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Novel sesquiterpenoids as tyrosine kinase inhibitors produced by *Stachybotrys chortarum*

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Abstract—In the course of screening for small-molecule inhibitors to Tyrosine kinase receptor seven novel K-76 derivatives (**1**–**7**) have been isolated from the fungal culture of *Stachybotrys chortarum*. The structures were elucidated by extensive mono- and bi- dimensional spectroscopy and mass spectrometry.

1. Introduction

Many diseases are characterized by persistent and unregulated angiogenesis. The recognition of the involvement of angiogenesis in major diseases has been accompanied by research to identify and develop angiogenesis inhibitors. Angiogenesis occurs in many stages generally classified in response to discrete targets in the angiogenesis cascade. Literature reports indicate that inhibitors of angiogenesis, working by diverse mechanisms, are beneficial in diseases such as cancer and metastasis,[1,2] ocular diseases,[3] arthritis[4,5] and hemangioma.[6]

In recent years, it has become clear that while angiogenesis is a complex multicellular phenomena, specific ligands and their receptors play a key role. In particular a combination of studies suggest that the Tie2 receptor (*T*yrosine kinase receptors with *i*mmunoglobulin and *E*GF homology domains) and its ligand are important in angiogenesis.

Based on the importance of Tie2 receptors, the inhibition of Tie2 activity is predicted to interrupt angiogenesis, providing disease-specific therapeutic effects. Recently, Lin et al.[7] have shown that exogenously administered soluble Tie2 receptor inhibited angiogenesis and cancer growth in animal models. Clearly, there is a need to develop potent inhibitors of the Tie2 receptor activity which will have sufficient activity to work in vivo at therapeutically acceptable concentrations.

In an effort to find new naturally occurring Tie2 inhibitors, we extended our search and found that extracts of *Stachybotrys chortarum* inhibited Tie2 receptor. Subsequent bioactivity-direct fractionation resulted in the isolation of a family of sesquiterpenoids of the triprenyl phenol type (**1**–**7**) with different side chains as the Tie2 principles. In this report, we describe the isolation of seven new K-76 derivatives, the elucidation of their structures and their biological activities as inhibitors of tyrosine kinase receptor from the cultured medium of *S. chortarum*.

2. Results and discussion

A fungus, taxonomically classified as *S. chortarum*, was isolated from a soil sample collected in Himalaya (India). This *S. chortarum* was grown in beef extract liquid culture. After 8 days of incubation, the fermentation broth was centrifuged and a portion of the mycelium cake was extracted with acetone. The acetone was evaporated and the remaining aqueous portion was extracted first with

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CH_2Cl_2 and then with ethylmethylketone. The organic solvent of this last extract was concentrated in vacuum to give a residue (2.6 g), whose HPLC indicated the presence as major components of K-76 and stachybotrydial previously isolated in our laboratories.

Bioassay and HPLC-guided fractionation of the ethylmethylketone extract by Biotage Flash 40 using a Silicagel cartridge afforded an active fraction (90 mg).

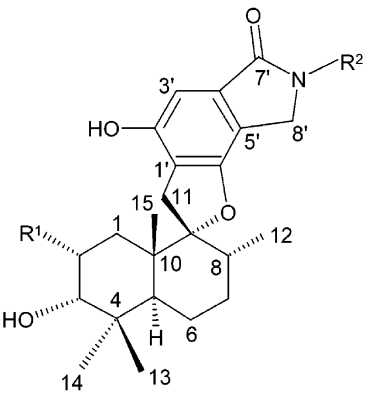
This active fraction was submitted to repeated reversed phase HPLC to give seven new metabolites (**1**–**7**), that based on their characteristic ^{1}H NMR signals could be identified as K-76 analogues. All of them incorporate in their structure a lactame ring.

The NMR data obtained from ^{1}H, ^{13}C, COSY, HMQC and HMBC experiments (see Tables 1 and 2 for ^{1}H and ^{13}C NMR data) showed that compounds **1** and **2** present substituted spiro[4-hydroxy-benzofuran-2(3*H*),1′-6′-hydroxy-2′,5′,5′,8′α-tetramethyl-decahydronaphthalene] structures. Thus, in compound **1**, four methyl carbons at δ_C 16.0 (C-12), 29.2 (C-13), 22.7 (C-14) and 16.3 (C-15), a methylene carbon at δ_C 32.9 (C-11), three quaternary carbons at δ_C 38.2 (C-4), 99.0 (C-9) and 42.8 (C-10), and two partial structures, C-1-C-2-C-3 and C-5-C-6-C-7-C-8-C-12, are identified, indicating the presence of a drimane skeleton.[8–11]

The ESI-MS (positive ion mode) of compound **1** showed a molecular ion peak at *m/z* 516 $[M+H]^+$ suggesting a molecular formula of $C_{28}H_{37}NO_8$. The signals at δ_H 7.36 (3′-H) and at δ_C 157.3 (C-6′), 155.6 (C-2′), 135.3 (C-4′), 118.2 (C-1′), 113.7 (C-5′) and 102.2 (C-3′) were assigned to the penta-substituted aromatic ring. The signals of C-1′, C-2′ and C-6′ were further long-range coupled to the geminal-coupled methylene signals at δ_H 3.12 and 3.52 (11-H_2), which were in turn long-range coupled to C-8, C-9 and C-10. The IR spectrum of **1** showed a band at 1690 cm^{-1}, which is consistent with the presence of α,β-unsaturated γ-lactam. In addition, a carbonyl signal at δ_C 169.9 (C-7′) and geminal-coupled methylene signals (δ_H 4.65 and 4.80; δ_C 45.2) were observed in the NMR spectra of **1**. Compound **1** has the following additional signals in the NMR spectra: a methine, two methylenes and two quaternary carbons at δ_C 173.9 and 175.1.

In the ^{1}H–^{1}H COSY spectrum, a proton at δ_H 5.66 (dd, δ_C 54.7), which correlated to the methylene carbon at 45.2 ppm and with two carbonyl at δ_C 169.9 and 177.9 in the HMBC spectrum, showed also correlations to one pair of methylene protons at δ_H 2.49 and 2.94 ppm, these protons in turn coupled with a methylene proton at δ_H 2.73 which are coupled to the carbonyl at δ_C 175.1 (C-13′). This spin system suggested a –N–CH(COOH)–CH_2–CH_2–COOH aminoacid substructure, for the side chain. Thus, we propose that **1** has a structure shown in Figure 1.

Compound **2** was isolated as a white solid. Its ESI-MS (positive ion mode) showed a molecular ion peak at *m/z* 488 $[M+H]^+$. The ^{13}C NMR spectrum of **2** displayed a total of 27 carbon signals, suggesting a molecular formula of $C_{27}H_{37}NO_7$. ^{1}H and ^{13}C NMR data (see Tables 1 and 2) confirmed the presence of the signals corresponding to the phenylspirodrimane skeleton with an α,β-unsaturated γ-lactam. The presence of a methyl carbon at 1.44 ppm (dd, δ_C 21.3) and a methyne group at 67.4 ppm (δ_H 5.06, dq) suggesting the presence of a threonine instead of glutamic acid in the structure of **2**.



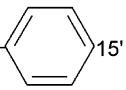
Compound	R1	R2
1	H	-CH(COOH)-CH_2-CH_2-COOH (9' 10' 11' 13')
2	H	-CH(COOH)-CH(OH)-CH_3
3	OH	-CH_2-CH_2-CH_2-COOH (9' 11' 13')
4	OH	-CH(COOH)-CH(CH_3)$_2$
5	OH	-CH(COOH)-CH_2-CH(CH_3)$_2$ (9' 13' 14')
6	OH	-CH(COOH)-CH(CH_3)-CH_2-CH_3
7	OH	-CH(COOH)-CH_2- (9')  15'

Figure 1. Structure of compound **1**–**7**.

The physico-chemical properties of compounds **3**–**7** were similar to each other and their NMR showed these metabolites have a hydroxyl substitution in the drimane moiety.

ESI-MS (positive ion mode) of compound **3** showed a molecular ion peak at *m/z* 488 $[M+H]^+$. ^{1}H and ^{13}C NMR data (see Tables 1 and 2) confirmed the presence of an additional hydroxyl group in the position C-2 of the drimane moiety. The presence of the following spin system –CH_2–CH_2–CH_2–X, in which X was a carboxylic group, as suggested by the methylene carbon chemical shift (-CH_2-COOH: δ_C 32.3) and the mass spectrometry data. Comparison of the spectroscopic data with those reported in the literature, indicated that **3** is the free acid of F-1839-F.[10]

Compound **4**, was isolated as a white amorphous solid, its ^{13}C NMR spectrum displayed a total of 28 carbon signals, its ESI-MS showed a molecular ion peak at *m/z* 502 $[M+H]^+$ suggesting a molecular formula of $C_{28}H_{39}NO_7$. After we assigned the signals corresponding to the phenylspirodrimane skeleton and the lactam, we confirmed

Table 1. ^{1}H NMR spectroscopic data for the compounds **1**–**7**. Spectra were recorded in pyridine-d_5 at 400 MHz and 25 °C. Chemical shifts are in ppm. Multiplicities are indicated with coupling constants in brackets (in Hz)

	δ_C and multiplicity						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1-H	2.33, dt (13.2, 3.3), 1.10, br. d (13.2)	2.33, dt (13.1, 3.2), 1.10, br. d (13.1)	2.43, t (11.8), 1.75, m	2.45, t (12.2), 1.78, m	2.46, t (12.1), 1.75, m	2.45, t (11.9), 1.76, m	2.41, t (12.5), 1.75, m
2-H	1.93, br. t (13.2), 1.65, m	1.92, br. t (13.1), 1.70, m	4.34, m	4.31, m	4.32, m	4.31, bd (11.5)	4.31, bd (11.5)
3-H	3.58, br. s	3.57, br. s	3.77, d (2.4)	3.73, d (2.4)	3.74, d (2.2)	3.73, br. s	3.74, br. s
5-H	2.66, dd (12.8, 2.5)	2.61, dd (12.7, 2.4)	2.58, dd (12.7, 2.1)	2.60, dd (13.3, 2.6)	2.62, dd (12.8, 2.2)	2.619, dd (12.7, 2.2)	2.59, dd (12.9, 2.4)
6-H	1.75, m, 1.45, m	1.70, m, 1.41, m	1.65, m, 1.42, m	1.60, m, 1.42, m	1.64, m, 1.42, m	1.64, m, 1.43, m	1.63, m, 1.42, m
7-H	1.70, m, 1.58, m	1.70, m, 1.60, m	1.60, m, 1.75, m	1.78, m, 1.60, m	1.55, m, 1.75, m	1.76, m, 1.57, m	1.75, m, 1.55, m
8-H	1.75, m	1.70, m	1.75, m	1.78, m	1.75, m	1.76, m	1.75, m
11-H	3.52, d (16.8), 3.12, d (16.8)	3.53, d (17.1), 3.12, d (17.1)	3.62, d (16.7), 3.13, d (16.7)	3.62, d (16.9), 3.13, d (16.9)	3.60, d (16.7), 3.12, d (16.7)	3.61, d (16.7), 3.13, d (16.7)	3.55, d (16.7), 3.05, d (16.7)
12-H	0.83, d (5.6)	0.81, d (5.9)	0.85, d (5.6)	0.85, d (6.6)	0.84, d (6.1)	0.85, d (5.2)	0.76, d (5.4)
13-H	1.21, s	1.17, s	1.29, s	1.22, s	1.25, s	1.22, s	1.25, s
14-H	0.89, s	0.87, s	0.94, s	0.91, s	0.92, s	0.91, s	0.91, s
15-H	0.97, s	0.96, s	1.04, s	1.03, s	1.03, s	1.03, s	1.01, s
3(-H	7.36, s	7.42, s	7.30, s	7.36, s	7.35, s	7.36, s	7.23, s
8(-H	4.80, d (16.4), 4.65, d (16.4)	5.38, d (17.5), 5.28, d (17.5)	4.15, d (16.5), 3.88, d (16.5)	4.95, d (16.9), 4.61, d (16.9)	4.82, d (16.5), 4.48, d (16.5)	4.99, d (16.7), 4.59, d (16.7)	4.72, d (16.2), 4.53, d (16.2)
9(-H	5.66, dd (10.8, 5.2)	5.61, d (4.0)	3.76, m, 3.48, m	5.21, d (9.9)	5.56, m	5.32, d (9.9)	5.83, dd (9.9, 5.2)
11(-H	2.73, m	5.06, dq (6.3, 4.0)	2.00, m	2.39, m	2.04, m, 1.92, m	2.22, m	3.76, dd (14.4, 5.2), 3.33, dd (14.4, 9.9)
12(-H	2.94, m, 2.49, m	1.44, d (6.3)	2.49, t (6.9)	1.11, d (6.7)	1.55, m	1.76, m, 1.43, m	---
13(-H				0.86, d (5.9)	0.94, d (6.5)	0.77, t (7.5)	7.41, d (7.9)
14(-H					0.85, d (6.6)	1.06, d (6.7)	7.19, m
15(-H							7.07, br. t (7.0)

Table 2. ^{13}C NMR spectroscopic data for the compounds **1**–**7**. The spectra were recorded in pyridine-d_5 at 100 MHz and 25 °C. Chemical shifts are in ppm

	δ_C and multiplicity						
C	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	24.8, t	24.8, t	34.0, t	34.0, t	34.0, t	33.9, t	33.9, t
2	26.0, t	26.0, t	66.3, d	66.4, d	66.4, d	66.4, d	66.3, d
3	75.0, d	74.9, d	79.1, d	79.2, d	79.2, d	79.2, d	79.1, d
4	38.2, s	38.2, s	38.9, s	38.9, s	38.9, s	38.9, s	38.9, s
5	40.5, d	40.3, d	39.9, d	39.9, d	40.0, d	39.9, d	40.0, d
6	21.4, t	21.4, t	21.2, t	21.2, t	21.2, t	21.1, t	21.2, t
7	31.6, t	31.5, t	31.6, t	31.6, t	31.5, t	31.5, t	31.5, t
8	37.5, d	37.4, d	37.1, d	37.2, d	37.3, d	37.2, d	37.2, d
9	99.0, s	98.8, s	98.5, s	98.8, s	98.8, s	98.8, s	98.7, s
10	42.8, s	42.8, s	43.9, s	43.9, s	43.9, s	43.9, s	43.8, s
11	32.9, t	32.9, t	33.0, t	33.0, t	33.0, t	33.0, t	33.0, t
12	16.0, q	16.0, q	15.9, q	15.9, q	16.0, q	15.9, q	15.9, q
13	29.2, q	29.1, q	29.5, q	29.4, q	29.5, q	29.4, q	29.4, q
14	22.7, q	22.7, q	22.4, q	22.4, q	22.4, q	22.4, q	22.4, q
15	16.3, q	16.3, q	17.2, q	17.3, q	17.3, q	17.3, q	17.3, q
1′	118.2, s	118.2, s	117.5, s	118.1, s	118.1, s	118.1, s	118.0, s
2′	155.6, s	155.4, s	155.5, s	155.6, s	155.6, s	155.6, s	155.5, s
3′	102.2, d	102.1, d	101.9, d	102.2, d	102.2, d	102.2, d	102.2, d
4′	135.3, s	134.8, s	135.3, s	134.8, s	135.3, s	135.0, s	135.3, s
5′	113.7, s	114.6, s	112.9, s	113.5, s	113.6, s	113.5, s	113.4, s
6′	157.3, s	157.2, s	156.9, s	157.1, s	157.1, s	157.1, s	157.0, s
7′	169.9, s	170.3, s	168.8, s	169.5, s	169.6, s	169.5, s	169.5, s
8′	45.2, t	47.6, t	47.4, t	45.5, t	44.9, t	45.6, t	45.5, t
9′	54.7, d	61.4, d	42.3, t	61.3, d	53.0, d	59.8, d	56.1, d
10′	173.9, s	173.2, s	—	173.4, s	174.7, s	173.6, s	173.6, s
11′	26.0, t	67.4, d	24.6, t	29.2, d	38.9, t	35.1, d	36.2, t
12′	31.9, t	21.3, q	32.3, t	19.9, q	25.3, d	25.9, t	138.5, s
13′	175.1, s		171.0, s	19.5, q	21.3, q	10.9, q	129.1, d
14′					23.2, q	16.1, q	128.8, d
15′							126.9, d

the presence of the signals in the ^1H NMR corresponding to two extra methyl as doublet (0.86 and 1.11 ppm respectively) attached at the same methyne, the presence also of an extra methyne group at 61.3 ppm (δ_H 5.21, dq) suggesting the presence of a valine as side chain.

The MS spectra of compounds **5** and **6** gave molecular ion peaks at *m/z* 515 $[M+H]^+$ indicating a molecular formula $C_{29}H_{41}NO_7$. This formula is 14 mass units larger than that of **4**, suggesting the presence of an extra methylene group. The ^1H and ^{13}C NMR spectra of **5** its very similar to those of compound **4**, with the exception of the presence of a extra methylene at δ_C 38.9 which correlated with a methyne proton δ_H 5.56 in the HMBC spectrum indicating the presence of a leucine instead of valine as side chain. The ^1H NMR spectrum of **6** showed a methyl doublet at δ_H 1.06 and a methyl triplet at δ_H 0.77 indicating the presence of an isoleucine instead of leucine as side chain.

Compound **7** was isolated as a pale yellow solid. The ^{13}C NMR spectrum of **7** displayed a total of 32 carbon signals, its ESI-MS showed a molecular ion peak at *m/z* 550 $[M+H]^+$ suggesting a molecular formula of $C_{32}H_{39}NO_7$. After we assigned the signals corresponding to the phenylspirodrimane skeleton and the lactam, we confirmed the presence in the ^1H NMR spectrum of signals at δ_H 7.41 (13′-H), 7.19 (14′-H) and 7.07 (15′-H) corresponding to an additional mono-substituted aromatic ring, the presence also of a methyne group at 36.2 ppm (δ_H 3.33, dd and 3.76 dd), a methyne group at 56.1 (δ_H 5.83, dd) and an extra quaternary carbon at δ_C 173.6 suggesting the presence of a phenylalanine as side chain.

3. Conclusions

Compounds **1**–**7** were identified as new K-76 derivatives, on the basis of their spectral data. In two independent experiments, all seven new compounds isolated from of *S. chortarum* reproducibly inhibited Tie2 kinase receptor. To measure the potency of these compounds as inhibitors of Tie2 the compounds were dissolved in DMSO. The percentage of inhibition was calculated assuming 100% activity for the controls. The IC_{50}s obtained for the different compounds are summarized in Table 3 and indicate that **4** is the most potent metabolite in this series.

Table 3. Activity data obtained for **1**–**7**

	IC_{50} (mM)
Compound **1**	>0.2
Compound **2**	0.031
Compound **3**	>0.4
Compound **4**	0.025
Compound **5**	0.097
Compound **6**	0.146
Compound **7**	0.046

4. Experimental

4.1. General methods

NMR spectra were recorded with a Jeol Alpha-400 NMR spectrometer (399.65 MHz for ^1H and 100.40 MHz for ^{13}C) using pyridine-d_5 as solvent. MS spectra were recorded on an Ion-trap Finigan LCQ. HRMS experiments were

performed on an APEX III FT-ICR MS (Bruker Daltonics, Billerica, MA). Data acquisition and data processing were performed using the XMASS software, version 6.1.2 (Bruker Daltonics). The HPLC separations were performed using a Beckman M126 pump equipped with a Beckman M168 UV/vis diode array detector (190–600 nm).

4.2. Microorganisms

The fungal strains were isolated from a soil collected in Himalaya (India). Working stocks were prepared on Potato Dextrose agar (22 g/L Dehydrated Potato, 20 g/L glucose and 17 g/L agar) slants stored at 4 °C. Slants were inoculated from long-term stocks kept at −196 °C or from freeze-dried cultures.

4.3. Fermentation

Fermentations in the bioreactors were prepared in three different steps; 250 mL flasks containing 30 mL of BGA1 medium (beef extract 0.5%, glycerol 1% and starch 2%, pH 6.5) were selected from freshly prepared plates and were fermented during 72 hours at 28 °C in orbital shakers (250 rpm). 25 mL of these broths were used as inocula for 400 mL fermentations, in BGA1 medium, contained in 2 L flasks. After 72 h growth under the above mentioned conditions, 800 mL of the resultant cultures were used to inoculate a 42 L MBR fermenter containing 20 L of BGA1 medium plus 0.02% SAG 471 Silicon Antifoam (Union Carbide). After the sterilization cycle at 121 °C for 45 min, the medium was cooled to 28 °C and inoculated. The fermenters were incubated at 28 °C and maintained at 0.5 bar overpressure with agitation speed of 300 rpm (75 m/min tip speed) and an air flow rate of 10 L air/min. Set point for pO_2 was adjusted to 80% and controlled by a cascade system from 300 to 750 rpm for agitation and from 10 to 40 L/min for aeration. The culture was harvested after 6 days.

4.4. Detection of inhibition of the tyrosine kinase portion of Tie2

The assay, based on inhibition of the incorporation of ^{33}P from ATP into the intracellular tyrosine kinase portion of Tie2 in an autophosphorylation reaction, was carried out in microtitre plate format (96 well) as described below.

Incubation buffer: 20 mM Tris at pH 8, 12 mM $MgCl_2$, 10 mM NaCl and 1 mM DTT (freshly added).

Wash buffer: 10 μM ATP in D-PBS.

Enzyme preparation. A partial cDNA clone for the Tie2 receptor was used to prepare the protein for the assay. A baculovirus expressed GST fusion for Tie2 kinase domain was constructed and expressed using the commercial vector pAcG1 (Pharmingen).

Ten microliters of inhibitor to be assayed diluted in water/DMSO were added to each well followed by 20 μL of protein (250 μg/mL, 5 μg/well) and 20 μL of ATP mixture (final concentration: 30 μM ATP (SIGMA A2383), 0.1 μCi/mL ^{33}P-ATP (Amersham AH9968) diluted in incubation buffer). Blanks were made up by adding 20 μL of buffer instead of protein. The plates were shook for 2 h at 30 °C in 96-well U-bottom polypropylene plates before aspirating onto the Millipore filter plates pre-wet with 100 μL of D-PBS per well. The filters were washed three times with 200 μL/well of wash buffer and harvested using the MultiSreen filtration system Vacuum Manifold from Millipore (MAVM 096 01). After removing the plastic base plate the filters were dried and placed in a plastic adapter (Packard 6005178). The radioactivity is measured by the addition of 50 μL/well of scintillation fluid (Miscroscint 0, Packard, 603611) in Top-Count.

4.5. Extraction and isolation

The culture broth (5 L) was centrifuged at 4000 rpm to separate the mycelium and the supernatant part. The mycelium cake was extracted with 300 mL of ketone at room temperature with orbital stirring (1 h at 400 rpm), the organic layer was centrifuged and the organic solvent was removed in vacuum. The aqueous portion obtained (300 mL) was extracted first with dichloromethane (2x400 mL), the remaining aqueous fraction was then extracted with ethylmethylketone (2×400 mL). The combined organic layers were mixed, dried over Na_2SO_4 and after filtration the organic solvent was removed under vacuum to yield 2.6 g of the organic residue. This organic extract (2 g) was dissolved in the minimum volume of $CHCl_3$, and then were purified on a Biotage Flash 40, using a Silicagel cartridge. The column (4.5×15 cm) was developed with a step gradient of $CHCl_3$ and MeOH: $CHCl_3$/MeOH (25:1, F1-F53), $CHCl_3$/MeOH (20:1, F54-F113), $CHCl_3$/MeOH (15:1, F114-F178), $CHCl_3$/MeOH (10:1, F179-F188), $CHCl_3$/MeOH (5:1, F189-F201) and $CHCl_3$/MeOH (1:1, F202-F274) at 10 mL/min. A total of 274 fractions of 8 mL each were obtained and monitored by analytical reversed phase HPLC. The most interesting fractions in basis of their composition were combined and evaporated to dryness. On the basis of the composition analysis we can conclude that only the active fraction **6** (F153-F194, 90 mg) contained metabolites that showed in the ESIMS spectra the presence in their structure of a lactame ring.

These compounds were isolated using reversed phase HPLC chromatography with a Kromasil C18 (5 μm, 250×10 mm) column and CH_3CN 0.1% TFA/H_2O 0.1% TFA as mobile phase from 50:50 (3 min) to 75:25 in 15 min. The flow rate was 4 mL/min, we injected 100 μL of a solution of 60 mg of the fraction described below, dissolved in 1 mL of MeOH previously filtrate by 0.45 μm, and the detection was performed using a diode array with a wavelength of 260±10 nm for the channel A and 306±10 nm for the channel B, the UV spectra were registered every 2 s from 200 to 600 nm. Peaks active in the Tie2 kinase assay were observed at retention times of 6.18, 8.97, 9.87, 11.15, 12.77, 13.47 and 14.32 min. After evaporating off the solvent from the fractions corresponding to these peaks 5.39, 5.87, 3.42, 5.58, 2.06, 3.55 and 5.25 mg, respectively, of residue were obtained. Final purification of these residues was carried out as follows.

4.5.1. Compound 1. The final purification of this compound

(from the fraction at 8.97 min retention time) was carried out using the reverse-phase HPLC column described above, eluted isocratically with CH_3CN 0.1% TFA/H_2O 0.1% TFA (45:55) at a flow rate of 4 mL/min while monitoring at 260 and 306 nm. Five milligrams of the sample, dissolved in 0.5 mL of MeOH and filtered through a 0.45 μm nylon membrane, were injected in 100 μL batches. Under these conditions, a peak appeared at a retention time of 14.83 min. After combining the fraction corresponding to this peak and eliminating the solvent 3.47 mg of compound **1** were obtained. HRMS (ESI): found *m/z* 516.2584 $[M+H]^+$ calculated for $C_{28}H_{38}NO_8$: 516.2592.

4.5.2. Compound 2. The final purification of this compound (from the fraction at 9.87 min retention time) was carried out using the reverse-phase HPLC column described above, eluted isocratically with CH_3CN/H_2O (45:55) at a flow rate of 4 mL/min while monitoring at 260 and 306 nm. Three milligrams of the sample dissolved in 0.5 mL of MeOH and filtered through a 0.45 μm nylon membrane were injected in batches of 100 μL. Under these conditions, a peak was observed at a retention time of 17.17 min. After combining the fractions from repeated injections corresponding to this peak and eliminating the solvent 1.25 mg of compound **2** were obtained. HRMS (ESI): found *m/z* 488.2650 $[M+H]^+$ calculated for $C_{27}H_{38}NO_7$: 488.2643.

4.5.3. Compound 3. The final purification of this compound (from the fraction at 6.18 min retention time) was carried out using the reverse-phase HPLC column described above, eluted isocratically with CH_3CN/H_2O (50:50) at a flow rate of 4 mL/min while monitoring at 260 and 306 nm. Five milligrams of the sample, dissolved in 0.5 mL of MeOH and filtered through a 0.45 μm nylon membrane, were injected in 100 μL batches. Under these conditions a peak was observed at 6.27 min. After collecting the fractions corresponding to this peak from repeated injections and eliminating the solvent, 2.06 mg of purified compound **3** were obtained. HRMS (ESI): found *m/z* 488.2637 $[M+H]^+$ calculated for $C_{27}H_{38}NO_7$: 488.2643.

4.5.4. Compound 4. The final purification of this compound (from the fraction at 11.15 min retention time) was carried out using the reverse-phase HPLC column described above, eluted isocratically with CH_3CN 0.1% TFA/H_2O 0.1% TFA (45:55) at a flow rate of 4 mL/min while monitoring at 260 and 306 nm. Five milligrams of the sample, dissolved in 0.5 mL of MeOH and filtered through a 0.45 μm nylon membrane, were injected in batches of 100 μL. Under these conditions, a peak was observed at a retention time of 21.26 min. After combining the fractions from repeated injections corresponding to this peak and eliminating the solvent 1.89 mg of compound **4** were obtained. HRMS (ESI): found *m/z* 502.2791 $[M+H]^+$ calculated for $C_{28}H_{40}NO_7$: 502.2799.

4.5.5. Compound 5. The final purification of this compound (from the fraction at 14.32 min retention time) was carried out using the reverse-phase HPLC column described above, eluted isocratically with CH_3CN 0.1% TFA/H_2O 0.1% TFA (50:50) at a flow rate of 4 mL/min. Five milligrams of the sample dissolved in 0.4 mL of MeOH and filtered through a nylon membrane were injected in batches of 100 μL while monitoring at 260 and 306 nm. Under these conditions, a peak was observed at a retention time of 21.74 min. After combining the fractions from repeated injections corresponding to this peak and eliminating the solvent 3.18 mg of compound **5** were obtained. HRMS (ESI): found *m/z* 516.2932 $[M+H]^+$ calculated for $C_{29}H_{42}NO_7$: 516.2956.

4.5.6. Compound 6. The final purification of this compound (from the fraction at 13.47 min retention time) was carried out using the reverse-phase HPLC column described above, eluted isocratically with CH_3CN 0.1% TFA/H_2O 0.1% TFA (50:50) at a flow rate of 4 mL/min. Three milligrams of the sample dissolved in 0.4 mL of MeOH and filtered through a nylon membrane were injected in batches of 100 μL while monitoring at 260 and 306 nm. Under these conditions, a peak was observed at a retention time of 19.43 min. After combining the fractions corresponding to this peak from repeated injections and eliminating the solvent 1.44 mg of compound **6** were obtained. HRMS (ESI): found *m/z* 516.2934 $[M+H]^+$ calculated for $C_{29}H_{42}NO_7$: 516.2956.

4.5.7. Compound 7. The final purification of this compound (from the fraction at 12.77 min retention time) was carried out using the reverse-phase HPLC column described above, eluted isocratically with CH_3CN 0.1% TFA/H_2O 0.1% TFA (50:50) at a flow rate of 4 mL/min while monitoring at 260 and 306 nm. 2 mg of sample, dissolved in 0.4 mL of MeOH and filtered through a 0.45 μm membrane, were injected in batches of 100 μL. Under these conditions, a peak was observed at a retention time of 17.69 min. After combining the fractions from repeated injections corresponding to this peak and eliminating the solvent 0.81 mg of compound **7** were obtained. HRMS (ESI): found *m/z* 550.2811 $[M+H]^+$ calculated for $C_{32}H_{40}NO_7$: 550.2799.

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