Blacpma
© 2014
Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 13 (4): 375 - 380
ISSN 0717 7917
www.blacpma.usach.cl

Artículo Original | Original Article
In memorian Professor Luis Astudillo, Universidad de Talca, Chile

Derivatives of 3H-spiro1-benzofuran-2, 1'-cyclohexanes: immunostimulants for veterinary use

[Derivados de 3H-espiro1-benzofurano-2, 1'-ciclohexanos: inmunoestimulantes de uso veterinario]

Brenda MODAK[1], Paulina TORRES[1], Carolina MASCAYANO[1], René TORRES[1], Alejandro URZÚA[1] & Beatriz VALENZUELA[2]

[1]*Departamento de Ciencias del Ambiente, Facultad de Química y Biología, &,*
[2]*Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile.*
Contactos | Contacts: **Brenda MODAK** - *E-mail address:* **brenda.modak@usach.cl**

Abstract: Four 3H-spiro1-benzofuran-2, 1'-cyclohexanes were synthesized from filifolinol, two of which are reported for the first time. Docking molecular studies were carried out to determine in silico whether these derivatives have similar immunostimulant activity to that reported for filifolinol, and its oxidation product, filifolinone. Through of the study of interactions of these compounds with the heterodimer of the protein present in teleost TLR1-TLR2, filifolinol, 3'-filifolinchloride and filifolinyl acetate shows similar interactions between them, allowing to predict that they would have similar immunostimulant activity, but different to filifolinone and filifolinane or that they would act by a different mechanisms.

Keywords: 3H-spiro1-benzofuran-2, 1'-cyclohexanes, filifolinol (1), molecular docking immunostimulant activity.

RESUMEN: Cuatro 3H-spiro1-benzofuran-2, 1'-ciclohexanos se sintetizaron a partir de filifolinol, dos de los cuales son reportados por primera vez. Se llevaron a cabo estudios de docking molecular para determinar in silico si estos derivados tienen actividad inmunoestimulante similar a la reportada para filifolinol y su producto de oxidación, filifolinona. A través del estudio de las interacciones de estos compuestos con el heterodímero de la proteína presente en teleósteos TLR1-TLR2 se estableció que el filifolinol, 3'-cloruro de filifolinilo y acetato de filifolinilo tienen interacciones similares con el heterodímero, lo que permite predecir que entre ellos tendrían una actividad similar, pero diferente a la de la filifolinona y filifolinano o que estos últimos actuarían por diferentes mecanismos.

Palabras clave: 3H-spiro1-benzofuran-2,1'-ciclohexanos, filifolinol, docking molecular actividad inmunoestimulante

Recibido | Received: May 20, 2014
Aceptado en versión corregida | Accepted in revised form: July 25, 2014
Publicado en línea | Published online: July 30, 2014
Declaración de intereses | Declaration of interests: This work was supported by Fondecyt Nº 1110295 and 1140261.
Este artículo puede ser citado como / This article must be cited as: B Modak, P Torres, C Mascayano, R Torres, A Urzúa, B Valenzuela. 2014. Derivatives of 3H-spiro1-benzofuran-2, 1'-cyclohexanes: immunostimulants for veterinary use. **Bol Latinoam Caribe Plant Med Aromat 13(4): 375 – 380.**

INTRODUCTION

Currently, the control of infectious diseases caused by viruses and bacteria that attack food-producing animals is focus on the use of antibiotics. However, the use of such drugs has generated great concern because the potential risk of emergence and spread of resistant organisms, as well as the generation of toxic waste, in products for human consumption and the environment (Rosenblatt-Farrell, 2009). Alternatively, many current researches consider the development of new pharmacological tools to stimulate the immune response.

Aquaculture is an export sector of great importance in Chile, which has been affected by major economic losses due to the occurrence of several emerging infectious diseases. These diseases can be optionally controlled using vaccines; however, one important limitation in the design of vaccines in this sector is the lack of immunostimulants and / or adjuvants to generate a good immune response.

On the other hand, the use of vaccines is limited in salmon fingerlings due to its small size and immaturity of their immune system, which prevents an adequate response. Therefore, to combat infectious diseases in salmons is necessary to develop new pharmacological tools to stimulate immune responses.

Heliotropium filifolium is a resinous bush that grows in desert environments and is characterized by the production of resin that covers their leaves and stems; as defence mechanism against adverse environmental conditions. Phytochemical studies of this species have shown that the resin is formed by minor amounts of flavonoids and large amounts of a series of unusual 3H-spiro1-benzofuran-2, 1'-cyclohexanes derivatives of mixed biosynthesis (Torres *et al.,* 1994; Urzúa *et al.,* 2001; Urzúa *et al.,* 2008).

Among the 3H-spiro 1-benzofuran-2, 1'-cyclohexanes derivatives, filifolinol (**1**), is by far the main component of this fraction. Early unpublished immunostimulant studies of filifolinol (**1**), evaluated through activation of lymphocytes in splenocytes and in dendritic cells of mouse bone marrow, showed that at a concentration of 10 mg/mL, filifolinol (**1**) was able to induce a significant increase in the percentage of dendritic cells (80%) and in lymphocytes T CD8 a 150% in relation to control.

Recent studies proposed the oxidation derivative of filifolinol (**1**); filifolinone (**2**), as a good candidate for studies of immunostimulant activity *in vivo*, since this compound increases the expression of MHC molecules class II promoting maturation of dendritic cells (Modak *et al.,* 2012). Also, the immunomodulatory effects of filifolinone (**2**) were studied *in vitro* using the SHK-1 cell line derived from leucocytes of salmon kidney head and *in vivo* in Atlantic salmon. For the study, the effect of this compound was evaluated in the expression of various cytokines. The results showed that filifolinone (**2**) increases the levels of expression of pro-inflammatory and anti-inflammatory cytokines. This suggests that filifolinone (**2**) is a potential alternative immunomodulator for veterinary uses (Valenzuela *et al.,* 2013).

In searching for new filifolinol (**1**) derivatives, with immunostimulatory activity two new compounds were synthesized. They correspond to filifolinane (**3**) and filifolinchloride (**4**) with structural variations in the C-3' of ring C. In addition, molecular docking studies were performed between toll-like receptors TLR2-TLR1 heterodimer and compounds (**1-5**),to establish a structure-immunostimulant activity relationship.

MATERIALS AND METHODS

Plant material

Heliotropium filifolium (Miers) Reiche was collected during the flowering season in Carrizal Bajo, Chile (III Region 28º 45' S, 70º 49' W) and identified by Dr. Sebastián Teiller.

Voucher specimens (ST-2214 SSCU) were deposited in the Herbarium of the Faculty of Biological Sciences of Catholic University of Chile, Santiago, Chile.

Extraction and isolation of Filifolinol (1)

The resin was obtained by immersion of the fresh plant in dichloromethane for 30-60 s at room temperature. The solvent was evaporated and the extract fractionated by column chromatography using hexane- ethyl acetate step gradient yielding filifolinol (**1**) (Urzua *et al.,* 2001).

Filifolinone (2)

Filifolinone (**2**) was obtained from filifolinol (**1**) by conventional oxidation with CrO_3 and was purified by column chromatography using benzene-ethyl acetate (Torres et al, 2002).

Filifolinane (3)

Filifolinol (**1**) (1g) dissolved in dichloromethane (5 mL) with 0.5 mL of N, N-dimethylformamide and

2.5 mL of thionyl chloride was refluxed for 1 h. The solvent was removed from the cold reaction mixture to give brown oil. After purification of the oil by column chromatography, using hexane- ethyl acetate step gradient, a pure white crystalline solid was obtained (yield, 50%). (mp 96-97ºC). $[\alpha]^{21}_{D}$: +1.09; IR (nujol) υ_{max} cm^{-1}:1716; ^{1}H-NMR see Table 1.

3'-Filifolinchloride (4)
Filifolinol (**1**) (1 g) dissolved in dichloromethane (5 mL) with 0.25 mL of pyridine was placed in a cold water bath. Thionyl chloride (2.5 mL) was added quickly and the mixture genteelly refluxed for 8 h. The cold reaction mixture was treated with 5mL of distilled water, extracted with diethyl ether and the extract was dried with anhydrous calcium chloride, filtered and the solvent removed to give a brown amorphous solid. After purification of the solid by column chromatography, using dichloromethane, a pure crystalline solid was obtained (yield, 13%) (mp 111-112ºC). $[\alpha]^{21}_{D}$: - 15.53; IR (nujol) υ_{max} cm^{-1}: 1720, 666; ^{1}H-NMR see Table 1.

***filifolinyl acetate* (5)**
Filifolinol (**1**) (2g) dissolved in 3 mL of acetic anhydride, with 0.4 mg of N, N-dimethylaminopyridine was stirred for 13 h at room temperature. The cold reaction mixture was treated with 5mL of distilled water, extracted with diethyl ether and the extract was dried with anhydrous calcium chloride, filtered and the solvent removed to give a brown amorphous solid. The solid was purified by preparative thin layer chromatography using hexane- ethyl acetate 9:1, a pure white crystalline solid was obtained (yield, 45%) (mp 127-128ºC). $[\alpha]^{21}_{D}$: - 4.69; IR (nujol) υ_{max} cm^{-1}: 1730, 1711; ^{1}H-NMR see Table 1.

Structural Determination
Melting points were determined on Kofler micro melting apparatus and are uncorrected. Optical rotations were measured on Perkin Elmer 241 polarimeter. IR spectra were obtained in Nujol on a Bruker IFS 66v instrument. ^{1}HNMR (400 MHz) spectra were recorded in $CDCl_3$ on Bruker Avance DRX400 spectrometer with TMS as internal standard. Filifolinol (**1**), filifolinone (**2**) and filifolinyl acetate (**5**) were identified by comparison of their spectroscopic data with those reported in the literature (Torres *et al.,* 2002; Urzúa *et al.,* 2008) and by TLC co-chromatography with authentic samples.

Molecular docking
All structures were built with the Gaussian view software. Restrained electrostatic potential (RESP) charges were obtained at the B3LYP/6-31G** level of theory employing the Gaussian 03 package (Frisch *et al.,* 2004) Docking studies was released between compounds **1-5** and crystal structure of Toll-like receptor TLR2-TLR1 heterodimer (PDB ID: 2Z81) at 1.8 Å of resolution was performed the AutoDock4 package (Morris *et al.,* 1998) using a Lamarckian algorithm and assuming total flexibility of 3H-spiro1-benzofuran-2,1'-cyclohexanes and partial flexibility of protein. The grid maps were made up of 60 x 60 x 60 points, with a grid-point spacing of 0.375 Å. The AutoTors option was used to define the ligand torsions and the docking results were then analyzed by a ranked cluster analysis, resulting in conformations with the highest overall binding energy (most negative-ΔG binding value). All the docking results were analyzed in VMD software (Phillips *et al.,* 2005).

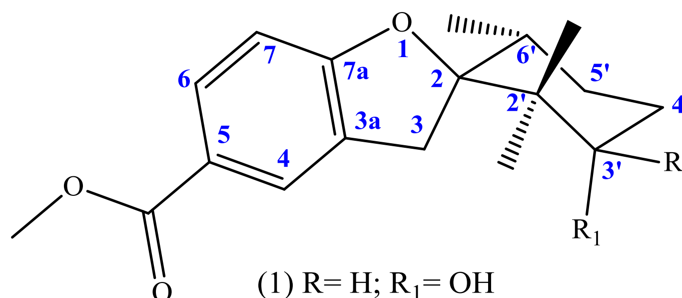
RESULTS AND DISCUSSION

Synthesis and identification of compounds
Because filifolinol (**1**) and in particular filifolinone (**2**), have shown interesting immunostimulating activity, looking to increase these activity, three compounds were synthesized with different substitution at C-3'. In order to modify the C-3'-OH of filifolinol (**1**), a reduction, chlorinated and acylated derivatives. The reduced and chlorinated compounds are reported for first time.

The IR spectra of compound **3** shows one intense peak at 1716 cm^{-1} assigned to the C=O stretching of the aromatic ester. The characteristic signal at 3500 cm^{-1} -OH in position 3' of starting compound filifolinol (**1**), is not observed indicating the substitution of the C-3'-hydroxyl group by -H. Finally, the ^{1}H-NMR spectrum (Table 1) was close similar to that of filifolinol (**1**) except for the signals of protons in C-3, shifted to lower field (δ 5.34, 5.47; J= 9.7 Hz) and a broad singlet at δ 3.01 (2H) corresponding to the protons attached to C-3'.

The IR spectrum of compound **4** showed two intense peaks at 1720 cm^{-1} assigned to the C = O stretching of an aromatic ester and 666 cm^{-1} assigned to the C-Cl stretching of an aliphatic chloride. In agreement with the replacement of the hydroxyl group for –Cl, the characteristic signal at 3500 cm^{-1} of C-3'-OH of filifolinol (**1**), was not observed. The reaction of filifolinol (**1**) with thionyl chloride and

pyridine occurs via an SN_2 mechanism (Smith, 2013) and produce inversion of the configuration at C-3’, leaving the -Cl in equatorial position, and the C-3’-H in axial position. Finally, the 1H-NMR spectrum (Table 1) was very similar to that of filifolinol (**1**), except for the signal at δ 3.69 m C-3'-H $_{(eq)}$ of filifolinol (**1**) shifted to δ 4.03 m C-3'-H$_{(ax)}$, due to the magnetic effect produced by the geminal Cl and the axial position.



(1) R= H; R_1= OH
(2) R+R_1= O
(3) R=R_1= H
(4) R= Cl; R_1= H
(5) R= H; R_1= OAc

Figure 1

Table 1
^{1}H-NMR data of compounds 1-5

C-H	(1)	(2)	(3)	(4)	(5)
C-7-H	6.70	6.76	6.75	7.28	6.72
C-6-H	7.81	7.86	7.84	6.71	7.83
C-4-H	7.79	7.79	7.80	7.84	7.81
OMe	3.84	3.86	3.86	3.86	3.86
C-3-Hα	3.62	2.78	5.47	3.47	3.48
C-3-Hβ	3.04	2.95	5.34	3.04	3.07
C-6’-H$_{(ax)}$	2.28	2.30	2.26	2.28	2.31
C-6’-Me$_{(eq)}$	0.78	0.90	0.72	0.79	0.78
C-5’-H$_{(ax)}$	1.38	1.32	1.86	1.97[a]	1.46
C-5’-H$_{(eq)}$	1.52	1.92	2.55	1.97[a]	1.51
C-4’-H$_{(ax)}$	1.61	2.72	1.56[a]	1.98[a]	1.66
C-4’-H$_{(eq)}$	1.97	2.76	1.56[a]	1.98[a]	1.91
C-3’-H$_{(eq)}$	3.69 (m)	-	3.01[b] (br s)	-	4.85 (br t, J= 2.8)
C-3’-H$_{(ax)}$	-	-	3.01[b] (br s)	4.03(m)	-
C-2’-Me$_{(eq)}$	1.45	1.34	1.22	1.22	1.22
C-2’-Me$_{(ax)}$	1.02	0.94	0.88	0.99	0.93
Ac-Me	-	-	-	-	2.31

[a] two hydrogens

Molecular docking
From the analyzed conformations, present interactions between filifolinol (**1**) and their derivatives with amino acids residues of the TLR1-TLR2 heterodimer were evaluated.

The docking results showed that filifolinol (**1**), 3'- filifolinchloride (**4**), filifolinone (**2**) and filifoly acetate (**5**) had non-covalent interactions with residues of the TLR2 segment. However, the filifolinane (**3**) interacted only with TLR1 segment. In addition, it was found that filifolinol (**1**) shown hydrogen bond between the hydroxyl and VAL-348 and π stacking with PHE-325. On the other hand, none interaction hydrogen bond or π stacking was observed between filifolinone (**2**) and TLR2 segment. The docking showed that filifolinone (**2**) had affinity with the TLR1 segment (figure 2), and the most interesting interactions were Van der Waals and π stacking with TYR-376 of TLR2 segment. Finally, 3'- filifolinchloride (**4**), filifolinone (**2**) and filifolyn acetate (**5**) , showed interactions with TLR1 segment. In addition, filifolyn acetate (**5**) showed hydrogen bond interaction between C-3'-acyl group and the hydroxyl group of PHE-325. Table 2 shows a summary of interactions found between compounds (**1-5**) and residues of the heterodimer.

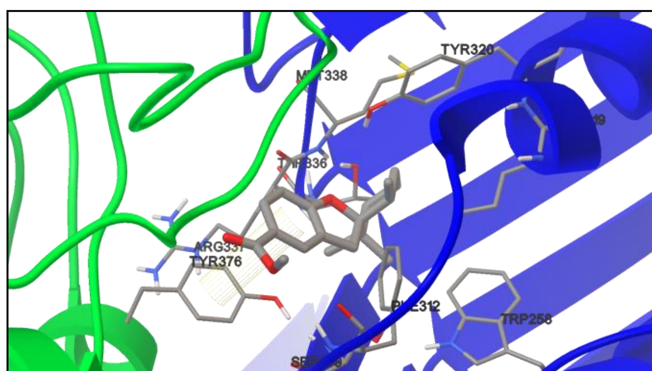


Figure 2
Main filifolinone (2)-TLR1 interactions.

Table 2
Summary of the most relevant interactions of compounds (1-5).

filifolinol(1)	filifolinone(2)	filifolinane(3)	3filifolinchloride(4)	filifolyn acetate (5)
ILE 319*	LEU 273	TRP 258**	ILE 319*	ILE 319*
PHE 325*	ASN 274	SER 309*	PHE 325*	PHE 325*
LEU 328	ASP 305	PHE 312**	LEU 328	TYR 326
LYS 347	VAL 309*	ILE 319*	VAL 343*	LEU 328
VAL 348*	LEU 312	TYR 320**	LYS 347	VAL 343*
PHE 349*	LEU 334	THR 336*	VAL 348*	VAL 348*
LEU 350*		ARG 337*	PHE 349*	PHE 349*
VAL 351		MET 338**	LEU 350*	LEU 350*.
LEU 355*		TYR 376	VAL 351	
LEU 367			LEU 359	
			LEU 365	
			LEU 367	
			TRP 386	

* TLR1 amino acid residues.
** Reported residues for TLR1-TLR2 heterodimer (Jin *et al.,* 2007)

CONCLUSION

The *in silico* ligand-receptor study shows that are common interactions between filifolinol (**1**), 3'-filifolinchloride (**4**) and filifolyn acetate (**5**) with the heterodimer TLR1-TLR2. This allows to predict that these compounds should be similar immunostimulant activity among them and different to filifolinone (**2**) or act by different mechanisms. In the same way, filifolinane (**3**), would have a totally different activity of filifolinol (**1**) and their derivatives.

ACKNOWLEDGEMENTS

This work was supported by Fondecyt Nº 1110295 and 1140261.

REFERENCES

Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Montgomery Jr J, Vreven T, Kudin KN, Burant JC, Millam JM, Iyengar SS, Tomasi J, Barone V, Men nucci B, Cossi M, Scalmani G, Rega N, Pe tersson GA, Nakatsuji H, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H, Klene M, Li X, Knox J E, Hratchian HP, Cross J B, Bakken V, Adamo C, Jaramillo J, Gomperts R, Stratmann RE, Yazyev O, Aus tin AJ, Cammi R, Pomelli C, Ochterski JW, Ayala PY, Morokuma K, Voth GA, Salvador P, Dannenberg JJ, Zakrzewski VG, Dapprich S, Daniels AD, Strain MC, Farkas O, Malick DK, Rabuck AD, Raghavachari K, Foresman JB, Ortiz JV, Cui Q, Baboul AG, Clifford S, Cioslowski J, Stefanov BB, Liu G, Liashenko A, Piskorz P, Komaromi I, Martin RL, Fox DJ, Keith T, Al-Laham MA, Peng CY, Na nayakkara A, Challacombe M, Gill PMW, Johnson B, Chen W, Wong MW, Gonzalez C, Pople JA. 2004. **Gaussian 03, Revi sion C.02**, Gaussian, Inc., Wallingford CT, USA.

Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, Lee, H, Lee JO. 2007. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptides. **Cell** 130: 1071 - 1082.

Modak B, Valenzuela B, Imarai M, Torres R. 2012. *In vitro* immunostimulant activity of the aro matic geranyl derivative filifolinone. **Bol Latinoam Caribe Plant Med Aromat** 11: 285 - 290.

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ. 1998. Automated docking using a lamarckian genetic algorithm and empirical binding free energy function. **J Comp Chem** 19: 1639 - 1662.

Phillips JC, Braun R, Wang W, Gumbart J, Taj khorshid E, Villa E, Chipot C, Skeel RD, Kale L, Schulten K. 2005. Scalable molecular dynamics with NAMD. **J Comp Chem** 26: 1781 - 1802.

Rosenblatt-Farrell N. 2009. El paisaje de la resisten cia de antibióticos. **Environ Health Perspect** 117: 244 - 250

Smith MB. 2013. **March's Advanced Organic Chemistry Reactions, Mechanisms, and Structure**, 7º Ed. John Wiley & Sons, Inc., Hoboken, New Jersey, USA.

Torres R, Villarroel L, Urzúa A, Delle Monache G, Delle Monache E, Gaics-Baitz E. 1994. Filifolinol, a rearranged geranyl aromatic derivative from the resinous exudates of *Heliotropium filifolium*. **Phytochemistry** 36: 249 - 250.

Torres R, Modak B, Urzúa A, Delle Monache F, Damonte E, Pujol C. 2002. Propiedades antivirales de compuestos naturals y semi-sintéticos de la resina de *Heliotropium filifolium*. **Bol Soc Chil Quim** 47: 259 - 263.

Urzúa A, Echeverría J, Rezende MC. Wilkens M. 2008. Antibacterial Properties of 3 H-Spiro[1-benzofuran-2,1'-cyclohexane] Derivatives from *Heliotropium filifolium*. **Molecules** 13: 2385 - 2393.

Urzúa A, Modak B, Torres R. 2001. Identification of the new aromatic geranyl derivative in the resinous exudates of *Heliotropium filifolium* (Boraginaceae). **Bol Soc Chil Quim** 46: 175 - 178.

Valenzuela B, Imarai M, Torres R, Modak B. 2013. Immunomodulatory effects of the aromatic geranyl derivative filifolinone tested by the induction of cytokine expression. **DCI** 41: 675 - 682