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Kirkinine, a New Daphnane Orthoester with Potent Neurotrophic Activity from *Synaptolepis kirkii*

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Abstract: The bioassay-guided fractionation of a dichloromethane extract from the roots of *Synaptolepis kirkii* using neuronal viability as a model allowed the isolation of the new daphnane orthoester kirkinine (**1a**) as a powerful neurotrophic constituent.

Daphnane diterpenoids are typical constituents of plants from the families Thymelaeaceae and Euphorbiaceace.[1] Early studies on these compounds focused on their powerful irritant and tumor-promoting activities,[1] but further investigations have disclosed a much wider and still largely untapped biological potential. Thus, resiniferatoxin from *Euphorbia resinifera* Berg. is a potent vanilloid in current clinical development to treat bladder hyper-reflexia and diabetic neuropathy,[2] while maprouneacin from *Maprounea africana* Muell. Arg. shows substantive *in vivo* antihyperglycemic activity[3] and yuanhuacin from *Daphne genkwa* Sieg. et Zucc. has been evaluated clinically as an abortifacient.[4] The use of daphnane diterpenoid-containing plants for a variety of conditions, including neurological problems,[5] prompted us to include them in an ongoing screening of plant extracts for neurotrophic activity. A dichloromethane extract from the roots of *Synaptolepis kirkii* Oliv. (Thymelaeaceae) showed potent activity in this assay and was further processed according to a bioassay-guided fractionation scheme. *S. kirkii* is a tropical plant endemic to southeastern Kenya and northeastern Tanzania, where a decoction from its roots is used against snakebites and for the management of epilepsy.[5,6] Previous studies led to the isolation of numerous phorbol and related esters,[7−9] some of which showed powerful skin irritancy as well as antineoplastic and immunostimulating activities.[8,10] We report here on the structure of kirkinine (**1a**), a new daphnane-type constituent endowed with potent neurotrophic activity.

Kirkinine (**1a**) was obtained as a colorless gum. LCMS provided the exact mass at *m*/*z* 673.3811 (calcd *m*/*z* 673.3952 $[M + H]^+$), suggesting $C_{38}H_{56}O_{10}$ as the molecular formula. The ^{13}C NMR ($CDCl_3$) spectrum showed 38 signals, sorted out through DEPT experiments into five methyls, 14 methylenes, 10 methines, and nine quaternary carbons. Carbonyl signals at δ 209.50 and 169.66 ppm were assigned to a ketone and an acetate function, respectively, in accordance with the presence of IR absorption bands at 1702 and 1739 cm^{-1} and an acetyl singlet at δ 1.99 in the ^1H NMR spectrum. Based on the comparative analysis of the ^1H and ^{13}C NMR spectra, the six olefinic carbons could be combined into an enone system, an exomethylene unit, and a disubstituted *E* double bond. As to the oxygenated carbons, a diagnostic low-field resonance (δ 117) suggested the presence of an orthoester group, a common structural feature within the daphnane diterpenoids.[1] The four oxymethine protons showed the pattern typically observed in 12-hydroxydaphnetoxin-type daphnanes [singlets for H-5

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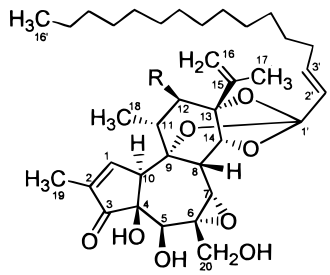
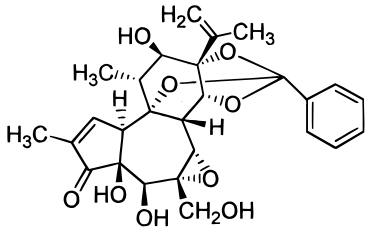


1a R= OAc
1b R= H



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(δ 4.26), H-7 (δ 3.55), and H-12 (δ 4.97), a narrow doublet for H-14 (δ 4.74, d, J = 2.6 Hz)],[1] strongly suggesting that **1a** is an analogue of 12-hydroxydaphnetoxin (**2**) having the 12-hydroxyl-acetylated and the phenyl group of the orthoester moiety replaced by an aliphatic chain. The virtual zero coupling between H-11 and H-12 is highly diagnostic of orthoester-type daphnanes having a β-oxygen function at C-12. In their C-12 epimeric compounds[3] as well as in C-12 oxygenated tiglianes[11] and resiniferonol-type daphnanes,[4] $J_{11,12}$ is instead large (8−11 Hz).

2D NMR spectra, including COSY, HMQC, and HMBC experiments, confirmed these data, identified the aliphatic orthoester moiety of **1a** as *E*-2-hexadecenoate, and allowed the unambiguous assignments of all quaternary carbons of the daphnane core. Diagnostic correlations with the orthoester carbon and the protons of the disubstituted olefin bond showed that these functionalities were adjacent to one another, while C-9 and C-4 (δ 78.2 and 72.1, respectively) could be differentiated on the basis of an HMBC correlation with the signal of H-14 (δ 4.74). Kirkinine (**1a**) is closely related to synaptolepsis factor K_7 (**1b**),[7] from which it differs only by the presence of an additional acetoxyl group at C-12.

When assayed in primary cultures of chick embryo dorsal root ganglion (DRG) neurons, kirkinine (**1a**) showed strong neurotrophic activity, promoting neuronal survival in a concentration-dependent fashion (Table 2). The potency observed was comparable to that of NGF (nerve growth factor). Compounds with neurotrophic activity are known,[12] but kirkinine (**1a**) is remarkable for its potency and structural diversity when compared with all other compounds endowed with NGF-like activity described so far.

Certain phorbol esters have been reported to support neuronal survival via a PKC-mediated mechanism,[13,14] and certain daphnane orthoesters can also activate this enzyme.[15] However, notwithstanding the presence of a common pharmacophore, specific ligands can differentially activate PKC subpathways and induce unique patterns of pharmacological responses.[16] Further studies on kirkinine will therefore be pivotal in order to define the structural elements and mechanism(s) underlying its remarkable neurotrophic activity and to better evaluate its biological potential.

Table 1. NMR Data of Kirkinine (**1a**)[a]

position	δ_C	δ_H	HMBC (H→C)
1	160.5 s	7.58 dd (1.8, 2.4)	2, 4, 10, 19
2	136.8 s		
3	209.5 s		
4	72.2 s		
5	72.0 s	4.26 s	3, 4, 6, 7
6	60.4 s		
7	64.2 d	3.55 s	5, 6, 8, 9, 14, 20
8	35.3 d	3.51 d (2.6)	6, 7, 9, 11, 14, 2'
9	78.2 s		
10	47.4 d	3.82 t (2.9)	1, 2, 9, 19
11	44.0 d	2.37 q (7.3)	9, 10, 12, 13, 18
12	78.8 d	4.97 s	9, 11, 13, 14, 18, O*C*OCH$_3$
13	83.5 s		
14	80.4 d	4.74 d (2.6)	7, 9, 15, 1'
15	143.1 s		
16	113.3 t	5.01 s	13, 15, 17
		4.95 s	
17	18.7 q	1.83 s	13, 15, 16
18	18.2 q	1.29 d (7.3)	9, 11, 12
19	9.9 q	1.79 dd (1.5, 1.1)	1, 2, 3
20	65.1 t	3.87 dd (12.6)	5, 6, 7
1′	116.7 s		
2′	122.8 d	5.61 d (15.6)	1′, 4′
3′	137.2 d	6.27 dt (15.6, 6.8)	1′, 2′, 4′, 5′
4′	31.9 t	2.07 m	2′, 3′, 5′, 6′
5′	28.2 t	1.40 dd (14.3, 7.3)	4′, 6′
6′−13′	29.7 t	1.25 m	
	29.7 t	1.25 m	
	29.6 t	1.25 m	
	29.6 t	1.25 m	
	29.5 t	1.25 m	
	29.4 t	1.25 m	
	29.3 t	1.25 m	
	29.3 t	1.25 m	
14′	31.8 t	1.25 m	
15′	22.7 t	1.25 m	
16′	14.1 q	0.88 t (6.8)	14′, 15′
O*C*OCH$_3$	169.7 s		
OCOC*H*$_3$	21.1 q	1.99 s	12, O*C*OCH$_3$

[a] δ Values in ppm were determined at 400 (^{1}H) and 100 (^{13}C) MHz in $CDCl_3$; δ_H and δ_C refer to $CHCl_3$ at 7.26 ppm and $CDCl_3$ at 77.0 ppm, respectively. J values in Hz are given in parantheses.

Table 2. Neurotrophic Activity of Kirkinine (**1a**) in DRG Culture

	concentration (nM)		
	7000	700	70
% max NGF activity	142	103	57

Experimental Section

General Experimental Procedures. The ^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC NMR spectra were obtained with an Avance 400 apparatus (Bruker). Mass spectra were measured with a LCT (Micromass), consisting of an Alliance 2690 LC (Waters) and an orthogonal accelerated time-of-flight MS analyzer, using a Z-spray interface and operated in positive-ion electrospray mode and controlled with Masslynx 3.3 software. Optical rotations were determined on an AA-10 automatic polarimeter (Optical Activity Ltd., Cambridge, UK). IR spectra were measured with a Nicolet Impact 410 FT-IR spectrometer (Thermo Optec, Madison, WI). Preparative scale LC was executed with a PROCHROM system (PROCHROM, Champigneulles, France), packed with Si gel 60 (20−45 μm, Amicon) at 60 bar. Preparative HPLC was carried out on a Hypersil BDS C_{18} (8 μm) column, controlled by Gilson UniPoint software to a Waters 3000 pump, a Gilson 402 syringe pump, 233 XL on-line column switching, and two Gilson 202 fraction collectors. All precoated TLC plates for both normal and reversed phase and the solvents used for isolation and purification were from Merck, Darmstadt, Germany.

Plant Material. Roots of *Synaptolepis kirkii* Oliv. were collected at the Arabako-Sokoke forest, Gede, Kenya, in January 1995. The plant was identified by S. G. Mathenge and F. P. Mudida. A voucher herbarium specimen (No. 839-95) was deposited at the Herbarium of the Department of Botany of Ghent University. The roots were air-dried and powdered mechanically.

Extraction and Isolation. Powdered roots (42 kg) were mixed with 4 L of water for 2 h and extracted with CH_2Cl_2 at room temperature (2 × 900 L). Removal of the solvent left a waxy residue (350 g), which was dissolved in MeOH−H_2O (9:1, 13 L) and extracted with hexane (4 × 13 L). The hexane phase was evaporated, affording 250 g of dark-brown solid material. The latter was dissolved in hexane−CH_2Cl_2 (2 L), filtered (P3 Büchner glass sintered funnel), and separated by LC (2 kg of Si gel G in a 53 × 11 cm i.d. column). Elution at 500 mL/min was started with hexane−CH_2Cl_2 from 100:0 to 0:100 in 180 min, with CH_2Cl_2−MeOH from 100:0 to 90:10 in 100 min and with EtOH for 70 min. The eluates were collected every 2 min and combined into 31 fractions according to their UV profiles. Fractions 23 and 24 were pooled (15.7 g) and further purified by RP-vacuum-layer chromatography (P2 Büchner funnel, Lichroprep C_{18}, 25−40 μm, 90 g), using mixtures of H_2O−acetonitrile, pure acetonitrile, and finally THF. Fractions eluted with pure acetonitrile (440 mg) were further fractionated by HPLC (Hypersil BDS C_{18}, 8 μm, 20 × 5 cm, i.d.; acetonitrile−H_2O 80:20 to 100:0 in 40 min, standing at 100:0 for 30 min; flow rate, 40 mL/min; fraction collection, 0.33 min per tube; detection, UV 235 nm; automatic injection, <40 mL). Altogether, 9.9 mg of **1a** was obtained.

Kirkinine (1a): colorless gum, $[\alpha]^{18}_D$ +0.06° (*c* 0.26, CH_2Cl_2); IR (KBr, film) ν_{max} 3451 (OH), 2924, 2854, 1739 (OC=O), 1702 (C=O), 1629 (C=C), 1229, 991 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESMS *m/z* 673.3811 (calcd for $C_{38}H_{56}O_{10}$+H, 673.3952).

Biological Activity. Dorsal root ganglia were dissected from White Leghorn chick embryos at embryonic day 10.[17] After dissociation and pre-plating, neurons were plated at 5 × 10^4 cells/mL into poly-L-ornithine and laminine-coated multiwell 96 plates in Basal Eagle medium containing 10% fetal calf serum. Immediately after plating, nerve growth factor (100 ng/mL) and **1a** were added to cells. Two days later, neuronal viability was assessed with Calcein-AM as described previously.[18]

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