Organic & Biomolecular Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/obc

Scalarane sesterterpenes from Thorectidae sponges as inhibitors of TDP-43 nuclear factor.

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Abstract. The analysis of two Thorectidae sponges samples, *Hyrtios* sp. and *Petrosaspongia* sp., collected at Fiji Islands, led to the isolation of five new scalarane derivatives along with fifteen known compounds. Their structures were elucidated on the basis of NMR and MS spectroscopic data. The small library of natural scalarane derivatives was investigated for their ability to modulate the activity of Trans-activation response DNA-binding protein of 43 kDa (TDP-43), a key factor in several neurodegenerative conditions and the study resulted in the identifications of potent inhibitors of TDP-43 protein.

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Introduction

Since the isolation in 1972 by Fattorusso *et al.* of the first member of the scalarane family of C-25 polycyclic terpenoids, scalarin[1], more than two hundred new members have been reported from marine sources.[2] These compounds appear to be restricted to Dictyoceratida sponges, especially those of the genera *Hyrtios, Hyatella*, *Phyllospongia*, *Smenospongia* and *Spongia*. The finding of scalarane derivatives in nudibranchs[3] could be ascribed to their dietary origin. Members of the family have been found to possess a wide range of biological activity including cytotoxic[4], enzyme inhibitory activity[5-7] and farnesoid X receptor (FXR) antagonistic activities.[8] In particular, heteronemin,[9] often found as major component of the reported sponges, was found to possess remarkable pharmacological activity.[10] Recent studies revealed that heteronemin inhibited the activation of the NF-κB pathway by downregulating the proteasome and triggering caspase-dependent apoptosis in K562 cells.[11] In addition, results from our laboratory have shown that heteronemin targets TDP-43, lowering its affinity towards its cognate nucleic acids.[12]

This finding prompted us to examine the organic extracts of two sponges belonging to the family Thorectidae, *Hyrtios* sp. and *Petrosaspongia* sp. available in our laboratories in order to extend the exploration of the binding capability to TDP-43 by additional members of the scalarane family for structure activity relationship studies.

The chemical investigation of the two sponges led to the isolation of five new sesterterpenes derivatives along with several members of scalarane family.

This paper reports the structural characterization of new compounds together with the analysis of their binding capability to TDP-43 assessed by bio-physical techniques as Surface Plasmon Resonance[13] and the Amplified Luminescent Proximity Homogeneous Assay Screen (AlphaScreen)[14].

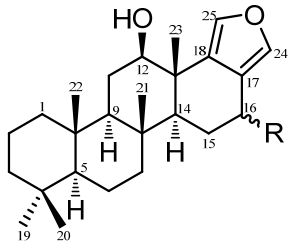
TDP-43 is a member of the superfamily of nuclear heterogeneous ribonucleoproteins (hnRNPs) and binds to specific sequences on DNA and RNA, and regulates different phases of RNA processing, including transcription, splicing, transport and stability. From a pharmacological point of view, TDP-43 shows a high tendency to aggregation and it is one of the major components of stress granules inclusions, affecting amyotrophic lateral sclerosis (ALS) and front-temporal lobar degeneration (FTLD).[15] Thus, the identification of novel compounds able to target TDP-43 and to affect its binding to DNA may lead to novel therapeutics since TDP-43-nucleic acid interaction is crucial in the development of neurological pathologies.

Results and discussion

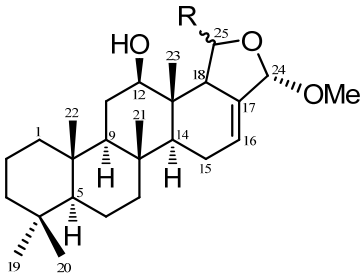
Structural studies

Sponge samples were collected using SCUBA at Fiji Islands during the 2007 field collection organized in the frame of CRISP project. The crude methanol extracts were subjected to solvent partitioning procedure, and the chloroform soluble material was fractionated by MPLC on silica gel followed by reverse phase HPLC.

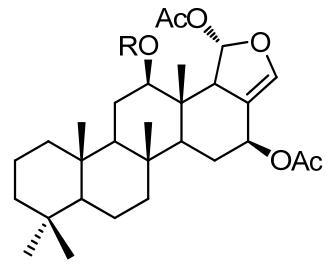
The chemical investigation of the first sample of the Fijian sponge *Hyrtios* sp. has led to the isolation of two new sesterterpenes (**1**, **2**), together with thirteen known compounds, scalarafuran (**3**),[9a,16] 12-deacetyl-*cis*-24α,25α-dimethoxyscalarin (**4**),[17] 12-deacetyl-*trans*-24α,25β-dimethoxyscalarin (**5**),[18] heteronemin (**6**),[9] heteronemin acetate (**7**),[18] 12-deacetyl-12-*epi*-deoxoscalarin (**8**),[4a] 12-*epi*-deoxoscalarin (**9**),[19] scalarolide (**10**),[16] 12-*epi*-scalaradial (**11**),[20] 12-deacetyl-12-*epi*-scalaradial (**12**),[21] 12-deacetyl-12,18-di*epi*-scalaradial (**13**),[16] hyrtiosal (**14**),[22] and methyl diacarnoate A (**15**)[23] (Figure 1). Known compounds were identified by comparison of their spectroscopic data with literature values, whereas the structures of unprecedented compounds **1** and **2**, were established as follows.



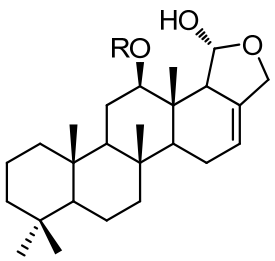
1 R= α-OAc
Scalarafuran **3** R= β-OAc



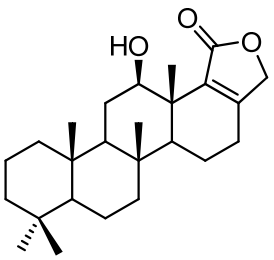
2 R= α-OH
4 R= α-OMe
5 R= β-OMe



Heteronemin **6** R=H
7 R=Ac



8 R=H
9 R=Ac



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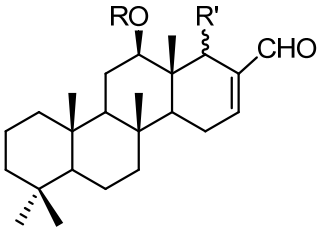
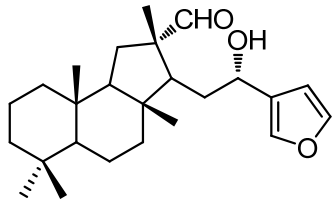
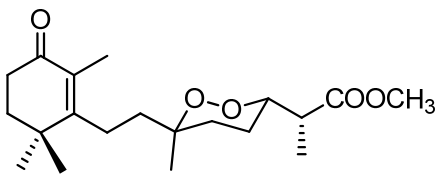


11 R=Ac R'= β-CHO
12 R=H R'= β-CHO
13 R=H R'= α-CHO



Hyrtiosal **14**



15

Figure 1. Known and new compounds (**1** and **2**) from *Hyrtios* sp.. The numbering system is referred to saturated scalarane skeleton.[2]

Compound **1** was isolated as a white amorphous solid with a molecular formula of $C_{27}H_{40}O_4$, as established from its HRESIMS. The ^{13}C NMR data revealed signals for 27 carbons (Table 1) including five methyls, seven methylenes, five methines, including two downfield signals (δ_C 64.9, d; 79.9, d). Also present four quaternary carbons, four olefinic carbons and one acyl carbonyl. The proton signals at δ_H 7.39 (br s) and 7.56 (br s) are representative of a 3,4 disubstituted furan moiety. These structural features and the chemical shifts of the remaining ^{13}C resonances (Table 1) are consistent with co-occuring scalarane terpenoids **3**-**14**, and, in particular showed a close similarity with those of scalarafuran (**3**).[9a,16] The ^1H and ^{13}C NMR data of **1** in comparison with those of **3** were almost identical except for the signal ascribed to H-16 appearing as a broad triplet (J = 2.7 Hz) at δ_H 5.88 instead of a triplet (J = 8.5 Hz) at δ_H 5.77, suggesting H-16 to be equatorial and, therefore, β-oriented. The α-orientation of the 16-acetoxy group determined a strong downfield shift of H-14 by *trans*-diaxial interaction. NOESY data and ^1H NMR constants confirmed the all-*trans* juncture of A-D rings and the β-orientation of the 12-hydroxy group. Therefore, the structure of **1** was concluded to be 16-*epi*-scalarafuran.[24]

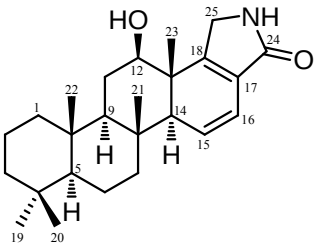
The molecular formula of **2** was deduced as $C_{26}H_{42}O_4$, on the basis of a molecular ion peak at *m/z* 419.3161 $[M + H]^+$ in the HRESIMS and ^{13}C NMR data. NMR data evidenced a close analogy with 12-deacetyl-*cis*-24α,25α-dimethoxyscalarin (**4**)[17] also present in the same collection of *Hyrtios*, and previously reported as semisynthetic[25] or natural component of sponges.[26] NMR (Table 1) and mass data indicated that one of two methoxy group in **4** was replaced by one hydroxy group in **2**. A combination of 2D NMR experiments readily identified compound **2** as the 25-hemiacetal derivative of **4**, with the same relative configurations. In particular, the chemical shift values of H-24 and H-25 (δ_H 5.10 and 5.13) were consistent with a 24,25-*cis* orientation,[17] whereas a dipolar coupling between H-25 and H_3-23 suggested an α-orientation of the hemiacetal hydroxyl group at C-25 and of the methoxy group at C-24.

Table 1. ^{1}H and ^{13}C NMR data (500 and 125 MHz, $CDCl_3$) of compounds **1** and **2**

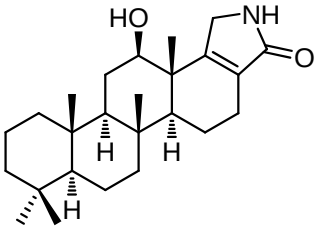
	1		**2**	
position	δ_H[a]	δ_C	δ_H[a]	δ_C
1	0.83 m	40.2	0.81 m	40.3
	1.71 m		1.71 m	
2	1.44 ovl	18.9	1.43 ovl	18.8
	1.58 ovl		1.59 ovl	
3	1.14 m	42.3	1.14 m	42.4
	1.39 m		1.36 m	
4	-	33.5	-	33.5
5	0.85 m	57.0	0.79 m	56.9
6	1.45 ovl	18.4	1.44 ovl	18.4
	1.60 ovl		1.60 ovl	
7	0.93 m	41.6	0.89 ovl	41.9
	1.82 ovl		1.66 m	
8	-	37.3	-	37.9
9	1.02 dd (12.8, 1.7)	59.1	0.90 ovl	59.3
10	-	38.0	-	37.8
11	1.53 ovl	28.3	1.42 ovl	25.7
	1.81 ovl		1.72 ovl	
12	3.69 dd (10.9, 4.7)	79.9	3.49 dd (11.0, 4.2)	81.9
12-OH			3.17 d (3.2)	
13	-	40.5	-	39.9
14	1.53 ovl	50.2	1.18 m	53.4
15	1.93 m	25.0	2.02 m	22.2
			2.10 m	
16	5.88 br t (2.7)	64.9	5.82 m	121.9
16-OCO$\underline{CH_3}$	2.05 s	21.9		
16-O$\underline{CO}CH_3$	-	171.2		
17	-	120.5	-	136.5
18	-	133.8	2.53 br s	56.9
19	0.88 s	33.6	0.84 s	33.5
20	0.84 s	21.5	0.81 s	21.6
21	0.88 s	16.6	0.85 s	16.7
22	0.91 s	17.8	0.92 s	17.5
23	1.16 s	18.8	0.81 s	9.2
24	7.39 br s	141.3	5.10 br s	103.7
25	7.56 br s	137.8	5.13 d (5.9)	105.3
24-OCH_3			3.39 s	54.4

[a]Coupling costants are in parentheses and given in hertz. ^{1}H and ^{13}C assignments aided by COSY, HSQC and HMBC experiments. Ovl: overlapped with other signals.

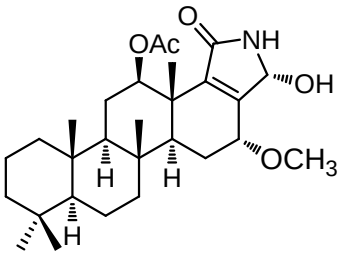
Chemical investigation of the $CHCl_3$-soluble fraction of the methanolic extract of the second sponge, *Petrosaspongia* sp. sample, has now led to the isolation of three new sesterterpenes lactams which we named petrosaspongiolactams A-C (**16**-**18**), together with five known compounds, scalarafuran (**3**),[9a,16] heteronemin (**6**),[9] hyrtiosal (**14**),[22] 16-*O*-deacetyl-16-*epi*-scalarolbutenolide (**19**),[27] and 12-*O*-acetyl-16-*O*-deacetyl-16-*epi*-scalarolbutenolide (**20**)[28] (Figure 2). Known compounds were identified by comparison of their spectroscopic data with literature values, whereas the structures of unprecedented compounds **16**-**18** were established as follows.



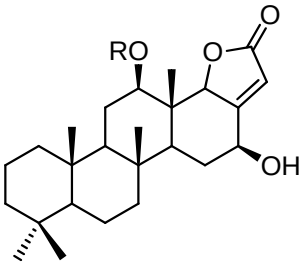
Petrosaspongiolactam A (**16**)



Petrosaspongiolactam B (**17**)



Petrosaspongiolactam C (**18**)



19 R=H
20 R=Ac

Figure 2. New (**16-18**) and known compounds from *Petrosaspongia* sp.

Petrosaspongiolactam A (**16**) had a molecular formula of $C_{25}H_{37}NO_2$ as established by HRESIMS and ^{13}C NMR data. The presence in the ^{1}HNMR spectrum of signals for five methyl singlets (δ_H 0.82, 0.85, 0.86, 0.96, and 1.03), and in the ^{13}C NMR spectrum of signals due to four methines, one of which appeared to be oxygenated, and four quaternary sp^3 carbons was indicative of a scalarane skeleton. Analysis of the NMR data also indicated the presence of an α,β conjugated acyl system (δ_C 172.7, s, 128.1, s, and 162.4, s), one disubstituted double bond (δ_H 5.97, dd, J = 9.5, 1.4 Hz, δ_C 128.9, and δ_H 6.36, dd, J = 9.5, 2.1 Hz, δ_C 118.7), one oxygen-bearing methine (δ_H 3.80, dd, J = 11.2, 3.8 Hz, δ_C 76.4), one nitrogen-bearing methylene (δ_H 4.18, s, δ_C 46.7) (Table 2). The analysis of the COSY, HSQC, HMBC, and NOESY spectra allowed to assign the ^{1}H and ^{13}C NMR signals corresponding to rings A–B–C of a scalarane-related compound bearing an hydroxy function at C-12, based on long-range correlations H_3-23/C-12 and H-11/C-12. The complete C-14/C-18/C-25 spin system containing a $\Delta^{15\text{-}16,17\text{-}18}$ conjugated diene system could be assigned from COSY data (Figure 3), that showed an allylic coupling between H-16 and H_2-25. The remaining acyl carbonyl evidenced by ^{13}C NMR spectrum and the nitrogen atom deduced by the molecular formula were arranged in a γ-lactam ring fused to C-17 and C-18 of ring D. The regiochemistry of the lactam ring was assigned on the basis of chemical shift considerations and from HMBC correlations. Indeed, the most deshielded olefin carbon (δ_C 162.4) was assigned to C-18 on the basis

of the long range correlations in the HMBC spectrum of this signal with H-12 (δ_H 3.80) and Me-23 (δ_H 0.96), and, therefore the acyl carbonyl was placed at C-24.

The ROESY correlations were in agreement with the all *trans*-fused A–B–C rings system and the axial (α) orientation of H-12 (cross peaks with H-9 and H-14) (Figure 3) which was confirmed by its coupling constants (dd, J = 11.2, 3.8 Hz).

The molecular formula $C_{25}H_{39}NO_2$, assigned to petrosaspongiolactam B (**17**) by HRESIMS, indicated that the compound is a dihydro-derivative of petrosaspongiolactam A (**16**). Analysis of the NMR data, in comparison with the parent compound **16**, clearly evidenced the absence in **17** of the olefinic signals assigned to $\Delta^{15,16}$ double bond replaced by the signals relative to two contiguous methylenes (δ_H 1.90, 1.60, δ_C 16.1, and δ_H 2.44, 2.15, δ_C 21.2) (Table 2 and Figure 3). Therefore petrosaspongiolactam B (**17**) was determined as 15,16 dihydro-derivative of petrosaspongiolactam A (**16**).

The molecular formula of petrosaspongiolactam C (**18**) was $C_{28}H_{43}NO_5$ as determined by combined HRESIMS and ^{13}C NMR analyses (Table 2). Three additional carbons, as compared to compounds **16** and **17**, were easily assigned to a methoxy group (δ_H 3.40, s; δ_C 57.7), and to one acetoxy group (δ_H 2.12, s; δ_C 21.7 and 171.3). The 2D NMR data allowed identifying a 12-acetoxy-16-methoxyscalarane framework based on the correlations of H-12 and H-16 with neighboring protons and carbons in the COSY (Figure 3) and HMBC data. A $\Delta^{17,18}$ double bond was inferred by heteronuclear long range correlations between H_3-23 at δ_H 1.20 and the quaternary olefinic carbon at δ_C 143.5 and between H-15 at δ_H 2.10 and the olefinic carbon at δ_C 153.5. The ^1H NMR spectrum also contained two mutually coupled signals relative to one exchangeable proton (δ_H 5.96) and to one carbinol proton (δ_H 5.44, δ_C 85.5). Even if, due to the paucity of the material the acyl carbonyl could not be detected by ^{13}C NMR analysis, a γ-hydroxy-γ-lactam ring fused to D ring was indirectly deduced by mass data. Furthermore, the ^{13}C chemical shifts of C-17 and C-18 indicated the location of the carbonyl at C-25 and defined the regiochemistry of the lactam, that was found to be opposite to that of petrosaspongiolactams A (**16**) and B (**17**). The axial and equatorial orientation, respectively, of H-12 [δ_H 4.96 (dd, J = 11.1, 4.5 Hz)] and H-16 [δ_H 4.00 (d, J = 3.9 Hz)] was suggested by coupling constant analysis. The NOE correlation between H-24 and H-16 revealed the β-orientation of H-24 (Figure 3). Petrosaspongiolactams A-C (**16-18**) represent further examples of the small group of nitrogen-containing scalaranes, which includes pyrrole-terpenes molliorins A-C[28,29,30] and hyatelactam.[31]

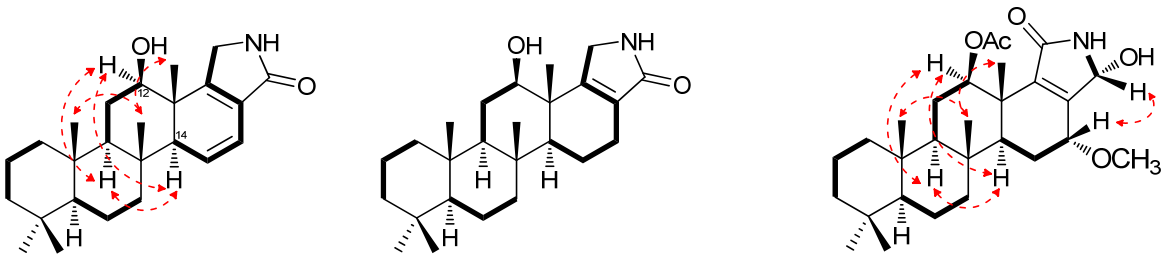


Figure 3. COSY connectivities (bold bonds) and NOESY (dashed arrows) correlations of petrosaspongiolactams A-C (**16**-**18**).

Table 2. ^{1}H and ^{13}C NMR data ($CDCl_3$) of Petrosaspongiolactams A-C (**16-18**)[a]

	16[b]		**17**[b]		**18**[b]	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.68 br d (12.5) 0.83 ovl	39.9	1.68 br d (12.4) 0.81 ovl	39.8	1.63 ovl 0.83 ovl	39.9
2	1.60 m 1.44 ovl	18.7	1.60 ovl 1.43 ovl	18.6	1.58 ovl 1.43 ovl	18.7
3	1.39 br dd (13.2, 1.5) 1.14 ddd (13.2, 13.2, 4.1)	42.2	1.38 br dd (12.8, 1.5) 1.13 ovl	42.1	1.37 br d (13.2) 1.13 ddd (13.2, 13.2, 4.1)	42.3
4	-	33.3	-	33.2	-	33.5
5	0.81 m	56.8	0.81 m	56.8	0.81 ovl	56.7
6	1.56 m 1.44 ovl	18.1	1.63 ovl 1.45 ovl	18.0	1.60 ovl 1.40 ovl	18.1
7	1.94 dt (12.7, 3.3) 0.92 m	40.9	1.85 m 0.93 m	41.3	1.81 m 0.96 m	41.7
8	-	37.5	-	37.6	-	37.0
9	0.97 br d (11.9)	58.1	0.91 ovl	58.3	1.01 m	58.0
10	-	37.4	-	37.7	-	37.6
11	1.80 br dd (12.8, 2.4) 1.51 m	28.1	1.79 br dd (12.7, 2.2) 1.53 m	28.1	1.78 br dd (12.5, 2.6) 1.54 ovl	25.4
12	3.80 dd (11.2, 3.8)	76.4	3.53 dd (11.1, 4.2)	76.7	4.96 dd (11.1, 4.5)	75.7
13	-	43.3	-	42.6	-	42.3
14	2.08 br s	56.9	1.10 m	54.6	1.44 ovl	50.3
15	5.97 dd (9.5, 1.4)	128.9	1.90 m 1.60 ovl	16.1	2.10 m 1.55 ovl	21.9
16	6.36 dd (9.5, 2.1)	118.7	2.44 m 2.15 m	21.2	4.00 d (3.9)	69.8
17	-	128.1	-	128.3	-	153.5
18	-	162.4	-	164.9	-	143.5
19	0.85 s	33.3	0.86 s	33.3	0.84 s	33.5
20	0.82 s	21.4	0.83 s	21.2	0.80 s	21.8
21	0.86 s	16.3	0.87 s	16.4	0.82 s	16.5
22	1.03 s	19.2	0.92 s	17.5	0.91 s	18.3
23	0.96 s	10.9	1.12 s	16.6	1.20 s	15.9
24	-	172.7	-	170.8	5.44 s	85.5
25	4.18 s	46.7	4.13 s	48.5	-	n.d
12-OCO$\underline{CH_3}$					2.12 s	21.7
12-O$\underline{CO}CH_3$					-	171.3
16-OCH_3					3.40 s	57.7
NH					5.96 br s	

[a] ^{1}H and ^{13}C NMR data at 400/100 MHz (**16**), 700/175 MHz (**17**) and 500/125 MHz (**18**) respectively. [b]Coupling constants are in parentheses and given in hertz. ^{1}H and ^{13}C assignments aided by COSY, HSQC, HMBC and ROESY experiments. Ovl: signals overlapped

Evaluation of TDP-43 affinity to scalarane sesterterpenes

The binding affinity between TDP-43 protein and scalarane sesterterpenes has been assessed by SPR biosensors analysis. The analysis has been achieved on all new compounds and on a large selection of the isolated known compounds.The binding of the free soluble molecules to TDP-43 was measured after protein immobilization on the CM-5 sensor chip, injecting the ligands on the enzyme modified sensor chip at different concentrations (from 10 nM to 10 μM). Figure 4 reports the sensorgrams of the most significant experiments. As expected, the increase of response units (RUs) in the association phase and the slope of the dissociation phase of the complex were clearly dependent on the analyte concentration (Figure 4).

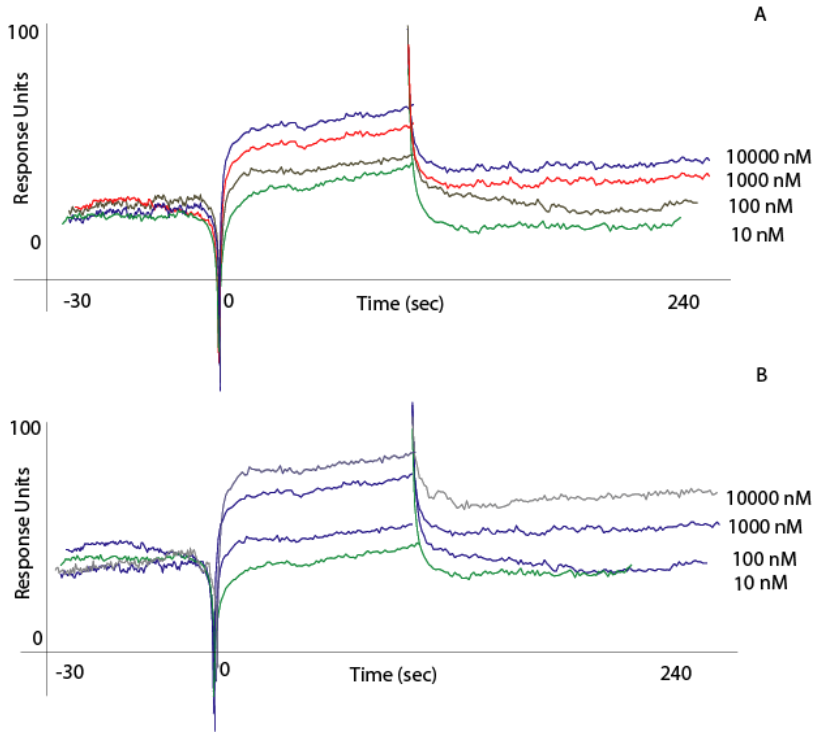


Figure 4. Panel A shows the sensorgrams gained from the injections of hyrtiosal (**14**) (0.01–10 μM from bottom to up) on TDP-43 modified chips. Panel B shows the sensorgrams gained from the injections of 12-deacetyl-12-*epi*-deoxoscalarin (**8**) (0.01–10 μM from bottom to up) on the modified chips.

On the basis of these experiments, dissociation constants (K_{DS}) have been calculated through the use of bioinformatics tools and are reported in Table 3.

Table 3. Dissociation constants (K_D) calculated for the complex between TDP-43 and natural products by SPR assays

Compound	K_D	s.d.
1	$50*10^{-6}$M	$30*10^{-6}$M
2	$2*10^{-6}$M	$16*10^{-6}$M
3	$19*10^{-6}$M	$16*10^{-6}$M
4	$4*10^{-6}$M	$1*10^{-6}$M
5	$80*10^{-6}$M	$40*10^{-6}$M
6	$0.3*10^{-6}$M	$0.1*10^{-6}$M
8	$7*10^{-6}$M	$5*10^{-6}$M
10	$22*10^{-6}$M	$8*10^{-6}$M
11	$21*10^{-6}$M	$5*10^{-6}$M
13	$16*10^{-6}$M	$12*10^{-6}$M
14	$2*10^{-6}$M	$0.6*10^{-6}$M
16	$6*10^{-6}$M	$8*10^{-6}$M
17	$29*10^{-6}$M	$22*10^{-6}$M
18	$35*10^{-6}$M	$28*10^{-6}$M
19	$9*10^{-6}$M	$3*10^{-6}$M
20	$12*10^{-6}$M	$10*10^{-6}$M

*Standard deviations have been calculated by four independent injections of natural compounds on TDP-43-containing sensor chips.

Thus, scalarane derivatives can be roughly distributed in two classes on the basis of their binding affinity towards TDP-43: high affine derivatives (K_D < 15μM) and low affine derivatives (K_D> 15μM). At the first class belongs heteronemin (**6**), compound **2,** hyrtiosal (**14**), 12-deacetyl-*cis*-24α,25α-dimethoxyscalarin (**4**), petrosaspongiolactam A (**16**), 12-deacetyl-12-*epi*-deoxoscalarin (**8**), 16-*O*-deacetyl-16-*epi*-scalarolbutenolide (**19**) and 12-*O*-acetyl-16-*O*-deacetyl-16-*epi*-scalarolbutenolide (**20**) whose mechanism of action has been examined in more details.

Analysis of the inhibition profile of TDP-43 binding to its cognate DNA mediated by scalarane sesterterpenes.

Since one of TDP-43 key functions is the binding to mRNA and DNA, thereby regulating their stability and translation, the effect of the most affine scalarane sesterterpenes on DNA-protein interaction has been deeply investigated. Indeed, gaining on the specific binding of a single strand (ss) DNA filament, called bt-TAR-32, to TDP-43[32] an Amplified Luminescent Proximity Homogeneous Assay Screening (AlphaScreen)[33] has been optimized, testing the most affine compounds reported above, Heteronemin (**6**) as positive control and petrosaspongiolactam C (**18**) as negative control.

In this experiment, TDP-43 was linked to anti-GST acceptor beads through its glutathione S-transferase (GST) tag and biotinylated (ss) DNA bt-TAR-32 was linked to streptavidin donor beads and the interaction between the counterparts has been favoured. A high AlphaScreen signal was measured due to the proximity of the mixture components allowing singlet oxygen transfer. Following, the selected scalarane sesterterpenes were pre-incubated at 25 μM with TDP-43-acceptor beads before adding ssDNA bound to donor beads, monitoring the luminescent signal.

As reported in Figure 5, compound **2**, 12-deacetyl-12-*epi*-deoxoscalarin (**8**), hyrtiosal (**14**) and petrosaspongiolactam A (**16**) showed a relevant inhibition of TDP-43 binding to its DNA target strand, comparable to Het, whereas 12-deacetyl-*cis*-24α,25α-dimethoxyscalarin (**4**), 16-*O*-deacetyl-16-*epi*-scalarolbutenolide **(19)** and 12-*O*-acetyl-16-*O*-deacetyl-16-*epi*-scalarolbutenolide (**20**) surprisingly did not affect this interaction, even if they were merely affine to TDP-43. This effect may be clarified postulating that these compounds bind TDP-43 in a protein region far from DNA binding site. As expected petrosaspongiolactam C (**18**) didn't affect protein-ligand binding.

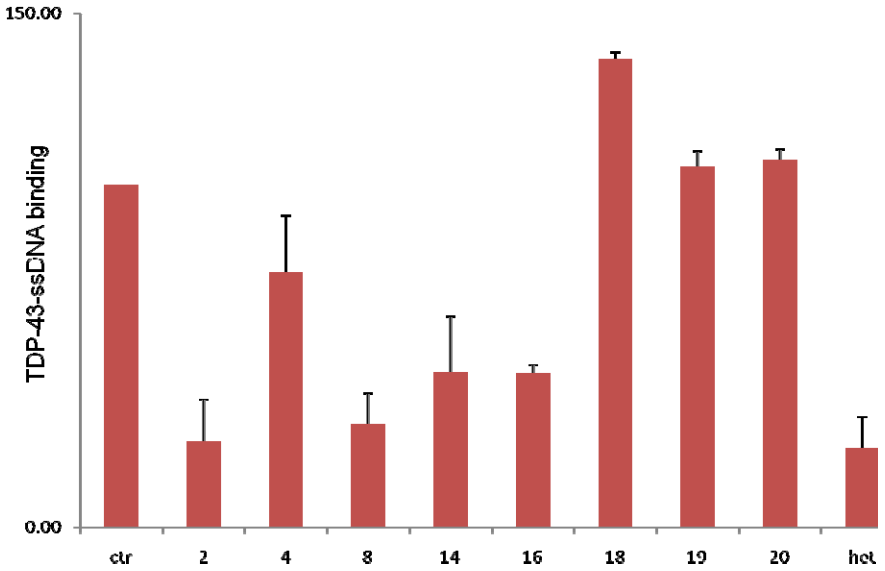


Figure 5. The histogram reports TDP-43-ssDNA binding percentage as measured by AlphaScreen assays. In the control (ctr) experiment, the binding between the counterparts has been measured and set as 100%. All the values relative to TDP-43-ssDNA binding, measured in presence of 25 μM of natural compounds, have been reported as percentage of control experiment. Standard deviation has been calculated by three independent measurements.

Then, the most active molecules were tested by AlphaScreen at several concentrations from 50 pM to 50 μM to deeply explore their inhibition profile. IC_{50} of 0.6 μM for petrosaspongiolactam A (**16**), 25 μM for hyrtiosal (**14**), 5 nM for 12-deacetyl-12-*epi*-deoxoscalarin (**8**) and 0.4 nM for compound **2** were then obtained using GraphPad Prism, revealing 12-deacetyl-12-*epi*-deoxoscalarin (**8**) and compound **2** as the most potent compounds in reducing the binding of TDP-43 to its cognate DNA (Figure 6). Since it has been reported that the inhibition of TDP-43-DNA binding promotes its cytoplasmic translocation and its tendency to aggregate[12], these compounds could be suitable as chemical probes in the study of TDP-43 aggregation state and its cellular localization.

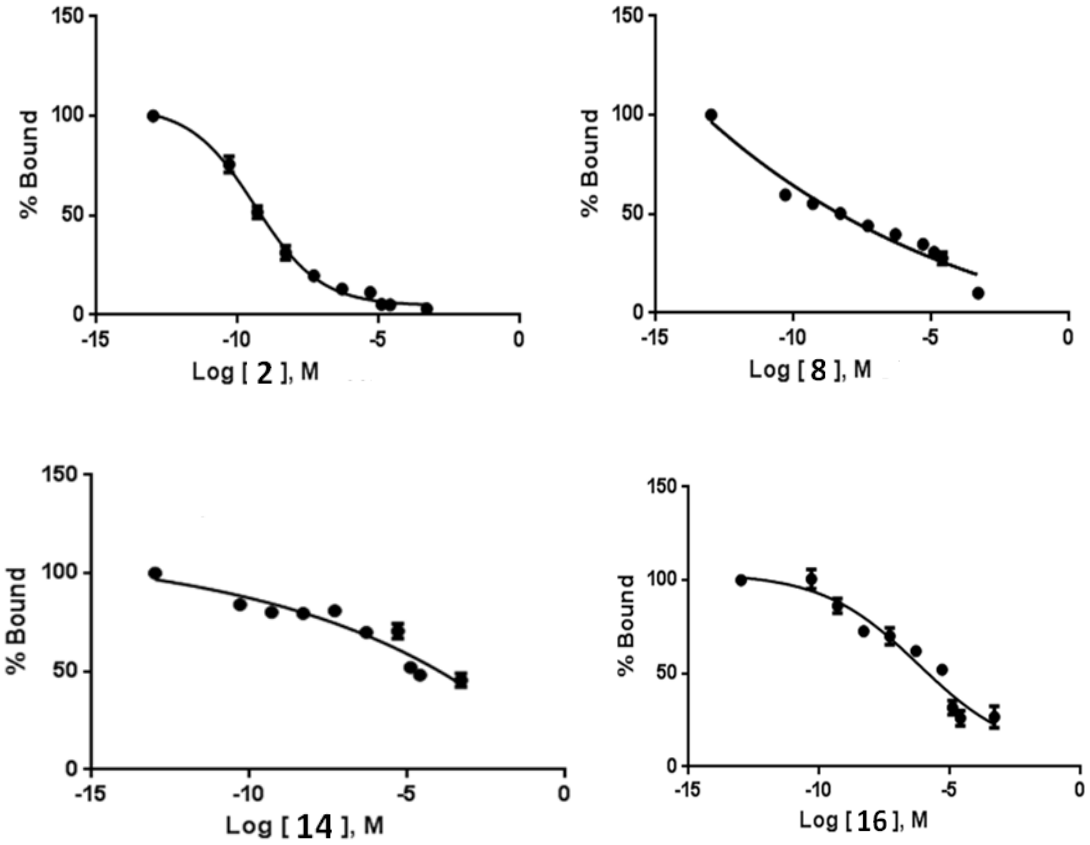


Figure 6: Inhibition of ss-DNA binding to TDP-43 by different concentrations of selected natural compounds (**2**, **8**, **14** and **16**) using AlphaScreen. Data are reported as percentage of ss-DNA bound

vs log [natural compounds]. Standard deviation has been calculated by three independent measurements.

Conclusion

Comparison of the chemical-physical data of all tested compounds relative to the binding affinity to TDP-43 and to the inhibition of the binding of TDP-43 to its DNA target strand could allow to draw some preliminary structure-activity relationship.

Previous studies on scalarane derivatives in the antitubercular and antiproliferative field indicated that the 16β-acetoxy group is a key pharmacophoric point in determining the biological activity.[10c] This group exerted a positive role also in the control of the binding of TDP-43 to its DNA target strand. Indeed, the inversion of configuration (compound **1** *vs* scalafuran **3**), the substitution with OH (compounds **19** and **20**) or with a α-methoxy group (petrosaspongiolactam C **18**) caused the loss of inhibitory properties on TDP-43. However, even important, the acetoxy group in the ring D is not essential for the reported activity, as compounds lacking this functionality were proved to retain inhibitory properties if the acetoxy group was replaced by an appropriate double bond (Δ^{16} in compounds **8** and **2** or Δ^{15} in petrosaspongiolactam A **16**) in the same ring. Moreover the tetracyclic scalarane derivatives of the scalaradial family compounds **11** and **13** were proved to show modest affinity toward the studied target, indicating the importance of an heterocyclic E ring for the inhibitory properties.

Experimental Section

General experimental procedures

Specific rotations were measured on a Perkin Elmer 243 B polarimeter. High-resolution ESIMS spectra were performed with a Micromass QTOF Micromass spectrometer. ESIMS experiments were performed on an Applied Biosystem API 2000 triplequadrupole mass spectrometer. NMR spectra were obtained on Varian Inova 400 MHz spectrometer (^{1}H at 400 MHz, ^{13}C at 100 MHz), Varian Inova 500 NMR spectrometer (^{1}H at 500 MHz, ^{13}C at 125 MHz, respectively) and Varian Inova 700 MHz spectrometer (^{1}H at 700 MHz, ^{13}C at 175 MHz, respectively) equipped with Sun hardware, δ (ppm), *J* in hertz, spectra referred to $CDCl_3$ (δ_H 7.27, δ_C 77.0) as an internal standard. Through-space ^{1}H connectivities were evidenced using a NOESY experiment with mixing times of 150 and 200 ms.

HPLC was performed using a Waters Model 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401. Silica gel (200-400 mesh) was used for flash

chromatography. The purities of compounds were determined to be greater than 95% by HPLC.

Sponge material and separation of compounds 1-15

The sponge R3308 was collected in Nagelelevu lagoon on the 19/05/2007 between 8 and 62 m deep using SCUBA and identified as *Hyrtios* sp. 2031 by Dr J. Hooper (Queensland Museum). A voucher specimen is deposited at the Queensland museum under the accessing number QM324690. The lyophilized material (138 g) was extracted with methanol (3 × 1.5 L) at room temperature and the crude methanolic extract (45.5 g) was subjected to a modified Kupchan's partitioning procedure[34] as follows. The methanol extract was dissolved in a mixture of MeOH/H_2O containing 10% H_2O and partitioned against *n*-hexane to give 6.2 g of the crude extract. The water content (% v/v) of the MeOH extract was adjusted to 30% and partitioned against $CHCl_3$ to give 11.3 g of the crude extract The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH (2.3 g).

The $CHCl_3$ extract (6.5 g) was fractionated in two runs by silica gel MPLC using a solvent gradient system from CH_2Cl_2 to MeOH.

Fraction eluted with CH_2Cl_2:MeOH 999:1 (114.6 mg) was purified by HPLC on a Nucleodur 100-5 C18 (5 µm; 10 mm i.d. × 250 mm) with MeOH:H_2O (92:8) as eluent (flow rate 3.5 mL/min) to give 4.5 mg of compound **1** (t_R=10.8 min), 29.6 mg of scalarafuran **3** (t_R=15.6 min), and 1.4 mg of compound **7** (t_R=15.9 min).

Fraction eluted with CH_2Cl_2:MeOH 995:5 (682.5 mg) was purified on a Nucleodur 100-5 C18 (5 µm; 10 mm i.d. x 250 mm) with MeOH:H_2O (90:10) as eluent (flow rate 3.5 mL/min) to give 3.7 mg of compound **15** (t_R=5.1 min), 2.6 mg of compound **13** (t_R=8.4 min), 2.0 mg of compound **11** (t_R=9 min), 111 mg of heteronemin **6** (t_R=11.1 min), 1.5 mg of scaloride **10** (t_R=15.3 min), 8.0 mg of compound **4** (t_R=16.8 min), 0.6 mg of compound **12** (t_R=30 min), 3.2 mg of compound **5** (t_R= 31.5 min), 2.1 mg of compound **2** (t_R=35.4 min).

Fraction eluted with CH_2Cl_2:MeOH 99:1 (534 mg) was purified on a Nucleodur 100-5 C18 (5 µm; 10 mm i.d. x 250 mm) with MeOH:H_2O (90:10) as eluent (flow rate 3.5 mL/min) to give 2.0 mg of compound **8 (**t_R=30 min) and 0.8 mg of compound **9** (t_R=33 min).

Compound **1**: white amorphous solid; $[\alpha]_D^{25}$ +34.7 (*c* 0.04, $CHCl_3$); ^1H and ^{13}C NMR data in $CDCl_3$ given in Table 1; ESI- MS: *m/z* 429.3 $[M+H]^+$. HRMS (ESI): calcd. for $C_{27}H_{41}O_4$: 429.3005; found 429.3009 $[M+H]^+$.

Compound **2**: white amorphous solid; $[\alpha]_D^{25}$ +157.1 (*c* 0.09, $CHCl_3$); ^1H and ^{13}C NMR data in $CDCl_3$ given in Table 1; ESI- MS: m/z 419.3 $[M+H]^+$. HRMS (ESI): calcd. for $C_{26}H_{42}O_4$: 419.3161; found 419.3170 $[M+H]^+$.

NMR data for compounds **3-15** as previously reported[16-23]

Sponge material and separation of compounds 16-20

The sponge R3244 was collected off Vanua Levu outer reef on the 12/05/2007 between 8 and 60 m deep using SCUBA and identified as *Petrosaspongia* sp. 2075 by Dr J. Hooper (Queensland Museum). A voucher specimen is deposited at the Queensland museum under the accessing number G324626.

The lyophilized material of *Petrosaspongia* sp. 2075 (263 g) was extracted with methanol (3 × 1.5 L) at room temperature and the crude methanolic extract (38 g) was subjected to a modified Kupchan's partitioning procedure[34] as previously described to obtained 3.4 g of *n*-hexane extract, 6.1 g of $CHCl_3$ extract and 3.0 g of *n*-BuOH extract. An aliquot of $CHCl_3$ extract (3.0 g) was chromatographed by silica gel MPLC using a solvent gradient system from CH_2Cl_2 to CH_2Cl_2:MeOH 1:1. Fractions eluted with CH_2Cl_2:MeOH 97:3 (558 mg) were further purified by HPLC on a Nucleodur 100-5 C18 (5 μm; 4.6 mm i.d. × 250 mm) with MeOH:H_2O (87:13) as eluent (flow rate 1.0 mL/min) to give 5.2 mg of 16-*O*-deacetyl-16-*epi*-scalarolbutenolide (**19**) (t_R=9.5 min), 6.8 mg of 12-*O*-acetyl-16-*O*-deacetyl-16-*epi*-scalarolbutenolide (**20**) (t_R=15.5 min), 60 mg of heteronemin (**6**) (t_R=24 min), 0.8 mg of hyrtiosal (**14**) (t_R=30 min) and 1.4 mg of scalarafuran (**3**) (t_R=38 min).

Fractions eluted with CH_2Cl_2:MeOH 95:5 (55 mg) were further purified by HPLC on a Nucleodur 100-5 C18 (5 μm; 4.6 mm i.d. × 250 mm) with MeOH:H_2O (85:15) as eluent (flow rate 1.0 mL/min) to give 4.8 mg of petrosaspongiolactam A (**16**) (t_R=27.5 min), 1.7 mg of petrosaspongiolactam B (**17**) (t_R=31 min), and 1.0 mg of petrosaspongiolactam C (**18**) (t_R=35 min).

NMR data for compounds **19** and **20** as previously reported.[27,28]

Petrosaspongiolactam A (**16**): white amorphous solid; $[\alpha]_D^{25}$ +14.9 (*c* 0.48, $CHCl_3$); ^1H and ^{13}C NMR data in $CDCl_3$ given in Table 2; ESI-MS: *m/z* 384.4 $[M+H]^+$. HRMS (ESI): calcd. for $C_{25}H_{38}NO_2$: 384.2903; found 384.2915 $[M+H]^+$.

Petrosaspongiolactam B (**17**): white amorphous solid; $[\alpha]_D^{25}$ +29 (*c* 0.06, $CHCl_3$); ^1H and ^{13}C NMR data in $CDCl_3$ given in Table 2; ESI-MS: *m/z* 386.3 $[M+H]^+$. HRMS (ESI): calcd. for $C_{25}H_{40}NO_2$: 386.3059; found 386.3064 $[M+H]^+$.

Petrosaspongiolactam C (**18**): white amorphous solid; $[\alpha]_D^{25}$ +8.9 (*c* 0.10, $CHCl_3$); ^1H and ^{13}C NMR data in $CDCl_3$ given in Table 2; ESI-MS: *m/z* 496.3 $[M+Na]^+$. HRMS (ESI): calcd. for $C_{28}H_{43}NNaO_5$: 496.3039; found 496.3045 $[M+Na]^+$.

TDP43–scalarane derivatives binding affinity by surface plasmon resonance

TDP43 was immobilized onto a CM5 sensor chip by using standard amine coupling procedures. 1X PBS was used as running buffer. The carboxymethyl dextran surface was activated with a 5 min

injection of a 1:1 ratio of EDC and NHS (100 mM at 5 µL/min). TDP43 was diluted to 30 ng/mL in potassium acetate (10 mM, pH 4.5) and injected onto the activated chip surface (flow rate 5 µL/min) until reaching ~15 000 RU. Remaining active groups were blocked with a 7 min injection of ethanolamine·HCl (1.0 M, pH 8.5) at 5 µL/min. For these biosensor experiments, scalarane derivatives (0.01–10 µM) were diluted in PBS containing 2 % DMSO. Each concentration was tested at least three times. Since they dissociated to baseline within a reasonable time, no regeneration was required. The interaction experiments were carried out at a flow rate of 10 µL/min over a 3 min injection time. The dissociation time was set at 600 s. Rate constant for association (ka), dissociation (kd) and the dissociation constant (K_D) were obtained by globally fitting data from all the injection of different concentrations of each compound by using BIAevaluation software (GE Healthcare) with a simple 1:1 Langmuir binding model.

Alpha-screen assays to monitor the effect of scalarane derivatives on TDP43 binding to bt-TAR32-DNA

TDP-43 with a glutathione S-transferase (GST) tag on the C-terminus was purchased from Abnova. Single-stranded DNA oligonucleotide was synthesized by LifeTechnologies and biotinylated (bt) at the 5′ end. The TAR-32 sequence is 5′-CTG CTT TTT GCC TGT ACT GGG TCT CTG TGG TT-3′ and corresponds to the first 32 nucleotides of the sequence identified by Ou et al. to bind to TDP-43. AlphaScreen GST detection kit was purchased from PerkinElmer Life Sciences. Assay mixtures contained TDP-43 at final concentration of 0.2 nM, bt-TAR-32 5 nM, 10 µg/mL of AlphaScreen streptavidin donor beads and anti-GST acceptor beads were diluted in a total volume of 40 µL assay buffer (25 mM Tris [pH 7.4], 0.1% chaps) in a 384-well plates. Assays were incubated in the dark at room temperature for 3 h to ensure the binding reaction was at equilibrium, and then the AlphaScreen signal was measured on EnSpire Alpha plate reader (PerkinElmer, Waltham, MA, USA). More in details, TDP-43 (0.2 nM) was preincubated with 20 µg/mL anti-GST acceptor beads in assay buffer for 30 min at room temperature and then added to assays containing 10 nM bt-TAR-32 also preincubated with 20 µg/mL streptavidin donor beads for 30 min at room temperature. After incubation in the dark at room temperature for 3 h to ensure the binding reaction was at equilibrium, the AlphaScreen signal was measured on a EnSpire Alpha plate reader. Different concentrations of bt-TAR-32 were tested to optimize the measurements. Then, scalarane derivatives at 25 µM and/or at variable concentration from 0.05 nM to 50 µM were preincubated with TDP-43 (0.2 nM) prebound to AlphaScreen anti-GST acceptor beads for 30 min at room temperature. DMSO concentrations were less than 1% in each sample. Assays were initiated by the addition of bt-TAR-32 at 10 nM prebound to AlphaScreen strepavidin donor beads. After

incubation in the dark at room temperature for 3 h, the AlphaScreen signal was measured. IC_{50} values were determined from nonlinear regression fits of the data in GraphPad Prism.

Acknowledgements

This work is part of the C2 component of the CRISP (Coral Reef Initiative in the South Pacific) project and granted by the Agence Française de Développement (grant CZZ 3012.01 T, AFD). We thank the Fiji Islands government for allowing us to collect in their country and K. Feussner (IAS, University of the South Pacific in Suva, Fiji) for his assistance in this project.

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