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Letter

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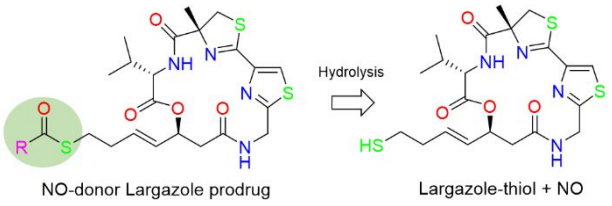
Synthesis and Antiproliferative Activity of Nitric Oxide-Donor Largazole Prodrugs

Matteo Borgini,[1] Claudio Zamperini,[1,2] Federica Poggialini,[1] Luca Ferrante,[4] Vincenzo Summa,[5,^] Maurizio Botta* [1,2,3, #] and Romano Di Fabio* [4,5]

1. Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy; 2. Lead Discovery Siena S.r.l., Castelnuovo Berardenga, 53019 Siena, Italy; 3. Biotechnology College of Science and Technology, Temple University, BioLife Science Building, Suite 333, 1900 North 12th Street, Philadelphia, Pennsylvania 19122, United States; 4. Promidis, Via Olgettina 60, 20132 Milano, Italy; 5. IRBM Science Park, Via Pontina Km 30.600, 00070 Pomezia, Italy. *These two authors share senior authorship.

KEYWORDS: Largazole, HDAC, nitric oxide, cancer, prodrug

ABSTRACT: The marine natural product Largazole is the most potent Class I HDAC inhibitor identified to date. Since its discovery, many research groups have been attracted by the structural complexity and the peculiar anticancer activity, due to its capability to discriminate between tumor cells and normal cells. Herein, we discuss the synthesis and the *in vitro* biological profile of hybrid analogues of Largazole, as dual HDAC inhibitor and nitric oxide (NO) donors, potentially useful as anti-cancer agents. In particular, the metabolic stability of the modified thioester moiety of Largazole, bearing the NO-donor function/s, the *in vitro* release of NO and the antiproliferative activity in tumor cell lines are presented.



Largazole is a potent and selective Class-I deacetylase (HDAC) inhibitor, isolated in 2008 from marine cyanobacteria *Symploca* sp.,[1] that showed a broad-spectrum growth-inhibitory activity against epithelial and fibroblastic tumor cell lines and a remarkable differential cytotoxicity profile over non-transformed cells.[1,2,3] The structure of Largazole is characterized by the presence of a structurally intriguing planar 16-membered depsipeptide core bearing a metabolically labile thioester side-chain, which, upon hydrolytic cleavage, liberates Largazole-thiol, the bioactive HDAC inhibitor species (Figure 1).[4]

HDACs are a family of epigenetic enzymes that catalyze the deacetylation of ε-N-Acetyl lysine in H3 and H4 histone tails, resulting in a tighter chromatin structure that inhibits transcription. Eighteen different HDAC isoforms have been identified to date, subdivided into 4 classes (I to IV): class I (HDAC-1, HDAC-2, HDAC-3, HDAC-8), class IIa (HDAC-4, HDAC-5, HDAC-7, HDAC-9) class IIb (HDAC-6, HDAC-10), class III (Sirtuin-1, Sirtuin-2, Sirtuin-3, Sirtuin-5, Sirtuin-6, Sirtuin-7) and Class IV (HDAC-11). With the only exception of Sirtuins (class III), the deacetylation of histone proteins is typically mediated by a Zn^{2+}-dependent mechanism.[5,6,7,8,9]

The inhibition of HDAC was found to induce cancer cell cycle arrest and cell death, reduce angiogenesis and modulate immune response. In particular, the pan-HDAC inhibitor of class I, II and IV SAHA (suberoylanilide hydroxamic acid, Vorinostat, Zolinza®),[10] depicted in Figure 1, was the first FDA-approved HDAC inhibitor for the treatment of refractory primary cutaneous T-cell lymphoma (CTCL).[11] Luesch and co-workers demonstrated that the antiproliferative activity of Largazole was specifically due to the inhibition of HDAC enzymes targeting Ac-H3 (Lys 9/14).[1] More recently, Largazole was hypothesized to play a relevant role also in the control of osteogenesis[12] and in liver fibrosis.[13]

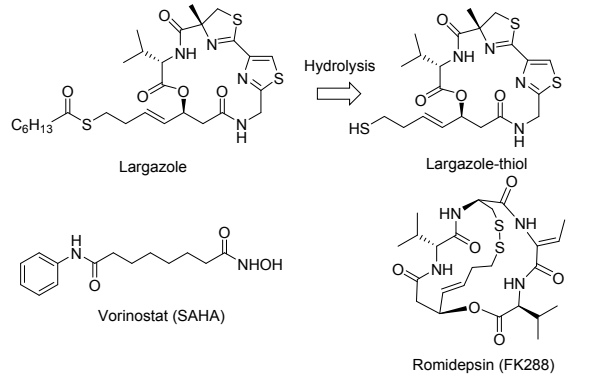


Figure 1. Chemical structures of Largazole, Largazole-SH and Vorinostat (SAHA)

In term of structure-activity relationship (SAR) and mode of action, the X-ray analysis of the co-crystal structure of the complex HDAC8-Largazole, demonstrated that the 16-membered depsipeptide core interacted as capping moiety with the surface rim of the enzyme, while the terminal thiol group, present in the pendant "warhead" of the macrocycle core, chelated the catalytic Zn^{2+}-containing catalytic domain of the enzyme with an ideal coordination geometry.[14] In fact, any

attempt to modify the thiol moiety resulted in the significant loss of activity.[15,16] Largazole shares some structure similarities with FK288 (Romidepsin, Istodax®, Figure 1),[17,18] a Class I HDAC inhibitor approved in 2009 by FDA for the of treatment of CTCL. This naturally occurring depsipeptide, upon metabolic reduction of the disulphide bond, releases the pharmacologically active species bearing the Zn^{2+}-binding thiol group.

Over the last decades, many research groups investigated the anticancer property of nitric oxide (NO) and its capability to overcome tumor cell resistance to conventional treatments.[19] NO is an endogenous and chemically reactive free radical gas, known as the smallest signaling molecule in living organisms. It is produced in mammals from amino acid L-arginine, oxygen and the cofactor tetrahydrobiopterin, by three distinct NO synthase isozymes, namely: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS).[20] NO is involved in the regulation of a plethora of physiological and pathological biochemical pathways in organs and tissues.[20,21,22] In particular, NO plays a key role as neurotransmitter at the synapsis level[23] acting as a key a mediator of learning, sleep, and feeding in the central nervous system. In addition, among other functions, it controls the vascular tone, regulates gene transcription and mRNA translation and produces post-translational modifications of proteins. Of note, NO shows dichotomous differential cellular response by distinct concentrations, either facilitating cancer events, including angiogenesis, apoptosis and metastasis,[23] or, at high concentrations (>200 nM), acting as a potent an anti-cancer agent.[19,23,24,25,26]

In the light of these evidence dual NO donors/HDAC inhibitors emerged over the last years as novel anti-cancer chemical entities, potentially more efficacious than selective HDAC inhibitors, owing to the capability of NO to specifically modulate function of some HDAC isoforms.[27] In particular, class I HDAC 2 has been found to be structurally modified by direct reaction with NO, either S-nitrosylation or Tyr-nitration reaction, or shuttled into the cell nucleus through the activation of protein phosphatase 2A.[28,29]

The first mixed NO-donor/HDAC inhibitor was reported in 2013, as a compound potentially useful for the treatment of cardiac hypertrophy and wound healing.[30] This novel hybrid molecule, obtained from the known HDAC inhibitor Entinostat by the introduction of a constitutive NO-donor furoxan moiety, showed a similar HDAC inhibitory profile as Entinostat, but an additive myogenic differentiation activity. Since then, different hybrid NO-donor/HDAC inhibitor derivatives, potentially useful as anti-cancer agents, have been reported in literature.[31] In particular, hybrids Doxorubicin-NO donors were found to be active against Doxorubicin-resistant human colon cancer cells (HT29-dx).[32] In addition, interesting results were obtained joining NO-donor moieties with anticancer platinum derivatives[33] and PepT1 inhibitors.[34] Finally, some hybrid HDAC inhibitor-NO donors exhibited an enhanced cytotoxic activity compared to HDAC inhibitors.[35]

Owing to this evidence, our aim was to obtain novel Largazole derivatives bearing one or more NO-donor functions at the metabolically labile thioester chain. These novel type of HDAC inhibitors, exploiting the prodrug character of the thioester moiety of Largazole, upon enzymatic hydrolysis would have efficiently released Largazole-thiol, the pharmacologically active species as HDAC inhibitor, and depending on the specific functionalization of the aliphatic thioester chain, produced one or more equivalents of NO (Figure 2).

To this aim, compounds **1** and **2** were synthesized and then sequentially evaluated in terms of capability to release NO *in vitro*, metabolic stability and antiproliferative activity in tumor cell lines with respect to Largazole.

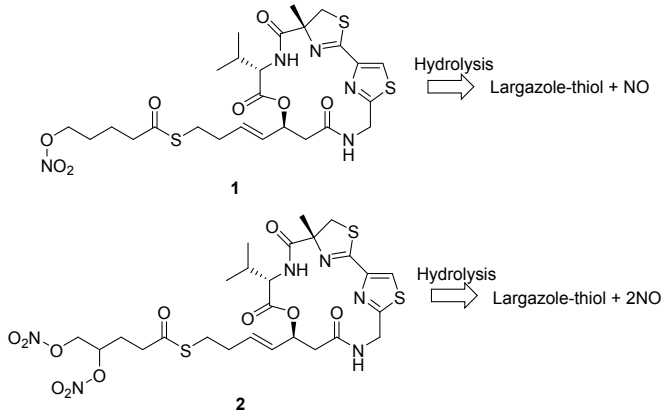


Figure 2. Chemical structures of Largazole analogues **1** and **2**.

The retrosynthetic analysis of compounds **1** and **2**, shown in Figure 3, suggested the disconnection of the olefin moiety, that could have then been obtained by cross-metathesis reaction between the known synthetic Largazole intermediate **3** and the terminal olefin derivative bearing suitable nitrate group/s.

Compound **3**, was prepared by condensation of fragments A and B, as depicted in Figure 3, following the same synthetic sequence reported in literature.[36] However, to obtain intermediate **3** in multigram scale, we specifically focused our attention on the optimization of the synthesis of fragment A (Scheme 1), which was efficiently prepared in seven synthetic steps and 63 % total yield from commercially available N-Boc glycine **4**. In particular, intermediate **6**, prepared in two steps and 82% yield from **4**, was transformed into the corresponding thiazole derivative with 3-bromopyruvic acid in THF at 50 °C, to get the corresponding free amino derivative, which was re-protected as N-Boc, affording **7** in 95% yield over two steps.

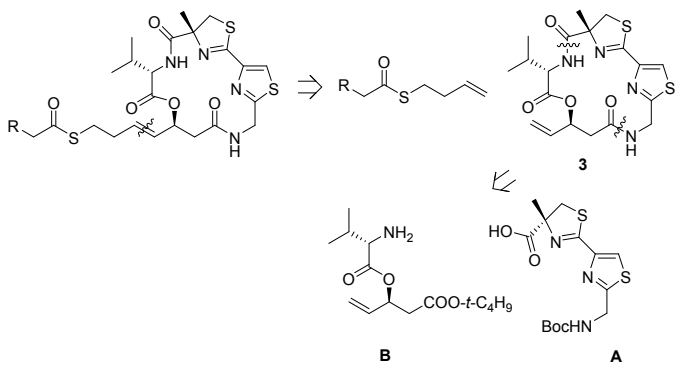
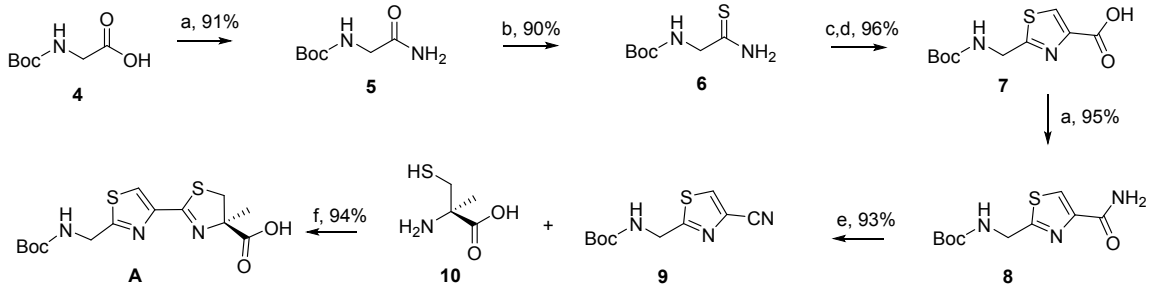


Figure 3. Retrosynthetic approach for the synthesis of NO-donor Largazole derivatives **1** and **2**.

Then, its carboxylic function was smoothly transformed into the corresponding nitrile group to get compound **8** which was condensed with α-methyl cysteine **10**, prepared from cysteine as reported in literature, affording target fragment A in 94% yield. The NO-donor thioester olefin derivative **14** was synthesized as shown in Scheme 2.

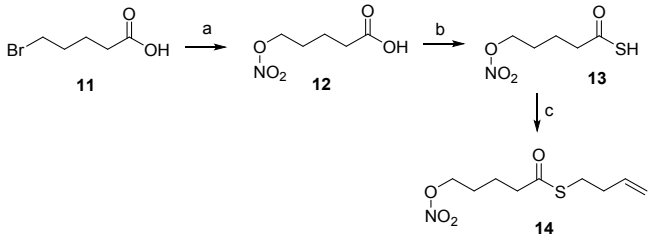
Scheme 1. Optimized synthesis of fragment **A**



Reagents and conditions: (a) isobutyl chloroformate, N-methyl morpholine, NH_4OH, THF, -20 °C, 1 h; (b) Lawesson's reagent, CH_2Cl_2, r.t.,15 h; (c) 3-bromopyruvic acid, THF, 50 °C, 1 h; (d) Boc_2O, 1N NaOH, dioxane, r.t., 40 min; (e) TFAA, diisopropylethyl amine, CH_2Cl_2, 0 °C, 1 h; (f) TEA, MeOH, reflux, 7 h.

In particular, 5-bromovaleric acid **11** was reacted with $AgNO_3$ in dry CH_3CN at 70 °C for 2 h to obtain intermediate **12**, which was then transformed into the corresponding thiocarboxylic acid derivative **13** by Lawesson's reagent in a microwave reactor at 60 °C. The final alkylation reaction with allyl bromide afforded the target olefin derivative **14** in 85% yield.

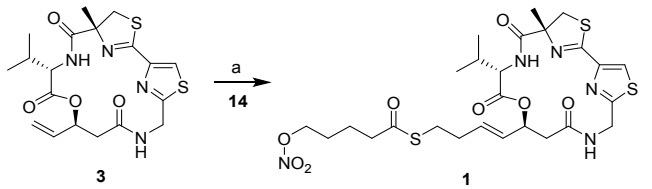
Scheme 2. Synthesis of the mono-nitrate olefin derivative **14**.



Reagents and conditions: (a) $AgNO_3$, dry CH_3CN, 70 °C, 1 h. (b) Lawesson's reagent, CH_2Cl_2, 60°C, 10 min, MW. (c) 4-bromo-1-butene, K_2CO_3, Acetone, 0 °C to 20°C, 30 min.

The following key intermolecular cross-metathesis reaction between intermediate **3** and compound **14**, performed with Grubbs 2nd generation ruthenium-based catalyst (Scheme 3), gave the target Largazole thioester analogue **1**, although only in 26% yield, due to the competitive homodimeric coupling reaction of the olefin derivative **14**, as confirmed by the isolation of large amount of unreacted olefin derivative **3**, after purification by flash chromatography.

Scheme 3. Cross-metathesis reaction.

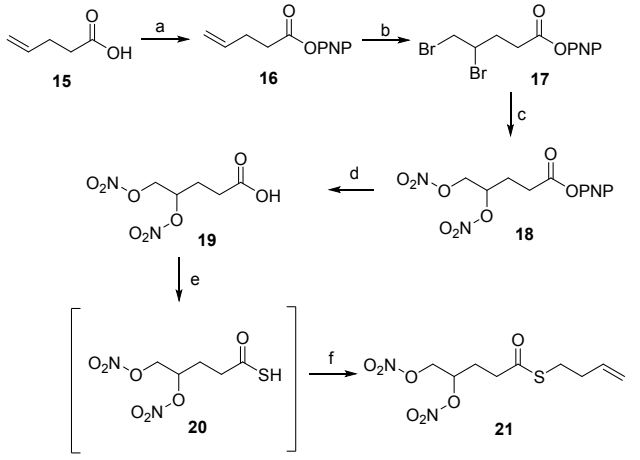


Reagent and conditions: Grubbs catalyst 2nd generation, dry CH_2Cl_2, 90 °C, 16 h.

As far as the synthesis of the corresponding bis-nitrate derivative **2** is concerned, the same synthetic sequence used from the preparation of **14** was attempted (Scheme 4). 4-Pentenoic acid **15** was protected as *p*-nitrophenol (PNP) ester. The following bromination reaction of intermediate **16** afforded compound **17** in 81% yield, which was easily converted into the corresponding bis-nitrate compound **18** in the presence of $AgNO_3$ in CH_3CN at 70 °C for 24 h. After the basic hydrolysis of the ester group, the resulting carboxylic acid **19** was transformed into the chemically labile thiocarboxylic acid derivative **20**, which was rapidly alkylated with 4-bromo-1-butene, to obtain compound **21** in 23% yield over two steps (Scheme 4). The following intermolecular cross-metathesis reaction with Largazole intermediate **3**, performed using the same reaction conditions set up for the synthesis of compound **1**, afforded this time only the side product derived from homodimeric coupling reaction of the olefin derivative **21**.

Due to this unexpected result, to obtain compound **2**, the synthetic approach was significantly modified (Scheme 5).

Scheme 4. Synthesis of bis-nitrate olefin **21**.

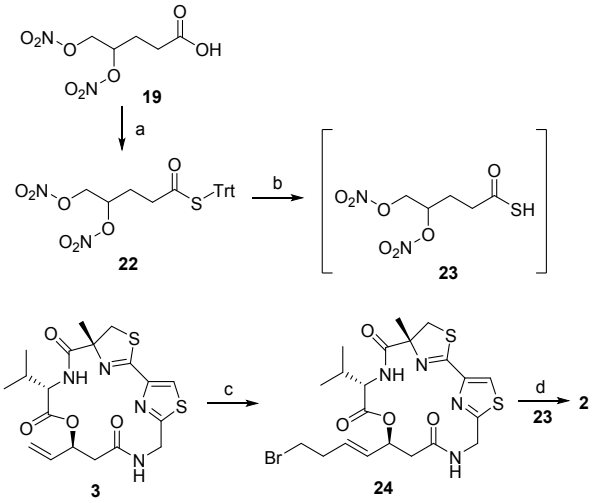


Reagents and conditions: (a) DIC, DMAP, CH_2Cl_2, 0 °C to r.t., 1h; (b) Br_2, CCl_4, r.t., 30 min; (c) $AgNO_3$, dry CH_3CN, 70 °C; 24 h. (d) NaOH 2N, THF/EtOH, 0 °C, 30 min; (e) Lawesson's reagent, CH_2Cl_2, 60 °C, 15 min, MW; (f) 4-bromo-1-butene, K_2CO_3, acetone, 0 °C to r.t., 30 min.

To this aim, the bis-nitrate carboxylic acid derivative **19** was transformed into the corresponding S-trityl thiocarboxylic ester derivative **22**.The following de-blocking reaction of the S-trityl protecting group, with TFA and triethylsilane in CH_2Cl_2 at room temperature for 1 h, afforded the corresponding chemically

labile thiocarboxylic acid **23**, which was then rapidly reacted with the bromo derivative **24**, smoothly synthesized from intermediate **3** by metathesis reaction in 42% yield, to get title compound **2**.

Scheme 5. Synthesis of compound **2**.



Reagents and conditions: (a) triphenylmethanethiol, DIC, DMAP, dry CH_2Cl_2, 0 °C to r.t., 4 h; (b) TFA, triethylsilane, dry CH_2Cl_2, 0 °C to r.t., 1 h; (c) 4-bromo-1-butene, Grubbs II, dry CH_2Cl_2, 90 °C, 16 h; (d) K_2CO_3, acetone, 0 °C to r.t., 4 h.

Largazole derivatives **1** and **2** were initially characterized in terms of capability to release NO, using the Griess method.[37] As shown in Figure 4, in the assay conditions, both compounds spontaneously produced NO.

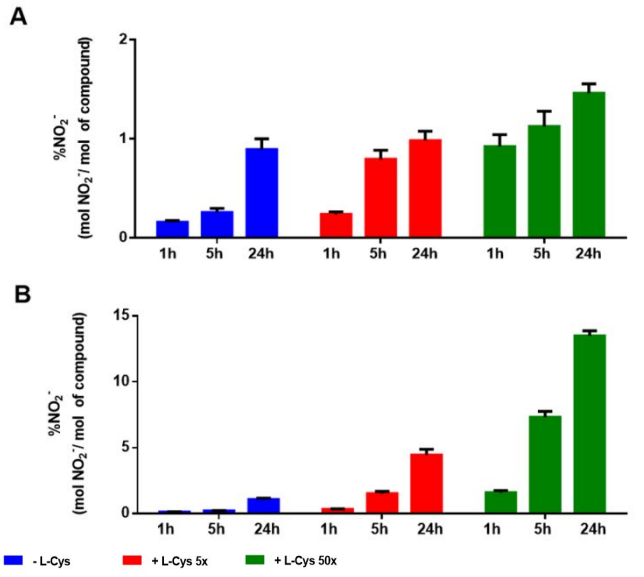


Figure *4*. NO release assay. Compounds **1** (A) and **2** (B) were incubated at 0.1 mM concentration in 50 mM phosphate buffer (pH=7.4) at 37°C, both in the absence and in presence of L-cysteine (0.5 mM and 5 mM). The yield of NO_2^- is expressed as % with respect to the initial concentration of compound at 1 h, 5 h and 24 h, respectively. The reported values are the average of three independent experiments.

As expected, the NO production was amplified in presence of increasing concentration of L-cysteine. The NO release was obviously more abundant for compound **2** than compound **1**, due to the presence of two NO-donor groups.

Once ascertained the capability of both compounds to efficiently release NO, their antiproliferative activity was evaluated against U-2OS (human osteosarcoma cell), Caco-2 (human colorectal adenocarcinoma cell) and IMR-32 (human neuroblastoma cell), using the parent compound Largazole as internal control. As shown in Table 1, compounds **1** and **2** showed a concentration and time-dependent inhibitory activity against tumor cell growth. In particular, as per the antiproliferative activity in the U-2OS cell lines, both compounds showed a relevant additive effect at 24 h with respect to the antiproliferative activity of Largazole, whereas, at 48 h and 72 h, the antiproliferative effect was similar to Largazole. The greater antiproliferative activity of compound **2** *vs* compound **1** at 24 h (pEC_{50} = 6.33 *vs* 5.71, respectively) is most likely due to the greater production of NO by compound **2** with respect to compound **1**.

Table 1. Cytotoxicity of Largazole, **1** and **2** against U-2OS, Caco-2, IMR-32 cell line.

		Compound[#]		
Cell line	Time	Largazole	**1**	**2**
U-2OS	24 h	4.69±0.12	5.71±0.15	6.33±0.10
	48 h	6.43±0.15	6.51±0.15	6.13±0.20
	72 h	6.48±0.10	6.77±0.09	6.00±0.70
CaCo-2	24 h	>2.0	4.95±0.19	5.04±0.24
	48 h	6.12±0.21	7.42±0.14	8.25±0.22
	72 h	7.84±0.09	8.09±0.16	8.35±0.16
IMR-32	24 h	7.46±0.16	7.82±0.17	7.53±0.12
	48 h	7.52±0.14	7.71±0,17	7.21±0,18
	72 h	7.91±0.08	7.92±0.06	7.30±0.14

[#] EC_{50} were determined as described in the Supplementary Information; they are the average value of n=3 independent experiments ± SEM.

This general trend is even more evident in the Caco-2 cell line, in which the improved antiproliferative activity of both compounds was more pronounced than that shown by Largazole at 24 h and 48 h and, in part, at 72 h. As anticipated, compound **2** was more potent than compound **1** at all timepoints. Conversely, in the IMR-32 cell line the antiproliferative activity was already evident at 24 h, whereas the additive effect of compound **1** and **2** with respect to the parent compound Largazole, was minimal or even absent.

To further explain the additive antiproliferative effect of compound **1** and **2** *vs* Largazole, their metabolic stability was assessed with respect to Largazole in the assay cell medium. As expected, a rapid hydrolysis of the thioester moiety was observed (Table 2, Supplementary Information). The chemical stability of these compounds was evaluated also in HEPES and DMSO, without observing chemical degradation, hence confirming the potential use of these compounds as prodrugs. Finally, the stability in human plasma profile was evaluated (Table 2, Supplementary Information), detecting the same rapid hydrolysis of the metabolically labile thioester side chain reported in literature for Largazole.[38,39]

CONCLUSIONS

Novel Largazole derivatives bearing one and two nitrate groups at the metabolically labile thioester side chain were efficiently synthesized. These compounds were endowed with dual activity profile, as consequence of the rapid liberation in cell medium of the HDAC inhibitor Largazole-thiol and the efficient production of NO. When characterized in terms of cytotoxicity in three different types of tumor cell lines, namely: U-2OS, Caco-2 and IRM-32, compounds **1** and **2** showed an additive antiproliferative activity compared to the parent compound Largazole, effect which was more pronounced in the U-2OS and Caco-2 cells than in IRM-32.

Additional antiproliferative studies are being performed in different type of cancer cell lines to further explore the anticancer potential of compounds **1** and **2**. The relative results will be reported in due course.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website http://pubs.acs.org. the synthetic procedures, the characterization chemical intermediates and final compounds and biological assay protocols are in details.

AUTHOR INFORMATION

Corresponding Author

*(R.D.F) Phone: +390249491702. Fax: +390249491712. E-mail: r.difabio@irbm.com

Author Contributions

All authors equally contributed to the preparation of this manuscript and gave approval to the final version.

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Notes

[#] This article is dedicated to the memory of Professor Maurizio Botta, deceased on August 2nd 2019, who spent his entire life in science aiming for improving the quality of the life of human beings.

[^] Present address: Department of Pharmacy, University of Naples “Federico II”, Via D. Montesano 49, 80131 Naples, Italy.

The authors declare no competing financial interests

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ABBREVIATIONS

HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; MW, microwave reactor; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; Trt, Trityl; DIC, N,N′-diisopropylcarbodiimide; DCM, dichloromethane; DMAP, 4-dimethylaminopyridine; DCE, 1,2-dichloroethane; TFA, trifluoroacetic acid

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