

# Synthesis of ST7612AA1, a Novel Oral HDAC Inhibitor, via Radical Thioacetic Acid Addition



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**Abstract: Background:** In the expanding field of anticancer drugs, HDAC inhibitors are playing an increasingly important role. To date, four/five HDAC inhibitors have been approved by FDA. All these compounds fit the widely accepted HDAC inhibitors pharmacophore model characterized by a cap group, a linker chain and a zinc binding group (ZBG), able to bind the  $Zn^{2+}$  ion in a pocket of the HDAC active site. Romidepsin, a natural compound, is the only thiol derivative. We have selected a new class of synthetic HDAC inhibitors, the thio- $\omega$ (lactam-carboxamide) derivatives, with **ST7612AA1** as drug candidate, pan-inhibitor active in the range of single- to two-digit nanomolar concentrations. Preliminary results of a synthetic optimization attempt towards a fast scale-up process are here proposed.



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**Methods:** in the four steps of synthesis, from unsaturated amino acid intermediate to the final product, we explored different synthetic conditions in order to have a transferable process for a scale-up synthetic laboratory.

**Results:** In the first step, isobutyl chloroformate was used and, after a simple work up with 1M HCl, **2** (96% yield) was obtained as a white solid, which was used directly in the next step. For thioacetic acid addition to the double bond of intermediate **2**, two different routes were possible, with addition reaction in the first (D') or last step (D). Reactions of **2** to give **5** or of **4** to give **ST7612AA1** were both performed in dioxane. Reactions were fast and did not need the usually advised radical quenching with cyclohexene. The corresponding products were obtained in good yields (step D', 89%; step D, 81%) after a flash chromatography.

**Conclusion:** **ST7612AA1**, a thiol derivative prodrug of **ST7464AA1**, is the first of a new generation of HDAC inhibitors, very potent, orally administered, and well tolerated. Here, we have identified a synthetic route, competitive, versatile and easily transferable to industrial processes.

**Keywords:** Thiol-based HDAC inhibitors, ST7612AA1, Thio- $\omega$ (lactam-carboxamide) derivatives, Thiol-ene reaction.

## 1. INTRODUCTION

In the expanding field of anticancer drugs, HDAC inhibitors are playing an increasingly important role [1]. To date, four HDAC inhibitors have been approved by the US Food and Drug Administration: **Vorinostat** (Merck, 2006), **Romidepsin** (Gloucester Pharmaceuticals now Celgene, 2009), **Belinostat** (TopoTarget now Onxeo, 2014) and **Panobinostat** (Novartis, 2015) for treatment of cutaneous T-cell lymphoma (CTCL), peripheral T-cell lymphoma (PTCL) and multiple myeloma. Recently, the Chinese Food and Drug Administration has also approved **Chidamide** (Shenzhen Chipscreen Biosciences, 2015) for the treatment of PTCL (Fig. 1). All of them are also being investigated for the treatment of different types of cancers as well as other diseases, as single agents and in combination therapies [2].

Moreover, approximately 15 new HDAC inhibitors are in different stages of clinical trials and an even greater number

of molecules are currently under preclinical investigation. It is worth noting that only over the last two years three HDAC inhibitors have been consecutively approved, which indicates the rapid development of the field of HDAC inhibitors.

All these compounds fit the widely accepted HDAC inhibitors pharmacophore model characterized by a cap group, a linker chain and a zinc binding group (ZBG), able to bind the  $Zn^{2+}$  ion in a pocket of the HDAC active site [1].

HDAC inhibitors obtained by total synthesis are hydroxamic acid or benzamide derivatives. Romidepsin, the only natural HDAC inhibitor, is a thiol derivative discovered from cultures of *Chromobacterium violaceum*, a Gram-negative bacterium isolated from a Japanese soil sample [3].

Benzamide-based inhibitors are usually selective for class I HDACs (1, 2, and 3) while hydroxamic acid-based inhibitors - more prevalent - are, unfortunately, metabolically unstable (i.e. with a short half-life) and show an off-target activity at other zinc-containing enzymes. Besides, they are more active in hematological malignancies than in solid tumors due to the peculiar pharmacokinetic profile [4]. In addition, many hydroxamic acid-based inhibitors have

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been shown to be Ames-positive and to cause chromosomal aberrations and this makes them suspected of genotoxicity [5].

Accordingly, the availability of novel inhibitors with nonhydroxamate ZBGs represents a highly desirable condition. The thiol and the mercaptoacetamide groups have been widely investigated in HDAC inhibitors and provided compounds with comparable HDAC inhibitory potency and cancer cell growth inhibition [6-10].

The role of thiols in the control of the cellular redox environment, essential for normal physiological function, is well-reported [11]. Oxidative stress is particularly prevalent in cancer where many malignant cell types possess an abnormal redox metabolism involving down-regulation of antioxidant enzymes and impaired mitochondrial function. Reactive oxygen species (ROS) play a role in many signal transduction pathways via the oxidation of redox sensitive cysteine residues. The range of cellular processes under redox regulation is extensive and includes both the proliferative and apoptotic pathways. Therefore, thiol-based HDAC inhibitors are of great interest for their potential synergistic pharmacological effects between ROS and histone acetylation.

### 1.1. ST7612AA1

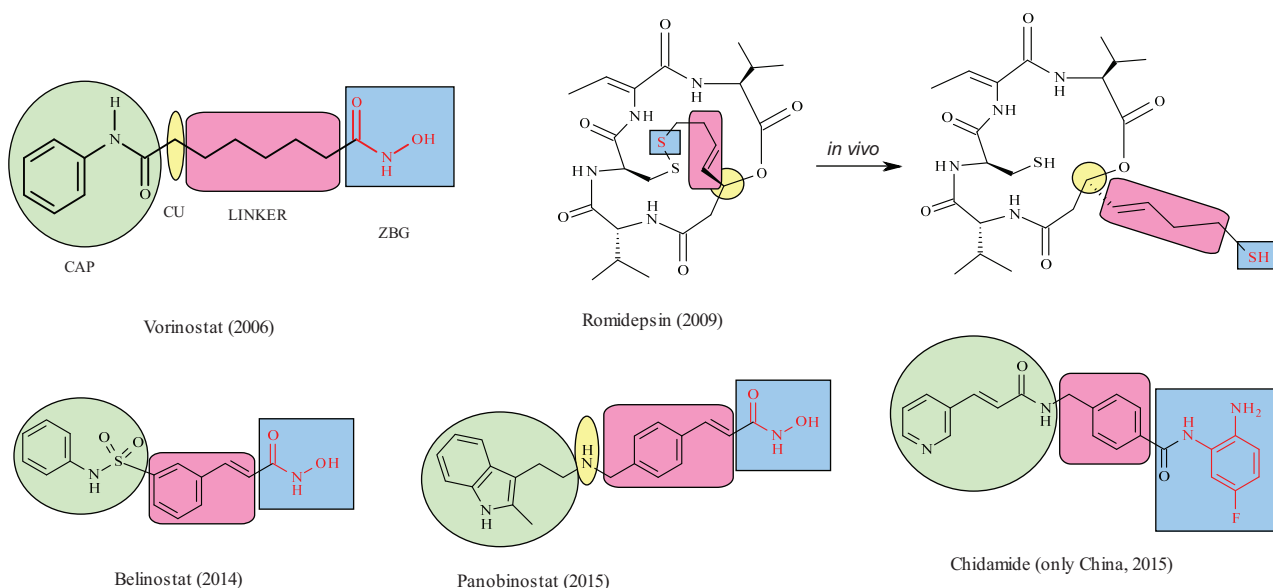
In Sigma-Tau, after more than a decade of research activity, a new class of synthetic HDAC inhibitors, the thio-

$\omega$ (lactam-carboxamide) derivatives, has been selected. The drug candidate stemming from this class is ST7612AA1, a  $\gamma$ -lactam derivative, pan-inhibitor active on eleven HDAC isoforms in the range of single- to two-digit nanomolar concentrations [12]. It is also active at sub-micromolar concentrations on a large number of tumor cell lines as well as effective in *in vivo* tumor models. After either oral or parenteral administration, ST7612AA1 showed a good tolerability, safety and negligible body weight loss, with a fast acetyl group hydrolysis to form the corresponding active drug (thiol) (Fig. 2) [13].

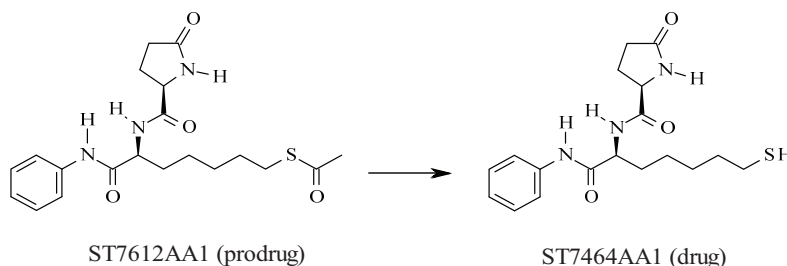
Recently, it has also been investigated as an HIV-1 latency reactivation agent where **ST7612AA1** showed to be a potent activator of latent HIV. The reactivation activity is exerted without activation or proliferation of CD4+ T cells, making this drug candidate useful for new potential therapies to eradicate the viral reservoirs [14].

However, it should be stated that these thiol-based HDAC inhibitors cannot be considered as simple bioisosteric analogues of their corresponding hydroxamic acid-based inhibitors [15]; indeed, a study on parasites highlighted an important difference between the two classes with, contrary to expectations, the hydroxamic acid derivatives more potent than the counterpart thiols [16].

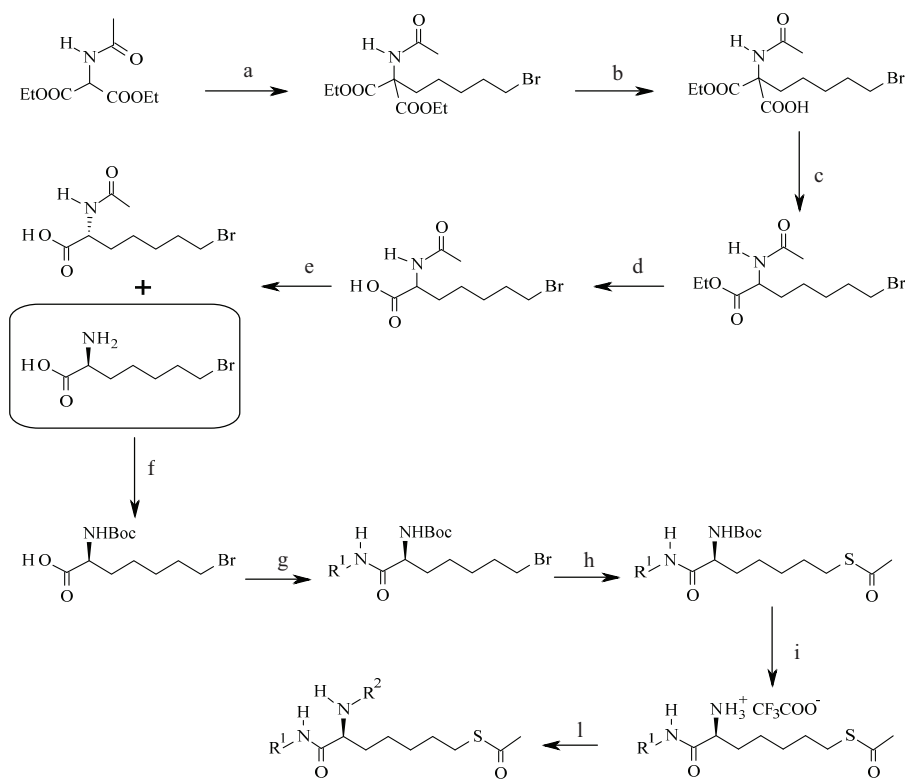
Overall, this new class of drugs represents a breakthrough in the field of HDAC inhibitors, and **ST7612AA1** is



**Fig. (1).** Structures of FDA approved HDAC inhibitors.



**Fig. (2).** Structures of **ST7612AA1** and the corresponding active drug after *in vivo* hydrolysis.



**Scheme 1.** Reagents and conditions: (a) 1) EtOH, EtONa, reflux, 30 min; 2) Br(CH<sub>2</sub>)<sub>5</sub>Br, reflux, 3h; (b) NaOH aq, EtOH, 0°C, 5h; (c) toluene, reflux, 4h; (d) NaOH aq, EtOH, 0°C, 4h; (e) aminoacylase, H<sub>2</sub>O, pH7, CoCl<sub>2</sub>·6H<sub>2</sub>O, 38°C, 24h; (f) (Boc)<sub>2</sub>O, Et<sub>3</sub>N, THF, H<sub>2</sub>O, r.t., 20 h; (g) R<sup>1</sup>NH<sub>2</sub>, EDCI, HOBr·H<sub>2</sub>O, THF, r.t.; (h) KSAC, EtOH, RT; (i) CF<sub>3</sub>COOH, CH<sub>2</sub>Cl<sub>2</sub>, r.t., 5h; (l) R<sup>2</sup>COOH, DMF, PyBOP, DIPEA, r.t., 20 h.

a drug candidate with a broad therapeutic potential. Thio- $\omega$ (lactam-carboxamide) derivatives could be obtained by different and versatile synthetic strategies; for example, our first approach drew a synthetic route with 12 steps, starting from diethyl acetamidomalonate, using an enzymatic stereoselective N-deacetylation to resolve racemic mixture (Scheme 1).

However, to get to the final step the enzymatic strategy could be avoided; **ST7612AA1** was obtainable through easily approachable and competitive industrial process.

Here we report our preliminary results of a synthetic optimization attempt towards a fast scale-up process.

## 2. MATERIALS AND METHODS

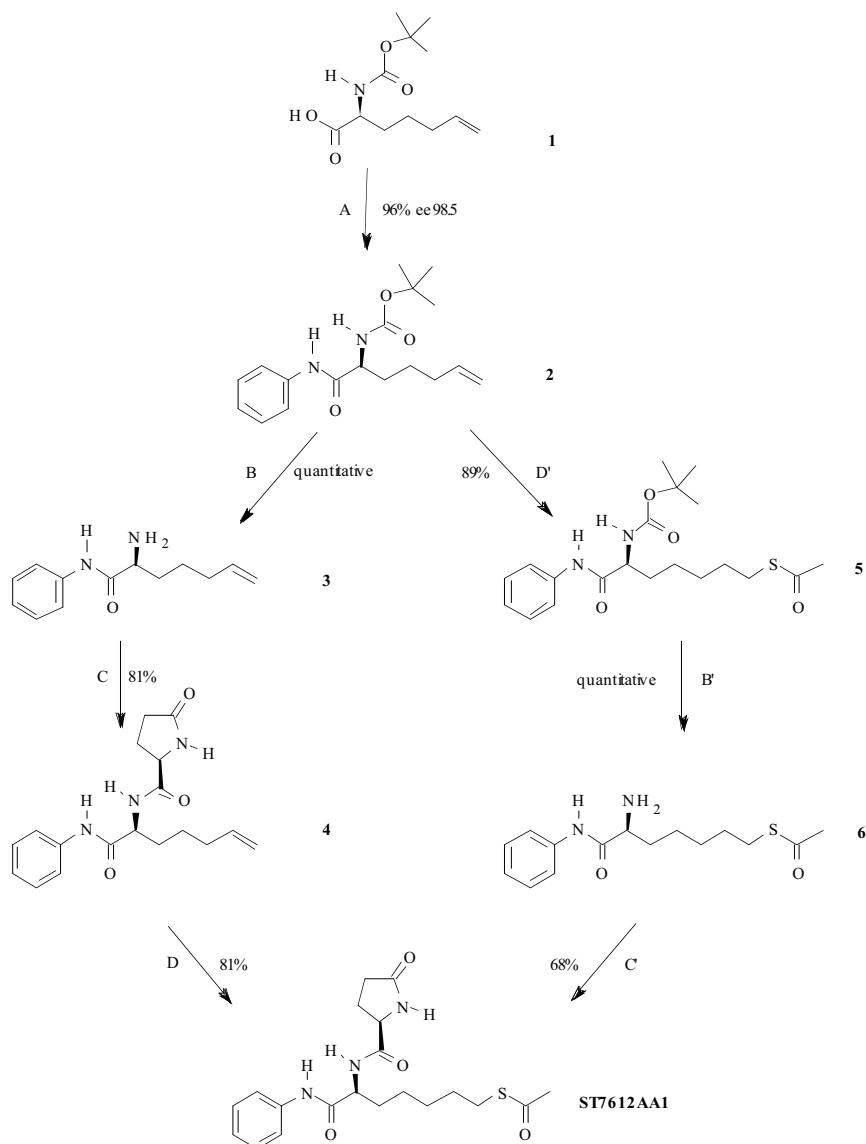
Reagents, solvents and anhydrous solvents were purchased from commercial suppliers and used without further purification. The exclusion of moisture from reagents and glassware was performed following standard techniques for manipulating air-sensitive compounds. Flash chromatography was performed on pre-packed silica gel columns (50  $\mu$ m particle size) with the indicated solvent systems. Thin layer chromatographies (TLCs) were performed on pre-coated, glass-backed silica gel plates (Merck 60F254). Visualization was performed under short-wavelength ultraviolet light or by iodine vapour staining into a iodine development chamber. Nuclear magnetic resonance spectra were recorded on Gemini spectrometers (Varian) at 300 or 500 MHz or on Bruker spectrometer at 300 Mhz. Peak positions are given in parts

per million downfield from tetramethylsilane as the internal standard; J values are expressed in hertz. Mass analyses were performed on Waters ZQ2000 spectrometer using electrospray (ES) technique. High resolution mass spectra were obtained by an Orbitrap spectrometer. HPLC analyses were performed on a Waters apparatus (HPLC Waters Alliance 2695 and PDA-UV detector 2996) equipped with a column Phenomenex Gemini C6-Phenyl (3  $\mu$ m, 150 x 4.6 mm).

### 2.1. Thioacetylation Approach to ST7612AA1 Synthesis

A thiol-ene process is the key step of the synthetic route for **ST7612AA1**, starting from the commercially available (S)-2-tert-butoxycarbonylamino-hept-6-enoic acid **1** (Scheme 2). The reaction between thiol groups and ene-carbon-carbon double bonds (reported since the early 1900s [17]) belongs to the world of the well-known “click chemistry” because of some of its features: in fact, it is characterized by high yields, regio- (and stereo-) specificity (anti-Markonikov products), oxygen- and water-insensitivity and it can be performed under very mild reaction conditions. It is commonly defined as “thiol click-chemistry” [18]. This reaction is known to proceed via radical or anionic chain mechanisms, named respectively “thiol-ene reaction” and “thiol Michael addition”.

In the last decades, the use of thermally sensitive radical initiators, such as AIBN (Azobisisobutyronitrile), for many thiol-ene reactions has emerged as necessary, particularly for the functionalization of biological materials [19-22] and for large-scale syntheses, where photoinitiation was not vi-



**Scheme 2.** Reagents and conditions: (A) Aniline, isobutyl chloroformate, N-methylmorpholine, DCM, r.t.; (B,B') DCM/TFA (4/1), 0°C – r.t.; (C) (2R)-5-oxopyrrolidine-2-carboxylic acid, isobutyl chloroformate, N-methylmorpholine, r.t.; (D,D') CH<sub>3</sub>COSH, AIBN, Dioxane, 75°C; (C') (2R)-5-oxopyrrolidine-2-carboxylic acid, DIPEA, PyBOP, DCM, r.t.

able and high temperatures and thermal initiators allowed achieving uniform bulk radical generation.

A great variety of thiols were used in many examples of AIBN-initiated thiol-ene reactions, such as radical construction of dendrimers functionalized with sugars, surface functionalization of semiconducting nanoparticles, and so on [18].

Moved by all these features towards scaling up of the process, we designed a **ST7612AA1** synthesis where the thioacetate group was built by an AIBN-catalyzed addition of thioacetic acid to a terminal double bond (Scheme 2, step D or D').

The first synthesis of **ST7612AA1**, on multi-mg scale, were performed in our laboratory. Now here report also the yields of a synthesis on ten-gram scale as it obtained from an outsourcing laboratory engaged in the preparation of the

product (A comprehensive comparison of yields, in Table S1; Supplementary Data).

## 2.2. Synthesis Optimization

Our synthesis started with the coupling of aniline with (S)-2-tert-butoxycarbonylamino-hept-6-enoic acid (Scheme 2, step A). We performed a little screening of different coupling reagents in order to compare reagents (cost), yields, and inversion of the chiral center (Table 1).

Chiral separation and enantiomeric excess (*ee*) determination were performed by HPLC on a chiral column (Phenomenex Lux Cellulose-2, 250\*4.6mm, 5  $\mu$ m) using n-hexane/isopropanol with 0.1% v/v diethyl amine as eluting mixture. In order to know the retention time of the two enantiomers, a racemic mixture of **1** was first used in the reaction with aniline (see Supplementary Material, Fig. S1)

**Table 1.** Step A – synthesis in Scheme 2. Different reaction conditions and corresponding yields.

Base	Coupling Reagent	Yield (%)	Note <sup>a</sup>	ee
-	EDC	87	No	98.5
N-methylmorpholine	isobutyl chloroformate	96	No	98.5
DIPEA	PyBOP	73	Yes	98.0
DIPEA	HBTU	78	Yes	96.5

<sup>a</sup> Chromatographic purification after work up.

### 2.3. Experimental

Only the optimization procedure with single steps is here reported. For a complete characterization of each intermediate and final product, the interested readers are encouraged to read our previously published full paper [12].

**Step A.** (*S*)-2-Tertbutoxycarbonylamino-hept-6-enoic acid (379 mg, 1.56 mmol) was dissolved in anhydrous DCM (8 mL) and the mixture was cooled at 0°C with an ice bath. Under stirring, N-methylmorpholine (169 mg, 1.67 mmol) and isobutyl chloroformate (228 mg, 1.67 mmol) were added. After 1 hour under stirring at 0°C, aniline (145 mg, 1.56 mmol) was added and the mixture was allowed to warm at room temperature. After 2 hours TLC monitoring showed complete conversion of the starting material; the mixture was diluted with DCM and washed two times with 1M HCl, two times with NaHCO<sub>3</sub>ss (saturated solution), and finally, with brine. The organic phase was dried on Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure to give **2** (477 mg, 96% yield) as a light brown solid that was used directly for the next step.

**Step B.** Compound **2** (472 mg, 1.48 mmol) was dissolved in DCM (16 mL) and the mixture was cooled at 0°C with an ice bath. Trifluoroacetic acid (4 mL, 52.24 mmol) was added under stirring and the mixture was allowed to warm at room temperature. After five hours, the conversion was complete and the solution was diluted with DCM, then washed two times with NaHCO<sub>3</sub>ss and brine. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure to give **3** (320 mg, 99%) as a brown glassy solid that was used directly for the next step.

**Step C.** (2*R*)-5-oxopyrrolidine-2-carboxylic acid (49 mg, 0.38 mmol) was dissolved in anhydrous DCM (2 mL) and the mixture was cooled at 0°C with an ice bath. Under stirring, N-methylmorpholine (40 mg, 0.40 mmol) and isobutyl chloroformate (54 mg, 0.40 mmol) were added. After one hour **3** (82 mg, 0.38 mmol) was added and the mixture was allowed to warm at room temperature. After 2 hours conversion was not complete and a premixed solution (0.5 mL in DCM) of (2*R*)-5-oxopyrrolidine-2-carboxylic acid (0.04 mmol) N-methylmorpholine (0.04 mmol) and isobutyl chloroformate (0.04 mmol) was added. After another hour, TLC showed complete conversion. The mixture was diluted with DCM and washed two times with 1M HCl, the same with NaHCO<sub>3</sub>ss and finally, with brine. The organic phase was dried on Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure to give **4** (100 mg, 81% yield) as a white solid that was used directly for the next step.

**Step D.** Compound **4** (121 mg, 0.37 mmol) was dissolved in dioxane (10 mL) and, under stirring, thioacetic acid (280 mg, 3.67 mmol) was added. The mixture was warmed at 75°C with an oil bath and AIBN (30 mg, 0.18 mmol) was added. After one-hour TLC monitoring showed complete conversion of **4**; the mixture was cooled at room temperature and then to 0°C and an excess of cyclohexene was added. The mixture was concentrated under reduced pressure, diluted with DCM and washed with 5% Na<sub>2</sub>CO<sub>3</sub> and brine. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude product was purified by chromatographic column (eluent DCM/MeOH = 95/5) to give **ST7612AA1** as a white solid (125 mg, 81 % yield).

**Step D'.** Compound **2** (143 mg, 0.45 mmol) was dissolved in dioxane (9 mL) and, under stirring, thioacetic acid (342 mg, 4.49 mmol) was added. The mixture was warmed at 75°C with an oil bath and AIBN (37 mg, 0.22 mmol) was added. After one-hour TLC monitoring showed complete conversion of **2**; the mixture was cooled at room temperature and then to 0°C and an excess of cyclohexene was added. The mixture was concentrated under reduced pressure, diluted with DCM and washed with 5% Na<sub>2</sub>CO<sub>3</sub> and brine. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under reduced pressure. The crude product was purified by chromatographic column (eluent cyclohexane/AcOEt = 80/20) to give **5** as a white solid (158 mg, 89 % yield).

**Step B'.** Compound **5** (158 mg) was dissolved in DCM (4 mL) and the mixture was cooled at 0°C with an ice bath. Trifluoroacetic acid (1 mL) was added under stirring and the mixture was allowed to warm at room temperature. After three hours the conversion was complete and the solution was diluted with DCM and concentrated under reduced pressure (three cycles of dilution and concentration) to give **6** as trifluoroacetate salt (163 mg, quantitative yield) as a brown glassy solid that was used directly for the next step.

**Step C'.** Compound **6** (163 mg, 0.4 mmol), (2*R*)-5-oxopyrrolidine-2-carboxylic acid (57 mg, 0.44 mmol) and DIPEA (155 mg, 1.20 mmol) were added to anhydrous DCM (3 mL) under stirring at room temperature. After complete dissolution, PyBOP (228 mg, 0.44 mmol) was added and the mixture was stirred at room temperature for 4 hours. Conversion was not complete but the mixture was worked up because of evidence of **6** degradation. The mixture was diluted with DCM and washed two times with 1M HCl, two times with NaHCO<sub>3</sub>ss and finally, with brine. The organic phase was dried on Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated under re-

duced pressure. The crude product was purified by chromatographic column (eluent AcOEt/MeOH = 95/5) to give **ST7612AA1** as a white solid (110 mg, 68 % yield).

### 3. RESULTS AND DISCUSSION

As shown in Table 1, this first step could be performed avoiding the use of expensive coupling reagents such as PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) or HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluoro-phosphate); EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide) and isobutyl chloroformate gave, surprisingly, about the same *ee* of PyBOP, which becomes even more advantageous considering that no chromatographic purification was needed after work up. Indeed, reaction with isobutyl chloroformate, after a simple work up with 1M HCl, saturated NaHCO<sub>3</sub> and brine, gave **2** (96% yield) as a white solid, which was used directly in the next step. Incidentally, HCl was preferable to the more commonly used citric acid, because of a more efficient extraction of N-methylmorpholine.

For thioacetic acid addition to the double bond of intermediate **2**, two different routes were possible, with addition reaction in the first (D') or last step (D) (Scheme 2). Reactions of **2** to give **5** or of **4** to give **ST7612AA1** were both performed in dioxane at 75°C with 0.5 equivalents of AIBN and 10 equivalents of thioacetic acid. Reactions were fast and did not need the usually advised radical quenching with cyclohexene. The corresponding products were obtained in good yields (step D', 89%; step D, 81%) after a flash chromatography.

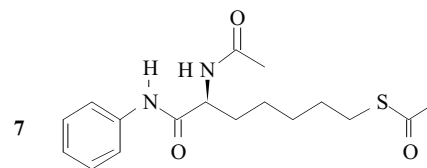
Concerning yields, we can observe that route D'-B'-C' was slightly worse than the other due to a poor yield in step C'. Besides, when using **6** (particularly as free base) for coupling to (2*R*)-5-oxopyrrolidine-2-carboxylic acid, a less polar byproduct was observed, subsequently identified as the N-acetylation derivative **7** (Fig. 3).

Indeed, it is known in literature that some attempts to deprotect S-acetate of 3-mercaptoproline derivatives, under basic conditions, resulted in an intramolecular trans-acetylation [23]. We therefore hypothesized that the organic base used in the coupling steps could be responsible for the same process, albeit giving an intermolecular reaction in this case. It must be said, however, that **7** was also observed in a reference sample for TLC, where **6** was simply stored in DCM at room temperature.

These concerns have pushed us to consider as favorite the alternative route, through the B-C-D steps, with the advantage to use, for step C, the cheaper conditions with isobutyl chloroformate and N-methylmorpholine.

#### Characterization Data

Thioacetic acid S-{(S)-6-[(R)-5-oxo-pyrrolidine-2-carbonyl]-amino]-6-phenylcarbonyl-hexyl} ester (**ST7612AA1**). <sup>1</sup>H NMR δ (500 MHz, DMSO-d<sub>6</sub>) 1.20-1.40 (m, 4H), 1.41-1.76 (m, 4H), 1.77-1.91 (m, 1H), 1.99-2.34 (m, 3H), 2.29 (s, 3H), 2.79 (t, *J* = 7.2 Hz, 2H), 4.04-4.14 (m, 1H), 4.35-4.47 (m, 1H), 7.03 (t, *J* = 7.3 Hz, 1H), 7.28 (t, *J* = 8.4 Hz, 2H), 7.57 (d, *J* = 7.6 Hz, 2H), 7.80 (s, 1H), 8.19 (d, *J* = 7.9 Hz, 1H), 10.06 (s, 1H);



**Fig. (3).** Structure of side product **7** from step C' of the synthetic procedure described in (Scheme 2).

<sup>13</sup>C NMR δ (126 MHz, DMSO-d<sub>6</sub>) 195.80, 177.84, 172.93, 171.02, 139.30, 129.21, 123.87, 119.74, 55.93, 53.60, 32.49, 31.06, 29.74, 29.46, 28.75, 28.21, 26.13, 25.28; *m/z* (ESI+, MeOH) 428.2 [M+Na]<sup>+</sup>; HRMS (ESI+, Orbitrap, MeOH) [M+Na]<sup>+</sup> C<sub>20</sub>H<sub>27</sub>N<sub>3</sub>O<sub>4</sub>S requires 428.1614, found 428.1602; HRMS (ESI-, Orbitrap, MeOH) [M-H]<sup>-</sup> C<sub>20</sub>H<sub>27</sub>N<sub>3</sub>O<sub>4</sub>S requires 404.1650, found 404.1656.

### CONCLUSIONS

In conclusion, **ST7612AA1**, prodrug of ST7464AA1, is the first of a new generation of HDAC inhibitors, very potent, orally administered, and well tolerated. It is a thiol derivative, pan-histone deacetylase inhibitor, active against a broad panel of cancer cell lines and *in vivo* tumor models. It was also an activator of latent HIV, potentially useful in HIV antiretroviral therapy.

In the present paper, the effects of different synthesis routes at lab scale for **ST7612AA1** synthesis were discussed. In Scheme 1 we reported our first synthetic way based on enzymatic resolution and thioacetylation by nucleophilic substitution with potassium thioacetate. Avoiding the enzymatic pathway (steps a-e), the highlighted intermediate in Scheme 1 could be obtained from the commercial 2-tert-butoxycarbonylaminohept-6-enoic acid by a simple hydrobromination. However, we have chosen the way of thioacetylation reaction by a radical mechanism, that yields the final product in only four steps, with high yields, easily, inexpensive and reproducible scalable process.

**ST7612AA1** is now completing the preclinical studies, with emphasis on evaluation of the pharmacokinetic and pharmacological properties, with the aim to identify the optimal dose and the identification of the best therapeutic indication. Once this phase will be ended, whether this new drug will enter in a clinical trials phase, it will be necessary a scale-up of the synthetic process. The results obtained from this study and here presented, will be an important starting point for this process.

### CONFLICT OF INTEREST

The authors Gianfranco Battistuzzi e Giuseppe Giannini are employees of Sigma-Tau I.F.R. SpA.

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Declared none.

### SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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