Notes

Structural Identification of an Anthrasteroid Hydrocarbon from the Sheep Tick *Ixodes ricinus*

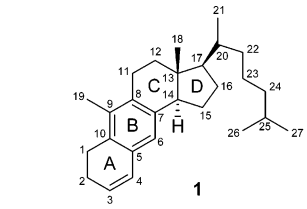
David Šaman,[†] Josef Cvačka,[†] Aleš Svatoš,[‡] Edwin A. P. Bouman,[§] and Blanka Kalinová*,[†]

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo nam. 2, CZ-166 10, Praha 6, Czech Republic, Max Planck Institute for Chemical Ecology, Hans-Knöll-Strasse 8, D-07745 Jena, Germany, and Institute of Parasitology, Academy of Sciences of the Czech Republic, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic

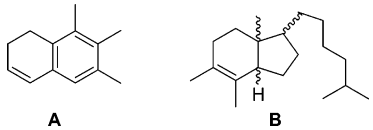
Received April 5, 2006

A new anthrasteroid hydrocarbon was isolated from cuticular integument of engorged female *Ixodes ricinus*, and its structure was determined by interpretation of spectroscopic and nuclear magnetic resonance data as 14α(*H*)-1(10→6)-*abeo*-cholesta-3,5,7,9(10)-tetraene (**1**).

Tick mating is a complex procedure regulated by sex pheromones. In the hard ticks (Ixodidae), at least three types of sex pheromones are involved in mating: an attractant sex pheromone, a mounting sex pheromone, and a genital sex pheromone.[1−3] The attractant sex pheromone is typically released from feeding females and stimulates searching behavior in males. The mounting sex pheromone is present on the surface of the engorged female tick's body and serves as a recognition cue for males searching for females. The genital sex pheromone provides a conspecific identification and induces spermatophore production in males. The sheep tick *Ixodes ricinus* (L.; Ixodidae, Prostriata) is an important vector for many serious human diseases. In a search for chemical compounds involved in *I. ricinus* reproductive behavior, the composition of the cuticular integument of *I. ricinus* was studied. We describe herein the isolation and identification of the monoaromatic anthrasteroid hydrocarbon (**1**) from engorged females of this tick.



1



A **B**

The chloroform cuticle extracts of *I. ricinus* males and engorged females were examined by GC-MS. Two distinct peaks appeared specifically in the chromatogram of the female extract (major peak **1**: 92%, t_R = 25.3 min; minor peak **2**: 8%, t_R = 19.9 min (Figure S1A, Supporting Information)). The minor peak **2** has not been completely characterized and will not be discussed further in this communication. The EIMS of the main compound **1** showed a molecular ion $M^{+\bullet}$ at *m*/*z* 364 (base peak). An accurate mass measurement provided the molecular formula, $C_{27}H_{40}$, which corresponds to eight double-bond equivalents. To determine the number of nonaromatic double bonds, hydrogenation was used. Compound **1** accepted two hydrogen atoms, suggesting the presence of one saturable double bond and one aromatic ring. The presence of an aromatic ring was indicated in the mass spectrum of **1**. An intense peak at *m*/*z* 362 (30%), and a less pronounced one at *m*/*z* 360 (4%), represented consecutive neutral losses of hydrogen molecules from the molecular ion at *m*/*z* 364. As the first loss was very intense (more intense than the loss of $CH_3^{\bullet}$, 4%), the formation of a condensed, two-ring aromatic system was inferred. Thus, the saturable double bond should be in a ring adjacent to the aromatic ring. The high intensity of the molecular ion (100%) also supported the assumption that an aromatic ring is present. The hydrogenation of **1** provided only one product (**3**; t_R = 24.7 min), eluting earlier than **1** (Figure S1C). When compared with **1**, the mass spectrum of this product showed a more intense loss of $CH_3^{\bullet}$ (e.g., 17%) and a peak at *m*/*z* 159; other masses were two mass units higher and the hydrogen neutral losses were less pronounced. No match of hydrogenated **1** with the NIST mass spectral library was found; however, the mass spectrum showed similarity with an anthrasteroid, 14α(*H*)-1(10→6)-*abeo*-cholesta-5,7,9(10)-triene, found in Cretaceous black shale.[8]

The ^{1}H NMR spectrum of **1** gave basic information about the presence of three aromatic/olefinic protons, one methyl attached to a double bond, three secondary methyls, one tertiary methyl, five protons with signals in the region 2.5−2.8 ppm, five others in the region 2.0−2.3 ppm, and unresolved protons with the intensity of 12 protons in the region 1.0−1.7 ppm. The obtained ^{13}C NMR spectrum confirmed the measured molecular compositions of **1**, with 27 carbon atoms in the molecule. On the basis of H,H-COSY, gHSQC, and g-HMBC 2D NMR spectra, two characteristic structural fragments, **A** and **B**, were determined. Their combination allowed us to formulate the hypothesis of an anthrasteroidal skeleton of **1**. Mass spectroscopic data observed for **1** agreed with those given in the literature for a similar compound, anthracholestatetraene, prepared synthetically by Tsuda and coauthors in 1963.[7] However, this paper did not provide any NMR data allowing comparison. Fragment **A** was described in detail by Bird at al., [4] and its ^{1}H NMR data fit quite well with those found for tick compound **1** (Table 1). Unfortunately, the ring containing the double bond in the fragment **A** (Scheme 1) is rather flexible, and that is the reason for the complex character of the ^{1}H NMR spectrum provided. Another compound with an anthrasteroidal skeleton was

* To whom correspondence should be addressed. Tel: (+420) 220 183 339. Fax: (+420) 220 183 582. E-mail: blanka@uochb.cas.cz.
† Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic.
‡ Max Planck Institute for Chemical Ecology, Jena.
§ Institute of Parasitology, Academy of Sciences of the Czech Republic.

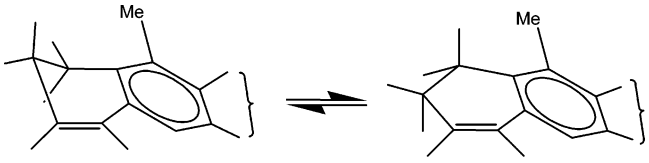
10.1021/np0680127 CCC: $33.50
Published on Web 08/05/2006

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of Compound **1** in $CDCl_3$[a]

position	δ_C	δ_H
1	23.8	2.69−2.8 (m)
2	23.4	2.31 (m)
3	126.7	5.94 (dt, 4.3, 4.3, 9.5)
4	128.4	6.4 (dt, 1.8, 1.8, 9.5)
5	131.2	
6	121.9	6.6 (bs)
7	137.7	
8	133.8	
9	132.7	
10	130.6	
11	26.2	2.69−2.8 (m)
12	37.2	1.65 (dddq, 3 × 0.5, 8.2, 10.7, 13.0)
		2.25 (ddd, 2.1, 7.1, 13.0)
13	41.8	
14	51.8	2.66 (m)
15	24.2	1.42−1.52 (m)
		2.05−2.1 (m)
16	28.9	1.42−1.52 (m)
		2.05−2.10 (m)
17	55.2	1.32 (m)
18	11.1	0.59 (d, 0.5)
19	14.5	2.14 (bs)
20	36.2	1.42−1.52 (m)
21	18.8	0.99 (d, 6.5)
22	36.1	1.05−1.11 (m)
		1.35−1.40 (m)
23	23.8	1.13−1.20 (m)
		1.35−1.40 (m)
24	39.5	1.13−1.20 (m)
25	28.3	1.53 (m)
26	22.6	1.88 (d, 6.6)
27	22.8	1.88 (d, 6.6)

[a] Chemical shifts are reported in ppm relative to the internal signal of TMS and $CDCl_3$ resonances at 77.0 ppm for ^{13}C NMR; multiplicity and *J* values in Hz are given in parentheses.

Scheme 1



described by Koshino and coauthors in 1989 in the grass fungus *Epichloe typhina*.[5] The structure of rings B, C, and D of this compound is identical with **1**, and corresponding ^{1}H and ^{13}C NMR data of these two compounds are similar (Table 1).

The terminal step in the structural identification of **1** was to characterize its side chain and its stereochemistry. The APT ^{13}C NMR spectrum of **1** suggested the presence of eight unassigned carbon atoms (five with odd and three with even numbers of protons) and three secondary methyl signals. Such data indicated the presence of a C_8H_{17} fragment, a characteristic feature of many steroids. Similarities between already published NMR data of many steroids[6] and the present NMR analysis of **1** clearly supported the conclusions that the side chain of **1** has a steroidal structure (Table 1).

To determine the stereochemistry of **1**, two different approaches were combined: inspection of the theoretical conformation based on molecular mechanics (MM+) calculations and the 2D-ROESY NMR experiments. There are two possibilities concerning the relative orientations of the C-18 methyl group and proton H-14 (Figure 1; Table S1, Supporting Information). When the *cis* orientation is considered, the expected angle between the aromatic ring and the C-13/C-18 bond is about 120° and the methyl group is deflected. In the case of a *trans* orientation, an angle of about 80° brings the C-18 methyl group significantly more into the field of the aromatic ring current. The calculated value of the chemical

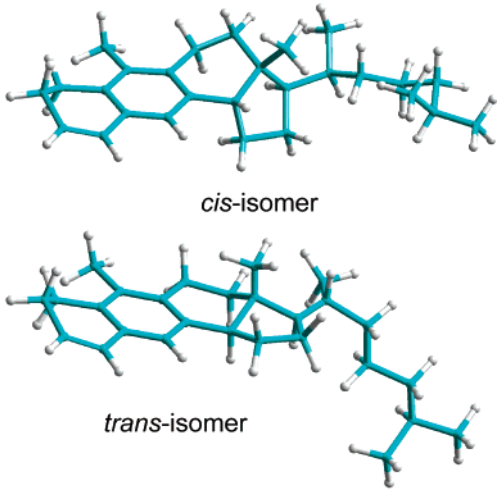


Figure 1.

shift of the methyl signal (δ 0.59) indicated that the C-18 methyl group and proton H-14 in **1** had a *trans* configuration. This assumption was supported by a 2D-ROESY NMR experiment (for the most important interactions see Table S1, Supporting Information). The structure of **1**, elucidated from NMR measurements, was consistent with the EIMS data discussed above. The expected position of the double bond in ring A was confirmed; *m/z* 251 resulted from C-8 aliphatic chain σ-cleavage, and the even mass *m/z* 224 ion resulted from a neutral loss of $C_{10}H_{20}$ from ring D. More intense *m/z* 209 and 197 ions represented trihydro-2,9-dimethylanthracenyl and trihydro-9-methylanthracenyl cations, respectively. The presence of an isopropyl group was indicated by an abundant ion at *m/z* 43. Analysis of all the experimental data led to the conclusion that the engorged-tick female-specific compound **1** has the structure 14α(*H*)-1(10→6)-*abeo*-cholesta-3,5,7,9(10)-tetraene.

Compounds with anthrasteroidal skeletons are quite rare in nature. Anthrasteroids found in Cretaceous black shales are considered specific geochemical markers of immature sea sediments.[8] Other anthrasteroidal structures were described among metabolites associated with mycelia of a new hybrid strain derived from *Penicillium citreo-viride*.[9] Our data provide the first example of an anthrasteroid in animals. The role and origin of **1** in *I. ricinus* biology is not known. Compound **1** is likely produced from cholesterol derivatives taken up with blood during feeding. Cuticle deposits of **1** might represent a way of removing excess amounts of such derivatives from the body fluid (detoxification) or a way to fight against pathogens (protection). Alternatively, the production of cuticular steroid hydrocarbons might represent a means of producing signals for intraspecific communication (pheromones). Female specificity of compound **1** supports the latter possibility but does not exclude other scenarios. In a closely related tick species, *I. persulcatus*, steroids such as cholesterol and its derivatives were found to be an integral part of lipophilic compounds on the surface of the tick's body.[10] Cholesteryl esters and cholesterol serve as contact sex pheromones in tick species such as *Boophilus microplus* (Canestrini)[11] and *Dermacentor variabilis* (Say)[12] and in other hard tick species.[3]

Experimental Section

General Experimental Procedures. GC-MS experiments were performed using a HP 6980 series gas chromatograph (Agilent) connected to a MasSpec magnetic sector-field mass spectrometer (Micromass, Manchester, UK). A nonpolar DB-5 MS column (30 m long, 0.25 mm internal diameter, film thickness 0.25 μm, J. & W. Scientific) was used for separations. The injector was operated in splitless mode at 220 °C. The source temperature was set to 200 °C; standard 70 eV spectra were recorded at 1 scan/s. The temperature of

the GC oven was programmed as follows: 60 °C, hold for 2 min; 10 °C/min to 320 °C and hold at 320 °C for 10 min. Helium was used as a carrier gas at a constant flow of 0.7 mL/min. The data were analyzed using OPUS software, and Wiley version 6 and NIST libraries were used for spectral data searches. HREIMS data were obtained on the same MasSpec instrument in positive ion mode using 70 eV ionization energy. A perfluorokerosine mixture was used as an internal standard.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE-500 spectrometer, equipped with a TXI 5 mm cryoprobe, in $CDCl_3$ (Aldrich, 99.9% D), using the signal of tetramethylsilane (δ 0.0 for ^{1}H NMR) or the central line of solvent signal (δ 77.00 for ^{13}C NMR) as references. Chemical shifts are given in ppm; coupling constants, in Hz. For 2D NMR experiments the standard pulse programs provided by the manufacturer of the spectrometer were used. The characteristic spectral width was 8 ppm for ^{1}H and 150 ppm for ^{13}C NMR spectra.

Animal Material. Adult males and females of *I. ricinus* were collected in the field. [Several voucher specimens have been deposited in the Laboratory of Natural Products, Institute of Organic Chemistry and Biochemistry (IR-2005-9-1).] No information about mating history of collected ticks was available, but since female *I. ricinus* are known to copulate off-host,[14,15] some of the males and females had probably mated. Females were allowed to feed on laboratory rabbits[13] until spontaneously detached from host. Males were not fed, since they do not exhibit adult feeding.

Extraction and Isolation. One hundred males and engorged females were placed separately in a Pasteur pipet-like glass column sealed on the bottom with precleaned cotton wool and extracted with $CHCl_3$ (10 mL; elution time ca. 10 min). Solvent from the extract was evaporated almost to dryness under a stream of argon. The crude extract was separated on precleaned glass plates (36 × 76 mm) coated with Adsorbosil-Plus (Applied Science Labs; a 0.2 mm layer with 12% gypsum) using hexane as the mobile phase. Spots were visualized by spraying with Rhodamine 6G solution (0.05% in ethanol). The major compound of interest (**1**) eluted as a sharp zone roughly in the middle of the plate (R_f = 0.58) of the female extract. The zone was scraped off the plate and extracted with 7 mL of freshly distilled diethyl ether. The solvent was evaporated to dryness under a stream of argon, and the residues were weighed. The residue (0.9 mg) was reconstituted in 90 μL of $CHCl_3$ and stored in a sealed glass ampule at −18 °C. The purity of the isolated compound (**1**) was higher than 99% (GC-MS).

Hydrogenation. Solvent from the tick extract was evaporated, and the residues were redissolved in ethyl acetate (0.5 mL). After adding Pt/C (10%) (ca. 2 mg) the reaction vial was evacuated and purged with hydrogen three times. The reaction was performed under vigorous stirring at normal pressure for 4 h. The black suspension was filtered through cotton wool in a Pasteur pipet, and the clear solution was analyzed by GC-MS as described above.

Compound 1: ^{1}H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 364 M^+, 100%), 362 ($M^+ - H_2$, 30), 360 ($M^+ - 2H_2$, 4), 349 ($M^+ - CH_3^\bullet$, 4), 251 ($M^+ - C_8H_{17}^\bullet$, 10), 225 (4), 224 ($M^+ - C_{10}H_{20}$, 17), 209 (35), 197 (21), 195 (16), 181 (7), 179 (9), 167 (5), 165 (7), 157 (17), 155 (7), 55 (5), 43 (15); HREIMS *m*/*z* 364.3125, calcd $C_{27}H_{40}$ 364.3130.

Acknowledgment. Financial support from the Institute of Organic Chemistry and Biochemistry, Prague (project Z40550506), the Grant Agency of the Czech Republic (206/04/0751), the Institute of Parasitology (Z60220518), and the Max-Planck-Gesellschaft is acknowledged. We thank to O. Blažek for skillful technical assistance and J. Erhart for providing *I. ricinus*. We also thank anonymous referees for valuable criticism of the manuscript.

Supporting Information Available: Reconstructed chromatograms at *m*/*z* 364 obtained for male, female, and hydrogenated female extracts and selected HMBC and ROESY correlations of **1** are available at http://pubs.asc.org.

References and Notes

(1) Sonenshine, D. E. *Biology of Ticks*; Oxford University Press: New York, 1991; Vol 1.
(2) Sonenshine, D. E.; Hamilton, J. G.; Phillips, J. S.; Lusby, W. R. In *Modern Acarology*; Dusbábek, F., Bukva, V., Eds.; Academia Praha and SPB Academic Publishing: The Hague, 1991; Vol. 1, pp 69−78.
(3) Hamilton J. G. C. *Parasitol. Today* **1992**, *8*, 130−133.
(4) Bird, T. G. C.; Felsky, G.; Meakins, G. D. *J. Chem. Soc., Perkin Trans. 1* **1978**, *1*, 1533−1537.
(5) Koshino, H.; Yoshihara, T.; Sakamura, S.; Shimanuki, T.; Sato, T.; Tajimi, A. *Phytochemistry* **1989**, *28*, 771−772.
(6) Eggert, H.; Djerassi, C. *J. Org. Chem.* **1981**, *46*, 5399−5401.
(7) Tsuda, K.; Hayatsu, R.; Steele, J. A.; Tanaka, O.; Mosettig, E. *J. Am. Chem. Soc.* **1963**, *85*, 1126−1131.
(8) Hussler, G.; Albrecht, P. *Nature* **1983**, *304*, 262−263.
(9) Nakada, T. and Yamamura, S. *Tetrahedron* **2000**, *56*, 2595−2602.
(10) Tkachev, A. V.; Dobrotvorsky, A. K.; Vjalkov, A. I.; Morozor, S. V. *Exp. Appl. Acarol.* **2000**, *24*, 145−158.
(11) de Bruyne, M.; Guerrin, P. M. *Arch. Insect Biochem. Physiol.* **1998**, *39*, 65−80.
(12) Hamilton, J. G. C.; Sonnenshine, D. E.; Lusby, W. R. *J. Insect Physiol.* **1990**, *35*, 873−879.
(13) Zemek, R.; Bouman, E. A. P.; Socha, R.; Dusbábek, F. *Exp. App. Acarol.* **2002**, *27*, 137−149.
(14) Babenko, L. V.; Bush, M. A.; Arumova, E. A.; Skadinsh, E. A. *Med. Parazitol. Parazit. Bol.* **1979**, *48*, 71−79.
(15) Mikulová, N.; Dusbábek, F.; Bouman, E. A. P. *Acarina* **2005**, *13*, 1−8.

NP0680127