A STUDY OF COMPLEX MIXTURES OF NATURAL SUBSTANCES BY THE DEFOCUSING AND DADI METHODS.

V. STEROID METABOLITES OF THE PHYTOPATHOGENIC FUNGUS *Verticillium dahliae*

R. R. Razakov, A. K. Kasimov, Kh. A. Aslanov, and A. S. Sadykov

UDC 543.51+543.8+547.92+576.809.8

The mixture of steroid metabolites of the phytopathogenic fungus *V. dahliae* have been studied by high- and low-resolution mass spectrometry, metastable defocusing, and the direct analysis of daughter ions (DADI). On the basis of the analysis of the spectral results obtained for the mixtures themselves and for the products of their tosylation the presence in the samples studied of mixtures of β-sitosterol, stigmasterol, and cholesterol has been established. Probable structures have been proposed for the components of the mixture with m/z values of the molecular ions of 426, 424, and 412.

In fungi, bacteria, and yeasts, steroids play the role of growth regulators and of protectors from the action of various toxic substances [1]. Until recently, the study of the steroids of microorganisms amounted to isolating the total fraction and then determining the structure and identifying the main components; the qualitative and quantitative compositions of the minor components remained unestablished.

The extremely low concentration and structural similarity of these compounds do not always permit the effective use of chromatographic and mass-spectrometric methods for their separation and study. This difficulty is partially overcome by modification, i.e., by the protection of the polar groups in these compounds with acyl and trimethylsilyl groups [2, 3].

As one of the promising methods of modifying the 3-hydroxy-Δ^5-steroids we have proposed the tosylation reaction [4]. The tosylates of these compounds, in contrast to those of other steroids, on being heated in the column of a chromatograph or in the ion source of a mass spectrometer are readily converted into the corresponding readily volatile 3,5-dienic anhydro derivatives. The molecular ions (M^+) of the latter possess a high stability to electron impact and give very specific patterns of the spectra in the direct analysis of daughter ions (DADI) [5, 6].

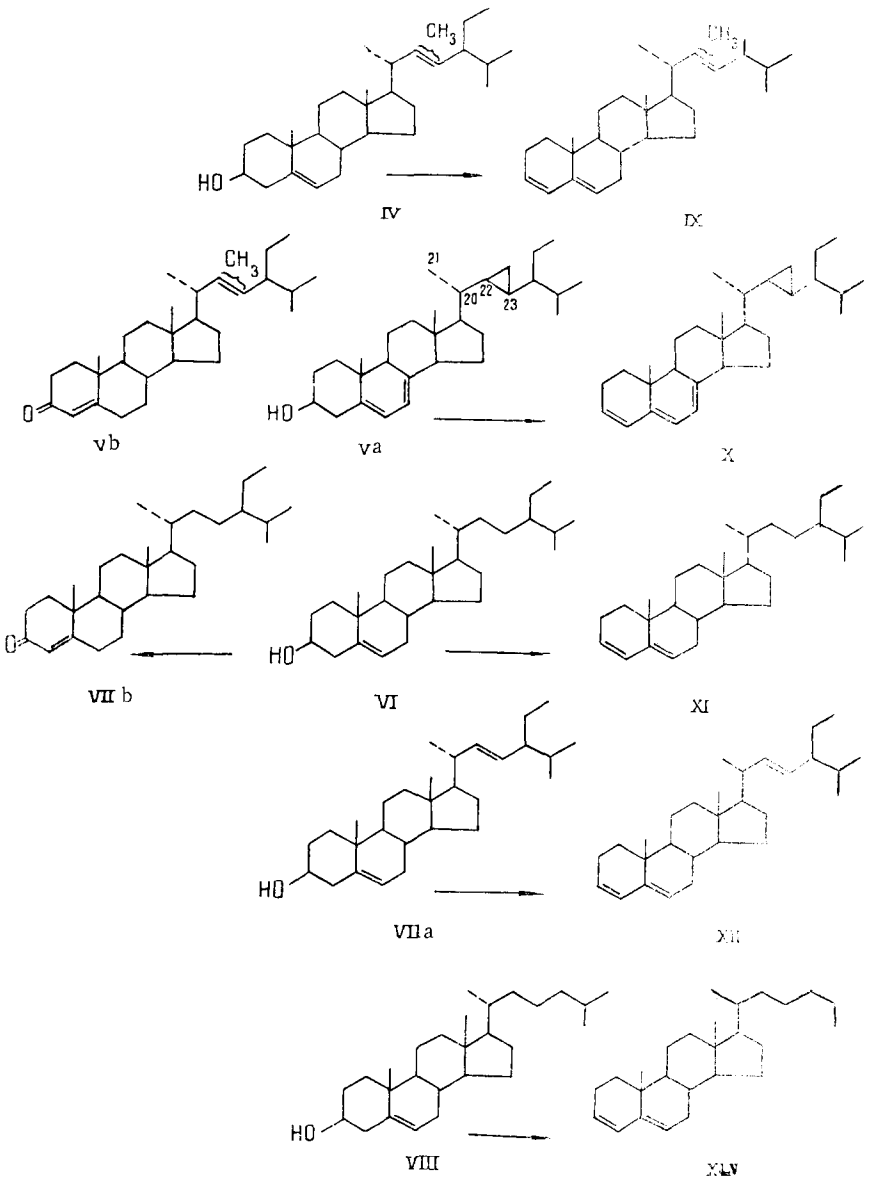
In its vital activity, the phytopathogenic fungus *Verticillium dahliae* secretes various metabolites belonging to the classes of lipids [7], phenolic compounds [8, 9], and steroids, and to other classes of compounds [7]. There is no information in the literature on the nature of the steroid metabolites. We have previously described minor components accompanying ergosterol — the main sterol of *V. dahliae* [4]. After the isolation of the ergosterol and its satellites, a number of components remain in the steroid fraction and it is the results of a study of these that are considered in the present paper.

The mass spectrum of a sample of the fraction under study showed, in the region of high mass, the peaks of ions with m/z 442 (I), 440 (II), 438 (III), 426 (IV), 424 (V), 414 (VI), 412 (VII) and 386 (VIII) (Fig. 1). The absence of a genetic link between these ions was established by the DADI and metastable defocusing methods, i.e., they were not daughter ions and had independent fragmentation pathways. It is obvious that these peaks corresponded to the molecular ions of the components of the mixture being studied. Because of the low intensity of the M^+ peaks of the minor components (I)-(III) it was impossible to obtain DADI spectra adequate for their analysis. The DADI spectra of M^+ ions of (IV) and (VII) proved to be identical with those of steroids that we had detected previously in the composition of the

Institute of Bioorganic Chemistry, Academy of Sciences of the Uzbek SSR, Tashkent. V. I. Lenin Tashkent State University. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 227-232, March-April, 1985. Original article submitted February 27, 1984.

 0009-3130/85/2102-0212$09.50

repellents of the beetle *Blaps deplonata* M. [10]. The identity of the components (VI) and (VIII) as β-sitosterol and cholesterol, respectively, was similarly shown.



In a comparison of the DADI spectrum of the M^+ ions of stigmasterol and component (VI) (Fig. 2a, b), similarities (the presence in them of peaks of ions with the same m/z values) and differences (increased intensities of the peaks of the ions with m/z 397, the presence of peaks of ions with m/z 124 and 289 in the spectrum of (VII) and their absence from the spectrum of stigmasterol) were found. This indicated that two substances with the same composition $C_{29}H_{48}O$ (in the high-resolution spectrum the peak of this ion was not split into a doublet) but having different structures corresponded to the ion with m/z 412 in the spectrum of a sample of the fraction of the metabolites of the fungus *V. dahliae* that is under study. There is no doubt that one of them was stigmasterol (VIIa). An additional conformation of this was the detection in the mass spectrum of a tosylated sample of the mixture under study (Fig. 3) of the peak of an ion with m/z 394, the DADI spectrum of which was identical with the spectrum of the anhydro derivative of stigmasterol [1]. It must be mentioned also that the m/z values of the M^+ peaks of (I-III) in the spectrum of the tosylated sample remained in unchanged form, which is possibly connected with the fact that these steroids do not belong to the 3β-hydroxy-Δ^5-steroid group. But the peaks of ions with m/z 426 (IV), 414 (VI), and 386 (VIII) in the spectrum of the tosylate appear in the form of M+ peaks of their anhydro derivatives with m/s values of 408 (IX), 396 (XI), and 368 (XIII), respectively.

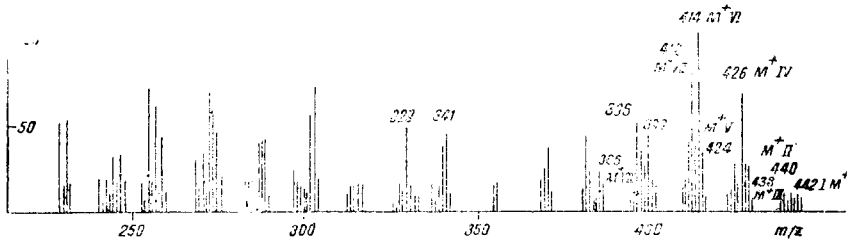


Fig. 1. Mass spectra of a mixture of the steroid metabolites of the fungus *V. dahliae*.

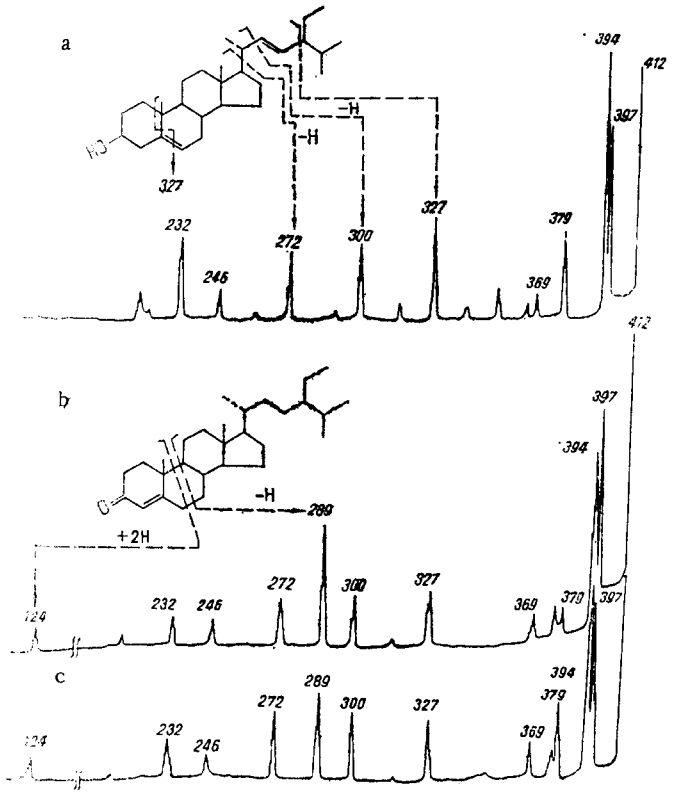


Fig. 2. DADI spectra of M^+ of stigmasterol (a) and of component (VII) (b)and the DADI spectrum of the M^+ ion with m/z 412 of the product of the oxidation of β-sitosterol (c).

So far as concerns the other component of the ion with m/z 412, the fact that it belonged to a 3-keto-Δ^4-steroid was shown by the peaks of ions with m/z 124 and 289 which arise on the decomposition of ring B [11]. In the formation of the first of them, the migration of two hydrogen atoms, and of the second that of one hydrogen atom, to the charged part of the molecule takes place (Fig. 2b). To confirm this we oxidized a sample under mild conditions (with hydrogen peroxide).

Previously, with the aid of DADI and defocusing methods we had established the presence in native samples isolated from various plant materials of β-sitosterol containing very small amounts of stigmasterol, campesterol, and cholesterol [12]. We therefore expected that the oxidation of β-sitosterol would, in part, form β-sitost-4-en-3-one (VIIb), the M^+ ion of which in the mass spectrum would be superposed on the M^+ peak of stigmasterol. In actual fact, in the mass spectrum of the product of the oxidation of the latter (although the pattern of the spectrum had a complex form) the peak of an ion with m/z 412 of considerable intensity appeared. The DADI spectrum of this ion was similar to that of the spectrum of M^+ of (VII) with respect both to the peaks of ions arising on the breakdown of ring A and to those arising on the cleavage of the side chain (Fig. 2b, c). Thus, in the mass spectrum of the sample under

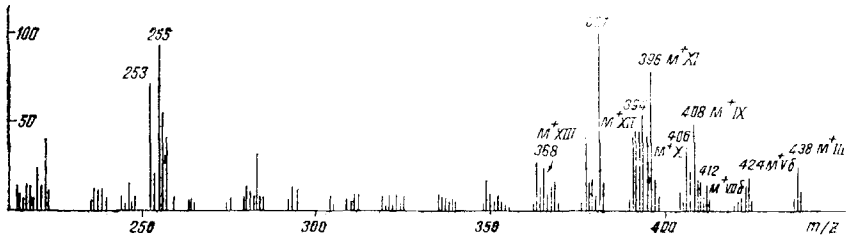


Fig. 3. Mass spectrum of the mixture of steroid metabolites after tosylation.

study the peak of the ion with m/z 412 corresponded to the M^+ peaks of two substances – stigmasterol (VIIa) and β-sitost-4-en-3-one (VIIb).

As already mentioned, in the mass spectrum of a tosylated sample of the mixture under study, component (IV) appeared in the form of an anhydro derivative with an m/z value of M^+ of 408 (IX), the DADI spectrum of this, like the spectra of anhydrosterols, being characterized by the peaks of ions arising on the splitting out of a methyl radical (ion with m/z 393), of the elements of the side chain (ions with m/z 365, 323, 282, and 255), and of rings D (ion with m/z 228) and C (ions with m/z 300 and 287). Previously, for sterol (IV) we proposed the probable structures (IVa) and (IVb) [10]. By the conversion of (IV) into its anhydro derivatives (IX) and the study of the DADI spectrum of the M^+ ion of the latter new information was obtained which confirmed that it is a 3β-hydroxy-Δ^5-steroid.

When the mixture being studied was tosylated, again separation into two components with m/z M^+ 424 took place. One of them underwent tosylation and appeared in the spectrum of the tosylate in the form of an M^+ peak corresponding to an anhydro derivative with a value of M^+ of 406. The DADI spectrum (Fig. 4a) of the latter, on the one hand, had a pattern similar to that of the M^+ spectrum of anhydroergosterol (it lacked the peaks of the ions arising in the breakdown of ring B). The side chain of this ion fragmented by cleavage of the C–C bonds, leading to ions with m/z 363, 321, 308, 280, and 253. On the other hand, the peaks of ions with m/z 308 and 296 – with even numbers – indicated the presence of a cyclopropane ring at C_{22}-C_{23} atoms [13]. It follows from what has been said that one of the components of the ion with m/z 424 had the structure (Va) and its anhydroderivative that of (X).

The other component with m/z M^+ 424 did not undergo tosylation, i.e., in the mass spectrum of the tosylate under study it appeared in the form of a peak with the initial value of m/z. In the DADI spectrum of this ion the peaks of ions characterizing the structures both of the side chain (the peaks of ions with m/z 381, 339, 298, and 217) and also the structures of rings A and B (peaks of ions with m/z 124 and 301) appeared (Fig. 4b). As already mentioned, the last two ions are characteristic for 3-keto-Δ^4-steroids. These facts permit us to suggest for the second component of the ion with m/z 424 the structure (Vb) as a product of the oxidation of the sterol (IV).

The detection of the unusual compounds (Va, b) and (VIb) in steroid metabolites deserves attention, since similar substances have not been detected hitherto in the products of the metabolism of fungi. This is connected to some extent with the methods of analysis used at the present time in the investigation of steroid metabolites. In the majority of cases, in such investigations the isolation of one of two main components is reported, and an assumption is made of the presence of other, minor components [14]. Nevertheless, many properties characteristic of these compounds (growth-regulating activity, protection from the harmful effects of exogeneous factors and phytotoxicity) can be ascribed just to the minor components. Their qualitative and quantitative compositions are possibly in dynamic equilibrium thanks to the unique enzyme systems of fungi which perform complex redox reactions. In these reactions the main components apparently participate as a reserve material.

The development and introduction of new methodological approaches to the investigation of such complex mixtures is opening up wide prospects in the understanding of the nature of such metabolites and elucidating their biological role in the functioning of the cells of microorganisms. The complex approach that we have proposed includes low- and high-resolution mass spectrometry, metastable defocusing, and the direct analysis of daughter ions in combination with conversion into anhydro and keto derivatives.

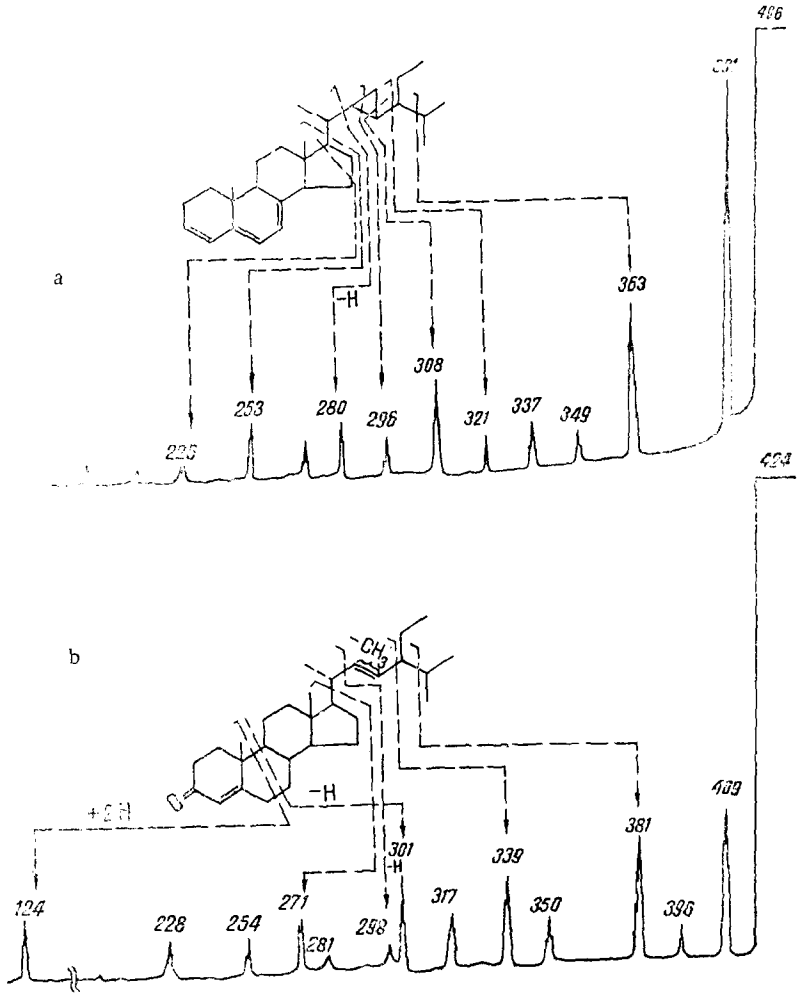


Fig. 4. DADI spectra of the M^+ ion of (X), the anhydro derivative of component (Va), (a) and of the M^+ ion of (Vb), (b).

EXPERIMENTAL

The spectra were taken on a MAT-311 mass spectrometer with a computer data-processing system. The conditions of recording the mass spectra and the DADI spectra and of defocusing were similar to those described previously [12]. The samples of mixtures of steroid metabolites isolated from the fungus *V. dahliae* were supplied by S. Z. Mukhamedzhanov and N. N. Stepanichenko, who took part in the discussion of the results obtained. The tosylation of the compounds was carried out by the procedure of Minale and Sodano [3]. The mixture being studied was oxidized by the addition of concentrated H_2O_2 with stirring for 1 h, followed by evaporation to dryness.

SUMMARY

1. The steroid metabolites of the phytopathogenic fungus *Verticillium dahliae* have been studied by low- and high-resolution mass spectrometry, the direct analysis of daughter ions, and metastable defocusing.

2. On the basis of an analysis of the spectral characteristics obtained for the sample under study and its tosylation products, the components of the mixture have been identified as β-sitosterol, stigmasterol, and cholesterol, and probable structures have been proposed for components with m/z values of the molecular ions of 424 and 412.

LITERATURE CITED

1. A. A. Akhrem and Titov, Steroids and Microorganisms [in Russian], Moscow (1970), p. 10.
2. J. Diekman and C. Djerassi, J. Org. Chem., 32, 1005 (1967).
3. L. Minale and G. Sodano, J. Chem. Soc., Perkin Trans. I, 1888 (1974).

4. A. K. Kasimov, Kh. A. Aslanov, and R. R. Razakov, Uzb. Khim. Zh., No. 5, 28 (1983).
5. D. H. Smith, C. Djerassi, K. H. Maurer, and U. Rapp, J. Am. Chem. Soc., 96, 3482 (1974).
6. R. R. Razakov, Kh. A. Aslanov, A. S. Sadykov, and B. V. Rozynov, Bioorg. Khim., 3, 600 (1977).
7. O. S. Otroshchenko, N. N. Stepanichenko, S. D. Gusakova, and S. Z. Mukhamedzhanov, Khim. Prir. Soedin., 275 (1979).
8. A. A. Bell, R. D. Stipanovic, and J. E. Puhalla, Tetrahedron, 32, 1353 (1976).
9. R. D. Stipanovic and A. A. Bell, J. Org. Chem., 41, 2468 (1976).
10. R. R. Razakov, A. K. Kasimov, Kh. A. Aslanov, and A. S. Sadykov, Bioorg. Khim., 6, 944 (1980).
11. F. J. Brown and C. Djerassi, J. Am. Chem. Soc., 102, 807 (1980).
12. R. R. Razakov, A. K. Kasimov, Kh. A. Aslanov, and A. S. Sadykov, Khim. Prir. Soedin., 81 (1981).
13. F. J. Schmitz and T. Pattabhiraman, J. Am. Chem. Soc., 92, 6073 (1970).
14. J. D. Welt and J. L. Laster, Lipids, 9, 575 (1974).

STEROID COMPOUNDS OF MARINE SPONGES.

IV. NEW STEROLS WITH UNUSUAL SIDE CHAINS FROM THE FUNGUS *Halichondria sp*$_1$

L. K. Shubina, T. N. Makar'eva,
A. I. Kalinovskii, and V. A. Stonik

UDC 547.92:639.29

Five new steroid alcohols have been isolated in the form of their acetates from extracts of the sponge *Halichondria sp*$_1$ by column chromatography on silica gel and they have been identified as 24-isopropyl-5α-cholest-22Z-en-3β-ol, 24-isopropylcholesta-5,22Z-dien-3β-ol, 24,24,26,26-tetramethylcholesta-5,25(27)-dien-3β-ol, 24,24,26,26-tetramethylcholesta-5,22E,25(27)trien-3β-ol, and 24-isopropenyl-25-methylcholesta-5,22E-dien-3β-ol. The structures were established by an analysis of spectral characteristics.

Continuing a study of sponge steroids [1] we have isolated five new sterols and have established their structures. The compounds, isolated in the form of their acetates (I)-(V) were the main components of the fraction of free sterols from a Vietnamese collection (Scientific-Research Ship "Professor Bogorov," 1982) of the sponge *Halichondria sp*$_1$. Their structures were established by an analysis of the results of chromato-mass spectrometry and a study of NMR spectra using experiments with differential spin decoupling and the recording of nuclear Overhauser effects (NOEs).

The sterol acetate (I) had a mass spectrum practically coinciding with that of the corresponding spectrum of the known [2] 24-isopropylcholesta-5,22E-dien-3β-ol acetate (VI). The values of the CH_3-18, CH_3-19, and H-6 chemical shifts in the ^{1}H NMR spectra of the two compounds also coincided. At the same time, the chemical shifts and the natures of the multiplicities of the H-22 and H-23 signals in the ^{1}H NMR spectra of (I) and (VI) differed. This gave grounds for assuming that (I) was the 22-cis isomer of the sterol (VI). In actual fact, on catalytic hydrogenation under the same conditions the two compounds gave the same derivative – 24-isopropyl-5α-cholestan-3β-ol acetate (VII). The 22, 23- position of the double bond in (I) was confirmed by experiments with differential spin decoupling (^{1}H NMR). When H-22 or CH_3-21 was irradiated, an H-20 multiplet (2.36 ppm) appeared. Irradiation at 2.36 ppm led to the degeneration of the CH_3-21 doublet into a singlet. Chemical shifts and multiplicities of the H-24, H-25, and H-28 signals in the ^{1}H NMR spectrum were determined similarly (Table 2). An analysis of the spin–spin coupling constants in the ^{1}H NMR spectrum of sterol (I) confirmed the hypothesis of the cis configuration of the 22,23- double bond. In actual fact, $J_{22,23}$ proved to be 11.5 Hz, while for related sterols with a trans-22,23-double bond this constant is usually

Pacific Ocean Institute of Bioorganic Chemistry, Far Eastern Scientific Center, Academy of Sciences of the USSR, Vladivostok. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 232-239, March-April, 1985. Original article submitted November 16, 1983.

