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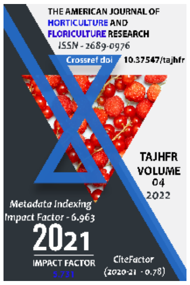
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Research Article

LOW MOLECULAR METABOLITES OF FUNGI. 13, 3-DIMETOXYSTACHIBOTRINIS STACHYBOTRYS CHARTARUM

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Lukhmon Kamolov
Researcher, Karshi State University Karshi, Uzbekistan

Sevara Tojiyeva
Researcher, Karshi State University Karshi, Uzbekistan

ABSTRACT

Stachybotrys chartarum produces a number of low molecular weight compounds. Column chromatography of the latter isolated the nitrogen-containing compound 3-methoxystachibotrin. The structure of 13,3-dimethoxystachibotrin was established on the basis of chemical transformations using the data of ЯМР^{1}H, ^{13}C, 2MЯМР^{1}H-^{1}H, ^{1}H -^{13}C chemical shift correlations (COSY), long-range ^{1}H -^{13}C interaction (HMBC) correlation, NOE measurements in a rotating coordinate system (ROESY), IR and mass- spectra of electron impact.

KEYWORDS

Stachybotrys chartarum; Stachybotrys lobulata type;chromatographic; fungi; stachybotriotoxins; staxibotrin;ЯМР^{1}H, ^{13}C, 2MЯМР^{1}H-^{1}H, ^{1}H -^{13}C chemical shift correlations (COSY), long-range ^{1}H -^{13}C interaction (HMBC) correlation, NOE measurements in a rotating coordinate system (ROESY), IR and mass- spectra of electron impact.

INTRODUCTION

Funguses belonging to the genus Stachybotrys Corda belong to the class of fungi Hyphomycetales, It belongs to the family Dematiaceae. Cellulose has transmitted this fungal disease Morphological and taxonomic characteristics of these fungi are given in [1], These kinds of fungi and their metabolites, can cause opportunistic infections in animals and humans that are sometimes very difficult to treat [2]. The metabolite profiles of Stachybotrys species consequently depend on time, substrate, and the individual biosynthetic potential of the respective strain [3].

The genus Stachybotrys includes more than 20 species. The most complete information about them:

Stachybotrys chartarum Bondorden – alternate staxibotris

Stachybotrys lobulata Berk - smallstaxibotris
Stachybotrys atra Corda – staxibotris black

In addition, two types of Stachybotrys chartarum are described:

Var. jateli pidopl.- species (poisonous species)

Var. atoksika pidopl. - species (non-toxic species). Single Stachybotrys lobulata type:

Var. macra pidopl. - species (large spore species.

After the etiological role of Stachybotryschartarum was determined, in 1938 it was commonly referred to as "H-3" (unknown equine disease) i.e. stachybotriotoxicosis in equine diseases [4,5]. Poisoning by horses Stachybotrys chartarum was first reported in Ukraine in 1931 by a substance grown in the feed and later by substances called stachybiotriotoxins. From 1937 to 1940, the disease expanded geographically. Stachybotrys chartarum fungus has been reported in Ukraine as well as in Moldova, Moldova, Bashkortostan, Eastern Siberia, Romania, Poland, Slovakia, and Hungary [1, 5].

In the same years, people with stachybotriotoxicosis as a result of consuming food contaminated with Stachybotrys were also identified [6]. In the future, the incidence of diseases in people who ate foods containing carbohydrates contaminated with microbes was higher [7, 8].

Cattle, sheep, pigs, rabbits, dogs, and chickens are practically infected with stachybotriotoxicosis [9 - 11]. In all cases, animal experiments have been found to be fatal, but horses are more susceptible to the disease.

The study of secondary metabolites of Stachybotrys chartarum of fungi belonging to the genus Stachybotrys revealed the etiological significance of stachybotriotoxicosis disease, primarily in animals and humns.

Several chemical laboratories in Kiev, Moscow, and Vilnius have conducted research on the problem of determining the chemical nature of stachybotriotoxins synthesized by the Stachybotrys chartarum fungus. It is noteworthy that the laboratory named after Academician D.H. Zelinsky was also involved in solving this problem. According to the publications, chemically significant results were obtained by the authors [11-13].

Stachybotrys chartarum infects cellulose-containing feed, producing mycotoxins that cause toxicosis in animals. The chemical nature of these mycotoxins has not been clarified. In this regard, we studied the low molecular weight metabolites of Stachybotrys chartarum. Column chromatography of the sum of the waste products of the latter, grown under laboratory conditions, isolated the predominant component in terms of content, which we called 3-matoxistachibotrin (I) (Fig. 1.). This article presents the results of establishing the structure of this compound.

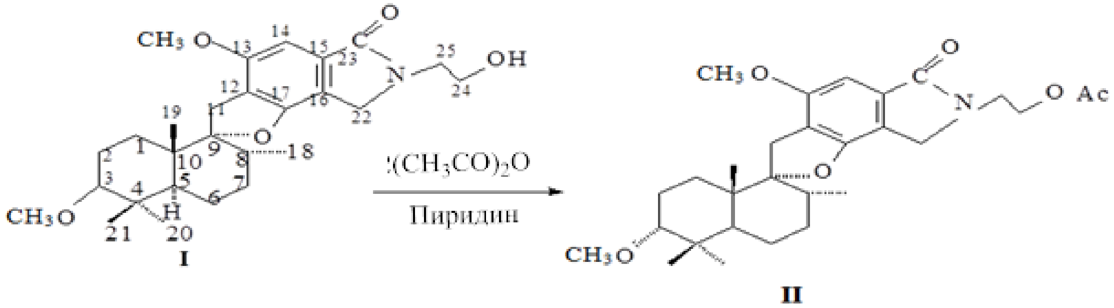

Fig. 1. Chemical modification of 13,3-dimatoxistachibotrin

THE MAIN RESULTS AND FINDINGS

General remarks. Thin layer chromatography (TLC) was performed on Silufol plates. The substances were detected on TLC by spraying with a 25% ethanol solution of phosphoric tungstic acid followed by heating for 5 min at 100–110 ° C.

For column chromatography, silica gels of the Silpearl and L brands, particle size 50-100 µm, were used. Silpearl was used to separate the metabolites of Stachybotrys chartarum. Purification and separation of the products of chemical transformations were carried out on columns with silica gel of grade L. The following solvent systems were used: I) benzene-methanol (10:1); 2) chloroform — methanol (100: 1); 3) chloroform-methanol (50: 1). Mass spectra and elemental compositions of ions were measured on an MX-1310 instrument at an ionizing voltage of 50 eV and a temperature of 100 °C. IR spectra were recorded on UR-20 and Perkin Elmer System 2000 FT spectrophotometers -IR to KBr.

^1H, ^{13}C, 2M ^1H-^1H, ^1H -^{13}C NMR spectra, chemical shift correlations (COSY) were recorded on a Bruker AM 400 instrument. ^{13}C NMR spectra were obtained with complete decoupling of C-H interactions and J-moducin. 2M NMR spectra of long-range ^1H-^{13}C interactions (HMBC) and NOE measurements in a rotating coordinate system (ROESY) were recorded on a Bruker AC 300 instrument. The spectra were recorded in deuteropyridine, unless otherwise indicated. All spectra were recorded using standard Bruker programs. ^1H NMR spectra of compounds I and 2 in deuteromethanol were obtained on a Tesla BS 567 A (o-HMDS) spectrometer.

Isolation of 13,3-dimethoxystachibotrin. Dry chloroform extract of Stacliybotrys chartarum strains (22.8 g) was loaded onto a column containing 300 g of Silpearl silica gel. The column was eluted sequentially with benzene and system I. When the column was eluted with system I, 40 mg of 13,3-dimethoxystachibotrin was isolated.

13,3-dimethoxystachibotrin (I), $C_{27}H_{39}NO_5$, m.p. 239 °C (from MeOH), $R_f = 0.60$, (TLC, silufol, syst. 1), [α] D24 = 13.5 ± 2 ° ($CHCl_3$-MeOH, 1: 1).

IR spectrum (KBr, ν, cm^{-1}): 3350-3140; 1675; 1650; 1630; 1475; 1360.

Mass spectrum, m/z (%): M^+ 457 (95)[$C_{27}H_{39}No_5$], 425 (17,5) [425, 2401; $C_{26}H_{35}NO_4$], 412 (100), 398 (11,3), 386 (16,3) [386, 2319; $C_{23}H_{32}NO_4$], 380 (10) [380, 2251; $C_{24}H_{30}NO_3$], 368 (2,8), 354 (2), 342 (1,9), 339 (1,8), 312(1,9), 300 (3), 287 (3,3), 274 (10), [274, 1097; $C_{15}H_{16}NO_4$], 260 (10), 256 (7,5), 242 (10), 234 (12,5), 223 (30), 221 (20), 189 (15),[189,1649;$C_{14}H_{21}$], 149 (12,5), 135(12,5),129(12,5),109(12,5).

24- monoacetate –13, 3-dimethoxystachibotrin (II) from I. 13,3-dimethoxystachibotrin (14 mg) was acetylated with 0.5 ml of acetic anhydride in 1 ml of absolute pyridine at room temperature for 15 min. After evaporating the solvents, the residue was chromatographed on a column eluting with system 3. 10 mg of amorphous monoacetate II, $C_{29}H_{41}NO_6$, R_f = 0.35 (TLC, silufol, $CHCl_3$-MeOH, 1:1) was isolated.

IR spectrum (KBr, ν, cm^{-1})1765; 1745; 1696; 1615; 1460; 1417; 1386; 1369.

Mass spectrum, m / z (%): M^+ 485 (50), 469 (25), 454 (100), 438 (9,2), 425 (13,1), 409 (5,3), 396 (7,8), 383 (6,9), 320 (6,9), 302 (2,6), 284 (5,3), 256 (19,7), 129 (35,7), 107 (15,7), 97 (36,8), 91 (18,4), 83 (27,6), 73 (42,1), 69 (65,8), 55 (100).

The elemental composition of 13,3-dimethoxystachibotrin was determined by high-resolution electron impact mass spectrometry - $C_{27}H_{39}NO_5$. ^{13}C NMR spectrum of compound I under study (Table 2) containing signals at 156.88; 155.39; 135.66; 117.60; 113.22; 101.80 ppm, indicates that the 13,3-dimethoxystachibotrin molecule includes a five-substituted benzene ring. Accordingly, in the ^1HNMR spectrum of compound I (Table 1) at 7.31 ppm. there is a one-proton singlet assigned to a single aromatic proton.

Table 1

Indices of NMR spectra ^1H, ^{13}C, ^1H-^1H COSY, HMQC, HMBC

13,3-dimethoxystachibotrin (I) and its diacetate II (δ, ppm, C_5D_5N, 0-TMS)

Atom C	Compound				
	I		II		
	δ_c	δ_H(J, Hz)	δ_c	δ_H(J, Hz)	HMBC (Atoms C)
1	24.72	α 2.35 тд (13; 3) β 1.13 дт (13; 3)	24.69	α 2.29 тд (14; 4) β 1,15 дт (14; 4)	
2	26,05	α 1,70 β 1,95 тт (13; 3)	26,06	α 1,75 β 1,95	
3	74,83	3,54	74,74	3,60	1; 5

4	38,21	-	38,23	-	
5	40,85	2,56 дд (13; 2,4)	40,44	2,56 дд (12,8; 2,6)	4; 10; 19; 21
6	21,33	α 1,55 β 1,42 кд (13; 3,5)	21,27	α 1,63 β 1,43 кд (13; 4)	
7	31,60	α 1,70 β 1,55	31,56	α 1,70 β 1,60	
8	37,34	1,75	37,29	1,75	
9	98,72	-	99,86	-	
10	42,75	-	42,78	-	
11	32,86	α 3,50 д (16,7) β 3,09 д (16,7)	32,69	α 3,37 д (17,1) β 2,95 д (17,1)	8; 9; 10; 12;13 17
12	117,60	-	124,25	-	
13	155.39	-	147.75	-	
14	101.80	7.31 с	108.55	7.39 с	12; 13; 16; 23
15	135.66	-	135.75	-	
16	113.22	-	119.05	-	
17	156.88	-	156.74	-	
18	15.91	0.80 д (5,8)	15.85	0.83 д (6,1)	7; 8; 9
19	16.21	0.97 с	16.17	0.96 с	1; 5; 9; 10
20	29.11	1.19 с	29.13	1.23 с	3; 4; 5; 21
21	22.72	0.88 с	22.71	0.91 с	3; 4; 5; 20
22	48.54	4.09; 4.35 д (16,7)	48.08	3.99; 4.29 д (17)	15; 16; 17; 23

23	169.09	-	167.85	-	
24	60.50	3.90 м (2H)	62.48	4.28 м (2H)	
25	45.95	3.65; 3.90 м	41.85	3.63; 3.88 м	
CH_3COO-13	-	-	20.71 168.62	2.37 с	
CH_3COO-24	-	-	20.63 170.55	1.90 с	
CH_3-O-3	73,65	0,87 с	73,64	0,86 с	

The spectrum of compound I-II was recorded in deuteropyridine. The chemical shifts given without multiplicities and SSCCs were determined from the 2M NMR spectra of ^1H-^1H, 1H-13C correlations of chemical shifts. Abbreviations: s-singlet, d-doublet, t-triplet, dd-doublet of doublets, td-triplet of doublets, dt-doublet of triplets, tt-triplet of triplets, cd-quartet of doublets, m-multiplet.

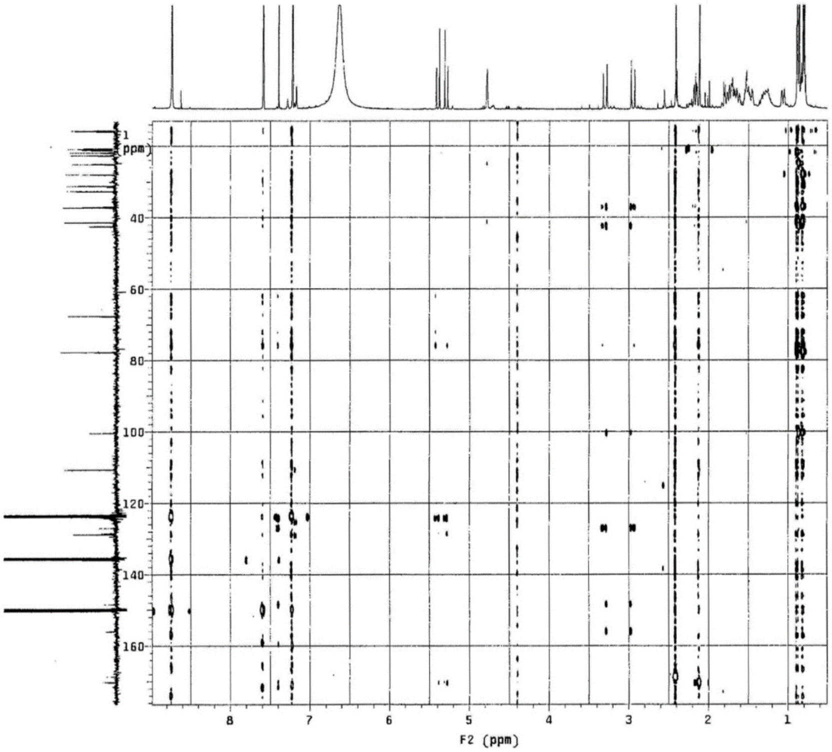

Fig. 1.2M NMR ^1H-^{13}C - spectrum of 13,3-dimethoxystachibotrinin deuteropyridine

Interpretation of the ^1H, ^{13}C, 2M ^1H -^1H and ^1H-^{13}C NMR spectra of chemical shift correlations (Fig. 1) allows constructing the fragmentary structure of 13,3-dimethoxystachibotrin shown in Fig. 2. The alicyclic part of the molecule consists of 15 carbon atoms and resembles the skeleton of the dryman sesquiterpenoid [14-22].

Acetylation of 13,3-dimethoxystachibotrin with acetic anhydride in pyridine gives diacetate III. The formation of diacetate suggests that there are two hydroxyl groups in the molecule of compound III. In the PMR spectrum of diacetate III, one of the acetyl groups resonates at 0.27 ppm, indicating the phenolic nature of the corresponding hydroxyl group. Indeed, the ^{13}C NMR spectrum shows a signal at 155.39 ppm belonging to the phenolic carbon atom.

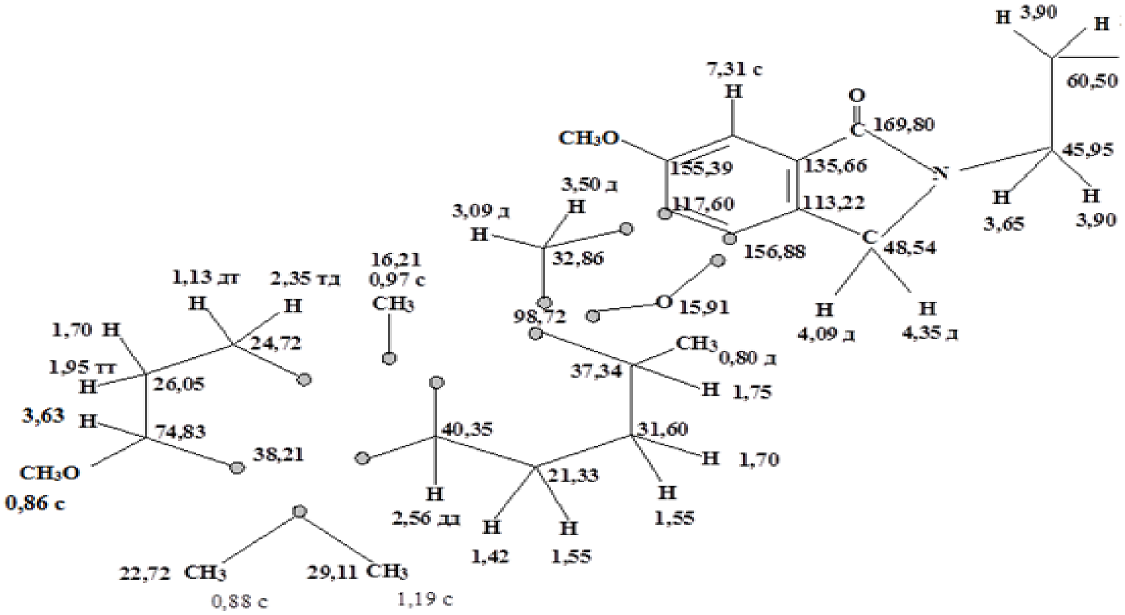

Fig. 2 Fragments of the structure of 13,3-dimethoxystachibotrin (I) according to ^1H, ^{13}C, 2M ЯМР ^1H -^1H и ^1H-^{13}C NMR and ^1H-^{13}C correlations of chemical shifts

In the mass spectrum of electron impact of 3-methoxystachibotrin, the maximum is the peak of the ion with m/z 412, which appears during the elimination of the hydroxymethyl radical (Scheme-2). The corresponding ions with m / z 454 and 496 are also formed during the mass spectrometric fragmentation of monoacetate II and diacetate III, but the peaks of these ions are not maximal. These facts indicate the presence of a secondary hydroxyl group in the molecule of compound I in the form of a hydroxymethyl function.

Signal at 169.09 ppm in the NMR spectrum ^{13}C of 13,3-dimethoxystachibotrin indicates that the compound under consideration contains an N - disubstituted carbamide or isomeric azomethinoxy group. The choice between them will be made later. The ester group is excluded by the IR spectrum of the discussed compound I, which contains an intense absorption band at 1675 cm $^{-1}$, as well as the fact that 13,3-dimethoxystachibotrin, as shown below, contains another oxygen function.

Thus, out of 5 oxygen atoms, 2 are part of the primary and phenolic hydroxyl groups, and the fourth function, the carbon atom of which resonates at 169.09 ppm. Chemical shifts of two aromatic carbon atoms are 155.39 and 156.88 ppm. in the NMR spectrum ^{13}C of 13,3-dimethoxystachybothrin indicate that, along with the phenolic hydroxyl group, one more oxygen function is associated with the benzene ring.

The presence in the same spectrum of a signal at 98.72 ppm, characteristic of an oxygen-bound quaternary carbon atom, indicates that the considered oxygen atom in the form of an epoxy function binds the alicyclic and aromatic parts of the molecule, namely, carbon atoms resonating at 156.88 and 98.72 ppm.

The appearance of ions with m\z 221 and 189 during fragmentation of 3-methoxystachibothrin under electron impact indicates that only a secondary methoxyl group is located in the sesquiterpenoid part of the molecule, including the C-23 atom, the function is attached to the benzene ring, and the primary hydroxyl group is located in the side chain.

The values of the chemical shifts of the three substituted carbon atoms of the benzene nucleus are 113.22; 117.60 and 135.66 ppm. allow us to conclude that these aromatic carbon atoms are linked to the substituents by a carbon-carbon bond.

In NMR spectrum of 13,3-dimethoxystachibotrin at 3.09 and 3.50 ppm. resonate two protons of the AB system, which in the 2M NMR ^{1}H - ^{13}C spectrum of correlations of chemical shifts correlate with a carbon atom resonating at 32.86 ppm. (C - 11). These protons of isolated methylene in the correlation spectrum of long-range ^{1}H - ^{13}C interactions (LMBC) (Table 2 and Fig. 3) correlate with three aromatic carbon atoms C-12 (117.60 ppm), C-13 (155.39 ppm), C-17 (156.88 ppm) and three carbon atoms of the terpenoid part of the molecule C - 8 (37.34 ppm), C - 9 (98.72 ppm), C -10 (42.75 ppm).

Table2.
Correlation spectrum ^{1}H - ^{13}C of long-range interactions (HMBC) 13,3-dimethoxystachibotrin (I) in deuteropyridine

Protons		Carbon atoms	
Position	**δ, ppm**	**Position**	**δ, ppm**
H-5	2,56	C-4 C-10 C-19 C-21	38,21 42,75 16,21 22,72
2H-11	3,09; 3,50	C-8 C-9 C-10 C-12 C-13 C-17	37,34 98,72 42,75 117,60 155,39 156,88

H-14	7,31	C-12 C-13 C-16 C-23	117,60 155,39 113,22 169,09
CH_3-18	0,80	C-7 C-8 C-9	31,60 37,34 98,72
CH_3-19	0,97	C-1 C-5 C-9 C-10	24,72 40,35 98,72 42,75
CH_3-20	1,19	C-3 C-4 C-5 C-21	74,83 38,21 40,35 22,72
CH_3-21	0,88	C-3 C-4 C-5 C-21	74,83 38,21 40,35 29,11
2H-22	4,09; 4,35	C-15 C-16 C-17 C-23	135,66 113,22 156,88 169,09

Therefore, it is this carbon atom of isolated methylene (C - 11) that binds the sesquiterpene part of the molecule to the aromatic atom resonating at 117.60 ppm. (C - 12), carbon-carbon bond. The epoxy function, linking carbon atoms C-9 (98.72 ppm) and C-17 (156.88 ppm), creates a spirobenzofuran system. In the same HMBC spectrum, the signal of the aromatic proton H-14 (7.31 ppm) correlates with carbon atoms C - 12 (117.60 ppm), C - 13 (155.39 ppm), indicating the location of the discussed proton at C - 14 (101.80 ppm). A similar conclusion also follows from a comparison of the ^1H NMR spectra of diacetate III. In the spectrum of diacetate III, the signal of the aromatic proton is shifted to a low field compared to that of 3-methoxystachibotrinan 0.27 ppm. Consequently, the aromatic proton and acetoxyl group in compound III are located vicinal. The aromatic hydrogen atom H-14 also correlates with the carbon atom C-23 (169.09 ppm). This means that the latter is at C -15. In the ^1H NMR spectrum of compound III, we observe single-proton doublets of the AB system at 4.09 and 4.35 ppm, which correlate with the signal of the carbon atom at 48.54 ppm. in the spectrum of 2M NMR ^1H-^{13}C correlations of chemical shifts. The considered protons in the NMBC spectrum of the same compound correlate with C-23. Therefore, the C-22 methylene group is attached to the C-16 atom.

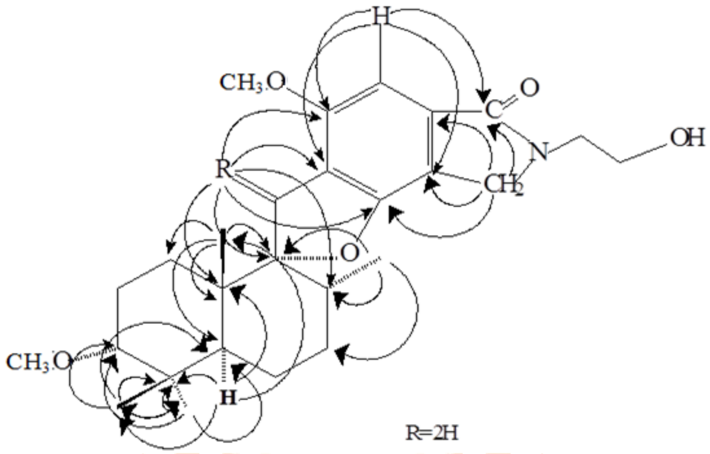

Fig. 3. HMBC spectrum of 13,3-dimethoxystachibotrin in deuteropyridine

Thus, two methylene groups remained unidentified, the protons of which, judging by the ^1H NMR spectrum, make up the AA'BB 'four-spin system, which goes over in the spectra of derivatives I-III to the AA'XX' system. The discussed protons in the 2M NMR spectrum of ^1H-^{13}C correlations of chemical shifts correlate with the signals of carbon atoms observed at 45.95 and 60.50 ppm. Taking into account the clarified part of the molecule, it follows from the elemental composition of the studied compound (**I**) $C_{27}H_{39}NO_5$ that 13,3-dimethoxystachibotrin must be one more cycle.

The recording of the ^1H and ^{13}C NMR spectra of 3-methoxystachibothrin and its diacetate III under the same conditions (C_5D_5N) shows that chemical shifts of 2H-24 protons, C-24, C-25 carbon atoms undergo a significant change upon going from 3-methoxystachibothrin to its diacetate III (table 1).

The articulation of the A / B rings and the conformations of the terpenoid part of the molecule of the new compound I were elucidated as follows.

These constants indicate the axial orientation of H-5, at least with respect to ring B, which is possible with the trans-junction of rings A and B. At the same time, the CSCR H-1α, H-1β, H-2β, H-3β, H -6β in the NMR spectra of compounds I-III (Table 1) determine the conformations of rings A and B as chairs $_{10}C^3$ and $_8C^5$, respectively (Fig. 5). In the ^1H-^1H NMR spectra of acetates II and III ($CDCl_3$) at 3.67 and 3.68 ppm. accordingly, the H-3 signal is observed in the form of a triplet with a VSWR ^3J = 3 Hz. The reported values of the chemical shifts H-13,3 indicate that the methoxyl group in monoacetate II is at C-13,3.

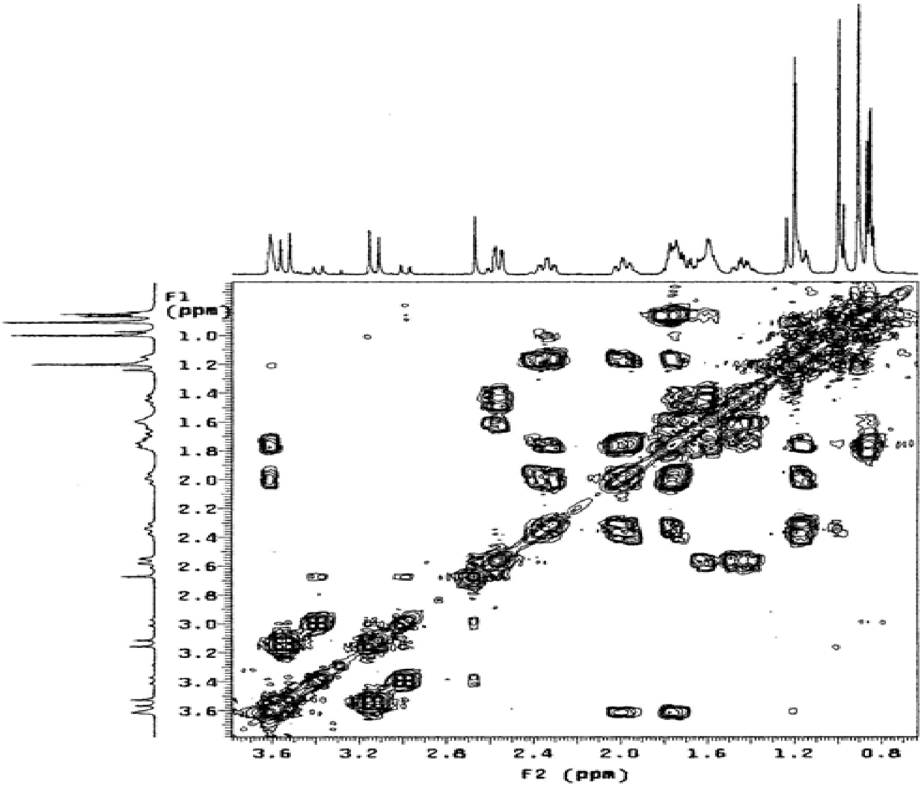

Fig. 4. ROESY spectrum of 13,3-dimethoxystachibothrin in deuteropyridine

CONCLUSION

These conclusions are also confirmed by the data of the ROESY spectrum of 13,3-dimethoxystachibotrin shown in Figure 4. Moreover, the detection of NOE between the CH_3-19 and H-8 protons in the same spectrum makes it possible to assign the latter β-, and the methyl group at C-8 (CH_3- 18) α - orientation.

The observation of NOE between antiperiplanar pairs of protons H-1α - H-2 β and H-5 - H-6β is a feature of the spectrum of ROESY 13,3-dimethoxystachibotrin and is in agreement with the established conformations of rings A and B. Thus, the presented experimental data allow us to conclude, that 13,3-dimethoxystachibotrin has the structure depicted by formula I.

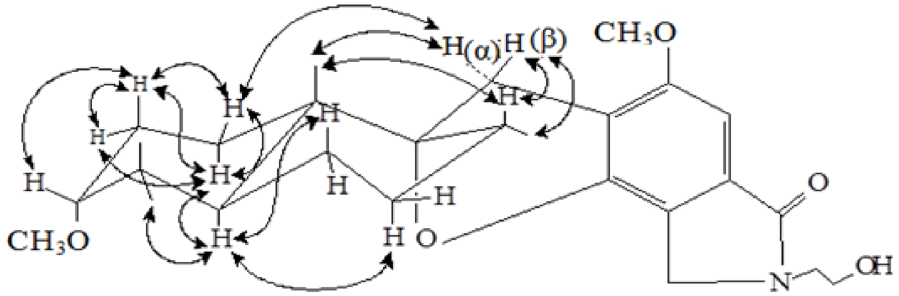

Fig. 5. The conformation of the terpenoid part of stachibotrin and the data of the ROESY spectrum in deuteropyridine

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