Recognition of very low concentrations of ATP by *Glossina tachinoides* Westwood

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Summary. Like many other blood feeders, *Glossina tachinoides* is stimulated to gorge by the presence of ATP in its diet. A concentration of 1.3×10^{-8} M ATP induces 50% feeding. The ability of *G. tachinoides* to detect ATP is the highest recorded so far among insects.
Key words. Tsetse flies; sensitivity to ATP; diet for mass-rearing.

A successful colony of *Glossina tachinoides* was recently established in the International Atomic Energy Agency's laboratories at Seibersdorf, Austria. The flies are maintained solely on fresh-frozen, thawed blood fed through a silicon membrane in a system previously described for *Glossina palpalis palpalis*[1]. As a standard procedure 10^{-3} M ATP is added to the thawed blood to secure a high percentage of engorgement by the flies. ATP is the most expensive component of the diet and attempts to reduce the price of the diet have been directed at optimizing the economy of its use. The significance of these efforts has received added emphasis because of the need to mass-rear *G. tachinoides* for use in tsetse control employing the sterile male technique.

Materials and methods. Teneral flies, 24–48-h old, were offered a series of concentrations of ATP, ADP or AMP, dissolved in water containing 0.15 M NaCl and 0.001 M $NaHCO_3$ and presented at 37 °C through a silicon membrane. After 15 min, percentage engorgement was determined and doses inducing 50% (ED_{50}) and 85% (ED_{85}) were calculated, using the log probit computerized procedure. The ED_{50} was used to compare gustatory sensitivity of *G. tachinoides* to that of other insects, while the ED_{85} is the dose which secures an effective level of feeding required to sustain satisfactory mass-rearing dynamics. As can be seen from the table, the ED_{50} of ATP for *G. tachinoides* females is 1.3×10^{-8} M while for the male it is 1.4×10^{-7} M. This level of sensitivity of detecting ATP is the highest recorded so far for any insect. It is about 40-fold higher than that of *Glossina palpalis palpalis*, and many orders of magnitude higher than that of *Rhodnius* or mosquitoes[2].

Part of the much higher sensitivity of *G. tachinoides* as compared to earlier data on *G. austeni*[3] and *G. morsitans*[4] can probably be attributed to the more suitable membrane used, as well as to the use of sodium bicarbonate which was found recently to have a very strong synergistic effect on the gorging response to ATP by mosquitoes[5,6].

Gorging response of *Glossina tachinoides* to adenine nucleotides*

Compound	No. flies	ED_{50} (μM)	ED_{85} (μM)
Females			
ATP	240	0.013 (0.007–0.024)	0.20 (0.10–0.42)
ADP	210	0.063 (0.034–0.15)	1.30 (0.50–3.8)
AMP	135	69.5 (36.4–133)	1060 (308–3640)
None	105	27% fed	
Males			
ATP	180	0.14 (0.12–0.19)	0.56 (0.31–1.02)
None	30	7% fed	

* Figures in parentheses are 95% confidence limits.

Of the three nucleotides tested with females ATP was by far the most effective. AMP is a cheaper compound but 5000-fold higher concentration is required to replace ATP, thus the use of this compound is surely not economic.

The ED_{85} of ATP is 2×10^{-7} M for females or 3.5×10^{-7} M for both sexes; an addition of 10^{-6} M ATP to the standard diet can secure 100% feeding, and would save more than 99% of the ATP used in the current diet regime.

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Symbioramide, a novel Ca^{2+}-ATPase activator from the cultured dinoflagellate *Symbiodinium* sp.

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Summary. A novel sphingosine derivative, symbioramide, has been isolated from the laboratory-cultured dinoflagellate *Symbiodinium* sp. as a sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activator, and its structure elucidated to be **1** on the basis of spectral and chemical means.
Key words. Dinoflagellate; symbioramide; *Symbiodinium* sp.; ceramide; Ca^{2+}-ATPase activator.

Recently, ceramides, already known as constituents of nerve tissue hydrolysates of mammalians[2], were isolated from extracts of some marine organisms such as sponges[3] or green[4] or red[5] algae. During our studies on bioactive substances from marine sources[6–9], we investigated extracts of the cultured dinoflagellate *Symbiodinium* sp. isolated from the

inside of gill cells of the Okinawan bivalve *Fragum* sp. In this paper we describe the isolation and structure elucidation of symbioramide (**1**), a new sphingosine derivative which activates SR Ca^{2+}-ATPase activity[10].

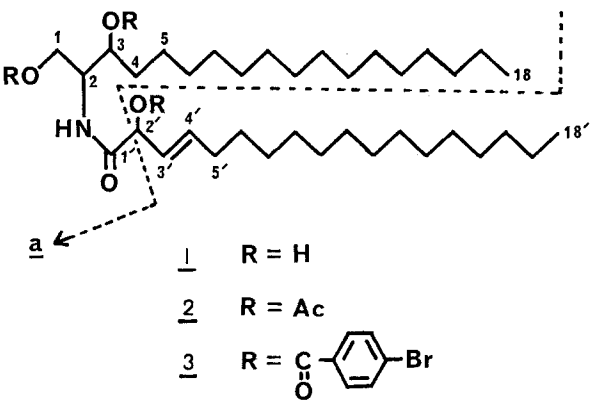
The SR Ca^{2+}-ATPase was prepared from rabbit skeletal white muscle by the method of Meissner et al.[11]. The technique of measurement of the Ca^{2+}-ATPase activity was carried out as previously described[10]. The dinoflagellate *Symbiodinium* sp. was grown unialgally in a seawater medium enriched with Provasoli's ES supplement[9] at 25 °C for four weeks. The cultured cells were harvested by centrifugation to yield 110 g of cells from 320 l of culture. The extracts of the harvested cells with methanol/toluene (3:1) were partitioned between toluene and water. The toluene soluble fraction was subjected to separation by silica-gel column chromatographies (methanol/chloroform, 5:95 and then benzene/acetone, 2:1) to give symbioramide (**1**)[12] as colorless crystals, mp 105–107 °C, $[\alpha]_D^{22}$ + 5.8° (c = 1, $CHCl_3$) in 0.026% yield (wet wt).

The IR spectrum of **1** provided evidence for alcohol (3270 cm^{-1}) and amide (1650 cm^{-1}) functionalities and the ^1H NMR spectrum indicated the presence of long aliphatic chain(s) (δ 1.28 br s, 50 H). On acetylation or *p*-bromobenzoylation, compound **1** afforded the triacetate (**2**)[13] or the tri-*p*-bromobenzoate (**3**)[14], respectively. Spin-spin decoupling experiments using the tri-*p*-bromobenzoate (**3**) suggested partial structures **A** and **B** (R = COC_6H_4Br). The ^{13}C NMR data of **2** supported the structures **A** and **B** (R = Ac). The FABMS spectra of **1**, **2**, and **3** gave $(M + H)^+$ ions at m/z 582, 708, and 1128, respectively. The EIMS spectrum of **1** afforded a characteristic ion at m/z 328 due to the fragment ion **a**. The high-resolution EIMS analysis of this fragment ion revealed the molecular composition of $C_{19}H_{38}O_3N$ (m/z 328.2860, Δ + 0.9 μm).

Acidic hydrolysis (1.2 M H_2SO_4 in 85% methanol) furnished methyl 2-hydroxyoctadec-3(*E*)-enoate[15], $[\alpha]_D^{28}$ − 16° (c = 1, $CHCl_3$), and 2(*S*)-amino-3(*R*)-hydroxyoctadecan-1-ol which was identified after acetylation as its triacetate[16], $[\alpha]_D^{22}$ + 14° (c = 0.1, $CHCl_3$). These observations led to the structure **1** for symbioramide, leaving the absolute configuration at C-2′ to be resolved.

A

B



1 R = H

2 R = Ac

3 R = C(=O)–C$_6$H$_4$–Br

The Ca^{2+}-ATPase in SR membrane plays a key role in muscle relaxation by energized Ca^{2+}-pumping from the cytoplasm into the lumen of SR[17]. Symbioramide **1** (10^{-4} M) activated SR Ca^{2+}-ATPase activity by 30%. This is the first example of SR Ca^{2+}-ATPase activator of marine origin. Symbioramide (**1**) may provide a valuable chemical tool for studies on the regulatory mechanisms of SR Ca^{2+}-pumping systems[10]. Symbioramide (**1**) also exhibited antileukemic activity against L1210 murine leukemia cells in vitro with an IC_{50} value of 9.5 μg/ml.

The α-hydroxy-β,γ-dehydro fatty acid contained in symbioramide (**1**) comes, to our knowledge, seldom from natural sources, although the ceramides of α-hydroxy fatty acids have been obtained from the sponge *Dysidea etheria*[3]. The dinoflagellate *Symbiodinium* sp. from which **1** has been isolated is a symbiont in the bivalve *Fragum* sp. The ceramides obtained from the sponge *D. etheria* may be of microbial origin[3], since sponges are known to possess symbiotic microorganisms such as dinoflagellates, cyanophytes, or bacteria.

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12 Colorless crystals, mp 105–107 °C; $[\alpha]_D^{22}$ + 5.8° (c = 1, $CHCl_3$); IR (KBr) 3270, 1650, 1535, 1470, and 1070 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.89 (6H, t, J = 7.0 Hz; H_3-18 and H_3-18′), 1.28 (50H, br s), 2.10 (2H, q, J = 7.3 Hz; H_2-5′), 3.81 (3H, m; H-1, H-2, and H-3), 4.05 (1H, d, J = 11.3 Hz; H′-1), 4.54 (1H, d, J = 7.3 Hz; H-2′), 5.57 (1H, dd, J = 15.3 and 7.3 Hz; H-3′), 5.92 (1H, dt, J = 15.3 and 7.3 Hz; H-4′), and 7.01 (1H, d, J = 7.2 Hz; NH); FABMS (positive ion, thioglycerol as a matrix) m/z 582 $(M + H)^+$; EIMS m/z 328 (fragment **a**), 310, 284, 270, and 253; Found m/z 328.2860, Calcd for $C_{19}H_{38}O_3N$: fragment **a**, 328.2851.

13 White solids, mp 75–78 °C; ^1H NMR ($CDCl_3$) δ 0.89 (6H, t, J = 6.9 Hz; H_3-18 and H_3-18′), 1.26 (50H, br s), 2.05 (3H, s; Ac), 2.08 (3H, s; Ac), 2.18 (3H, s; Ac), 4.04 (1H, m; H-1), 4.32 (1H, m; H′-1), 4.36 (1H, m; H-2), 4.91 (1H, m; H-3), 5.49 (1H, d, J = 7.2 Hz; H-2′), 5.53 (1H, m; H-3′), 5.91 (1H, dt, J = 14.5 and 6.7 Hz; H-4′), and 6.55 (1H, d, J = 8.8 Hz; NH); ^{13}C NMR ($CDCl_3$) δ 14.00 q (2C), 20.59 q, 20.83 q, 20.89 q, 22.58 t, 25.28 t, 28.61 t, 29.14 t, 29.38 t, 29.61 t (19C), 31.42 t, 31.83 t, 32.24 t, 50.68 d, 62.09 t, 73.86 d, 74.44 d, 122.90 d, 137.65 d, 168.44 s (2C), 169.73 s, and 170.67 s; FABMS (positive ion, thioglycerol as a matrix) m/z 708 $(M + H)^+$; EIMS m/z 412 (fragment **a**), 370, 352, and 310; Found m/z 412.3078. Calcd for $C_{23}H_{42}O_5N$: fragment **a**, 412.3063.

14 White solids, mp 57–59 °C; ^1H NMR ($CDCl_3$) δ 0.88 (6H, t, J = 6.9 Hz; H_3-18 and H_3-18′), 1.25 (50H, br s), 1.76 (1H, m; H-4), 1.86 (1H, m; H′-4), 2.05 (2H, q, J = 7.1 Hz; H_2-5′), 4.44 (1H, dd, J = 11.5 and 4.0 Hz; H-1), 4.55 (1H, dd, J = 11.5 and 5.6 Hz; H′-1), 4.60 (1H, m; H-2), 5.24 (1H, m; H-3), 5.64 (1H, dd, J = 15.4 and 7.1 Hz; H-3′), 5.75 (1H, d, J = 7.1 Hz; H-2′), 5.98 (1H, dt, J = 15.4 and 7.1 Hz; H-4′), 7.04 (1H, d, J = 8.6 Hz; NH), 7.40, 7.49, 7.52, 7.60, 7.71, and 7.86 (each 2H, d, J = 8.5 Hz; aromatic protons); FABMS (positive ion, thioglycerol as a matrix) m/z 1134, 1132, 1130, and 1128 $(M + H)^+$.

15 Colorless oil; $[\alpha]_D^{28} - 16°$ ($c = 1$, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.81 (3H, t, $J = 6.9$ Hz; H_3-18'), 1.18 (24H, br s), 1.99 (2H, q, $J = 7.2$ Hz; H_2-5'), 2.77 (1H, d, $J = 5.9$ Hz; OH-2'), 4.54 (1H, t, $J = 5.9$ Hz; H-2'), 5.43 (1H, dd, $J = 15.3$ and 5.9 Hz; H-3'), and 5.81 (1H, dt, $J = 15.3$ and 7.2 Hz; H-4'); EIMS m/z 253 $(M - COOMe)^+$.

16 White crystals, mp 92–94 °C [lit.[3] mp 100.5–101 °C]; $[\alpha]_D^{22} + 14°$ ($c = 0.1$, $CHCl_3$) [lit.[3] $[\alpha]_D + 15.7°$ ($c = 0.86$, $CHCl_3$)]; ^1H NMR ($CDCl_3$) δ 0.89 (3H, t, $J = 6.9$ Hz; H_3-18), 1.27 (26H, br s), 1.60 (2H, m; H_2-4), 2.10 (3H, s; Ac), 2.07 (3H, s; Ac), 2.08 (3H, s; Ac), 4.07 (1H, dd, $J = 11.6$ and 3.9 Hz; H-1), 4.25 (1H, dd, $J = 11.6$ and 6.1 Hz; H'-1), 4.40 (1H, m; H-2), 4.91 (1H, m; H-3), 5.84 (1H, d, $J = 9.0$ Hz; NH); EIMS m/z 428 (M + 1), 368, 294, and 144.

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Use of oven-dried blood for in vitro feeding of tsetse flies

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Summary. Comparison of the survival, fecundity and offspring size of *Glossina palpalis palpalis* females fed reconstituted oven-dried blood, fresh, frozen/thawed, or reconstituted freeze-dried blood showed that oven-drying at 45 °C does not diminish the nutritional quality of blood. The significance of this finding is discussed with a view to optimizing costs and conditions of blood-diet storage and transportation in the context of mass-rearing of tsetse flies.
Key words. Tsetse flies; blood feeding; oven-dried blood; mass-rearing.

Mass-rearing of tsetse flies, for release in tsetse control programs employing the Sterile Insect Technique (SIT), is dependent on the availability of a suitable source of food for the flies. Laboratory colonies of tsetse flies have so far been fed either in vivo on a variety of host animals[1] or in vitro on various types of blood presented underneath suitable types of membranes[2,3].

The use of fresh blood for in vitro feeding of tsetse flies is hampered by limitations imposed by storage costs and nutritional quality deterioration and necessitates a regular supply of fresh blood. An added complication is the variable nutritional quality arising from genetic, environmental and physiological effects on blood composition and from the influence of chemical, physical and microbiological agents on blood during collection, handling and storage. Therefore routine quality control tests are conducted to ascertain the suitability of particular batches of blood before use in feeding flies in the main colony. The fulfilment of this requirement has been made possible through increasing the shelf-life of blood by a variety of methods. Wetzel and Luger[4] were the first to use blood, stored deep-frozen, for in vitro feeding of tsetse flies. Later Wetzel[5] investigated freeze-drying of blood as a further improvement in conditions of storage and transportation of blood suitable for feeding tsetse flies in large numbers.

Freezing and freeze-drying involve high operational costs and require a reliable electricity source. In the current study we investigated the use of low-temperature oven-drying as a cheaper method of preparing dried blood suitable for in vitro feeding of tsetse flies.

Material and methods. Bovine and porcine blood were collected by venipuncture from animals at a local abattoir and defibrinated by mechanical agitation during collection[3]. Each batch and type of blood was used to prepare four different diets: 1) Fresh defibrinated bovine (FBB) or porcine (FPB) blood was dispensed into capped vials in volume aliquots sufficient for one day's feeding, irradiated (1 KGy) and stored at 4 °C until used for feeding. 2) Fresh defibrinated blood was dispensed as described above, irradiated (1 KGy) and stored at – 20 °C until used. Just before feeding, vials containing frozen bovine (FFBB) or porcine (FFPB) were thawed out in warm water (ca 40 °C). 3) Defibrinated bovine or porcine bloods were each freeze-dried using the procedure described by Wetzel[5]. The freeze-dried bloods were stored vacuum-sealed in aluminium bags at room temperature. To prepare the diets appropriate amounts of freeze-dried bovine (FdBB) or porcine (FdPB) blood were weighed and added to distilled water (23.25%, W/V). The rehydrated bloods were dispensed into stoppered vials, irradiated (1 KGy) and stored (up to 45 days) at 4 °C until used in feeding tests. 4) Blood to be oven-dried was mixed (1:4, V/V) with distilled water and dispensed into 250-ml glass petri dishes. This dilution procedure decreased the viscosity of blood and prevented the formation of a film of hard-dried material which would insulate and conceal undried blood underneath. The petri dishes containing the diluted blood were placed in an oven equipped with an air fan and the samples were dried at 45 °C. The blood took 30–40 h to dry. The dried product was stored in aluminium bags until required. To prepare diets appropriate amounts of the oven-dried bovine (OdBB) or porcine (OdPB) blood were weighed and mixed (23.25%, W/V) with distilled water. The rehydrated blood was dispensed into stoppered vials, irradiated (1 kGy) and stored at 4 °C until used for feeding.

Flies and feeding tests. Freshly emerged *Glossina palpalis palpalis* females were obtained from a stock colony routinely maintained at this laboratory under conditions described by Van der Vloedt[6]. Each diet was supplemented with ATP (10^{-3} M) and tested on 30 newly emerged females in three

Performance of *G. p. palpalis* fed different types of whole blood diets.*

Diet	% Survival		Pupae produced per female		Mean puparial weight
	25 days	35 days	25 days	35 days	(mg) ± SD
Bovine blood					
FBB	95.0	93.4	0.54	1.52	30.54 ± 3.01
FFBB	83.3	73.3	0.62	1.17	27.71 ± 4.07
FdBB	98.4	85.0	0.72	1.32	29.21 ± 2.90
OdBB	93.3	81.7	0.72	1.48	29.63 ± 3.19
Porcine blood					
FPB	93.3	80.0	0.53	0.90	28.96 ± 4.11
FFPB	96.6	88.9	0.63	1.15	31.15 ± 3.26
FdPB	95.6	91.1	0.55	1.51	25.72 ± 4.19
OdPB	93.3	83.3	0.73	1.37	30.13 ± 4.08

* Abbreviations of diets FBB, FFBB, FdBB, OdBB, FPB, FFPB and OdPB are as described under "Materials and methods". Data are a mean of results obtained for 3 different batches of each type of blood.