutrophils stimulated or not with the ionophore to achieve a final composition of chloroform–methanol–water (1:2:1, v/v/v). This solution was incubated for 24 h at 4°C with shaking. Chloroform and water (1 volume of each) were added to achieve phase separation and the lower chloroform phase was collected and evaporated to dryness under nitrogen.

Hydrolysis and derivatization

1-O-Alkyl-2-acyl-*sn*-glycerol or 1,2-diacyl-*sn*-glycerol derivatives of phospholipids, either synthetic or present in supernatants from PMN, were obtained on hydrolysis with PLC according to ref. 6: 1 ml of Tris buffer containing 50 m*M* Ca^{2+} (pH 8) and 100 μg/l PLC was added to the dry residues obtained after Bligh and Dyer extraction. Samples containing PLC alone were used as controls. Incubation was carried out for 1 h at room temperature with constant shaking, following which the lipids were extracted with dichloromethane. The organic phase was collected and transferred into a 5-ml vial and dried under nitrogen. The dry residues were converted into HFB derivatives by addition of 1 μl of heptafluorobutyric anhydride in 50 μl of triethylamine in toluene (5%, v/v). The vial was stoppered and incubated at 70°C for 10 min. Water and toluene (0.2 ml of each) were added and the mixture was shaken for 1 min. The organic layer was collected and washed with 0.2 ml of aqueous ammonia solution (5%, v/v), followed by centrifugation at 500 *g* for 10 min at 4°C to achieve phase separation. The organic phase was collected and immediately injected into the chromatograph.

Gas chromatographic and mass spectrometric procedures

GC was performed on a Shimadzu GC 9A instrument (Touzart et Matignon, Vitry, France) using an OV-1 fused-silica capillary column (25 m × 0.25 mm I.D.) maintained at 240°C. A 1-μl sample was injected in a Ross injector maintained at 270°C using a siliconized needle. The capillary head pressure was set at 0.4 bar with helium as the carrier gas. For electron-capture detection, nitrogen was used as an additional gas at a flow-rate of 30 ml/min. The 10-mCi ^{63}Ni detector was



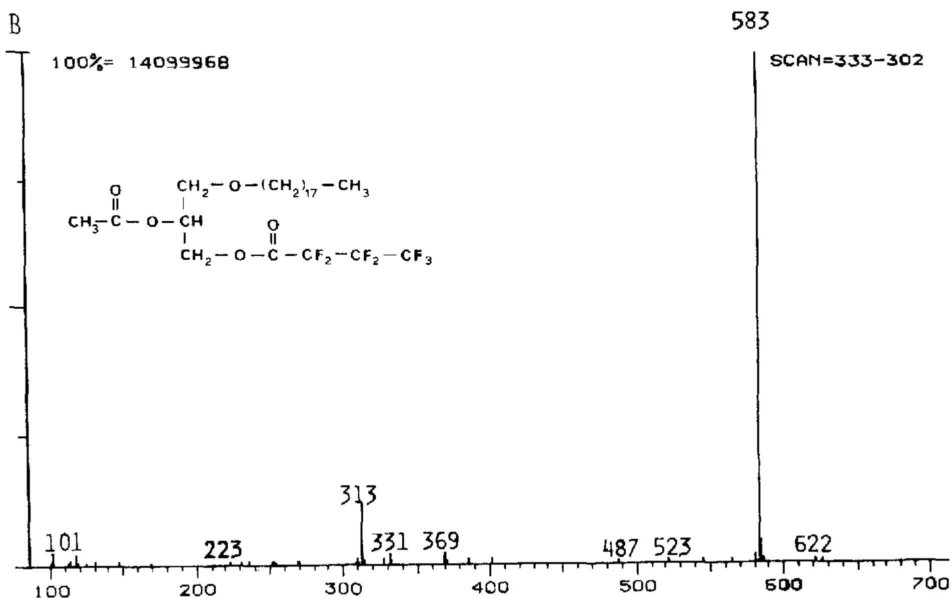


Fig. 1.

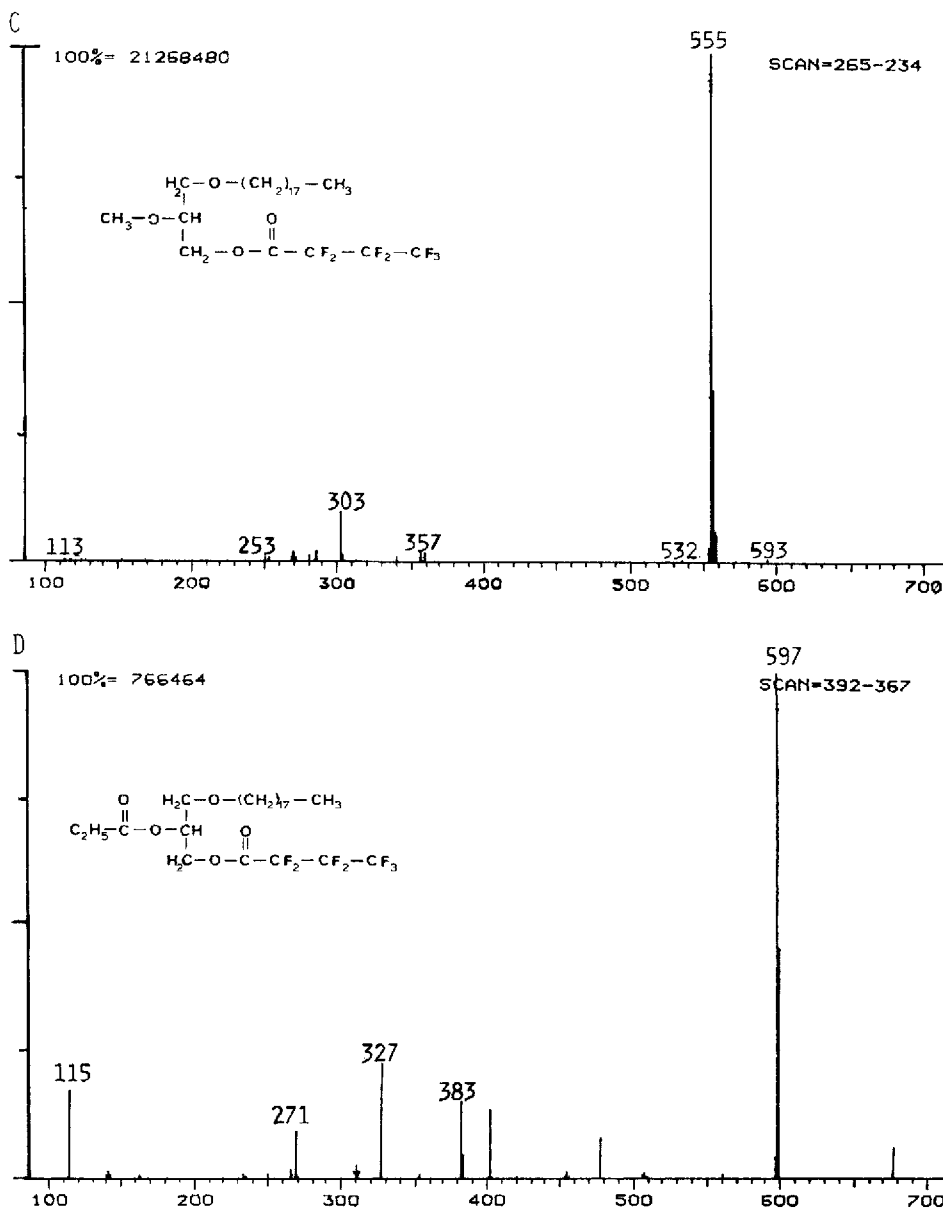


Fig. 1. Mass spectra of HFB derivatives of paf-acether and analogues. GC–MS: OV-1 column, injector temperature 270°C, column temperature 240°C, isobutane as chemical ionization agent. (A) PAF C_{16} HFB; (B) PAF C_{18} HFB; (C) OMe PAF HFB; (D) Propio PAF C_{18} HFB.

maintained at 300°C. For GC–MS, a Girdel 30 gas chromatograph (Delsi, Suresnes, France) with the column directly connected to the mass spectrometer ion source (70°C) was used. The R10-10C mass spectrometer (Nermag, Rueil Malmaison, France) was operated in the chemical ionization mode using isobutane (0.2 Torr) as the reagent gas.

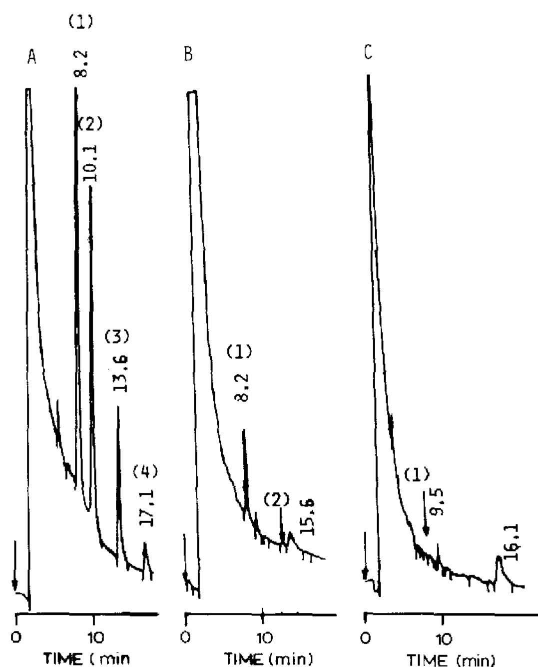


Fig. 2. (A) GC of reference heptafluorobutyrate derivatives. Peaks: 1 = PAF C_{16} HFB (200 ng/ml); 2 = OMe PAF HFB (200 ng/ml); 3 = PAF C_{18} HFB (100 ng/ml); 4 = Propio PAF C_{18} HFB (50 ng/ml). (B) Chromatogram obtained from stimulated neutrophils. Peaks: 1 = PAF C_{16} HFB (50 ng/ml); 2 = PAF C_{18} HFB (internal standard was not introduced). (C) Chromatogram obtained from non-stimulated neutrophils (internal standard was not introduced).

RESULTS

GC–MS of heptafluorobutyrate derivatives of paf-acether and analogues

In a first series of experiments, the hydrolysis products of synthetic paf-acether and analogues with PLC were analysed by GC–MS after conversion into HFB derivatives. The mass spectra of the HFB derivatives of hydrolysed PAF C_{16}, PAF C_{18}, OMe PAF C_{18} and propio PAF C_{18} with PLC are shown in Fig. 1A, B, C and D, respectively. The structures of these products were confirmed. All spectra were characterized by the base peak (M+1) at m/z 555 (PAF C_{16}), 583 (PAF C_{18}), 555 (OMe PAF C_{18}) and 597 (propio PAF C_{18}). The only other important ion present in the spectra is due to the loss of the alkyl ether side-chain at the C-1 position: m/z 313 (PAF C_{16}), 313 (PAF C_{18}), 285 (OMe PAF C_{18}) and 327 (propio PAF C_{18}). After identification of the products by MS, the partition ratio (k') for each of the compounds was calculated. The following k' values for the HFB derivatives were obtained: PAF C_{16}, 3.6 ± 0.4 ($t_R = 9$ min); OMe PAF C_{18}, 4.2 ± 0.3 ($t_R = 10$ min); PAF C_{18}, 5.4 ± 0.4 ($t_R = 12$ min); propio PAF C_{18}, 8.4 ± 0.5 ($t_R = 19.5$ min) (t_R = retention time).

GC with electron-capture detection of HFB derivatives of paf-acether and analogues

In a second series of experiments, the various compounds were analysed separately by GC using the same type of column (OV-1) with electron-capture detec-

tion. The following k' values were obtained: PAF C_{16} HFB, 3.4; OMe PAF C_{18} HFB, 4.1; PAF C_{18} HFB, 5.6; propio PAF C_{18} HFB, 8.2. The column allowed a good separation of a mixture of the various derivatives with a good selectivity between C_{16}, C_{18} paf-acether HFB, OMe PAF C_{18} HFB and propio PAF C_{18} HFB (Fig. 2A). Using OMe PAF C_{18} HFB as an internal standard, the quantitative relationship between the amount of PAF C_{16} HFB derivative injected and peak height was investigated. The calibration graph obtained was linear over the range 20–320 pg injected with a correlation coefficient of 0.998. The limit of detection, set at the amount of PAF C_{16} HFB giving a peak with a signal-to-noise ratio of $> 3:1$, was 20 pg injected. The reproducibility of the method was determined with a sample of physiological fluid ($n=5$) spiked with 50 ng of PAF C_{16}. The coefficients of variation were below 1.5 and 5.1% for the intra- and inter-analysis assays, respectively.

Prior to applying this method to biological samples, the recovery of the extraction step, which could be a limiting factor for sensitivity, was determined. The extraction yield was measured using 1-O-[^{3}H]hexadecyl-2-O-acetyl-*sn*-glycero-3-phosphocholine added to unstimulated cells under the same experimental conditions, as described below, to stimulate the cells. The recovery of radioactivity was $76 \pm 10\%$ ($n=15$) and this variability indicated that the use of an internal standard was required.

The hydrolysis step could also be another important limiting factor. The yield of this step was evaluated by comparison between the peak height obtained from a PAF C_{16} solution hydrolysed by PLC and then derivatized with HFBA versus the peak height for the same molar amount of the synthetic expected product (1-O-hexadecyl-2-acetyl-*sn*-glycerol HFB derivative). The yield was $98.2 \pm 1.0\%$ ($n=6$).

Application to supernatants from PMN

The GC method with electron-capture detection was validated after extraction and derivatization of supernatants from human PMN stimulated or not with ionophore. A single product corresponding to the PAF C_{16} HFB derivative was observed on analysing extracts from stimulated PMN (Fig. 2B). No peak corresponding to PAF C_{18} was detected. Neither PAF C_{16} HFB nor other paf-acether analogues were detected on analysing supernatants from unstimulated PMN (Fig. 2C).

DISCUSSION

The purpose of this study was to develop a simple, sensitive and specific assay for the determination of the various molecular species of paf-acether. As PAF homologues and analogues exhibit different biological activities [16], the measurement of activity alone with a bioassay does not reflect the real molecular concentration of paf-acether. In order to increase the specificity and sensitivity of the analysis, several workers have proposed physicochemical methods such as GC coupled with MS [19,20]. As phospholipids are not intrinsically volatile, GC requires the elimination of the phosphocholine polar head group. As chemical

methods are unspecific, it was necessary to hydrolyse the phospholipids using a selective enzyme (PLC) prior to derivatization of the free hydroxy group formed. One approach to identifying the derivatives is to form sufficiently stable compounds, detectable by MS with a soft ionization technique. In this respect, the HFB derivatives increase the stability of the molecular ion formed, using isobutane as a chemical ionization reagent. As electron-capture detection is more amenable to automation, we developed a highly specific chromatographic method using a fused-silica capillary column. A good isothermal separation of the compounds was achieved using a non-polar silicone phase.

The selectivity with respect to other lipids such as sphingomyelin and phosphatidylcholine derivatives (PAF derivatives differentiated by the nature of the C-1 or C-2 substituents) was also evaluated by injecting hydrolysed synthetic mixtures (results not shown), thus confirming the absence of interferences with PAF C_{16} HFB and PAF C_{18} HFB. The selectivity of the method was also confirmed by the analysis of stimulated and non-stimulated neutrophils (Fig. 2B and C).

The results appeared to be satisfactory because (1) the purification was sufficient, (2) the chromatograms were not overloaded and (3) the threshold of sensitivity for PAF C_{16} was 20 pg injected.

Ramesha and Pickett [20] recently reported the suitability of the acylation of 1-O-alkyl-2-acetyl-*sn*-glycerol with pentafluorobenzoyl chloride for the quantification of 10 pg by GC–negative-ion chemical ionization MS. In our hand, the pentafluorobenzene derivatives do not give better sensitivity than the HFB derivatives when electron-capture detection is used.

According to the previously described pathway for the biosynthesis [23–25] of paf-acether, involving acetyl transferase, several molecular species of this mediator could be present in supernatants from stimulated cells and organs. The possible active structural analogues could differ in the length of the hdyrocarbon chain at the C-1 position (PAF C_{16-19}) and in the nature of the acyl group at the C-2 position (acetyl or propionyl). In contrast to other reports [9], only C_{16} paf-acether was found in supernatants from ionophore-stimulated PMN. The reason for this difference is unknown.

CONCLUSION

We have reported on the quantification of paf-acether by GC–ECD using 1-O-alkyl-2-acetyl-*sn*-glycerol heptafluorobutyrate derivatives. The method allows the specific detection and quantification of small amounts (20 pg) of the various possible analogues of paf-acether. This method should be applicable to the detection of different molecular species of paf-acether synthetized by various stimulated cells and organs. In addition, the high efficiency of the chromatographic system and the selectivity of the detection do not imply the systematic use of GC–MS. This technique may also become a useful tool for the assessment and characterization of paf-acether.

ACKNOWLEDGEMENT

This work was supported by a grant from the University of Paris XI, AI 8420.

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