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STUDY OF THE RELEASE OF FORMALDEHYDE, RESPONSIBLE FOR THE IRRITATION CAUSED IN POST-SURGERY PATIENTS, FROM A PHARMACEUTICAL PACKAGE

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(Received July 18, 1983).

Summary

The organic solvent, formaldehyde, present in a suture contained in a plastic pharmaceutical package, was identified using Gas Chromatography and Mass Spectroscopy. A possible mode of its release is also suggested.

Key Words: Formaldehyde, Suture Package, Gas Chromatographic and Mass Spectroscopic Determination.

Introduction

The determination of residual solvents in plastics packaging materials in various food and pharmaceutical applications has received wide attention over the past few years [1,2,3,4]. The presence of such residual solvents becomes alarming when these compounds are toxic and/or carcinogenic in nature. Such compounds upon contact, may cause a wide variety of problems ranging from simple irritation to carcinogenesis to exposed tissues [5-13]. Such solvents are used: during the application of different coatings in laminates and/or during the printing procedure with organic inks.

Recent methods of analysis of such volatile residuals in packaging materials include the "head space" technique [1,14] in combination with Gas Chromatography and Mass Spectroscopy [4,15].

A particular suture contained in a pharmaceutical plastic package was reported to cause repeatedly an irritation in post surgery patients. The residual solvents retained in the package content were suspected as the sourse of the irritation and so this present work was undertaken with the objectives:

1) to identify the compound/compounds which may have caused the irritation.
2) to determine the source releasing such compound/compounds.
3) to study the mode of release of such compound/compounds.

Materials and Methods

Suture sample packages were provided by a Midwestern supplier. The suture package consisted of the outer package film laminate and the package content which in turn consisted of the inner folder and the suture itself.

Analysis of volatile compounds present in both parts of the package was performed separately by use of the "hot Jar" technique in combination with Gas Chromatography and Mass Spectroscopy.

Sample preparation-analysis

Samples were cut into small pieces of dimensions (1 × 1) cm and placed in glass serum vials of 26ml capacity. The vials were immediately closed with teflon coated stoppers, sealed with aluminum crimp caps, evacuated to 15 in. Hg and heated for 1 hr at temperatures ranging from 60-180^{0}C, to release compounds in the headspace.

Aliquots (1ml) from the headspace of the vials were then withdrawn, using a 1ml gas tight syringe equipped with a two way luer valve and introduced into the GC/FID and GC/MS system respectively.

In addition to samples, negative controls were used to monitor the interfering compounds released from the teflon coated stopper.

GC/FID Analysis

The headspace samples from heated vials were analysed on a Varian model 3700 equipped with a· dual flame ionization detector.

Gas Chromatographie Conditions:
St. Phase: Chromosorb 104, 60-80 Mesh. 3′ × 1/8′′
Col. Temp. 150^{0}C, isothermal
Inj. port. temp. 180^{0}C
Detect. temp.: 240^{0}C
Carrier gas: Nitrogen 60ml/min

GC/Mass Spectrometry Analysis

Headspace samples from heated vials were analysed using a Hewlett Packard model 5992 A GC/MS instrument systm. Selected. Ion Monitor mode program was used.

GC/MS Conditions:

St. Phase: Carbowax 20M, 60-80 Mesh, 6′ × 1/4′′
Col. Temp: 75^{0}C, isothermal
Inj. port. Temp: 150^{0}C
Det. Temp.: 240^{0}C
Dwell Time: 100 msec
Carrier gas: Helium, 15ml/min
Ions monitored 29,30,31 m/e

Results

Responses and retention times for both parts of the package obtained by GC/MS analysis are shown in Figures 1,2 and 3. As it can be seen from the head space profiles in Figures 1,2 and 3 there are two compounds released from both the outer package (Fig. 2) and the package content (Fig. 3) eluting at retention times 0.4 and 3.0 min respectively that predominate concentration wise. Since formalde-

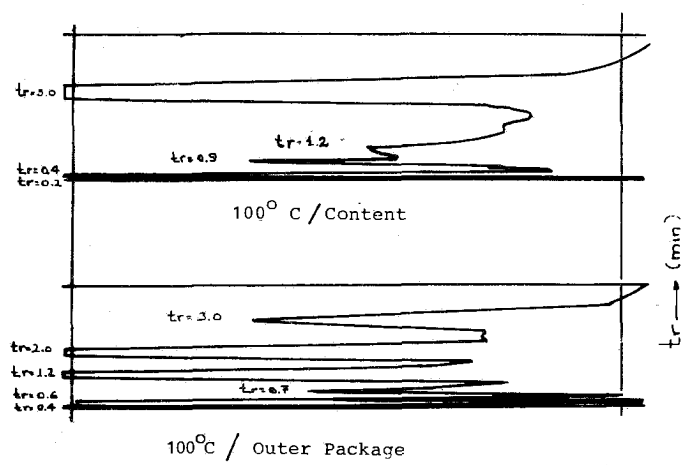

FIG. 1: *Chromatogram of Headspace Analysis of a) Content Using GC/FID b) Outer Package.*

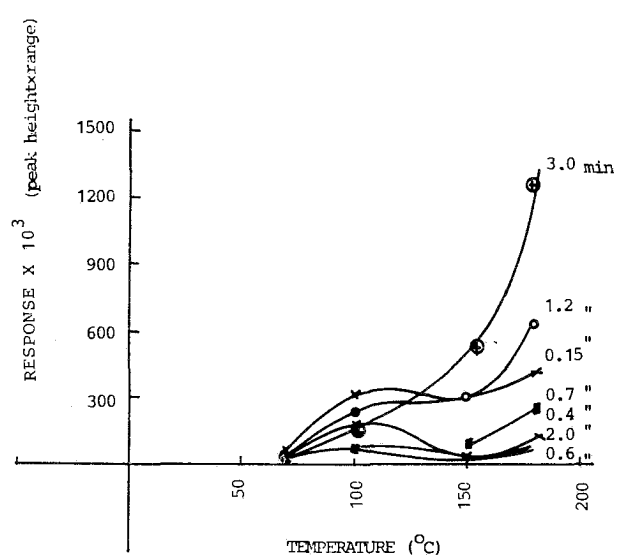

FIG. 2: *GC/FID Analysis of Compounds Released from the Outer Package at Various Temperatures. Values Next to Curves Correspond to Respective $t_{\bar{R}}$ of Compounds Eluted.*

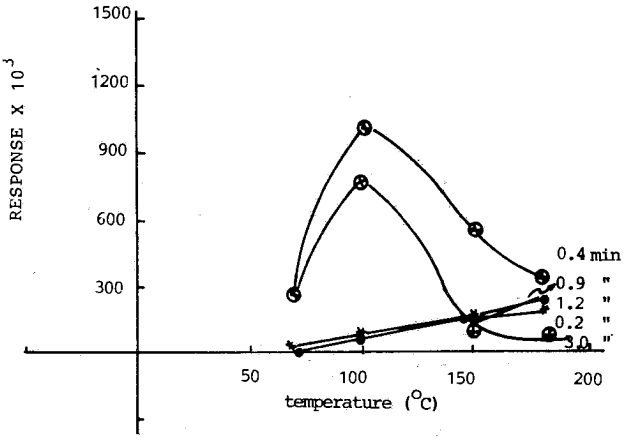

FIG. 3: *GC/FID Analysis of Compounds Released from the Content of the Package at Various Temperatures.*

hyde was suspected as the source of irritation, release of this compound was focused upon. The elution time for standard formaldehyde using GC/FID was 0.4 min. To confirm the compound of tr = 0.4 min as formaldehyde, the same experiment was carried out using GC/MS.

Data for both parts of the package obtained by GC/MS analysis are shown in Table I and Figures 4 and 5.

The retention time for standard formaldehyde using GC/MS was 0.6 min. Ions 29 and 30 were monitored and the ratio between them 10:8 was used for verification purposes.

GC/MS chromatograms (Figures 4 and 5) confirm the presence of formaldehyde in the package content. By plotting the response of m/e for ion 30 versus the heating temperature (Table I), a curve shown in Figure 6 is obtainded, identical to that corresponding to the compound having tr = 0.4 in Figure 3.

TABLE I: Detection of Formaldehyde Released from the Content and the Outer package, Using SIM Mode of GC/MS.

Sample	Response of ions at t_R = 0.6 min		
	29 m/e	30 m/e	31 m/e
Content heated at 60°C	91,058	63,218	4,323
Content heated at 100°C	162,598	116,773	8,399
Content heated at 150°C	70,005	60,317	4,212
Content heated at 180°C	12,057	14,992	7,412

Outer package heated at 60°C, 100°C, 150°C and 180°C gave no response.

Negative control heated at 60°C, 100°C, 150°C, 150°C and 180°C gave no response.

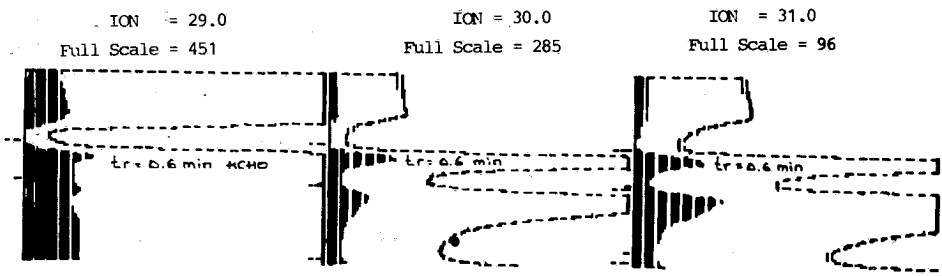

FIG. 4: *Standard formaldehyde solution (contains 15% methanol) under SIM mode.*

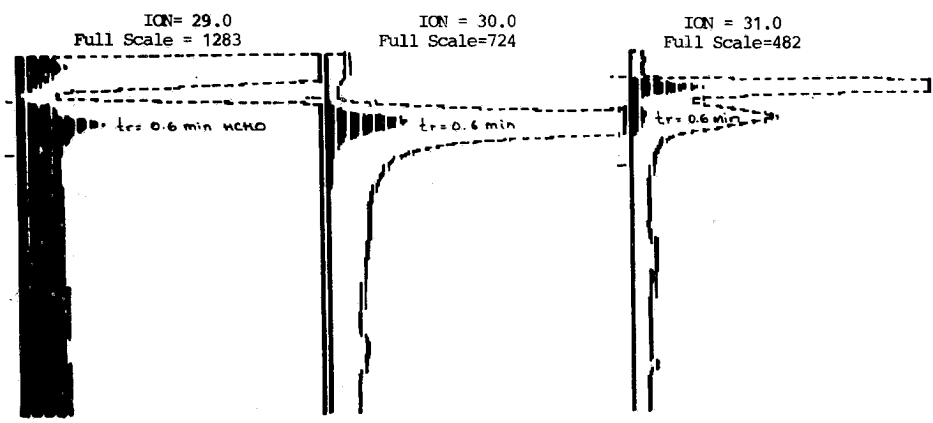

FIG. 5: *Head space sample of package content under SIM mode.*

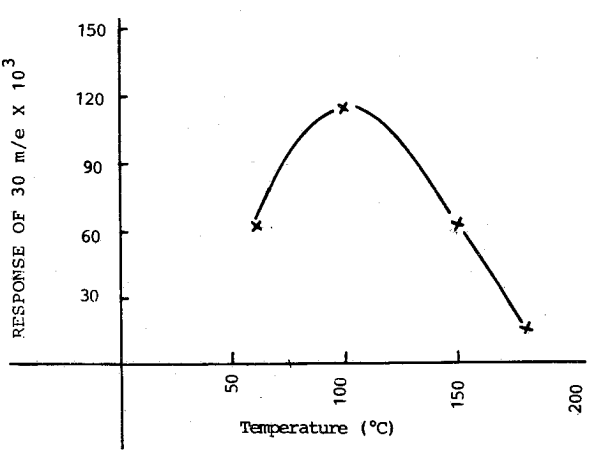

FIG. 6: *Monitoring of Formaldehyde Released from Package at Various Temperatures.*

The GC/MS chromatogram of the outer package showed no indication for the presence of formaldehyde. The compound with retention time 0.4 min being released from the outer package during the GC/FID analysis did not give the characteristic 29:30 ion ratio of 10:8.

The contents of the package were further divided into suture and the inner folder. The same experiment was repeated using GC/FID.

Results shown in Table II and Figures 7,8 and 9 indicate that formaldehyde is being released from the suture and at a temperature around 100-125^{0}C it reaches maximum concentration. Decrease in formaldehyde at temperatures higher than this region seems to be the results of a side reaction.

TABLE II: Detection of Formaldehyde Released from the Suture and the Inner Folder, using SIM Mode of GC/MS.

Sample	Response of ions at t_R=0.6 min 29 m/e	30 m/e	31 m/e
Suture heated at 60^{0}C	100,214	80,820	5,322
Suture heated at 100^{0}C	207,015	166,331	17,215
Suture heated at 125^{0}C	320,476	256,823	28,109
Suture heated at 150^{0}C	250,141	200,461	20,336
Inner folder heated at 60^{0}C	—	—	—
Inner folder heated at 100^{0}C	—	86,971	4,170
Inner folder heated at 125^{0}C	—	58,750	3,431
Inner folder heated at 150^{0}C	—	30,576	3,489

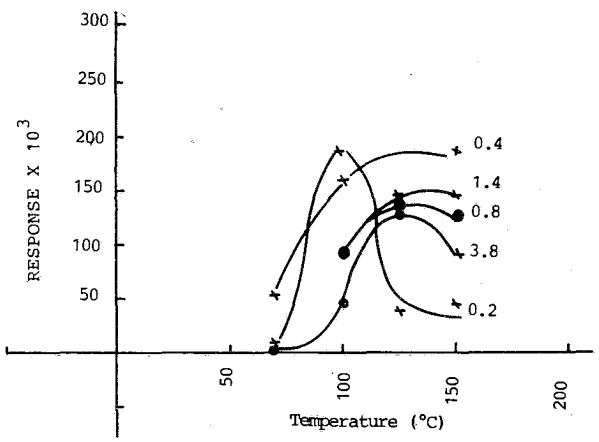

FIG. 7: *GC/FID Analysis of Compounds Released from the Suture at Various Temperatures.*

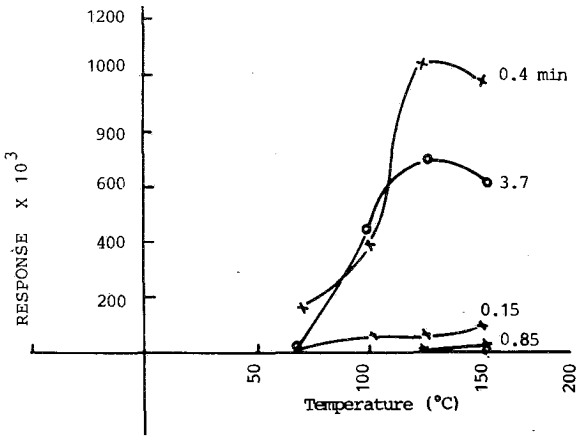

FIG. 8: *GC/FID Analysis of Compounds Released from Inner Folder at Various Temperatures.*

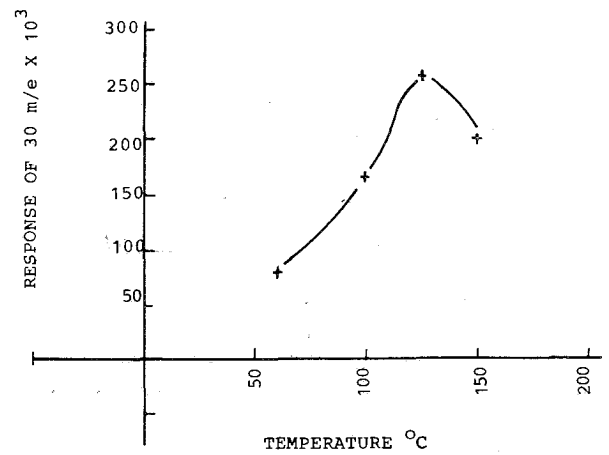

FIG. 9: *Monitoring of Formaldehyde Released from Suture at Various Temperatures, using GC/MS.*

Using IR scan, the plastic outer layer of the laminated package was identified as PET (Polyethylene terephthalate).

With the purpose to study the mode of formaldehyde release from the suture, the former was placed in the previously described serum vials and heated at temperatures of 100°C and 150°C. At 30 min intervals for 3 hours, a headspace sample of 0.5 ml was injected into the GC/MS.

Results of the time course study are shown in Figure 10. It is shown that the rate of formaldehyde release does not change during 3 hours heating at 100°C and 150°C and does not reach equilibrium. This indicates that formaldehyde is probably released by evaporation or reaction. However in the actual case of the post-surgery

patient it is most likely that formaldehyde is being released by evaporation rather than by reaction since direct environment and conditions minimize such possibility.

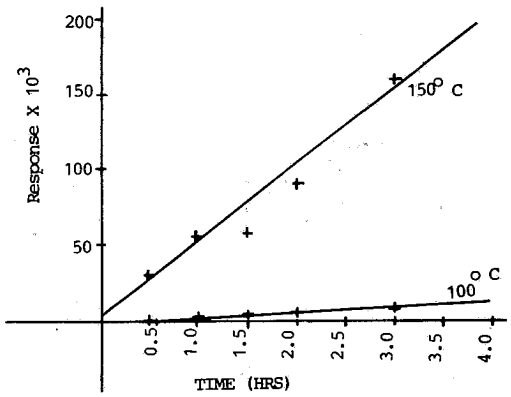

FIG. 10: *Time Course Study of Formaldehyde Release.*

Περίληψη

Μελέτη της απελευθέρωσης φορμαλδεΰδης, υπεύθυνης για ερεθισμούς που προκαλούνται μετεγχειρητικά, από την φαρμακευτική συσκευασία.

Η σημασία του προσδιορισμού καταλοίπων διαλυτών σε πλαστικά υλικά που χρησιμοποιούνται στη συσκευασία τροφίμων και φαρμακευτικών ειδών, έχει τα τελευταία χρόνια αρκετά τονιστεί, ιδιαίτερα σε περιπτώσεις όπου οι ουσίες αυτές, είτε είναι τοξικές, είτε προκαλούν τοπικούς ερεθισμούς σε όσους χρησιμοποιούν τα προϊόντα αυτά.

Η Αέρια Χρωματογραφία και πρόσφατα ο συνδυασμός Αέριας Χρωματογραφίας/Φασματοσκοπίας Μάζης έχουν χρησιμοποιηθεί επιτυχώς στον προσδιορισμό τέτοιων καταλοίπων διαλυτών. Σ'αυτή την εργασία ταυτοποιήθηκε η φορμαλδεΰδη, υπεύθυνη για τον ερεθισμό που προκάλεσε σε ασθενείς, σε μετεγχειρητικό στάδιο και προτείνεται ένας πιθανός τρόπος απελευθέρωσής της από το υλικό του ράμματος.

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PRELIMINARY COMMUNICATION

Chimika Chronika, New Series, 13, 149-154 (1984)

ON THE SPHINGOPHOSPHONOLIPIDS OF "PELAGIA NOCTILUCA"

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(Received August 8, 1983)

Summary

The polar lipids of the cnidaria scyphozoan *Pelagia noctiluca* were isolated and fractionated by column chromatography on silicic acid. The column fractions were studied by TLC combined with quantitative analysis of total, and alkali stable phosphorus and phosphonate -phosphorus. Phosphonolipids constitute 24% of the total lipid phosphorus and their thin-layer chromatographic behavior is similar to that of C-AEP and C-MAEP of different molecular species (di-, tri-, and tetra-hydroxy derivatives). Also major components of the polar lipids of *P. noctiluca* are phosphatidyl choline: 37.75%, phosphatidyl ethanolamine: 12% and cardiolipin: 6.5% (of total lipid P).

Key Words: ceramide aminoethyl phosphonate, cardiolipin, phosphatidyl choline, phosphatidyl ethanolamine, invertebrates, medusa, lipid column chromatography.

Abbreviations

TLC, Thin Layer Chromatography; Car, Cardiolipin; Sps, Sphingosine; Cer, Cerebrosides; Crm, Ceramides; Sph, Sphingomyelin; PE, Phosphatidyl ethanolamine; PC, Phosphatidyl choline; PI, Phosphatidyl inositol; MAS, Mild Alkaline Stable components; F.A., Fatty acid; n-PrOH, n-propanol; Et. Ac, Ethyl acetate; P, Phosphorus; A.A., Acetic acid; MeOH, Methanol.

Introduction

Biological molecules with a direct carbon to phosphorus bond have been discovered with considerable delay [1]. Lipid bound phosphonate was first found in a sea anemone as a sphingolipid ceramide aminoethyl phosphonate [2,3]. Several other phosphonate lipids have been identified (and characterized) in nature [4,5]. The lipid from the sea anemone *A. Elegantissima* [3] was shown to contain sphingosine as the major base and palmitic acid as the major fatty acid. The lipids of many marine

invertebrates, of some terrestrial animals and of human body have been studied and it was established that they have the carbon-phosphorus bond. However the lipids of "medusae" were studied mainly with emphasis in their fatty acids, which are composed of a highly complex mixture 70-80% unsaturated but the phosphonolipids were largely neglected[6]. It is interesting that the ceramide aminoethylphosphonates in sea anemones and shell fish differ in complexity of the composition of fatty acids and long chain bases respectively (i.e. the fatty acid composition is complex in the sea anemones and simple in shellfish, whereas long chain base[7] are complex in shellfish and simple in sea anemones).

Materials-Methods

A. Materials and reagents.

Collection of the organism: The cnidarian scyphozoa *Pelagia noctiluca* were collected at the beginning of May in the area of Saronic bay, Greece. Immediately the scyphozoan were extracted alive. Silica gel G type 60 was purchased from MERCK, Car, Sps, Cer, Crm and silica gel Mallinckrodt, 100 mesh, for column chromatography from SERVA. PE and PC were isolated in our laboratoty from egg yolk. PI was purchased from SIGMA. Sph was isolated from bovine brain. TLC was performed on glass chromatoplates coated with silica gel G, (thickness, O.25 mm) and activated 1h at 120^{0}C. The centrifuge was SUPERSPEED SORVALL RC 2-B. Evaporation of solutions were made in a rotary flash evaporator (laboratory-Glass Instrumen. Corp. New York 31. Serial N^{0}FE 1518).

B. Extraction of lipids.

The lipids were extracted by modification of the Bligh and Dyer method[8]. 21 skyphozoan ("jelly fishes") *P. Noctiluca* were homogenized separately with a 3-fold volume of $CHCl_3$/MeOH (1:2, V/V) by OMNI MIXER (0^0 C, 5′ at medium speed). 1 volume $CHCl_3$ was added to the homogenate and the solvent was removed by centrifugation (0^{0}C, 10′, 3000 rpm). 1 Volume $CHCl_3$/MeOH (1:1, V/V) was added to the residual material and it was rehomogenized and centrifuged as above. To the combined extracts 1 volume H_2O and 1 Volume $CHCl_3$ were added and after mild mixing, it was left quiet (12h) for phase separation, (The upper Water-methanol phase was kept for the study of acidic lipids). The lower $CHCl_3$ phase (including the fluffy layer) was evaporated to dryness in the rotary evaporator and it was redissolved in 50ml $CHCl_3$/MeOH (9:1), ("Crude lipids").

C. Analytical methods.

Phosphorus was determined by the Long-Staples method[9]. Phosphonolipids were determined[12] by a modification of Aalbers-Bieber[10] method.

D. Isolation and purification of the crude lipids.

A portion of crude lipids were evaporated to dryness, redissolved in a minimal

amount of $CHCl_3$/MeOH (98:2, V/V) and applied to a column (1.1 × 10 cm) packed with 2g of silicic acid activated at 120°C overnight, mixed with 2g of Hyflo Super Cel. Stepwise elution was performed with solvent mixtures of increasing polarity as indicating in table I. The elution was monitored by T.L.C. with C/M/W (65:25:4, V/V/V).

E. Thin layer chromatography.

Analytical TLC of lipids was performed on silica gel G layers using the following systems (by volume): C/M/W 65:25:4 or 90:10:1 or 30:8:1, n-ProH/W 7:3, n-ProH/28% NH_3/W 6:2:1 or 15:1:5, C/M/AA/W 50:25:6:2, and C/M/c. NH_3 60:35:5.

F. Mild alkaline hydrolysis.

To 1-16 mg of lipid, 1 ml 0.1N KOH (in 90% methanol) was added in a screwcapped tube with teflon lined cap. The tube was left at 35° - 40°C with occasional mixing for 2-3 h. The solution was acidified with 0.2ml 1 N HCl, chloroform (2 ml) and water (0.5 ml) were added, mixed well and centrifuged. The lower chloroform layer was withdrawn washed with 1 ml MeOH/W (1:2, V/V) and taken to dryness in a stream of nitrogen.

G. Acid catalyzed methanolysis.

The method of Sweeley and Moscatelli has been used (15h, 2N HCl-MeOH 80°C) for degradation of ceramide-phosphonolipids[11].

Results - Discussion

21 medusae *P. noctiluca* were extracted alive according to Bligh-Dyer. A portion of the chloroform layer was fractionated by column chromatography on silicic acid as shown in Table I. The column fractions were studied by TLC, accompanied by quantitative analysis of total and alkali stable phosphorus, as well as of the phosphonate phosphorus. The first volumes of chloroform/MeOH (9:1, V/V) eluted a phospholipid (6.7% of total phosphorus) which cochromatographed with cardiolipin and 95.4% of its P was rendered water soluble after mild alkaline hydrolysis (see fig. 1). The last volumes of chloroform/MeOH (9:1, V/V) eluted two major components (30% of total lipid P). The first one cochromatographed with PE and the next (composed of two spots, very close to each other) had chromatographic mobility similar to that of PC (Fig. 2). After mild alkaline hydrolysis the first (PE-like) component was hydrolyzed completely, in contrast to the second one. Namely, 40% of lipid-P from this column fraction (possibly PC) was hydrolyzed (milk-alkali labile) whereas 60% of the MAS-components (i.e. 10.8% of total P) which was positive to ninhydrin was determined as phosphonolipid (see Table I, Fig. 2 and Fig. 3). The first volumes of chloroform/MeOH (4:1, V/V) eluted ninhydrin positive compounds corresponding to 26% of total

TABLE I: Column Chromatographic Conditions and Analytical Data

Fraction Number	Solvent System	Bed Volumes	Column Fraction Total-P*	MAS components Total-P**	MAS components Phospho-nate-P***	Hydrolyzed components Total-P**
1	C/M, 98/2	8	0.8	—	—	—
2	C/M, 9/1	1.5	6.7	4.6	—	95.4
3	C/M, 9/1	3.5	30.0	60.0	60.0	40.0
4	C/M, 4/1	4	26.0	50.0	95.0	50.0
5	C/M, 4/1	4	33.0	25.0	8.0	75.0
6	EtAc/M, 3/2	5	1.0	—	—	—
7	C/M, 4/1	8	0.8	—	—	
8	MeOH	3				

* As % of total-P of lipid extract.

** As % of total-P of column fraction.

*** As % of total-P of MAS components.

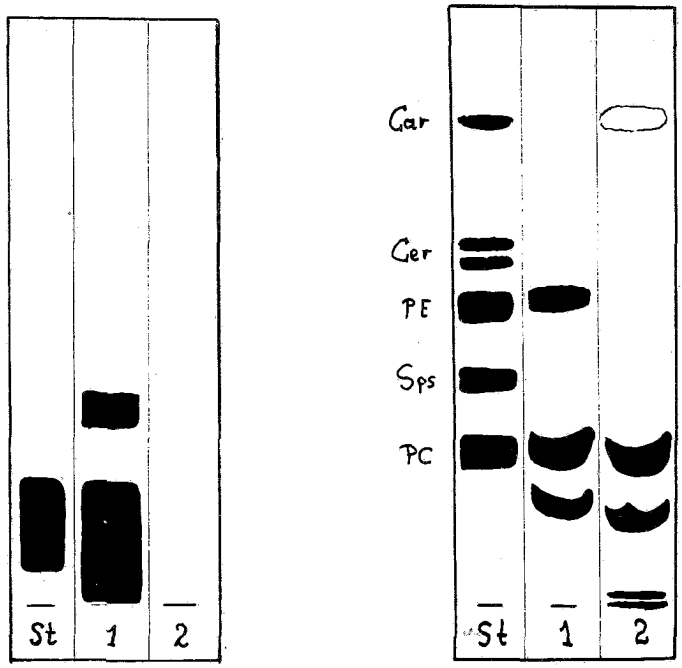

FIG. 1. *TLC of the intact (1)* and MAS *(2) components of column fraction No 2 along with cardiolipin standard (st). The chromatogram was developed with chloroform/MeOH/water (90:10:1 V/V/V). The spots were detected by i) iodine vapor ii) spraying with molybdenum blue reagent.*

FIG. 2. *TLC of the intact (1) and MAS (2) components of column fraction No 3 along with PC, PE, Cer, Sps standards (st). The chromatogram was developed with chloroform/MeOH/Water (65:25:4 V/V/V). The fraction's spots were detected by i) iodine vapor ii) spraying with molybdenum blue reagent iii) sprayring with ninhydrin.*

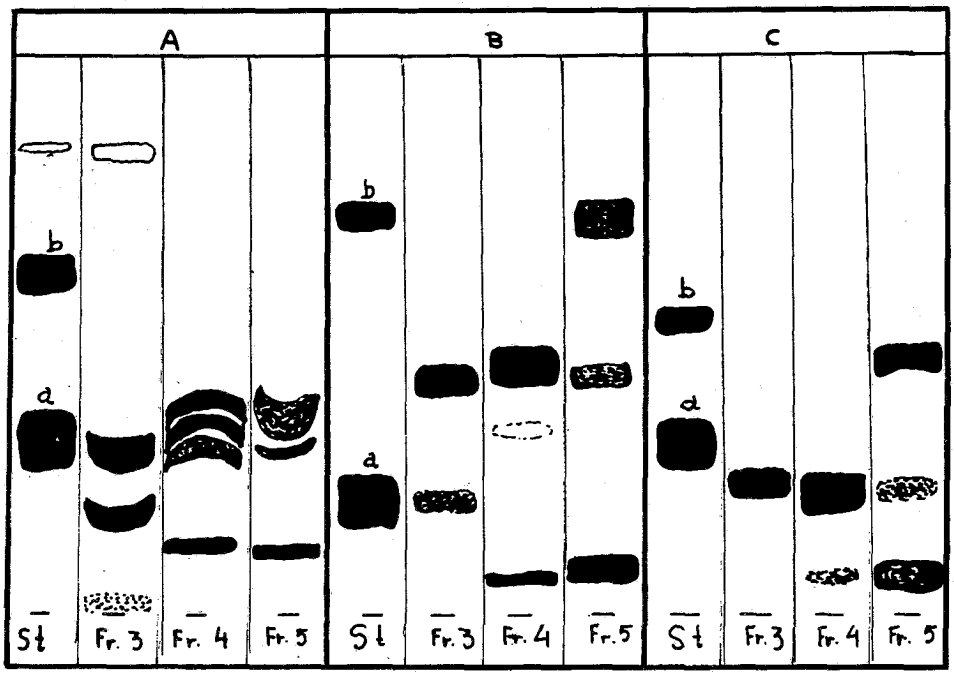

FIG. 3. *TLC of the MAS components of the column fractions No. 3,4,5 along with PC (a) and PE (b) standards (st). The chromatograms was developed with A: chloroform/MeOH/Water (65:25:4, V/V/V) B: chloroform/MeOH/A.A./Water (50:25:6:2, V/V/V/V) C: chloroform/MeOH/conc. ammonia (60:35:5, V/V/V) The spots were detected by i) iodine vapor ii) spraying with ninhydrin iii) spraying with molybdenum blue reagent.*

lipids. Of this fraction 50% was hydrolyzed by mild alkaline hydrolysis and the unhydrolyzed (MAS) compound contained 95% phosphonolipids (see Fig. 3, Fig. 4). The last bed volumes of chloroform/MeOH (4:1, V/V) eluted one ninhydrine positive spot accounting for 33% of total lipid-P, 25% of it (8.25% of total lipid-P) was unhydrolyzed by mild alkaline hydrolysis (MAS).

Another interesting point of the fraction which was eluted with the last bed volumes of chloroform/MeOH (4:1, V/V) has appeared to contain two molecular species of sphingophosphonolipids (see Fig. 3, B and C).

A portion of the MAS-lipids of the column fraction No. 4, containing 270 γ P was further purified by preparative T.L.C.

The purified lipids of its major spot were completely hydrolyzed by acid catalyzed methanolysis for 15h. The fatty acid methyl esters were extracted with light petroleum ether and are analyzed by TLC and gas chromatography.

The remaining methanolic layer contains the long chain bases and the aminoalkyl phosphonic acids, which will be isolated and identified in the future.

By summarizing the data mentioned above, it is evident that 24% of the phosphorus-containing lipids of *P. noctiluca* are sphingo-phosphonolipids (see Table I).

Περίληψη

Μελέτη των Σφιγγοφωσφονολιποειδών της "Pelagia noctiluca"

Τα πολικά λιποειδή του κνιδόζωου *"Pelagia noctiluca"* (συνομοτ. σκυφόζωα) απομονώθηκαν με εκχύλιση και κλασματώθηκαν σε χρωματογραφική στήλη πυριτικού οξέος. Από τη στήλη ανακτήθηκαν 8 κλάσματα (πιν. I), που μελετήθηκαν χωριστά με χρωματογραφία λεπτής στιβάδας (Σχ. 1-3), καθώς και με αναλυτικούς προσδιορισμούς ολικού φωσφόρου, φωσφόρου λιποειδών ανθεκτικών σε ήπια αλκαλική υδρόλυση και φωσφόνο-λιποειδών. Κύρια συστατικά των πολικών λιποειδών του κνιδόζωου είναι η φωσφατιδυλοχολίνη (37,75%), φωσφατιδυλαιθανολαμίνη (12%) καρδιολιπίνη (6,4%) και τρία τουλάχιστον σφιγγο-φωσφονολιποειδή που αντιπροσωπεύουν τα 24% του συνολικού λιποειδικού φωσφόρου (βλ. και Πιν. I). Τα φωσφολιποειδή αυτά εκλούονται από τη στήλη με τα τελευταία κλάσματα χλωροφορμίου-μεθανόλης, 9:1 (V/V) και με χλωροφόρμιο-μεθανόλη, 4:1 (V/V). Η χρωματογραφική τους συμπεριφορά είναι όμοια με εκείνη του C-AEP και C-MAEP διαφόρων μοριακών τύπων (δι-, τρι- και τετρα-υδρόξυ παράγωγα).

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SYNTHESE ET ETUDE PHARMACOLOGIQUE DE QUELQUES a - DIALKYLAMINOMETHYL γ - LACTONES TRICYCLIQUES.

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Résumé

L'étude pharmacologique des dérivés aminés des γ - lactones n'a commencé que récemment. Le présent mémoire se porte à la synthèse des γ - lactones α - dialkylaminométhyl tricycliques dont l'étude pharmacologique a révélé une activité antireserpinique.

Key Words: 3 - Dialkylamino methyl 9b - Methyl 3a, 4, 5, 9b Tetrahydronaphtalene [1,2 - b] Furan 2(3H) - one.

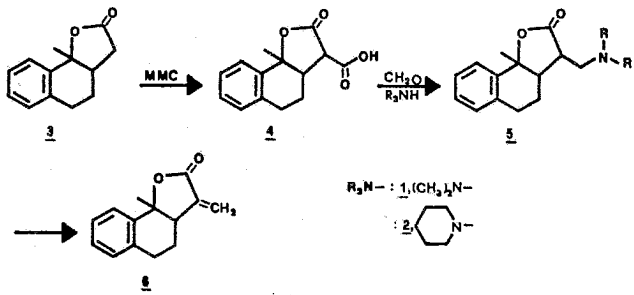
Introduction

Le squelette de la γ- butyrolactone est souvent rencontré dans les molécules des produits naturelles; on le trouve chez certains sesquiterpènes et alcaloides du groupe de la pilocarpine et chez certains hétérosides cardiotoniques. Bien que les propriétés pharmacologiques des substances des catégories précédentes soient dépuis longtemps étudiées, en particulier l'activité anticancereuse alkylante des α - méthylène γ - butyrolactones sesquiterpéniques[1-7], pourtant très peu de choses sont connues en ce qui concerne l'activité pharmacologique des γ - butyrolactones qui possèdent une fonction amine. Nous pouvons citer le cas des γ - spirolactones et des γ-butyrolactones ayant des propriétés neuroleptiques et antireserpiniques intenses[8,9].

Dans le présent travail nous décrivons la synthèse et l'étude de l'activitè antireserpinique des γ - lactones α - dialkylaminométhyl tricycliques du type 5.

La synthèse de ces composès est effectuée selon le schéma I par carboxylation de la lactone 3 avec le carbonate de méthyl et de magnésium (MMC)[10-13]; on obtient ainsi l'acide 4 qui par réaction de Mannich fournit les dérivés aminés 5 transformés par la suite à leurs chlorhydrates. Les bases 5 sont instables et se transforment facilement même à la température ambiante au dérivé méthylénique 6.

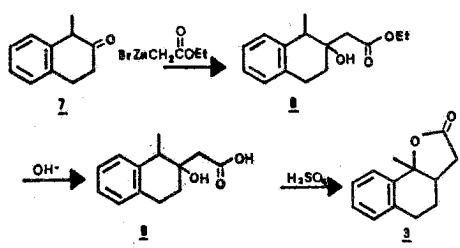
La lactone 3 est préparée selon le schéma II à partir de la naphtalénone 7 qui à



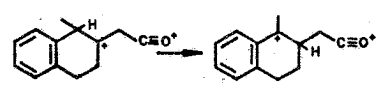
SCHEMA I

son tour est préparée par méthylation de l'énamine avec la pyrrolidine de la 2(1H) dihydro - 3,4 naphtalénone[14].

Ainsi la cétone 7 réagit selon Reformatsky pour donner l'hydroxy ester 8 qui par saponification fournit l'acide 9; ce dernier est aisement transformé en lactone 3 au sein de l'acide sulfurique concentré[15-19]. La structure de cette lactone est démontrée par l'analyse élémentaire, par son spectre à l'infrarouge où la fréquence du carbonyle est caractéristique, ainsi que par son spectre de RMN où le méthyle apparaît comme singulet tandis qu'au spectre de l'hydroxy acide 9 apparaît comme doublet. Cela prouve qu'une transposition d'un ion hydride a lieu à la position 2 et qu'un déplacement du centre électropositif du carbocation à la position 1 se passe au sein de l'acide sulfurique (schèma III).



SCHEMA II



SCHEMA III

Par ailleurs le spectre de RMN de l'acide 4 permet de conclure que le proton de la position 3 qui apparait comme doublet ($\delta = 3{,}6$ ppm $J = 11$ Hz) se trouve en position trans diaxiale avec le proton de la position 3a[20].

Les composés 1 et 2 de formule générale 5 ont été étudiés pour leur activité entireserpinique. Avec l'Imipramine nous obtenons une protection de 64% vis à vis de la reserpine ($p<0{,}01$ test de Mann-Whitney), avec le composé 2 une protection de 38% ($p<0{,}05$) et avec le composé 1 15% (non singnificative).

D'apres les résultats obtenus il semble que le dérivé pipéridinique en bout de chaine posséde une activité qui reste imprecise avec le dérivé diméthylaminique. L'explication la plus plausible que nous puissions avancer à propos de cette différence d'activité entre les deux composés est que le noyau pipéridinique augmente la lipophilie et la basicité de la molécule et par voie de conséquence son

activité. Des explications analogues ont été données dans le passé à propos des neuroleptiques[21,22].

Partie Experimentale

Les points de fusion ont été pris à l'appareil de Büchi et ne sont pas corrigés. Les spectres I.R. ont été enregistrés sur un appareil Perkin - Elmer 177. Les spectres de RMN ont été réalisés sur un appareil Varian FT - 80A en utilisant la TMS comme référence interne. Les microanalyses ont été réalisées par le service central de microanalyse de C.N.R.S. de Thiais.

Acide (méthyl - 1 hydroxy - 2) 1,2,3,4 tétrahydronaphtalène acétique 9.

On fait réagir 46, 1 g (0,70 gratom) de Zn^0 en poudre activée avec un mélange de 91,8 g (0,57 mole) de méthyl-1 tétraloné- 2, 117,6 g de bromoacétate d'éthyle (0,7 mole) et 400 cm^3 de benzène anhydre de facon à maintenir une ébullition douce en ajoutant le mélange sur le métal et en chauffant s'il est nécessaire. On complète la réaction en chauffant à reflux pendant 8h, refroidit dans la glace et décompose avec l'acide sulfurique à 10%. Après avoir lavé la couche organique à l'eau bicarbonatée et à l'eau, on la sèche et évapore le solvant. La distillation sous pression réduite du résidu fournit un mélange azéotropique d'hydroxy ester et de méthyl - 1 tétralone - 2 (97 g) que l'on saponifie avec un excès de solution hydroalcoolique de KOH à 10%. Après évaporation de l'éthanol sous pression rèduite on ajoute de l'eau et extrait quatre fois à l'éther. La couche éthérée nous fournit après évaporation du solvant et distillation sous vide 25,4 g de méthyl - 1 tétralone - 2 ($E_{0,5} = 95 - 96^0$).

La couche aqueuse est acidifiée avec HCl à 20% sous refroidissement énergique. On obtient l'hydroxy acide avec un rendement de 40%.

$F = 130^0$ (éther -n - pentane), IR (nujol) ν(OH) 3490 cm^{-1}, ν(C = 0) 1680 cm^{-1}, RMN ($CDCL_3$) δ 1,35 ppm (d, 3H, CH_3), 2,6 (s, 2H, CH_2COOH), 2,7 à 3,2, (m, 3H, 1,4 —H) 6,8 (s, 1H, OH), 7,1 (m, 4H, 5,6,7,8 -H aromatiques). Analyse ($C_{13}H_{16}O_3$)% Calc. C: 70,90 H: 7,27 % Tr. C = 70,91 H = 7,18.

Méthyl - 9b tétrahydro - 3a,4,5,9b, naphtalène [1,2 - b] furanone - 2(3H). 3

6g d'hydroxy acide 9 (0,027 mole) sont traités sous agitation et refroidissement avec un mélange de 60 cm^3 d'acide sulfurique conc. et de 15 gouttes d'acide sulfurique fumant pendant 2h. On verse le mélange dans de la glace pilée et extrait à l'éther. La couche organique est lavée successivement à l'eau carbonatée et à l'eau et séchée sur sulfate de sodium. Après filtration sur alumine on obtient la lactone 3 avec un rendement de 60%. $F = 45^0$, IR (film) ν(C = O) 1765 cm^{-1}, RMN ($CDCl_3$) δ 1,5 ppm (s, 3H, CH_3) 1,5 à 2,1 (m, 3H, 3a, 4-H), 2,15 à 2,95 (m, 4H, 3,5-H), 6,7 à 7,5 (m, 4H, 6,7,8,9-H aromatiques). Analyse ($C_{13}H_{14}O_2$) % Calc. C = 77,22 H = 6,93% Tr. C = 77,13 H = 6,91.

Acide méthyl - 9b oxo - 2 héxahydro - 2, 3, 3a 4,5,9b naphtalène [1,2 - b] furan carboxylique - 3 4.

3,8g de lactone 3 (0,018 mole) sont ajoutés dans un mélange de 40 cm^3 de carbonate de méthyl et de magnésium 2M dans la DMF et de 200 cm^3 de DMF anhydre. On porte à léger reflux pendant 8h.

On décompose avec de la glace et de l'acide chlorhydrique à 30%, extrait l'acide au chlorure de méthylène, lave la couche organique à l'eau, la sèche sur sulfate de sodium et évapore le solvant. L'acide qui cristallise lentement dans l'éther est lavé avec un mélange éther - n - pentane 5/1.

Rdt = 42%, F = 137^0(déc.), IR (nujol) ν(C=0) γ lactone: 1760 cm^{-1}, ν(C=0) carboxyle 1700 cm^{-1}, RMN ($CDCl_3$) δ 1,78 ppm (s, 3H, CH_3), 1,85 à 3,35 (très complèxe m, 5H, 3a, 4,5 — H), 3,50 (d, 1H, J = 11Hz, 3 - H), 6,9 à 7,7 (m, 4H, 6,7,8,9 — H aromatiques). Analyse ($C_{14}H_{14}O_4$)% Calc. C = 68,29 H = 5,69% Tr. C = 68,19 H = 5,69.

Diméthylamino méthyl - 3 méthyl - 9b tétrahydro - 3a, 4, 5, 9b naphtalène furan [1,2 - b] one - 2(3H). 1.

1g d'acide 4(0,004 mole) est mélangé avec 10 cm^3 d'éthanol et chauffé vers 40^0. On ajoute sous agitation 0,006 mole de diméthylamine en solution éthanolique saturée. On refroidit dans la glace et ajoute très lentement (en 1 heure) 1,3g de solution de formol à 35% (0,015 mole). On contine à agiter pendant 2h à T = 20^0. On ajoute alors 70cm^3 d'eau froide et extrait rapidement avec 3 × 30cm^3 de chloroforme. On lave la couche organique avec 4 × 20cm^3 d'eau froide, la sèche (toujours à froid) sur sulfate de sodium et acidifie avec de l'éthanol saturé d'acidechlorhydrique. On évapore totalement sous vide les solvants et ajoute de l'éther anhydre. Le chlorhydrate précipite. Après filtration du précipité, dans le filtrat on isole après évaporation du solvant le dérivé méthylénique 6 (F = 77^0, IR (nujol) ν(C=0) γ lactone 1760 cm^{-1}, ν(C=C) 1650cm^{-1} RMN ($CDCl_3$) δ 1,78 ppm (s, 3H, CH_3), 1,9 à 3,3 (très complèxe m, 5H, 3a, 4, 5 — H), 5,65 (d, 1H, AMX, $J_{AX} \simeq$ 3Hz $>CH_AH_M$) 6,25 (d, 1H, AMX, $J_{MX} \simeq$ 3Hz, $>CH_AH_M$), 6,8 à 7,7 (m, 4H, 6, 7, 8, 9 — H aromatiques). Analyse ($C_{14}H_{14}O_2$)% Calc. C = 78,50 H = 6,54% Tr. C = 78,23 H = 6,58). Son rendement varie très largement selon la vitesse de l'addition du formol, la durée de la réaction et surtoût la température à laquelle on effectue la réaction. Une transformation quantitative en dérivé méthylénique est effectuée lorsqu'on réalise la réaction vers 50^0 durant quelques heures. Rdt en dérivé méthylénique, 45% Rdt en chlorhydrate 1: 48%. F(éthanol - éther): 203^0, IR (nujol) 2385cm^{-1} $\nu(NH^+)$, ν(C=0) lactone 1755cm^{-1}, RMN ($CDCl_3$) δ 1,78 ppm (s, 3H, CH_3), 2, 12 à 2,5 (m, 3H, 3a, 4 - H), 2,62 (m, 1H, 3 - H) 2,87 (s, 6H, diméthylamino), 3,37 (m, 2H, 5 - H), 7,18 à 7,41 (m, 4H, 6, 7, 8, 9 - H aromatiques).

Analyse ($C_{16}H_{22}NO_2Cl$) % Calc. C = 64,97, H = 7,44, N = 4,73, Cl = 12,01% Tr. C = 64,92, H = 7,50, N = 4,62, Cl = 12,16.

De la même facon on prépare le chlorhydrate 2 Rdt en méthylénique 6: 46%. Rdt en chlorhydrate 2: 46%, IR (nujol) $\nu(NH^+)$ 2440cm^{-1}, ν(C=0) γ lactone

1755cm^{-1}, RMN ($CDCl_3$) δ 1,85 ppm (s, 3H, CH_3), 2,75 (m, 1H, 3 - H), 3,31 (m2H, 5 - H), 7 à 7,62 (m, 4H, 6, 7, 8, 9 - H aromatiques), 9,37 (s, large 1H, NH^+). Analyse ($C_{19}H_{26}NO_2Cl$) % Calc. C = 67,95; H = 7,74% Tr. C = 67,73 H = 8,06.

La réaction de Mannich avec la diéthylamine fournit la base 5 ($R_2N = (C_2H_5)_2N$) avec un faible rendement.

L'étude de l'antagonisme à la reserpine a été effectuée selon le protocole proposé par C. Gouret et J. Thomas[23]. Ce protocole utilisé pour la selection des antidepresseurs, consiste à rechercher une éventuelle protection vis à vis du ptosis ou occlusion des paupières provoqué chez la souris et le rat par injection de reserpine. Nous avons utilisé des rats Wistar de 180-200 g. Cinq lots de neuf animaux ont été formés. Le premier lot recoit deux injections i.p. de 5μl g^{-1} de rat d'eau distillée à 30 minutes d'intervalle (lot temoin). Le deuxième lot recoit une injection d'eau distillée et 30 min après une injection de reserpine (2mg kg^{-1} en i.p.) (lot de référence). Le troisième recoit de l'imipramine (20mg kg^{-1} en i.p.). Le quatrième recoit le produit No 1 (20mg kg^{-1} i.p.) et la reserpine (2mg kg^{-1} i.p.) et le cinquième le produit No 2 (20mg kg^{-1} i.p.) et la reserpine (2mg kg^{-1} i.p.).

Pour toutes les administrations i.p. le volume d'injection est toujours 5μl g^{-1} de rat. On evalue la protection des produits administrés avant la reserpine (imipramine, composé 1, composé 2) par comparaison des ptosis des lots 5,4 et 3 avec le lot de référence 2 selon la formule:

$$p = \text{protection} = 100 \times \frac{\text{ptosis du lot 2-ptosis des traités 3,4,5}}{\text{ptosis du lot 2}}$$

Etude de la toxicité chez le rat.

Rats Wistar de 200-220 g.

Convulsion à partir de 100mg kg^{-1} pour le K_1 et K_2. Les animaux meurent après une bouffée de violentes convulsions à partir de 200mg kg^{-1} pour le K_2 et de 300mg.Kg^{-1} pour le K_1.

A 500mg kg^{-1} la survenue des convulsions et la mort est très rapide pour le k_2 (5-10 min) et plus lente pour le K_1 (20-30 min).

Ce profil toxicologique présente une analogie assez forte avec celui des antidepresseurs tricycliques comme l'imipramine[24].

Summary

Synthesis of amino derivatives of tricyclic γ-lactones with antireserpinic activity.

The pharmacological study of amino derivatives of γ-lactones started recentyly. This study reports the synthesis of tricyclic a-dialkylaminomethyl-γ-lactones, which showed antireserpinic activity.

Περίληψη

«Σύνθεση αμινικών παραγώγων τρικυκλικών γ-λακτονών με αντιρεσερπική δράση».

Η φαρμακολογική μελέτη αμινοπαραγώγων των γ-λακτονών άρχισε πρόσφατα. Η παρούσα εργασία αναφέρεται στην σύνθεση τρικυκλικών α-διαλκυλάμινομεθυλο γ-λακτονών των οποίων η φαρμακολογική μελέτη έδειξε αντιρεσερπινική δράση.

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REGIOSELECTIVITY IN THE 1,3-DIPOLAR CYCLOADDITION REACTIONS OF BENZONITRILE OXIDE AND DIPHENYLNITRILIMINE WITH CINNAMIC ESTERS

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Summary

The effect of the substituents on the regioselectivity of the reactions of several substituted cinnamic esters with benzonitrile oxide and diphenylnitrilimine is studied. In all cases, the two possible regioisomers are obtained in various ratios. The differences in the proportions of the two regioisomers are discussed on the basis of frontier orbitals of the reacting species.

Key Words: Nitrile Oxides, Nitrile Imines, Cinnamic Esters, Cycloaddition Reactions.

Introduction

The 1,3-dipolar cycloaddition reactions of both nitrile oxides and nitrile imines with unsymmetrically substituted ethylenic or acetylenic dipolarophiles are usually regioselective, that is the formation of one of the possible regioisomers is favored[1]. The nature of the substituents of both the dipolarophile and 1,3-dipole has a great effect on the regioselectivity of the reactions. Thus in some cases both the two possible regioisomers are obtained whereas in others only one. The regioselectivity of the 1,3-dipolar cycloaddition reactions was for a long time an unsolved problem, since it could not be explained by the classical resonance theory[2,3,4,5]. This problem is now approached by the use of pertrubation theory as it has been applied approximately by Houk and his coworkers[6,7].

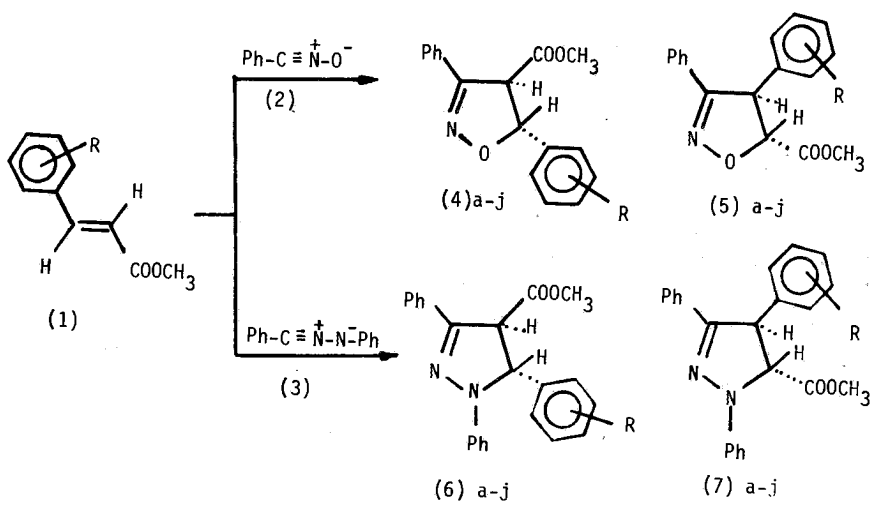
The regioselectivity is explained on the basis of the favorable interaction of frontier molecular orbitals. Reactions of nitrile oxides and nitrile imines with electron-rich alkenes are LUMO dipole controlled reactions according to Houk's and Sustmann's[8] terminology. This frontier orbital interaction leads to 5-substituted isoxazolines and pyrazolines respectively, as the larger coefficients of these frontier orbitals interact this way. In the reaction of nitrile oxides and nitrile imines with electron-deficient dipolarophiles both LUMO dipole and HOMO

dipole interactions influence the cycloaddition and the regioselectivity is reduced and in some cases is reversed that is the formation of 4-substituted derivatives is favored.

The substituted cinnamic esters (1) used in this work are electron-deficient dipolarophiles. Reactions of α,β-unsaturated esters with nitrile oxides[9,10,11,12], nitrile imines[13,14,15,16,17] and diazoalkanes[18] are already known. Although the influence of 1,3-dipole substituents on the regiochemistry of these reactions has been studied to some extent[9,11], very little is known about the influence of dipolarophile substituents[10,13]. The purpose of this paper is to estimate this effect and these results are of some importance for the understanding on the problem of regioselectivity.

Results and discussion

All the reactions were carried out under the same conditions. Benzonitrile oxide (2) (abbreviated BNO) and diphenylnitrilimine (3) (abbreviated DPNI) were liberated "in situ" at room temperature from the corresponding benzhydroxamoyl chloride and N-α-chloro-benzylidene-N-phenyl hydrazide with triethylamine in the presence of dipolarophile in anhydrous benzene solution. Reactions with BNO gave the two regioisomeric isoxazolines (4) and (5) and reactions with DPNI the two regioisomeric pyrazolines (6) and (7) according to the equations.



(a): R = H; (b): R = p-Cl; (c): R = p-CH_3; (d): R = p-CH_3O;

(e): R = p-NO_2; (f): (R = o-Cl; (g): R = o-CH_3O; (h): R = o-NO_2;

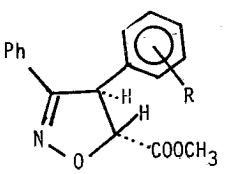
(i): R = m-Cl; (j): R = m-NO_2.

Some reactions with DPNI were also repeated at higher temperature 80° (reflux in benzene) without any change in the ratio of the two regioisomers. The yields as it was shown with TLC were almost quantitative. The ratio of the two regioisomers

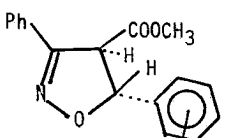
(4), (5) and (6), (7) was determined in the crude reaction mixture by proton NMR spectroscopy from the differences in the chemical shifts of the isoxazoline and pyrazoline protons respectively of the 4-and 5-positions in accordance with bibliographic data [10,13]. The ratios of the two isomers and the chemical shifts of the 4-and 5-position isoxazoline and pyrazoline ring protons for the obtained isoxazolines and pyrazolines are given in Tables I and II respectively.

From the results of Table II it comes out that the reactions with DPNI show very low regioselectivity and the two regioisomers are formed in almost equal amounts. All the substituents regardless of their position and their kind (electron acceptors or electron donors) reduce the regioselectivity in comparison with the unsubstituted cinnamic ester.

TABLE I: Ratios of the isomeric isoxazolines (4) and (5) and chemical shifts of 4- and 5-isoxazoline ring protons.



(4)



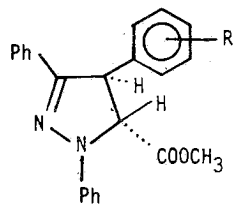
(5)

R	Compd	Chemical shifts (δ, $CDCl_3$)		Compd	Chemical Shifts (δ, $CDCl_3$)		Yield
		4-H	5-H		4-H	5-H	(4):(5)
H	4a	4.49	6.00	5a	4.95	5.10	82:18*
p-Cl	4b	4.43	5.94	5b	4.88	5.05	80:20
p-CH_3	4c	4.45	5.92	5c	4.88	5.05	80:20
p-CH_3O	4d	4.47	5.90	5d	4.88	5.02	90:10
p-NO_2	4e	4.47	6.07	5e	4.95	5.22	72:28
o-Cl	4f	4.38	6.28	5f	4.86	5.63	71:29
o-CH_3O	4g	4.33	6.15	5g	4.88	5.48	75:25
o-NO_2	4h	4.42	6.54	5h	4.95	5.78	62:38
m-Cl	4i	4.48	5.94	5i	4.88	5.11	75:25
m-NO_2	4j	4.47	6.05	5j	4.95	5.20	79:21

*Huisgen [10] and coworkers give for the same reaction the ratio 67:33.

Reactions with BNO show remarkably higher regioselectivity than reactions with DPNI. Formation of the 4-carbomethoxy derivative (4) is favored in all cases. Electron donors substituents in p-position increase the proportion of (4) isomer while electron acceptors substituents reduce it. Substituents in m-position have very low effect on the regioselectivity, whereas substituents in o-position

TABLE II: Ratios of the isomeric pyrazolines (6) and (7) and chemical shifts of 4- and 5-pyrazoline ring protons.



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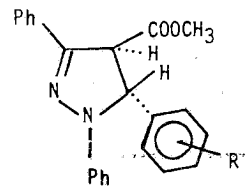


(7)

R	Compd	Chemical shifts (δ, $CDCl_3$)		Compd	Chemical shifts (δ, $CDCl_3$)		Yield
		4-H	5-H		4-H	5-H	(4):(5)
H	6a	4.19	5.53	7a	4.63	4.76	38:62*
p-Cl	6b	4.18	5.51	7b	4.63	4.75	47:53*
p-CH_3	6c	4.19	5.52	7c	4.63	4.76	44:56
p-CH_3O	6d	4.18	5.48	7d	4.60	4.73	50:50*
p-NO_2	6e	4.20	5.63	7e	4.65	4.92	55:45
o-Cl	6f	4.17	6.02	7f	4.62	5.38	47:53
o-CH_3O	6g	4.11	5.87	7g	4.58	5.25	47:53
o-NO_2	6h	6.28	6.17	7h	4.67	5.54	44:56
m-Cl	6i	4.17	5.53	7i	4.61	4.78	44:56
m-NO_2	6j	4.22	5.66	6j	4.68	4.93	54:46

*Huisgen and coworkers give for the same reactions[13].
(6a) : (7a) 33:67
(6b) : (7b) 38:62
(6d) : (7d) 50:50

regardless of their kind increase the proportion of 5-carbomethoxy derivative (5). (Table I).

The regiochemistry of these reactions can be explained on the basis of the favorable interaction of frontier molecular orbitals of the reacting species. Substituents generally cause some changes to the energy of the frontier molecular orbitals and to the magnitude of the coefficients of the atomic orbitals. In order to estimate these changes some theoretical calculations (CNDO/2) have been done for the esters 1a, 1d, and 1e. The interatomic bond distances and angles used for the calculations were chosen from data for analogous systems. Esters 1d and 1e have been chosen for the calculations since nitro and methoxy group in p-position as strong electron acceptor and donor respectively must have the greatest electronic influence on the double bond orbitals. The results of these calculations are given in Table III along with the corresponding known[6] values for BNO and the

TABLE III: Frontier orbital energies (in eV) and coefficients for dipoles and dipolarophiles

	HOMO			LUMO		
	Coefficients	$E_{calc.}$	$E_{est.}$	Coefficients	$E_{calc.}$	$E_{est.}$
Ph-CNO*	$C_C = -0.438$ $C_O = 0.602$	-11.028	-10	$C_C = 0.326$ $C_O = 0.253$	2.193	-1
HC = NNH*	$C_C = -0.591$ $C_N = 0.801$	-10.947	-	$C_C = 0.597$ $C_N = 0.401$	4.53	-
Ph-C = N-N-Ph**	$C_C < C_N$	-	-7.5	$C_C > C_N$	-	-0.5
C_6H_5-$\overset{\beta}{C}$H=$\overset{\alpha}{C}$H-$COOCH_3$	$C_\alpha = 0.472$ $C_\beta = 0.342$	-11.9	-9	$C_\alpha = -0.435$ $C_\beta = 0.475$	1.439	0
p-O_2N-C_2H_4-CH=CH-$COOCH_3$	$C_\alpha = 0.452$ $C_\beta = 0.354$	-12.6	-9.7	$C_\alpha = -0.429$ $C_\beta = 0.411$	0.457	-1
p-CH_3O-C_6H_4-CH=CH-$COOCH_3$	$C_\alpha = 0.439$ $C_\beta = 0.278$	-11.4	-8.5	$C_\alpha = -0.430$ $C_\beta = 0.479$	1.51	0.1

*According to Houk's calculations[6]

**In a non planar most stable conformation of HC=NNH the biggest coefficient is to be located on the carbon atom in HOMO[20].

unsubstituted nitrile imine. Calculations for DPNI have not been done because there are no experimental data for its geometry. The used values are estimated from the corresponding unsubstituted derivative according to Houk's approximation[6]. Even for the calculated energy values some estimations are usually made according to experimental data, since they are much higher than the real values. The values of the coefficients obtained by CNDO/2 are usually considered satisfactory.

The experimentally determined[6] frontier molecular orbital energies of methyl acrylate are $E_{HOMO} = -10.75$ eV and $E_{LUMO} = -0.8$ eV. Conjugated substituents as phenyl, destabilize HOMO by ~ 1.5 eV and stabilize LUMO by ~ 0.5 eV according to Houk's approximation. Thus the HOMO and LUMO energies of methyl cinnamate are estimated to be $E_{HOMO} = -9.2$ eV and $E_{LUMO} = -1.3$ eV. The HOMO energy of methyl cinnamate was found experimentally[19] $E_{HOMO} = -8.63$ eV, whereas the LUMO energy calculated on the basis of $\pi\pi^*$ absorption[19] was found $E_{LUMO} = 0.6$ eV. Correlation of the above values gives $E_{HOMO} = -9$ eV and $E_{LUMO} = 0$ eV as acceptable values for methyl cinnamate at least for qualitative estimations. The estimated in the same manner, values for methyl p-methoxy cinnamate are $E_{HOMO} = -8.5$ eV and $E_{LUMO} = 0.1$ eV and for methyl p-nitro-cinnamate $E_{HOMO} = -9.7$ eV and $E_{LUMO} = -1$ eV. Using the values of Table III the diagramm of interaction of frontier molecular orbitals of methyl cinnamate - BNO and methyl cinnamate - DPNI was made, given in Figure 1.

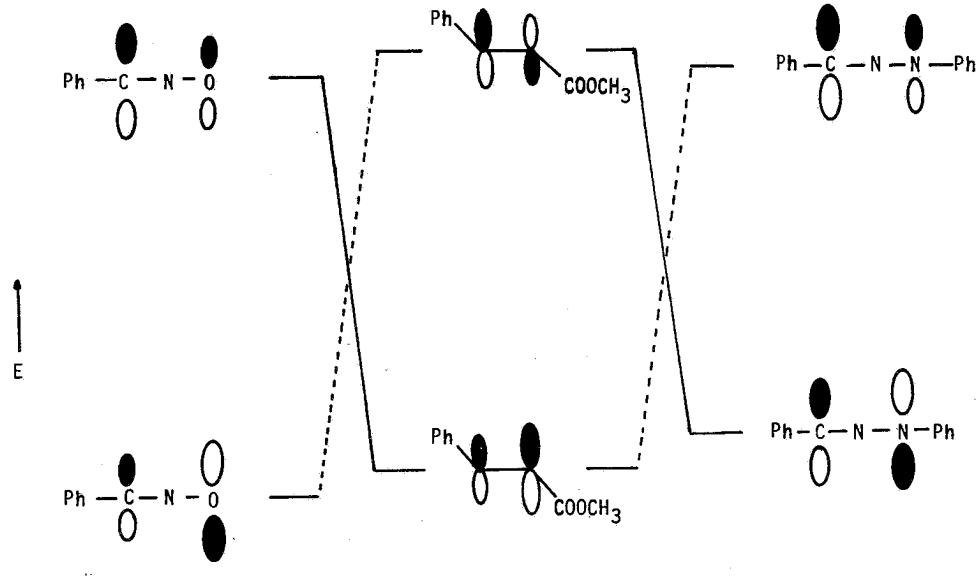


FIG. 1: *Frontier Molecular Orbital Interaction of Methyl cinnamate with BNO and DPNI.*

From Figure 1 it results that for the system methyl cinnamate-BNO the main interaction, that is the interaction which corresponds to the lower energy difference, is LUMO dipole-HOMO dipolarophile. This interaction leads to the

formation of the 4-carbomethoxy derivative (4a) whereas HOMO dipole - LUMO dipolarophile interaction leads to the formation of both regioisomers (4a) and (5a) with a slight favor for the (4a) derivative, because the orbital coefficients of dipolarophile LUMO are almost equal. Since the LUMO dipole-HOMO dipolarophile interaction is the main one the reaction is regioselective and the formation of (4a) is favored. A methoxy substituent at p-position destabilizes the HOMO of dipolarophile so the reaction is almost completely controlled by LUMO dipole-HOMO dipolarophile interaction (lower energy difference) and the regioselectivity is higher. A nitro substituent at p-position stabilizes the HOMO of dipolarophile so the HOMO dipole-LUMO dipolarophile interaction controlls to a slightly higher extent the reaction than in the other two cases. But while in the cases of methyl cinnamate and methyl p-methoxy-cinnamate HOMO dipole-LUMO dipolarophile interaction leads to both regioisomers with a slight favor for the 4-carbomethoxy derivative, in the case of methyl p-nitro-cinnamate this interaction leads also to both regioisomers but with a slight favor for the 5-carbomethoxy derivative, because the magnitude of orbital coefficients of dipolarophile LUMO is reversed. Application of the pertrubation equation (1)[1] gives a quantitative measure of all these factors which affect the regioselectivity:

$$\Delta E = \frac{[C_a C'_d \beta_{ad} + C_c C'_e \beta_{ce}]^2}{E_I} + \frac{[C'_a C_d \beta_{ad} + C'_c C_e \beta_{ce}]^2}{E_{II}} \qquad (1)$$

Where: ΔE = Stabilization energy for the interaction at one direction

$$E_I = E_{\text{HOMO dipole}} - E_{\text{LUMO dipolarophile}}$$

$$E_{II} = E_{\text{HOMO dipolarophile}} - E_{\text{LUMO dipole}}$$

C and C′ are atomic orbital coefficients of the atoms which interact in the HOMO and LUMO respectively, β is the resonance integral which depends not only on the distance of the atoms but also on their nature $\beta_{C\text{-}C} > \beta_{C\text{-}N} > \beta_{C\text{-}O}$. For a reasonable distance of 1.75 Å for a transition state these resonance integrals have the following values: $\beta_{C\text{-}C} = 6.22$ eV, $\beta_{C\text{-}N} = 5.83$ eV and $\beta_{C\text{-}O} = 5.38$ eV. Equation (1) was applied only for the interaction of the frontier molecular orbitals.

The calculated stabilization energies for the three pairs of regioisomers (4a) - (5a), (4d)-(5d) and (4e)-(5e) are given in Table IV. The differences between the calculated stabilization energies for each pair of regioisomers show that reaction with methyl p-methoxycinnamate must have the higher regioselectivity whereas reaction with methyl p-nitro-cinnamate the lowest, in accordance with the experimental data.

The other two p-substituted cinnamic esters which bear moderate electron donors show the same behaviour with the unsubstituted cinnamic ester. No essential changes are also observed with the m-substituted cinnamic esters. o-Substituted cinnamic esters show the same regularity in the regioselectivity order. The regioselectivity is reduced from methoxy to nitro derivative. However, an

TABLE IV: Stabilization energies (in eV) for the regioisomeric pairs 4a-5a, 4d-5d and 4e-5e.

Compds	R	4-CO_2CH_3 isomer	5-CO_2CH_3 isomer	
		ΔE	ΔE	ΔΔE
4a-5a	H	0.9947	0.9537	0.0410
4d-5d	4-CH_3O	0.9487	0.9003	0.0484
4e-5e	4-NO_2	0.9199	0.9047	0.0152

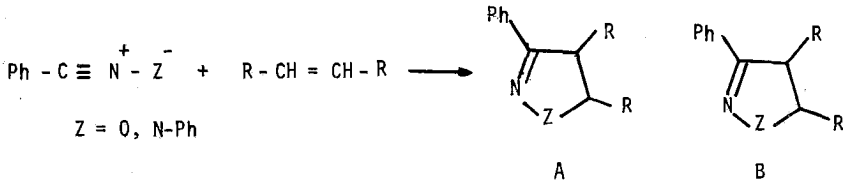
increased proportion of 5-carbomethoxy derivative is obtained with all o-substituted cinnamic esters. This increase cannot be attributed to steric factors because in the 5-carbomethoxy derivative the two aryl groups are near to each other. A possiple explanation is that the o-substituent causes a small out of the ethylenic double bond plane rotation of the aryl ring which results to some hindered conjugation of the aryl group with the double bond. So the system begins to look to some extent like methyl acrylate which gives with BNO almost exclusively the 5-carbomethoxy derivative [10].

On the contrary to the reactions of cinnamic esters with BNO, reactions with DPNI show very low regioselectivity. As it results from Figure 1, reaction of methyl cinnamate with DPNI is HOMO dipole controlled. This interaction leads as in the case of BNO to both regioisomers because the orbital coefficients of LUMO dipolarophile are not sufficiently different. A small favor for the 5-carbomethoxy derivative is observed in the most cases which can be explained if it is accepted that C_C coefficient is bigger than C_{NPh} coefficient in the HOMO of nitrile imine. This view [20,21] is used also for the explanation of regioselectivity in the reactions of DPNI with α,β-unsaturated ketones [22]. In the reaction with methyl p-nitro cinnamate a reversion of the regioselectivity is observed as in the case with BNO.

The observed regioselectivity can be also explained using the method of additivity [23]. The stabilization energy differences between the two regioisomeric transition states of the reactions of BNO and DPNI with several mono-substituted alkenes have been calculated using the second order pertrubation method [23]. According to the additivity method the energy difference for a reaction with a disubstituted alkene can be calculated by a simple algebraic addition of the corresponding values for the monosubstituted alkenes. Otherwise second order calculations are too complicated. The calculated values are given in Table V.

As it results from Table V the calculated energy diffference values are in complete accordance with the experimental data. The magnitude order of $\Delta E \sim 1{,}5$ Kcal for the reaction with BNO shows that both regioisomers must be formed, but the one in higher proportion than the other. The positive sign shows that A must be in a higher proportion as it really happens. The regioselectivity also must be reduced from the p-methoxy derivative to the p-nitro derivative. The magnitude order of $\Delta E \sim 0.8$ Kcal for the reaction with DPNI shows that the reaction has very low regioselectivity and the two regioisomers must be formed in practice in

TABLE V: Differences of the Stability Energies for the Formation of the Regioisomers A and B.



(R-CH = CH-R)	C_6H_5-C$^+$= N-O$^-$ (Z = O)	C_6H_5-C$^+$= N-N$^-$-C_6H_5 (Z = N-C_6H_5)
C_6H_5-CH = CH_2	-3.901[a]	-3.502[a]
p-CH_3O-C_6H_4-CH = CH_2	-4.209[a]	-3.226[a]
p-O_2N-C_6H_4-CH = CH_2	-3.580[a]	-3.375[a]
CH_3O_2C-CH = CH_2	-2.232[a]	-2.621[a]
C_6H_5-CH = CH-CO_2CH_3	1.669	0.881
p-CH_3O-C_6H_4-CH = CH-CO_2CH_3	1.977	0.605
p-O_2N-C_6H_4-CH = CH-CO_2CH_3	1.348	0.754

(a) The energy differences for the reactions with mono-substituted alkenes are given by J. Bastide and H. Rousseau[23]

almost equal amounts. Substituents regardless of their kind reduce furthermore the regioselectivity.

Experimental.

NMR spectra (60 MHz, Me_4Si internal standard, δ values) were recorded on Varian A60A spectrometer. Substituted cinnamic acids were prepared by a standard procedure[24] from malonic acid and the corresponding substituted benzaldehyde in the presence of triethylamine. All these cinnamic acids are known compounds from the literature. Cinnamic acids were esterified with excess of methanol in the presence of a catalytic amount of sulfuric acid. The physical and spectral data of the obtained esters were in accordance with those found in the literature. Benzhydroximic acid chloride was prepared[25] by chlorination of benzaldoxime and benzhydrazidoyl chloride from benzoic acid phenyl hydrazide and PCl_5[26].

All the reactions were carried out by the following standard procedure.

Methyl cinnamate (1 eq.) and either benzhydroximic acid chloride or benzhydrazidoyl chloride (1.2 eq.) in anhydrous benzene solution were treated with triethylamine (1.5 eq.) with stirring at room temperature. The reaction was monitored by TLC. After the reaction was completed the crude reaction mixture

was filtered to remove the insoluble triethylamine hydrochloride and the filtrate was evaporated in vaccuo. The relative amounts of the two regioisomers were measured by NMR after intergration of the signals of 4- and 5-isoxazoline or pyrazoline ring protons.

Περίληψη

Regio - εκλεκτικότητα στις αντιδράσεις 1,3 διπολικής κυκλοπροσθήκης του βενζονιτριλοξειδίου και της διφαινυλονιτριλιμίνης με κινναμωμικούς εστέρες.

Οι αντιδράσεις βενζονιτριλοξειδίου με διάφορους υποκατεστημένους κινναμωμικούς εστέρες εμφανίζουν μια αξιόλογη regio-εκλεκτικότητα. Τα κύρια προϊόντα της αντιδράσεως είναι οι 4-καρβομεθοξυ-ισοξαζολίνες σχηματίζονται όμως σε μικρότερη αναλογία και οι regio-ισομερείς 5-καρβομεθοξυ-ισοξαζολίνες. Η σχετική αναλογία των δυο regio-ισομερών επηρεάζεται από τη φύση των υποκαταστατών στο αρύλιο του κινναμωμικού εστέρα. Οι δότες γενικά αυξάνουν το ποσοστό του 4-καρβομεθοξυ-ισομερούς ενώ οι δέκτες αυξάνουν σχετικά το ποσοστό του 5-καρβομεθοξυ-ισομερούς.

Αντίθετα οι αντιδράσεις της διφαινυλονιτριλιμίνης με τα ίδια διπολόφιλα δεν εμφανίζουν αξιόλογη regio-εκλεκτικότητα. Πάντως με εξαίρεση τα μ-και π-νιτρο-παράγωγα παρατηρείται μια μικρή υπεροχή στις 5-καρβομεθοξυ-πυραζολίνες. Γενικά οι υποκατεστάτες ανεξάρτητα από τη φύση τους ελαττώνουν τη regio-εκλεκτικότητα.

Η διπολόφιλη συμπεριφορά των υποκατεστημένων κινναμωμικών εστέρων τόσο με βενζονιτριλοξείδιο όσο και με διφαινυλονιτριλιμίνη ερμηνεύεται με θεωρητικούς υπολογισμούς και προβλέψεις στα μετωπικά τροχιακά των αντιδρώντων συστημάτων με τη βοήθεια της θεωρίας της διαταράξεως. Τα πειραματικά δεδομένα βρίσκονται σε ικανοποιητική συμφωνία με τις θεωρητικές προβλέψεις.

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PRELIMINARY COMMUNICATION

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ELUCIDATION OF THE VARIABLE EFFECT OF VITAMIN C ON EXPERIMENTAL MALIGNANT TUMORS IN WISTAR RATS

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Summary

Previously, we reported that vitamin C has a variable effect on experimentally induced tumors in Wistar rats, ranging from inhibition to stimulation of their growth. Two kinds of malignant tumors were used as experimental models: 1. Transplanted Borstel sarcoma cells, and 2. Benzo (a) pyrene (BaP) induced tumors.

The present studies have further revealed that the beneficial or the stimulating effect of vitamin C is related to the histological type of the induced tumors. The most pronounced beneficial effect of vitamin C was found in BaP induced fibrosarcomas, where the Carcinogenic potency (Cp) of the carcinogen was reduced from 58.8 to 40.2. In cases of BaP induced rhabdomyosarcoma, the reduction of the Cp was from 51.5 to 45.4.

Contrarywise, BaP induced undifferentiated sarcoma and mixed tumors vitamin C showed a stimulating effect, enhancing the Cp of BaP from 48 to 52.5 and from 52.6 to 56.9 respectively.

Further investigations are necessary with a greater number of rats for the statistical confirmation of these results.

Key Words: Vitamin C, malignant tumors, variable effect.

Abbreviations: BaP, Benzo (a) pyrene; Cp, Carcinogenic potency; Ap, Anticarcinogenic potency; PAH, Polycyclic Aromatic Hydrocarbons.

Introduction

The present experimental results concerning the effect of vitamin C for the prevention and treatment of malignant diseases are conflicting. A number of

publications reported the beneficial effect of vitamin C against several types of animal [1,2,3] and human cancer [4,5,6,7].

At the same time various publications pointed out that vitamin C could not supress tumor induction caused by carcinogens or stop the growth of transplantable and DMBA induced [9] tumors. Also high doses of vitamin C did not benefit patients with advanced cancer [10].

Recently, further investigations revealed that vitamin C, according to the histological type of the BaP induced tumors, can either inhibit tumor growth by reducing the Cp of BaP, or stimulate tumor growth by enhancing the Cp of the Polycyclic Aromatic Hydrocarbons (PAH) [11].

The aim of the present investigation was to evaluate under which conditions vitamin C could promote or inhibit tumor growth and cell malignancy.

Materials and methods

Materials

Induction of BaP tumors

Two groups of 30 female Wistar rats each, were used.

Benzo (a) pyrene (FLUKA Swiss) mol. weight 252, was dissolved in tricaprylin shortly before administration. One ml of the carcinogenic solution containing 10.08 mg BaP (252×4) was s.c. injected.

To the drinking water of the 1st Group, 2g% sugar was added.

To the drinking water of the 2nd Group, 2g% sugar + 2.5g vitamin C (HOFFMANN La ROCHE Basel, Swiss, and MERCK AG. Darmstadt, F.R. Germany) was added.

Four rats died at the beginning of the experiment (three from Group 1, and one from Group 2) and have not been considered for the final evaluation of the results.

Transplantation of experimental fibrosarcoma tumors

Two groups of seven week old male Wistar rats were used.

The first group consisting of eight rats kept in separate cages without additional vitamin C but to whom were given standard food and drinking water containing 2g% sugar.

The second group was given vitamin C 2.5g% + 2g sugar in drinking water. Twenty rats were distributed in separate cages with standard food as in group 1.

Nine weeks later 0.2ml of fibrosarcoma WR 3413 cell suspension in Hanks solution containing 35mg tumor cells were injected s.c. in the dorsal area of each animal.

The fibrosarcoma cells originated from a polyomavirus tumor (BB/T2Berlin-Buch, Graffi). They were subsequently transplanted in the Borstel Institute [12].

The surface of the tumor growth was measured weekly. All rats were sacrified ten weeks after the fibrosarcoma cell transplantation for the histological examination of the tumors.

Methods

Determination of the Carcinogenic potency (Cp) of Benzo (a) pyrene

The carcinogenic potency (Cp) of BaP was determined by taking into consideration the two constant parameters of malignancy, specifically, (a) tumors incidence in % and (b) divided with the mean survival time of the animals multiplied by 100.

$$Cp_{BaP} = \frac{\text{Tumor incidence in \%}}{\text{mean survival time}} \times 100$$

Calculation of the Antineoplastic potency (Ap) of Vitamin C

The Antineoplastic potency (Ap) of Vitamin C was calculated according to the method described by Kallistratos and Fasske[13].

$$Ap = Cp_{BaP} - Cp_{BaP+Vit.C}$$

In order to consider a compound as an Anticarcinogen, the Ap-value must be smaller than the Cp of the carcinogen tested.

Histological examinations

After the death of the rats due to the BaP induced tumor malignancy, and also all rats sacrified ten weeks after the fibrosarcoma cell transplantation, an autopsy was undertaken in order to detect any pathological alterations of the organs or metastases. The tumors were removed as completely as possible for measuring the tumor weight. A part of the tumor was fixed in 8% formalin for the histological examination.

Results

The oral administration of approximately 525 mg vitamin C/rat/day to the 2nd Group of rats causes a prolongation of their life compared with the 1st Group without vitamin C (Chart 1).

The mean survival time of the BaP Group 1 without vitamin C was 191.5 days, and the tumor incidence 100%.

Consequently, the Cp of BaP under the mentioned experimental conditions was calculated as follows:

$$Cp_{BaP} = \frac{100}{191.5} \times 100 = 52.2$$

Likewise, the mean survival time of the Vitamin C second Group was 227.5 days, a fact indicating that the rats receiving more than 0.5g vitamin C per day,

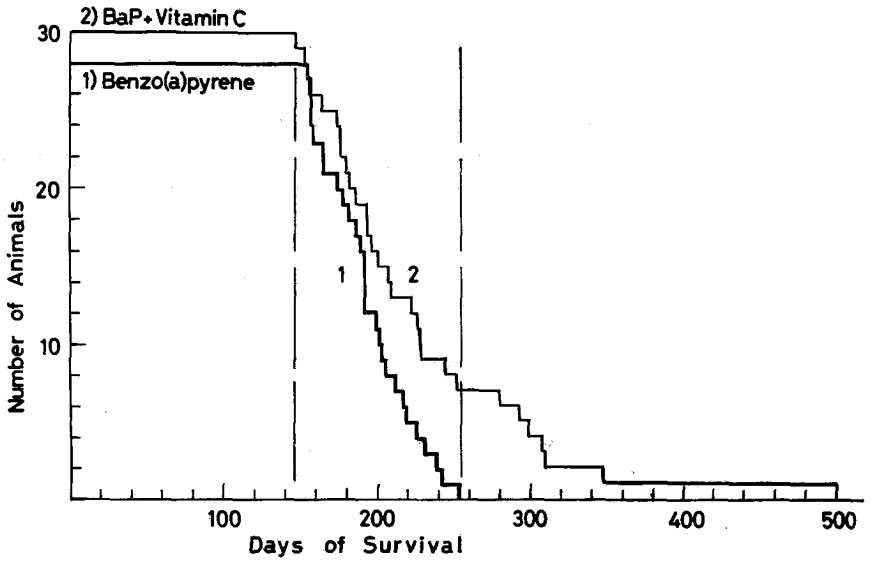


CHART. 1. *Life prolongation of Wistar rats treated with a single s.c. injection of 10mg BaP and simultaneous oral administration of vitamin C (Group 2). Control Group 1 without vitamin C.*

corresponding to approximately a total amount of more than 120g for the duration of the experiment until their death, lived 36 days longer. The Carcinogenic potency of BaP in the presence of vitamin C was determined as follows:

$$Cp_{BaP+Vit.C} = \frac{100}{227.5} \times 100 = 43.9$$

Consequently, ascorbic acid decreased the Cp of BaP exactly 8.3 units. Therefore, the Anticarcinogenic potency (Ap) of vitamin C can be evaluated either as a difference of $52.2 - 43.9 = 8.3$ or as a ratio $52.2 : 43.9 = 1.19$

By comparing the mean survival time of the two groups, it is obvious that the group treated with vitamin C had a prolongation of life of 36 days, which is statistically significant according to the t-student test $p < 0.05$.

Furthermore, the effect of vitamin C on BaP induced tumors became more clear, if by the analysis of the mortality curve the experimental data for four rats where ascorbic acid was ineffective are neglected. In that case, the statistical evaluation for the remaining 26 rats is more significant $p \simeq 0.001$.

It must be pointed out that the Anticarcinogenic potency (Ap) of vitamin C is not constant for all types of malignancy but varies considerably. If, instead of calculating the Cp for the total number of tumors, the Cp of each histological type of induced tumor is calculated separately, then the diverse action of vitamin C on malignancy becames more evident. For example, in case of fibrosarcoma induced tumors, the mean survival time of this BaP sub-group was 170 days instead of 191.5 days (Table I). However, the mean survival time of the vitamin C fibrosarcoma sub-group was 248.5 days, instead of 227.5 days

TABLE I: The mean survival time, tumor weights, histology, Carcinogenic potency (Cp) and statistical evaluation of the BaP induced tumors in Wistar rats, with and without the oral-administration of Vitamin C.

GROUPS	HISTOLOGICAL TYPE OF TUMORS				TOTAL Nr of BaP Induced Tumors
	Fibrosarcoma	Rhabdomyo-Sarcoma	Undiff. Sarcoma	Mixed Tumors	
1 GROUP					
(BaP in Tricaprylin	n 9	n 7	n 10	n 1	n 27
mean survival time in days	170	194	208	190	191.5
Standard deviation	17.9	33.8	26.3	—	27.8
tumor weights in gram	138	84	84	180	110
(min-max weights in g)	(35-270)	(40-160)	(25-150)		(25-270)
Cp_{BaP}	58.8	51.5	48	52.6	52.2
2 GROUP					
(BaP in Tricaprylin + vit. C	n 16	n 6	n 4	n 3	n 29
mean survival time in days	248.5	220	194	175.66	227.5
Standard deviation	86.9	66.2	27.5	25.1	75.2
tumor weights in gram	86.5	98.3	52.9	97.3	86.5
(min-max weights in g)	(18-158)	(56-150)	(18-73)	(25-140)	(18-158)
$Cp_{BaP+vit.C}$	40.2	45.4	52.5	56.9	43.9
t(1 – 2)	3.48	0.84	0.85	—	2.40
p	<0.01	>0.05	>0.05	—	<0.05

compared with the total number of all vitamin C induced tumors. From these experimental results, it is obvious that the rats of the fibrosarcoma sub-group lived 78.5 days longer with vitamin C than without the Antineoplastic agent, a fact which is statistically significant. Consequently, the Cp of BaP + vitamin C for the fibrosarcoma sub-group was 40.2. This means that the Antineoplastic potency of vitamin C in induced fibrosarcoma was 58.8 – 40.2 = 18.6 units, instead of 8.3 units of the total tumors. These results reveal that vitamin C shows until now its highest Ap in fibrosarcoma (Chart 2).

For the remaining histological types the number of induced tumors were not sufficient for a statistical evaluation. By six and seven induced rhabdomyosarcomas with and without vitamin C, the Cp of BaP was decreased from 51.5 to 45.4, which corresponds approximately to the Cp-values for the total number of BaP induced tumors, (Table I).

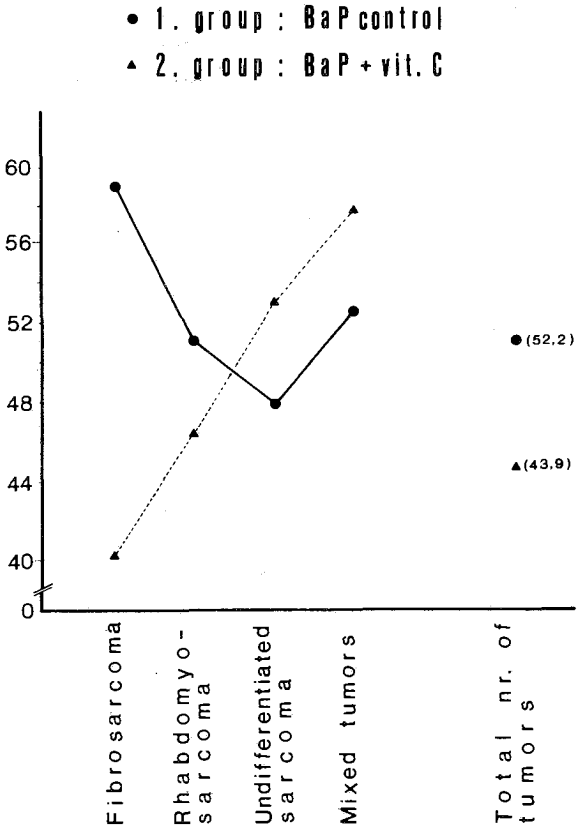


CHART 2. *The Carcinogenic potency (Cp) of BaP induced tumors in relation to their histological type. The highest Cp-value 58.8 of BaP was found by fibrosarcoma where vitamin C is most effective reducing it to 40.2. These experimental results are statistically significant. Furthermore, the Ap of vitamin C is decreased almost linearly from rhabdomyosarcoma towards undifferentiated sarcoma and mixed tumors where it expresses its stimulating action, a fact which needs to be statistically confirmed. The Cp of BaP for the total number of tumors is with and without vitamin C 43.9 and 52.2 respectively.*

Contrarywise, in the sub-group of undifferentiated sarcoma and mixed tumors, vitamin C shows a stimulating effect enhancing the Cp of BaP from 48 to 52.5 and from 52.6 to 56.9 respectively. Due to the small number of tumors, these results need also a statistical confirmation.

Also the tumor weights were generally smaller in the vitamin C group with a mean weight of 86.5g compared with the BaP group without vitamin C, with a mean weight of 110g. (Figure 1).

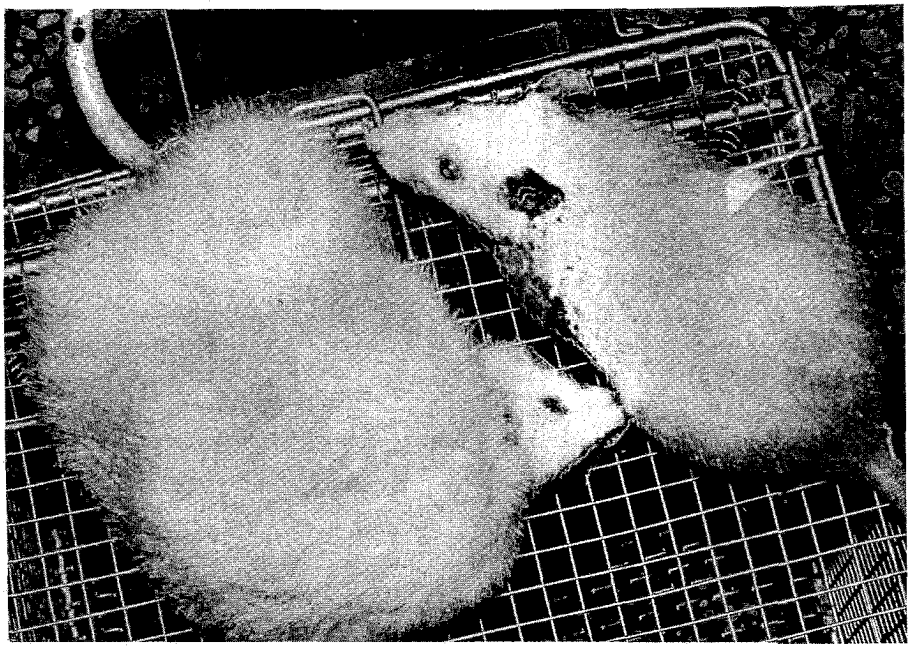


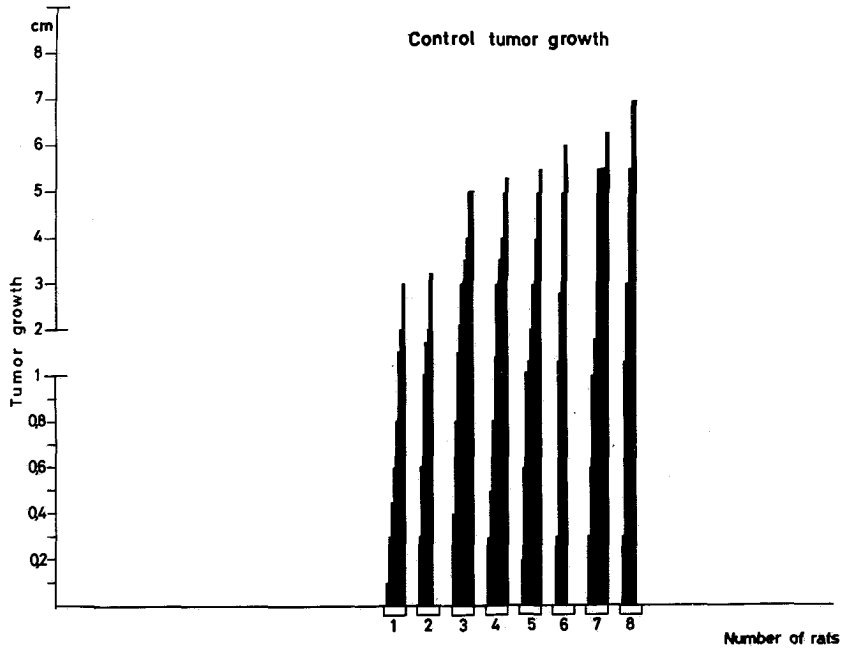
FIG. 1: *A characteristic photo of Benzo(a)pyrene (10mg) tumor induction in Wistar rats. Left: Without vitamin C, tumor weight 250g. right: simultaneous oral administrastion of about 500mg/day vitamin C, tumor weight 25g.*

The effect of vitamin C on transplanted fibrosarcoma tumors

Transplantation of standardized fibrosarcoma cell suspensions into the Wistar rats caused the development of rapidly growing tumors in all treated animals. After ten weeks tumors reached a diameter between 3-7 cm before the animals were sacrified for the histological examination (Chart 3a).

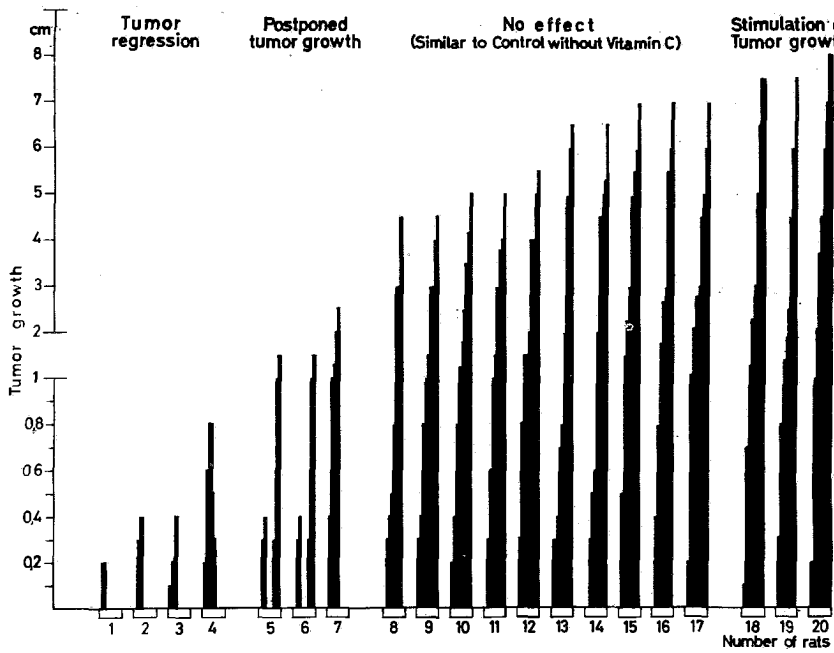
The effect of vitamin C on transplanted fibrosarcoma cells in 20 rats is demonstrated in Chart 3b. Due to the controversing effect of vitamin C, the results are not uniform, but vary all the way from total tumor regression up to stimulation of tumor growth. They can be classified into four categories:

3a



3a. *Tumor growth in cm, a record of each week (ordinates) of the transplanted fibrosarcoma cells by eight Wistar rats (abscissas) of the control group without vitamin C. The length of the white boxes corresponding to each rat represent ten weeks of the duration of the experiment, beginning from the day of malignant cell transplantation. All rats after ten weeks developed tumors varying from 3-7cm in length.*

3b



3b. *Tumor growth of transplanted fibrosarcoma cells by twenty Wistar rats, with simultaneous oral administration of vitamin C. All rats developed tumors after a few weeks. In four rats a total tumor regression was observed, and in three further rats a postponed tumor growth. Contrarywise, ascorbic acid had no effect at all in ten rats and in three further cases probably tumor stimulation was provoked.*

I. Total tumor regression	4 rats	20%
II. Delayed tumor growth	3 rats	15%
III. No effect	10 rats	50%
IV. Stimulation of tumor growth	3 rats	15%

Even in this type of transplanted malignant cells, the controversing effect of vitamin C from total regression all the way up to stimulation of tumor growth, is evident. Of course these results need also a statistical confirmation with a greater number of animals.

Discussion

The stimulating effect of vitamin C on tumor growth was reported almost half a century ago [15], and was confirmed by subsequent investigations [16].

Likewise, the opposite action of vitamin C, that means its inhibitory effect on malignant tumors has also been confirmed during the last decate [4,5,17]. Furthermore ascorbic acid prevents nitrosamine formation [18] and inhibits tumor induction caused by carcinogens such as Aflatoxins or Polycyclic Aromatic Hydrocarbons [13].

This variable effect on malignant cells is not an exclusive property of vitamin C, but even some cytostatics can also behave like carcinogens. For example Neocarzinostatin, an antitumor protein, can sometimes act as a carcinogenic agent and induce tumors of the kidneys such as hypernephroma [19]. Also the cyclophosphamide Endoxan can provoke cytological alterations, such as polynuclear cells etc [20,21].

Various opposing effects of vitamin C on the two investigated experimental malignant tumor kinds are classified in Table II.

Furthermore, the simultaneous BaP induction of mixed tumors such as fibrosarcoma and rhabdomyosarcoma was enhanced in the presence of vitamin C by a ratio at 3:1 (Figure 2)

The investigations of the effect of vitamin C on experimental malignant tumors in rats were accomplished through the uninterrupted administration of high doses of ascorbic acid to the rats both during the day and during the night. The reason was that a beneficial effect could only be expected in cases where the malignant cells are continuously under the influence of high concentrations of vitamin C. This was achieved by adding 2% sugar to the vitamin C solution of the drinking water of the rats. The presence of sugar is important for three main reasons: (a)The Wistar rats and other laboratory animals do not like the acidic taste of the vitamin C solutions, and therefore the sugar gives a sweet taste which the animals prefer.

TABLE II. The controversing effect of Vitamin C on experimental malignant tumors in Wistar rats.

TUMOR TYPE	INHIBITION←VITAMIN C→STIMULATION	
1. Transplanted fibrosarcoma cells	Total regression of tumors	Stimulation of tumor growth
2. Benzo(a)pyrene induced tumors	Decrease of the Cp fibrosarcoma rhabdomyosarcoma	Increase of the Cp undifferentiated sarcoma mixed tumors
	prolongation of life	acceleration of death
		increased incidence of metastases and mixed tumors

[1]Twenty rats. Vitamin C orally.
[2]Twenty nine rats. Vit. C orally.

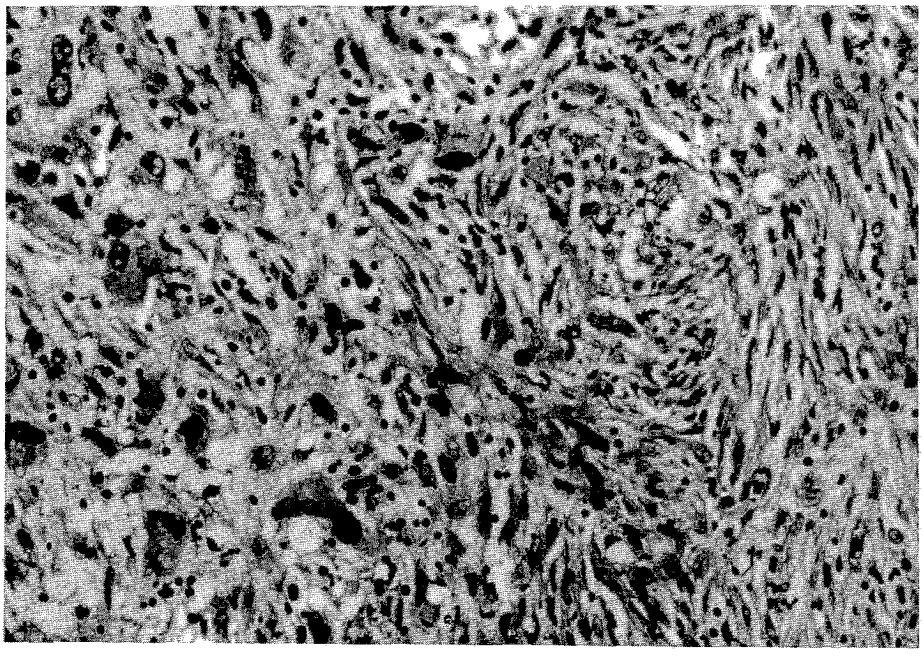


FIG. 2: *Histology Nr. K. 131. Simultaneous BaP induction of mixed tumors in Wistar rats which were enhanced with Vitamin C. Right: the fibrosarcoma part and left: the rhabdomyosarcoma section of the tumor. (Haemalaun-Eosin stain, magnification* × *420).*

(b) Sugar solutions make rats thirsty and consequently they drink both more fluid and more frequently during the day and at night, thus increasing the daily amount of vitamin C which can be orally administered. (c) The calories of the sugar might be beneficial to the organism in cases of tumor cachexy.

The oral administration of high doses of vitamin C to the tumor bearing rats was partially successful in decreasing the Carcinogenic potency of Benzo(a)pyrene-induced fibrosarcoma and rhabdomyosarcoma tumors, and in prolonging their survival time.

On the contrary, in cases of BaP-induced undifferentiated sarcoma and mixed tumors, vitamin C had a stimulating effect on tumor malignancy, and consequently decreased the survival time of the corresponding rats. Similar opposing results were observed with the transplanted fibrosarcoma tumors.

These preliminary data are suggestive for the variable effect of the vitamin C for the two kinds of investigated malignant tumors, but supplementary studies could probably confirm that this impression is actually correct.

In conclusion, these experiments revealed that the inhibitory or the stimulating effect of vitamin C is related to the histological type of the BaP-induced tumors, which also defines its inconsistent action on malignant cells.

The reported investigations should be taken into consideration for an eventual clinical application of high doses of vitamin C, because they proved its contradictory effect on various types of tumors. A further systematic elucidation of the histological types of tumors which are susceptible to vitamin C, would contribute to the definition of the right indications of vitamin C for the prevention and treatment of malignant diseases.

Περίληψη

Διαλεύκανση της παράδοξης δράσης της βιταμίνης C, σε πειραματικούς κακοήθεις όγκους, των επιμύων Wistar.

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Έχει αναφερθεί κατά το παρελθόν ότι η βιταμίνη C, έχει μια παράδοξη δράση στους πειραματικούς όγκους των επιμύων Wistar, που κυμαίνεται από πλήρη εξαφάνιση των όγκων μέχρι αύξηση της κακοήθειας τους. Για την διαλεύκανση του φαινομένου αυτού, χρησιμοποιήθηκαν δύο είδη όγκων σαν πειραματικά πρότυπα:

Πρώτον, μεταμοσχευθέντα κακοήθη κύτταρα του τύπου σαρκώματος Borstel και δεύτερον αυτόχθονες κακοήθεις όγκοι, που προκλήθηκαν από ενέσεις βενζο(α)πυρενίου.

Οι παρούσες πειραματικές εργασίες απέδειξαν ότι η αναστολή ή η αύξηση της κακοήθειας που προέρχεται από την δράση της βιταμίνης, C, εξαρτάται εν μέρει από τον ιστολογικό τύπο του σχηματιθέντος όγκου.

Η μεγαλύτερη ανασταλτική δράση της βιταμίνης C, βρέθηκε στα σχηματισθέντα ινοσαρκώματα όπου η καρκινογόνος ισχύς του βενζο(α)πυρενίου ελαττώθηκε από 58.8 σε 40.2. Αυτή η διαφορά είναι και στατιστικά σημαντική.

Στην περίπτωση των σχηματισθέντων ραβδομυοσαρκωμάτων η ελάττωση της καρκινογόνου ισχύος του BaP ήταν από 51.5 σε 45.4.

Αντίθετα στα σχηματισθέντα από βενζο(α)πυρένιο αδιαφοροποίητα σαρκώματα και στους μικτούς όγκους, η βιταμίνη C, αυξάνει την κακοήθεια του καρκινογόνου από 48 σε 52.5 και από 52.6 σε 56.9, αντίστοιχα.

Συμπληρωματικές πειραματικές εργασίες με μεγαλύτερο αριθμό πειραματοζώων θα τεκμηριώσουν στατιστικά και μερικά από τα αναφερθέντα αποτελέσματα.

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SHORT PAPER

Chimika Chronika, New Series, 13, 185-191 (1984)

CYCLOADDITION REACTIONS OF DIBENZALACETONE WITH SOME 1,3-DIPOLES

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(Received November 29,1983)

Abbreviations

FMO = Frontier Molecular Orbitals
DPNI = Diphenylnitrile imine
MNO = Mesitonitrile oxide
BNY = Benzonitrile - 4 - nitrobenzylide
DBA = Dibenzalacetone
BNO = Benzonitrile oxide

Results and Discussion

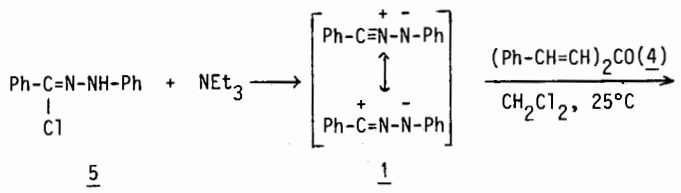
In connection with the previous works [1,2] on the cycloaddition between several 1,3-dipoles and enonic systems we have undertaken the present study on the cycloaddition of diphenylnitrile imine (DPNI,**1**), mesitonitrile oxide (MNO, **2**) and benzonitrile - 4 - nitrobenzylide (BNY, **3**) with dibenzalacetone (DBA, **4**). We have found that DPNI and MNO attack the ethylene double bonds of the dipolarophile, in agreement with similar studies [3,4] on the cycloaddition of those 1,3-dipoles with α,β-unsaturated ketones. On the contrary the BNY attacks the carbonyl double bond of the DBA.

DPNI and BNY are generated in situ. All the dipoles were prepared by known procedures [5,6,7].

The reactions were carried out at room temperature, whereas the reaction time was 30-40 hours. In all reactions the dipoles were in excess (3:1).

The products **7** and **8** show in ir absorptions at 1720cm^{-1} (νC=O) and at 1600cm^{-1} (νC=N). The oxazoline **9** shows no carbonyl absorption, but only a C=N stretching vibration at 1600cm^{-1}.

The nmr spectra of the compounds **7** and **8** are very useful for the study of the regioselectivity of these cycloadditions. Thus, the observed values for the four



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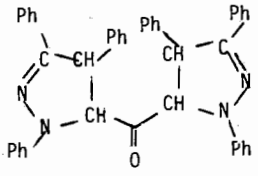

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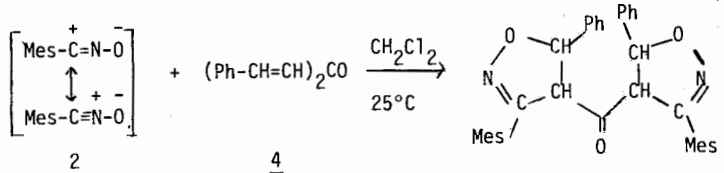

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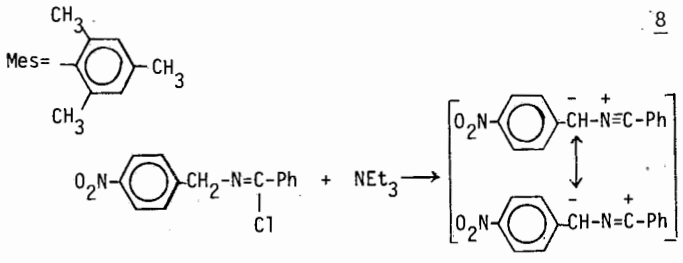

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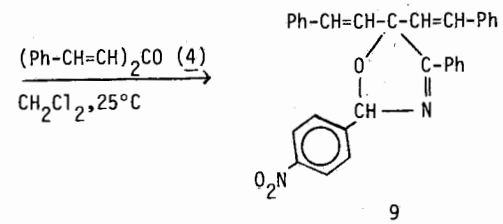

9

protons of the pyrazoline and isoxazoline rings in connection with the literature data for reactions between α,β-unsaturated ketones and DPNI and benzonitrile oxide (BNO)[3,4] are in agreement with the proposed structures of the cycloadducts **7**

and **8** (a 5-acylpyrazoline and a 4-acyl-isoxazoline respectively). In the compound **7** the H_4 and H_5 protons resonate at δ 4.55 and 4.85 their difference in chemical shift being small and equal to 0.3ppm, in agreement with previous data[3]. In contrary, in the compound 8 the protons H_4, H_5 resonate at δ 3.71 and 6.08 the difference in chemical shift being equal to 2.37 ppm. In the case of a compound with an opposite regioselectivity this difference according to the literature[3,4] should be much smaller like in the compound 7. The nmr spectrum of the oxazoline **9** gave signals for the two protons adjacent to the phenyl groups of the double bonds at 8.10 δ and for the protons of the phenyl groups at 7.01-7.43 δ. The signals for the other two protons of the double bonds must be in the region of the aromatic protons.

All the products **7-9** show in the mass spectra ion peaks corresponding to the retro-1,3-dipolar cycloaddition and to other decomposition fragments of the pyrazoline, isoxazoline and oxazoline ring. A peak corresponding to the molecular ion is present only in the mass spectra of the products **7** and **8** (Table).

The formation of these cycloadducts **7,8,9** can be explained after consideration of the Frontier Molecular Orbitals (FMO) of the reacting species[3,8-10]. Figure 1 shows the estimated frontier orbital energies of DPNI, BNO and α,β-unsaturated ketones. It results from the energy differences that the reaction with DPNI is HOMO-dipole controlled, whereas the reaction of BNO with α,β-unsaturated ketones is LUMO-dipole controlled.

The regioselectivity of the reactions is explained considering the orbital interactions[3,10] like that in Figures 2,3.

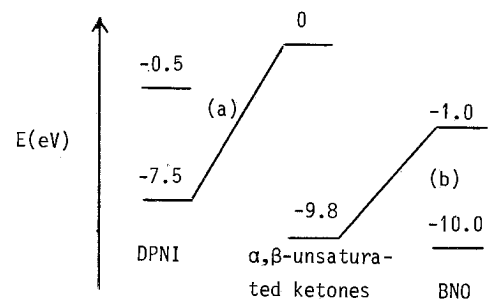

FIG. 1: *Estimated frontier orbital energies of DPNI, BNO and α,β-unsaturated ketones, dominant interaction for DPNI is (a) and for BNO (b)*

It should be noticed however that the stereochemistry of the products **7** and **8** is not known and this problem is under further consideration.

The BNY attacks the carbonyl double bond of the DBA. The reactions of the nitrile ylides with the electron deficient dipolarophiles are HOMO-dipole controlled[9]. We can explain the regioselectivity of the reaction considering the atomic orbital parameters and the FMO interaction between the nitrile ylide and the carbonyl double bond[9] of DBA (Figure 4).

The suggested regioselectivity in the reaction of BNY with DBA is in agreement

TABLE

Analytical and Spectral Data of **7,8,9**

Compound	Mp ^{0}C	Yield %	Molecular Formula	Analysis % Calcd/Found C	H	N	
7	235-237	16	$C_{43}H_{34}N_4O$	82.39	5.50	8.99	ir (Nujol): 1600(C=N), 1720(C=O) cm^{-1};
			M.W.: 622	82.67	5.54	8.78	nmr(deuteriochloroform): 6.70-7.60(m), 4.55(d), 4.85(d)δ; 622(10)$M^{+}\cdot$, 234(5), 194(98)
8	246-248	34	$C_{37}H_{36}N_2O_3$	79.83	6.52	5.03	ir (Nujol): 1600(C=N), 1720(C=O) cm^{-1};
			M.W.: 556	79.72	6.52	5.03	nmr(deuteriochloroform): 6.91-7.50(m), 6.50(s), 6.08(d), 3.71(d), 2.10(s), 1.75(s)δ; 556(18)$M^{+}\cdot$, 234(63), 161(38)
9	272-273	20	$C_{31}H_{24}N_2O_3$	78.79	5.12	5.93	ir (Nujol): 1600cm^{-1} (C=N);
			M.W.: 472	78.80	4.89	6.23	nmr(dimethylsulfoxide-d): 8.10(d), 7.01-7.43(m)δ; $M^{+}\cdot$, 234(6), 238(40)

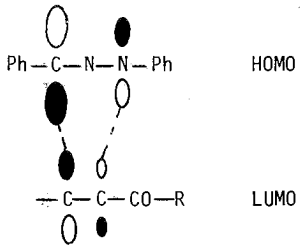

Figure 2: HOMO-LUMO interaction between DPNI and DBA

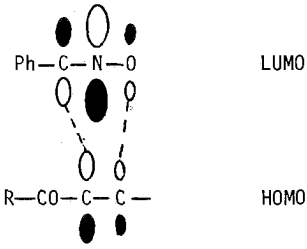

Figure 3: HOMO-LUMO interaction between BNO and DBA

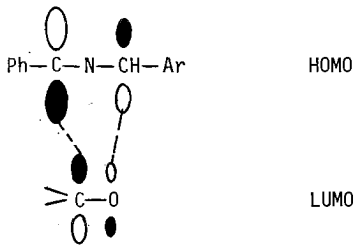

FIG. 4: *HOMO-LUMO interaction between BNY and the carbonyl double bond of DBA*

with the results in other studies on the cycloaddition of BNY with similar dipolarophiles[11].

The above data as well as other taken from the literature[3,4,11] are found in agreement with the proposed structures.

Experimental

All melting points are uncorrected and they were obtained with a hot stage apparatus. Ir spectra were obtained with a Perkin-Elmer 297 spectrometer, nmr spectra, reported in δ units (TMS), were reported with a Varian A60A spectrometer, whereas mass spectra were measured with a Hittachi-Perkin-Elmer Model RMU-6L spectrometer with an ionization energy of 70 eV.

DPNI **1** and BNY **3** were liberated in situ from N-phenyl-benzhydrazidoyl chloride[5] **5** and N-(4-nitrobenzyl)-benzoic acid imidochloride[6] **6** respectively.

MNO **2** was prepared from mesitylaldoxime[7].

DBA **4** was prepared from benzaldehyde and acetone.

Cycloaddition reactions of DBA **4** *with DPNI* **1** *and BNY* **3**

Triethylamine (10 mmol) was added to a dichloromethane solution (10 ml) of DBA **4** and the chlorides **5** and **6** (6 mmol) respectively. The reaction mixture was

stirred at room temperature (30 hours). Then the triethylamine hydrochloride was removed by filtration and after evaporation of the solvent the oily residue was chromatographed on silica gel and eluted with chloroform. The reaction products were further purified by recrystallization with dichloromethane/hexane mixture. Analytical and spectral data are summarized in the table.

Cycloaddition reaction of DBA **4** *with MNO* **2**

MNO (6 mmol) was added to a dichloromethane solution of DBA (2 mmol). The reaction mixture was stirred at room temperature for 40 hours. Then the solvent was evaporated. The reaction product was purified by recrystallization with dichloromethane/hexane mixture. Analytical and spectral data are given in the table.

Abstract

The cycloaddition of diphenylnitrile imine **1,** mesitonitrile oxide **2** and benzonitrile-4-nitrobenzylide **3** with dibenzalacetone 4 leads to the formation of cycloadducts 7,8 and 9 respectively in moderate yields (16-34%). The reactions are examined on the basis of the frontier molecular orbitals of the reacting species.

Key Words: Diphenylnitrile imine, mesitonitrile oxide, benzonitrile-4-nitrobenzylide, dibenzalacetone.

Περίληψη

Αντιδράσεις κυκλοπροσθήκης διβενζαλακετόνης με ορισμένα 1,3-δίπολα

Από τις αντιδράσεις κυκλοπροσθήκης της διφαινυλονιτριλιμίνης **1,** του μεσιτονιτριλοξειδίου **2** και του 4-νιτροφαινυλο-βενζονιτριλυδίου **3** με τη διβενζαλακετόνη **4** προκύπτουν τα προϊόντα **7,8,9** αντίστοιχα και σε μέτριες αποδόσεις (16-34%). Η προσθήκη της διφαινυλονιτριλιμίνης και του μεσιτονιτριλοξειδίου στη διβενζαλακετόνη έγινε στους δύο αιθυλενικούς διπλούς δεσμούς της κετόνης, ενώ του νιτριλυλιδίου στον καρβονυλικό διπλό δεσμό της. Οι αντιδράσεις εξετάζονται με θεώρηση των μετωπικών μοριακών τροχιακών των αντιδρώντων συστατικών.

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SHORT PAPER

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REACTIONS OF SILVER AND THALLIUM (I) SALTS OF PHENYLNITROACETONITRILE WITH t-BUTYL HALIDES

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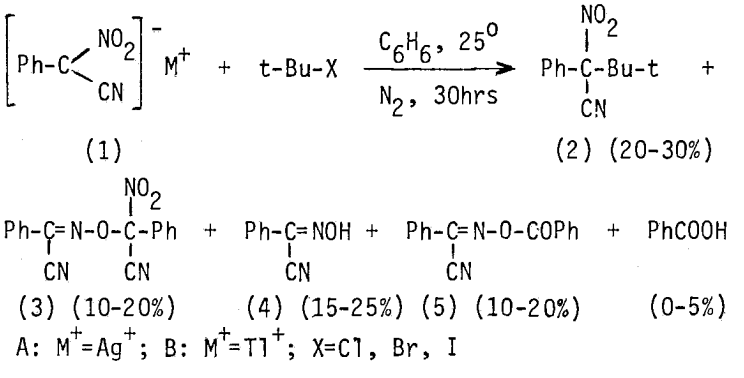
(Received January 13, 1984)

The title reactions lead to the formation of several products (2-5).Some mechanistic aspects for these complicated reactions are offered.

Key words: Silver and Thallium (I) Salts of phenylnitroacetonitrile. t-Butyl halides.

It has been shown previously that the reaction of silver salt of phenylnitroacetonitrile with chlorotriphenyl-methane[2] and with carbon disulfide[3] gives the unusual products of α,α′-bis(triphenylmethaneazo)-stilbene and O-(phenylcyanonitromethyl)-2-hydroxyimino-2-phenyl-acetonitrile respectively. As further work we have undertaken the study of the reaction of silver and thallium (I) salts (1A) and (1B) with t-butyl halides, in order to investigate whether products analogous to bis-azostilbene are formed.

The reactions of silver (1A) and thallium (1B) salt with t-butyl halides (chloride, bromide, iodide) took place in benzene, at room temperature, under an atmosphere of nitrogen and vigorous strirring. We have found that several products are formed, namely the C-alkylation product (2), the O-(phenylcyanonitromethyl)-hydroxyimino-phenylacetonitrile (3), the hydroxyimino-phenyl-acetonitrile (4) with its O-benzoyl derivative (5) and benzoic acid, but without any isolation of a product analogous to methaneazostilbene.



(1) (2) (20-30%) (3) (10-20%) (4) (15-25%) (5) (10-20%) (0-5%)

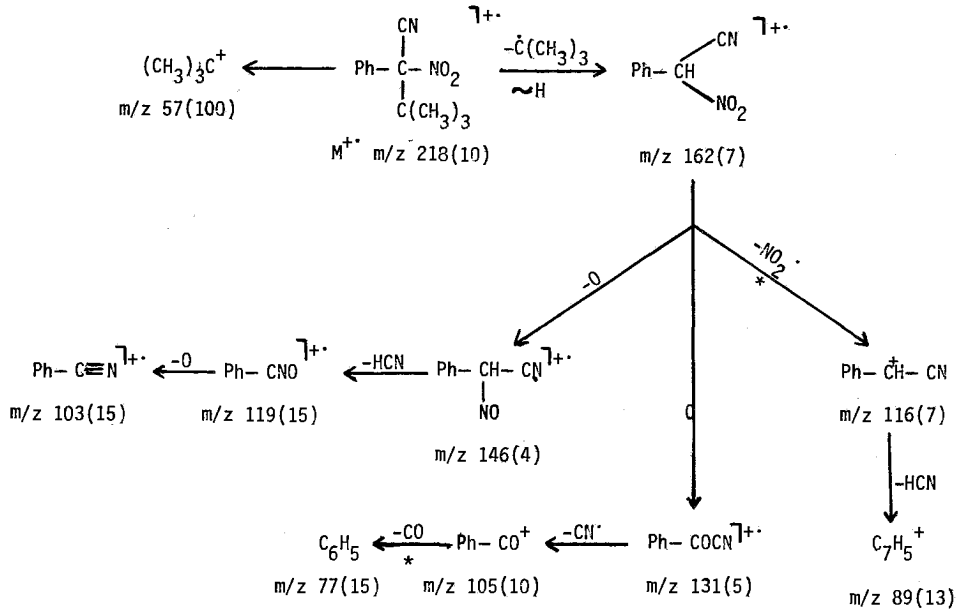
A: $M^+=Ag^+$; B: $M^+=Tl^+$; X=Cl, Br, I

We have also found that all halides (X=Cl, Br, I) gave the same products with some variations in their yields. Small is also the influence of the metal (silver or thallium) the only difference being an increase in the yield of the product (3) from 10% with silver salt to 20% with thallium salt and a parallel decrease in the yield of (5) from 20% with silver salt to 10% with thallium salt. At higher temperature (55-60 ^{0}C) decomposition of the salt is observed[4] with formation of benzoic acid and of the product (5), whereas at lower temperature (-18 ^{0}C) in toluene the system is unreactive.

In all reactions no detectable O-alkylation product (nitronic ester) was observed, a fact suggesting that the reaction is rather under thermodynamic control[5].

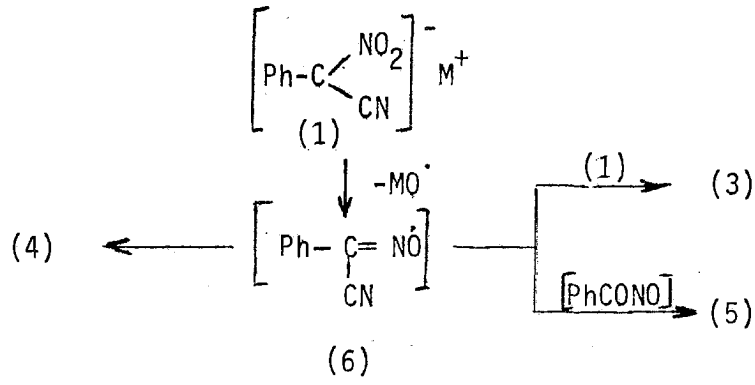
It is of interest to note that the reaction[6] of silver salt (1A) with benzydryl bromide gave the corresponding C-alkylation product, hydroxyiminoacetonitrile (4) and benzophenone, whereas the reaction[7] of the same salt (1A) with methyl iodide and benzyl chloride afforted the corresponding unstable nitronic esters.

The mass spectrum of the compound 2 shows a low intensity peak for the molecular ion $M^{+\cdot}$. Other prominent peaks are those corresponding to the ions $[M+H-Bu(t)]^+$, $[C_6H_5COCN]^+$, $C_6H_5CO^+$, whereas the base peak corresponds to the ion $(CH_3)_3C^+$. A general fragmentation pattern is given in Scheme I.



Scheme I

Concerning the reaction mechanism it is suggested[3] that a species like (6) could explain the reaction products (3-5) as below, whereas further decomposition[4,8] of the unstable intermediate (PhCONO) could lead to the formation of benzoic acid.



Experimental

All melting points are uncorrected and they are obtained with a Kofler hot stage apparatus. IR spectra were obtained with a Perkin-Elmer Model 257, whereas NMR spectra reported in δ units with a Varian Associates A-60A spectrometer with TMS as internal reference. The mass spectra were obtained with a Hitachi-Perkin-Elmer Model RMU-6L spectrometer, with ionization energy 70 eV.

Silver (1A) and Thallium (I) Salt (1B) of Phenylnitroacetonitrile

Both salts were prepared[2,7] from the corresponding sodium salt with addition of the appropriate aqueous solution of silver nitrate or thallium (I) nitrate. The salts were stored in the dark and in a dessicator. The IR spectra (Nujol) of (1A) and (1B) showed peaks at 3050, 2200, 1490, 1330, 675 cm^{-1} and at 3050, 2220, 1500, 675 cm^{-1} respectively.

Reaction of the Salts (1A), (1B) with t-Butyl Halides. General Procedure

t-Butyl halide (5 mmole) in dry benzene (10 ml) was added slowly to a stirred suspension of the salt (1A) or (1B) (5 mmole) in dry benzene (15 ml) at room temperature, under a nitrogen atmosphere. After completion of addition the mixture was stirred for 30 hrs and filtered. The filtrate was evaporated under reduced pressure and the oily residue was chromatographed on a silica gel columm (eluent benzene) to give the following products:

I. 3,3-Dimethyl-2-phenyl-2-nitro-butyronitrile (2), oil (30%, from 1A and t-butyl chloride). IR spectrum (liquid) showed peaks at 3050, 2970, 2220, 1580, 1370 cm^{-1}, whereas NMR (CCl_4) at 1.6(9H, s), 7.55(3H, m), 8.15δ(2H, m). For its mass spectrum see Scheme I.

II. O-(Phenyl-cyano-nitromethyl)-2-hydroxyimino-2-phenyl-acetonitrile (3) (20%, from 1B and t-butyl iodide), m.p. 120-122 °C (lit[3]. m.p. 122-123 °C).

III. 2-Hydroxyimino-2-phenyl-acetonitrile (4) (25%, from 1A and t-butyl bromide), m.p. 127-129 ^{0}C (lit[7]. m.p. 129.5 ^{0}C).

IV. O-Benzoyl-2-hydroxyimino-2-phenyl-acetonitrile (5) (20%, from 1A and t-butyl chloride), m.p. 135-137 ^{0}C (lit[9]. m.p. 138-139 ^{0}C).

V. Benzoic acid (5%, from 1A and t-butyl bromide), m.p. 122-123 ^{0}C.

Περίληψη

Αντιδράσεις Άλατος Αργύρου και Θαλλίου (I) του Φαινυλονιτροακετονιτριλίου με τρ. -Βουτυλο-αλογονίδια.

Τα άλατα αργύρου (1A) και θαλλίου (1B) αντιδρούν με τρ.-βουτυλο-αλογονίδια και εκτός από το προϊόν C-αλκυλιώσεως (2) δίνουν και διάφορα άλλα προϊόντα (3-5), καθώς και βενζοϊκό οξύ σε ποικίλλουσες αποδόσεις. Εξετάζεται το φάσμα μαζών της ενώσεως (2) και προτείνεται ένα γενικό μηχανιστικό σχήμα για την πορεία αυτών των πολυπλόκων αντιδράσεων.

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