143. Boophiline, an Antimicrobial Sterol Amide from the Cattle Tick *Boophilus microplus*

by **Olivier Potterat** and **Kurt Hostettmann***

Institut de Pharmacognosie et Phytochimie, Université de Lausanne, BEP, CH-1015 Lausanne

and **Alexandra Höltzel** and **Günther Jung**

Institut für Organische Chemie der Universität, Auf der Morgenstelle 18, D-72076 Tübingen

and **Peter-Allan Diehl**

Institut de Zoologie, Université de Neuchâtel, Chantemerle 22, CH-2007 Neuchâtel 7

and **Orlando Petrini**[1])

Microbiology Institute, ETH-Zentrum, CH-8092 Zurich

(22. VII. 97)

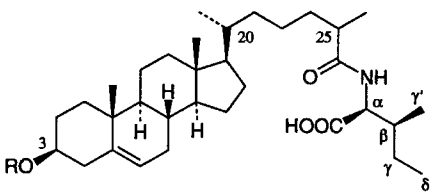
Boophiline (**1**), a new sterol amide was isolated from the cattle tick *Boophilus microplus* (Ixodidae). The structure was assigned as *N*-[3-(sulfooxy)-25ξ-cholest-5-en-26-oyl]-L-isoleucine by detailed 2D NMR investigations in conjunction with FAB mass spectrometry and acidic hydrolyses. Complete assignment of the diastereotopic methylene protons of the ring system could be deduced from the NMR data. In agar dilution assays, **1** exhibited antifungal properties against *Cladosporium cucumerinum* and antibacterial activity against *Bacillus subtilis* and *Escherichia coli*.

Introduction. – The cattle tick *Boophilus microplus* (Ixodidae) is an economically important pest affecting stock farming in most tropical and subtropical regions of the world [1]. It can transmit highly pathogenic agents such as the protozoa *Babesia bigemina, Babesia bovis*, and *Anaplasma marginale*. These ticks also cause anemia, weight loss, reduced milk production, secondary infections, and diminished values of hide.

It is known that ticks and their eggs are susceptible to attack by microorganisms. However, eggs or adults of *Boophilus microplus* are normally not infected by fungi when alive. On the other hand, we observed on several occasions during egg-laying in humid conditions that, while dead females were rapidly overgrown by fungi, the surrounding eggs were not infested. This prompted us to search for antimicrobial principles in this acarine. In a preliminary experiment, a crude $CHCl_3$/MeOH 2:1 extract of eggs inhibited the growth of the entomopathogenic fungi *Metarrhizium anisopliae* and *Beauveria bassiana*, and of the saprophitic fungus *Aspergillus niger*. Using a TLC bioautographic assay against *Cladosporium cucumerinum* [2], we detected the presence of at least one antifungal compound in both egg and female extracts of *Boophilus microplus*. Antibacterial properties were also revealed against *Bacillus subtilis* in an agar overlay TLC assay

[1]) Present address: *Pharmaton SA*, P.O. Box, CH-6903 Lugano.

[3]. The active compound appeared as a purple spot when TLC plates were sprayed with *Godin*'s reagent [4]. We report here on the isolation and characterization of this metabolite, *i.e.* compound **1**, which proved to be a new cholesterol[2]) sulfate derivative linked to a L-isoleucine residue through an amide bond.



1 R = SO_3H

2 R = H

Results and Discussion. – Whole female ticks were extracted with CH_2Cl_2/MeOH 1:2 at room temperature. Fractionation of the crude extract by a combination of silica gel and reversed-phase (*RP-18*) chromatography afforded compound **1**, named boophiline (see *Exper. Part*). The continuous-flow fast-atom-bombardment (CF-FAB) MS of **1** exhibited a $[M - H]^-$ pseudomolecular ion at *m/z* 608. From the ^{13}C-NMR and DEPT data, the molecular formula of **1** could be deduced as being $C_{33}H_{55}NO_7X$ (X = P, S). The presence of a P-atom in the molecule was excluded by ^{31}P-NMR. The ^{13}C-NMR and DEPT data indicated a cholesterol-derived structural unit. Further, they suggested the presence of an amino acid which was identified as L-isoleucine by GC-MS after acidic hydrolysis (6N HCl, 110°). Following partial hydrolysis (2N HCl, 2 h) of **1**, compound **2** could be isolated quantitatively from the mixture, with a 80 amu lower relative molecular mass than that of **1**. This pointed towards the cleavage of a sulfate group.

The combinatorial use of data deduced from homo- and heteronuclear two-dimensional NMR spectra (TOCSY, P.E.COSY, HSQC, and HMBC) of **1** allowed the assignment of the ^{1}H and ^{13}C resonances of the sterol moiety and the L-isoleucine unit, with the exception of the resonances of the methylene groups of the sterol side chain because of severe signal overlapping. To achieve in this case an assignment independent of chemical-shift comparisons with cholesterol, a 2D HMQC-TOCSY [5] spectrum of **1** was acquired which made possible an unambiguous assignment (*Table 1*).

The 1D ^{1}H-NMR spectrum of **1** in (D_6)dimethyl sulfoxide exhibits a br. *d* at 7.29 ppm and a *m* at 4.07 ppm which can easily be assigned to NH and to H–C(α) of L-isoleucine, respectively. The sterol unit gives rise to two resonances which appear clearly separated from the rest of the signals. One of the two corresponds to the olefinic proton H–C(6) (5.25 ppm), the other one belongs to the methine proton H–C(3) (3.81 ppm). The rest of the signals forms a crowded region between 0.80 and 2.35 ppm (the so-called methylene envelope), with the Me resonances showing up as intense peaks at higher field. One 'primary', three 'secondary', and two 'tertiary' Me groups are detected.

---

[2]) Based upon biosynthetic considerations, the configuration of cholesterol was adopted for the description of compound **1** throughout this paper, although only the relative configuration of the sterol moiety was established from the spectral data.

Table 1. *^1H and ^{13}C-NMR Chemical Shifts and HMBC Data of* **1** *in (D_6)DMSO.* δ in ppm.

	$\delta(^1H)$	$\delta(^{13}C)$	HMBC connectivities to
H_α–C(1)	0.94	36.9	C(2), C(3), C(19)
H_β–C(1)	1.76		C(2), C(3), C(5), C(10), C(19)
H_α–C(2)	1.85	28.7	C(1), C(3), C(10)
H_β–C(2)	1.38		C(1), C(3)
H_α–C(3)	3.81	75.2	–
H_α–C(4)	2.35	39.3	C(2), C(3), C(5), C(6), C(10)
H_β–C(4)	2.11		C(3), C(5), C(6), C(10)
C(5)	–	140.7	–
H–C(6)	5.25	121.0	C(4), C(7), C(8), C(10)
H_α–C(7)	1.47	31.4	C(5), C(6), C(8), C(9), C(14)
H_β–C(7)	1.89		C(5), C(6), C(8), C(9)
H_β–C(8)	1.36	31.4	C(7), C(9), C(14), C(15)
H_α–C(9)	0.85	49.6	C(10)
C(10)	–	36.0	–
H_α–C(11)	1.46	20.5	C(8), C(9), C(12), C(13)
H_β–C(11)	1.37		C(8), C(9), C(13)
H_α–C(12)	1.09	39.3	C(11), C(13), C(17), C(18)
H_β–C(12)	1.92		C(9), C(11), C(13), C(14), C(18)
C(13)	–	41.8	–
H_α–C(14)	0.95	56.2	C(8), C(9), C(13), C(15), C(17), C(18)
H_α–C(15)	1.51	23.8	C(8), C(13), C(14), C(17)
H_β–C(15)	1.03		C(8), C(14), C(16)
H_α–C(16)	1.73	27.6	C(13), C(15), C(17)
H_β–C(16)	1.20		C(15), C(20)
H_α–C(17)	1.05	55.1	C(12), C(13), C(14), C(16), C(18), C(20), C(21)
Me(18)	0.62	11.6	C(12), C(13), C(14), C(17)
Me(19)	0.92	19.0	C(1), C(5), C(9)
H–C(20)	1.33	35.0	C(17), C(21)
Me(21)	0.86	18.5	C(20)
CH_2(22)	0.98/1.29	35.5	C(21), C(22)
CH_2(23)	1.08/1.23	23.0	–
CH_2(24)	1.19/1.37	34.9	C(22), C(23), C(25), C(26), C(27)
H–C(25)	2.34	39.7	C(24), C(26), C(27)
C(26)	–	174.8	–
Me(27)	0.92	17.9	C(24), C(25), C(26)
NH	7.29	–	–
COOH	–	176.0	–
H–C(α)	4.07	57.4	C(26), COOH, C(β), C(γ), C(γ')
H–C(β)	1.77	37.1	C(α), C(γ), C(γ'), C(δ)
CH_2(γ)	1.13/1.44	24.7	C(α), C(β), C(γ'), C(δ)
Me(γ')	0.81	15.8	C(α), C(β), C(γ)
Me(δ)	0.80	11.5	C(β), C(γ)

The 2D HMQC-TOCSY technique combines the advantages of large dispersion in the indirect (^{13}C) dimension with the in-phase absorptive nature of TOCSY peaks, thus avoiding signal overlapping and cancellation of cross-peaks due to antiphase splitting in both dimensions. Application of a very short mixing time (16 ms) for the spin-lock resulted in a magnetization transfer reaching only the protons of the next neighbored CH_n fragment.

The linkage between the sterol side chain and L-isoleucine was elucidated by means of several HMBC connectivities (*Table 1*), the most prominent of them being cross-peaks from H–C(25) of the sterol side chain and H–C(α) of L-isoleucine to the carbonyl C(26) atom. Thus, boophiline (**1**) consists of a cholesterol unit with one of the original Me groups at C(25) being replaced by a carbonyl group which is involved in an amide bond to L-isoleucine. A sulfate group substitutes the OH group at C(3), which is reflected by ^{13}C-NMR chemical shift differences of *ca.* 3 ppm for C(2), C(3), and C(4) as compared to cholesterol. Based upon biosynthetic considerations, the relative configuration at C(20) is assumed to be that found in cholesterol; however, no definitive spectral evidence is available. The configuration at C(25) could not be established.

The diastereotopic methylene protons in the sterol ring system could be assigned by combining two complementary approaches. First, ^3J(H,H) coupling constants were extracted from the P.E.COSY spectrum (*Table 2*). The relative positions of vicinal protons in six-membered C-rings can be distinguished by their coupling constants (^3J(H,H) = 8–15 Hz for axial position, ^3J(H,H) = 2–8 Hz for equatorial-axial or equatorial-equatorial position). By this means, the prochirality assignment of the methylene groups of rings A and B was achieved.

Table 2. *^{3}J(H,H) Coupling Constants of* **1** *in (D_6)DMSO*

			^3J(H,H) [Hz][a])
Ring A	H_α–C(3)	H_β–C(2)	10.0
	H_α–C(3)	H_α–C(2)	5.0
	H_α–C(3)	H_β–C(4)	11.7
	H_α–C(3)	H_α–C(4)	4.9[b])
	H_β–C(2)	H_β–C(1)	3.5
Ring B	H–C(6)	H_α–C(7)	2.2[b])
	H–C(6)	H_β–C(7)	5.3[b])
Rings A/B	H–C(4)	H–C(6)	2.2[b])
Ring C	H_β–C(11)	H_β–C(12)	4.0
Ring D	H_β–C(15)	H_β–C(16)	6.5
Ile	NH	H–C(α)	8.3[b])
	H–C(α)	H–C(β)	5.1[b])
	H–C(β)	Me(γ')	6.7
	H–C(β)	CH_2(γ)	5.0
	CH_2(γ)	Me(δ)	6.7
	H_a–C(γ)	H_b–C(γ)	13.5

[a]) The coupling constants were extracted from a P.E.COSY spectrum. The accuracy is estimated to be ± 0.5 Hz.
[b]) These coupling constants were also found in the resolution-enhanced 1D NMR spectrum, the accuracy being ± 0.2 Hz. We renounced to determine further coupling constants from the resolution-enhanced 1D NMR spectrum because of their ambiguity concerning the coupling partner.

The above-mentioned signal overlapping in the P.E.COSY spectrum did not allow the extraction of sufficient ^3J(H,H) coupling constants for an assignment of all diastereotopic methylene protons in the sterol skeleton, so that additional information from interproton distances was necessary. A model of the molecule **1** was built and minimized with the aid of standard algorithms from the program INSIGHT II (*Biosym Inc.*, San Diego, CA, USA). The model-derived distances were compared to cross-peak intensities of a jump-symmetrized (JS)-ROESY [6] spectrum. This recently developed variant of the conventional ROESY technique overcomes the drawbacks from artifacts of TOCSY or COSY type and allows a quantification of interproton distances for molecules exhibiting only weak NOEs ($\omega_o\tau_c \approx 0$).

The presented stereoheterotopicity assignment in the sterol ring system is consistent with the rule that axial H-atoms in cyclohexane rings almost always show smaller ^{1}H-NMR chemical shifts than equatorial H-atoms at the same C-atom ($\delta_e > \delta_a$) [7]. The data agree well with the previously published stereoheterotopicity assignment of cholesterol [8]. Signal overlapping in all homonuclear spectra made a similar approach for the assignment of the diastereotopic methylene protons in the side chain and the $CH_2(\gamma)$ protons of L-isoleucine impossible. Moreover, chemical-shift comparisons with cholesterol which could be crucial are hazardous due to the unknown influence of the carbonyl group C(26)=O.

The antifungal activity of **1** was determined against *Cladosporium cucumerinum* and *Candida albicans*. In TLC bioautographic assays, while 1 μg of **1** showed inhibitory activity against *Cladosporium cucumerinum*, 10 μg were completely inactive against *Candida albicans*. The minimum inhibitory concentration (*MIC*) of **1** against *Cladosporium cucumerinum* in an agar dilution assay was found to be 10 μg/ml. Miconazole, used as a positive control, was active at 10 μg/ml in this assay. Using agar dilution tests, **1** proved to be also active against the bacteria *Bacillus subtilis* (*Gram*-positive) and *Escherichia coli* (*Gram*-negative), with *MIC* values of 10 and 5 μg/ml, respectively. By comparison, the antibiotic chloramphenicol was active against both bacteria at 1 and 10 μg/ml, respectively.

In Nature, steroids linked to amino-acid residues are mainly found as bile constituents, in which acids derived from cholesterol are conjugated with glycine or taurine [9]. In addition, bufotoxins in which various steroidal genins are esterified at C(3) with suberoylamino-acid residues occur in toad species (*Bufo* spp.) [10]. Sterol sulfates, rare in plants, have been reported from various animal sources, in particular marine organisms. Some of these compounds were found to exhibit antimicrobial properties. Thus, squalamine, an aminosterol antibiotic isolated from the shark *Squalus acanthias* exhibited potent antimicrobial activity against fungi, protozoa, and both *Gram*-positive and *Gram*-negative bacteria [11]. Sterol sulfates with antibacterial and antifungal properties have been also reported from marine sponges [12–14] and echinoderms [15].

Ticks, in particular *Boophilus microplus*, are known to contain large amounts of free and esterified cholesterol [16]. On the other hand, it is to our knowledge the first report of a sterol sulfate in these acarines. No report on antimicrobial metabolites in *B. microplus* was found in the literature. Antibacterial substances have been detected in the dermal-gland secretion from *Amblyoma variegatum*, but the structure of the active constituents has not been explored [17]. Boophiline (**1**) could play a significant role in the defense of the cattle tick against microbial infection. At the same time, it gives new insight into cholesterol metabolism in ticks.

This work was supported by the *Swiss National Science Foundation*. We are grateful to Drs. *J. F. Graf* and *J. Jonczy* (*Novartis*, CH-1566 St. Aubin) for their generous supply of ticks and to Ms. *M. Vlimant* for her help for their collection. Thanks are also due to Dr. *P. J. Fisher* (Department of Biological Sciences, University of Exeter, England) for carrying out some preliminary biotests on extracts and to Mr. *G. Nicholson* (Institut für Organische Chemie, Universität Tübingen, Germany) for performing the amino-acid analysis.

Experimental Part

General. TLC: Silica gel *60* F_{254} sheets (*Merck*); $CHCl_3$/MeOH/H_2O 65:35:5 (system *1*); detection with *Godin*'s reagent [4]. M.p.: *Mettler-FP-80/82* hot-stage apparatus; uncorrected. $[\alpha]_D^{20}$: *Perkin-Elmer-241* polarimeter. IR Spectra: *Perkin-Elmer-781* spectrometer. FAB-MS and continuous-flow FAB-MS (CF-FAB-MS): *Finnigan-MAT-TSQ-700* triple-stage quadrupole instrument equipped with a *70-BioProbe* accessory (*Finnigan MAT*); source temp. r.t., probe tip 50°, PAB gun 4 kV and 1.2 mA, Xe gas used for bombardment, negative-ion mode.

Cattle Ticks. Engorged ticks *Boophilus microplus* Canestrini (strain Biarra) were obtained from a laboratory colony at the *Novartis-Agricultural Research Station* (St. Aubin, Switzerland). This tick is reared on the backs of young Simmental steers in closed stables at 23° and 60–70% r.h.

Extraction and Isolation of N-*(3-Sulfo-25ξ-cholest-5-en-26-oyl)*-L-*isoleucine* (*Boophiline*; **1**). Whole female ticks (863 g) were extracted 3 times for 24 h with CH_2Cl_2/MeOH 1:2 (1.8 l). The crude extract (22.2 g) was fractionated by column chromatography (CC; silica gel, $CHCl_3$/MeOH/H_2O 65:35:5). A 2.0-g portion of the active fraction (5 g) was further separated by CC (silica gel, $CHCl_3$/MeOH/H_2O 70:35:1) to give 600 mg which were further purified by reversed-phase CC (*RP-18* cartridges, MeOH/H_2O mixtures of increasing MeOH content). Elution with MeOH/H_2O 80:20 provided crude active compound (125 mg). Purification of a portion (100 mg) of this material by reversed-phase medium-pressure liquid chromatography (MPLC; *RP-18*, MeOH/H_2O 4:6 → 6:4 (0.05% CF_3COOH)), followed by CC (silica gel, $CHCl_3$/MeOH/H_2O 70:35:1) provided 25 mg of pure **1**. Amorphous white powder. M.p. 185–190°. TLC (silica gel, system *1*): R_f 0.24. $[\alpha]_D^{20} = -29$ (DMSO, $c = 0.5$). IR (KBr): 3400, 2930, 2860, 1635, 1585, 1440, 1385, 1245, 1220, 985. ^{1}H- and ^{13}C-NMR: *Tables 1* and *2*. CF-FAB-MS (negative-ion mode, matrix 5% glycerol): 608 ($[M - H]^-$).

Amino-Acid Analysis. Compound **1** was hydrolyzed in 6N HCl at 110° for 24 h. The hydrolysate was derivatized to propyl esters and trifluoroacetamides and analyzed by GC/MS on a glass-capillary column (25 m × 0.25 mm i.d.) coated with *Chirasil-Val* as stationary chiral phase [18]. The *Carlo-Erba-2900* gas chromatograph was coupled to a *Finnigan-MAT-112-S* mass spectrometer with electron-impact ionization.

NMR Spectroscopy. 1D ^{1}H-NMR and 2D NMR spectra of **1**: *Bruker-AMX2-600* spectrometer (*Bruker Analytische Messtechnik GmbH*, Karlsruhe, Germany; ^{1}H at 600.13 MHz, ^{13}C at 150.9 MHz); soln. of **1** (9.8 mg) in (D_6)DMSO (0.5 ml) at 300 K; δ in ppm rel. to solvent signal (δ(^{1}H) 2.48 ppm, δ(^{13}C) 39.5 ppm), J in Hz; homonuclear TOCSY [19] [20], P.E.COSY [21] [22], and JS-ROESY [6] and heteronuclear HSQC [23], HMBC [24], and HMQC-TOCSY [5] experiments; for TOCSY and HMQC-TOCSY spectra, a clean-MLEV-17 spin-lock sequence was applied; *Bruker Aspect* station, 5-mm inverse ^{1}H/^{13}C/^{15}N-triple resonance probe with *z*-gradients, *IRIS-Indigo* workstation (*Silicon Graphics Inc.*, Mountain View, CA, USA) using the software UXNMR (*Bruker*). ^{31}P-NMR Spectrum of **1**: *Bruker DRX250* (^{1}H at 250.13 MHz, ^{31}P at 101.26 MHz) 5-mm QNP (^{13}C,^{19}F,^{31}P/^{1}H) probe; ^{1}H-decoupling; no signal, except that of the standard (orthophosphoric acid). ^{13}C-NMR and DEPT spectra as well as the ^{1}H-NMR spectrum of **2**: *Varian VXR-200* instrument at 50.3 and 200 MHz, resp.

Antimicrobial Activity. Bioautography with *Cladosporium cucumerinum* was performed on Al-backed TLC sheets [2]. Glass-backed TLC plates were used for agar overlay bioautography against *Candida albicans* [25] and *Bacillus subtilis* [3]. *MIC* values were determined using the agar dilution methodology in *LB* (*Luria-Bertani*) medium. Incubation was at 30° for 16 h (*B. subtilis* and *E. coli*) or 5 d (*Cladosporium cucumerinum*). Tests were carried out in duplicate.

N-*(3-Hydroxy-25ξ-cholest-5-en-26-oyl)*-L-*isoleucine* (**2**). Compound **1** (20 mg) was refluxed in 2N HCl for 2 h. After cooling, the mixture was extracted with AcOEt and the org. layer evaporated: 15 mg of **2**. Amorphous white powder. ^{1}H-NMR (CD_3OD): 5.24 (br. *d*, $J = 4.6$, H–C(6)); 4.39 (*d*, $J = 5.7$, H–C(α)); 3.40 (*m*, H–C(3)); 1.01 (*s*, Me(19)); 0.71 (*s*, Me(18)); other signals unresolved. ^{13}C-NMR (CD_3OD): 179.6 (C(26), COOH)[3]); 142.2 (C(5)); 122.4 (C(6)); 72.4 (C(3)); 58.2, 57.2 (C(17), C(14))[4]); 51.8 (C(9)); 43.5, 41.2 (C(4), C(12)); 43.0 (C(13));

[3]) Only one signal was observed in the carbonyl region which was tentatively assigned to both C=O.

[4]) C(α) not observed or obscured.

41.8 (C(25)); 38.6, 37.1, 36.1 (C(1), C(22), C(24)); 38.3 (C(β)); 37.7 (C(10)); 37.0 (C(20)); 33.3 (C(8)); 33.0, 32.3, 29.3 (C(7), C(2), C(16)); 26.3, 25.3, 25.0 (C(γ), C(23), C(15)); 22.2 (C(11)); 19.9, 19.3, 18.4 (C(19), C(21), C(27)); 16.3 (C(δ)); 12.3, 11.8 (C(18), C(γ')). FAB-MS (negative-ion mode, matrix glycerol): 528 ([M – H]$^-$).

REFERENCES

[1] D. E. Sonenshine, 'Tick Pheromones', in 'Biology of Ticks', Ed. D. E. Sonenshine, Oxford University Press, New York–Oxford, 1991, pp. 331–369.
[2] A. L. Homans, A. Fuchs, *J. Chromatogr.* **1970**, *51*, 327.
[3] M. O. Hamburger, G. A. Cordell, *J. Nat. Prod.* **1987**, *50*, 19.
[4] P. Godin, *Nature (London)* **1954**, *174*, 134.
[5] L. Lerner, A. Bax, *J. Magn. Reson.* **1986**, *69*, 375.
[6] J. Schleucher, J. Quant, S. J. Glaser, C. Griesinger, *J. Magn. Reson., Ser. A* **1995**, *112*, 144.
[7] E. Breitmaier, 'Structure Elucidation by NMR in Organic Chemistry', J. Wiley & Sons, New York, 1993, p. 58.
[8] W. K. Wilson, R. M. Sumpter, J. J. Warren, P. S. Rogers, B. Ruang, G. J. Schroepfer, *J. Lipid Res.* **1996**, *37*, 1529.
[9] T. Hoshita, 'Bile Alcohols and Primitive Bile Acids', in 'Sterols and Bile Acids', Eds. H. Danielsson and J. Sjövall, Elsevier Science Publishers, Amsterdam, 1985, p. 279.
[10] G. R. Pettit, Y. Kamano, P. Drasar, M. Inoue, J. C. Knight, *J. Org. Chem.* **1987**, *52*, 3573.
[11] K. S. Moore, S. Wehrli, H. Roder, M. Rogers, J. N. Forrest, Jr., D. McCrimmon, M. Zasloff, *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1354.
[12] N. Fusetani, M. Takahashi, S. Matsunaga, *Tetrahedron* **1994**, *50*, 7765.
[13] T. Nakatsu, R. P. Walker, J. E. Thompson, D. J. Faulkner, *Experientia* **1983**, *39*, 759.
[14] S. Kanazawa, N. Fusetani, S. Matsunaga, *Tetrahedron* **1992**, *48*, 5467.
[15] I. Bruno, L. Minale, R. Riccio, L. Cariello, T. Higa, J. Tanaka, *J. Nat. Prod.* **1993**, *56*, 1057.
[16] L. M. Cherry, *Insect Biochem.* **1976**, *6*, 587.
[17] C. Pavis, H. Mauleon, N. Barre, M. Maibeche, *J. Chem. Ecol.* **1994**, *20*, 1495.
[18] H. Frank, G. Nicholson, E. Bayer, *J. Chromatogr. Sci.* **1977**, *15*, 174.
[19] A. Bax, D. G. Davis, *J. Magn. Reson.* **1985**, *65*, 355.
[20] C. Griesinger, G. Otting, K. Wüthrich, R. R. Ernst, *J. Am. Chem. Soc.* **1988**, *110*, 7870.
[21] L. Mueller, *J. Magn. Reson.* **1987**, *72*, 191.
[22] A. Bax, L. Lerner, *J. Magn. Reson.* **1988**, *79*, 429.
[23] G. Bodenhausen, D. J. Reuben, *Chem. Phys. Lett.* **1980**, *69*, 185.
[24] A. Bax, M. F. Summers, *J. Am. Chem. Soc.* **1986**, *108*, 2093.
[25] L. Rahalison, M. O. Hamburger, M. Monod, E. Frenk, K. Hostettmann, *Phytochem. Anal.* **1991**, *2*, 199.