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Biological activities of substituted trichostatic acid derivatives

CÉDRIC CHARRIER[a], JOËLLE ROCHE[b], JEAN-PIERRE GESSON[a] and PHILIPPE BERTRAND[a]*

[a]Synthèse et Réactivité des Substances Naturelles, CNRS UMR 6514, Université de Poitiers: 40 Avenue du Recteur Pineau, Poitiers, F-86022, France

[b]Institut de Physiologie et Biologie Cellulaires, CNRS UMR 6187, Université de Poitiers: 40 Avenue du Recteur Pineau, Poitiers, F-86022, France

e-mail: philippe.bertrand@univ-poitiers.fr

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Abstract. New substituted trichostatic acid derivatives have been synthesized and evaluated for their biological activities towards the H661 non-small lung cancer cell line. These syntheses were achieved by alkylation of propiophenones to introduce the side chain with a terminal precursor of hydroxamic acid and aminobenzamide derivatives. The first fluorinated derivatives of trichostatic acid are described, such as 6-fluoro trichostatin A, with antiproliferative activities in the micromolar range and with histone deacetylase inhibitory activity.

Keywords. Trichostatic acid; HDAC; inhibitor.

1. Introduction

Reversible chemical modifications of DNA and histones are important cellular events that promote activation or repression of DNA transcription and are responsible for epigenetic modifications that are altered during cancer progression.[1,2] Histone acetylation, involved in chromatin decondensation, is associated to active chromatin and is mediated by Histone Acetyl Transferases (HAT). On the contrary, histone deacetylation by Histone Deacetylases (HDAC) leads to a transcriptionally inactive and condensed form of chromatin,[3–6] In addition, histones are not the only substrates for HAT/HDAC as more than 50 target proteins with diverse functions have been described and include for example, tubulin and p53.[7] Overexpression of HDAC has been reported in cancers and HDAC inhibition has emerged as a promising anti-cancer treatment.[8,9] The eighteen HDAC identified in humans are classified in four classes, class I, II and IV being zinc dependent metalloproteins, while class III is NAD dependent.[5] Several programs were initiated worldwide to develop HDAC inhibitors (HDACi) with particular focus on inhibitors targeting the zinc dependent classes for anti-cancer applications. Natural products were isolated from fungus like trichostatin A (TSA) **1**. Synthetic compounds, like SAHA **2**,[10,11] MS-275 **3**, and NVP-LAQ824 **4** (figure 1) have reached clinical trials.[12] SAHA was recently approved for treatment of cutaneous T-cell lymphoma.[13] TSA[14] is well known as a HDACi.[15,16] TSA has been synthesized in either racemic[17] or enantiopure[18] form, and enantioselective preparation of trichostatin D, a O-α-glucoside derivative of TSA,[19] has also been described.

In this article we described the synthesis of new trichostatic acid derivatives and their evaluation as antiproliferative agents and HDAC inhibitors. In a previous work we described indanone analogues of TSA **5a** and **6a**[20] (figure 2) with antiproliferative activities on H661 lung cancer cells (IC_{50} = 0·3 and 2·5 μM respectively) and HDACi property. We planned to compare the activity of these reported compounds to new 6-substituted TSA analogues with no additional ring constraint.

2. Experimental

Abbreviations used: PE: petroleum ether, EtOAc: ethyl acetate, THF : tetrahydrofuran, TBTU: tetrafluoroborate O-(benzotriazol-1-yl-1,1,3,3-tetramethyl)-uronium, EDC: 1-[3(dimethylamino)propyl]-3-ethyl-carbodiimide hydrochloride.

*For correspondence

2.1 *1-(4-Dimethylaminophenyl)-propan-1-one* **9**

Method A: To a solution of aldehyde **7** (3·05 g, 20·5 mmol) in THF (20 ml) was added a THF solution of BrMgEt freshly prepared (31 ml, 61·5 mmol, 2N in THF). The mixture was refluxed 4 h under nitrogen and then cooled. A saturated solution of NH_4Cl was then added to obtain neutralization (15 ml). The resulting mixture was extracted (EtOAc, 3 × 100 ml) and the combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to give the crude alcohol that was purified (flash chromatography on silica, EtOAc : PE 10 : 90). The alcohol **8** was obtained as a colourless oil (3·6 g, 98%).

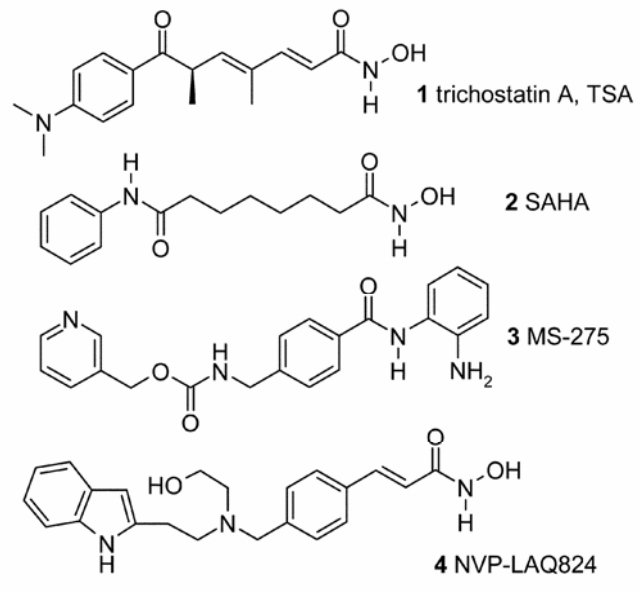


Figure 1. Natural and synthetic HDACi.

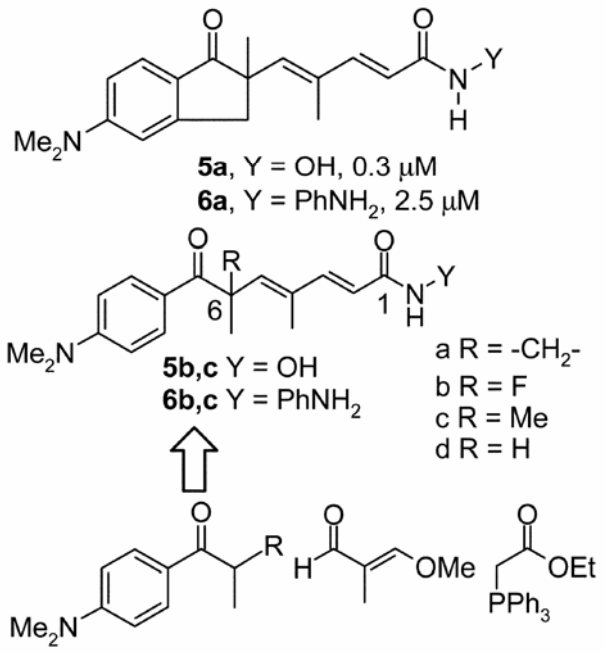


Figure 2. Targeted TSA derivatives.

The intermediate alcohol **8** (3·6 g, 20 mmol) was dissolved in tBuOH (20 ml) and tBuOK was added (2·24 g, 20 mmol) prior to benzophenone (3·64 g, 20 mmol). The solution was refluxed for 4 h. The resulting mixture was diluted with water (50 ml) and extracted with EtOAc (3 × 100 ml). The organic layers were combined and dried ($MgSO_4$). Purification (flash chromatography EtOAc : PE 10 : 90) gave the pure ketone **9** as a solid (2·13 g, 60%).

Method B: To a solution of nitrile **10** (3 g, 20·5 mmol) in THF (20 ml) was added a THF solution of BrMgEt freshly prepared (31 ml, 61·5 mmol, 2N in THF), followed by addition of CuBr (60 mg). The mixture was refluxed 4 h under nitrogen then cooled. Water was added (15 ml), followed by careful addition of H_2SO_4 (1N, 50 ml). The mixture was refluxed for 1 h and then cooled. Neutralization with NaOH was followed with extraction with EtOAc (3 × 100 ml). The organic layer was dried ($MgSO_4$) and the solvent removed under reduced pressure. The ketone **9** was obtained as a solid (3·6 g, 98%) with satisfactory purity for the next step.

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·18 (*d*, 6H, J = 6·6 Hz), 3·0 (*s*, 6H), 3·5 (*q*, 1H, J = 6·8 Hz), 6·60 (*d*, 2H, J = 9·0 Hz), 7·9 (*d*, 2H, J = 9·0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 19·5, 34·1, 39·6, 110·6, 123·6, 130·3, 153·1, 201·9.

2.2 *1-(4-Dimethylaminophenyl)-2-methyl-propan-1-one* **13c**

Prepared as mentioned above from nitrile **10** (3 g, 20·5 mmol), THF (20 ml) and a THF solution of BrMgiPr freshly prepared (31 ml, 61·5 mmol, 2N in THF) as a solid (3·5 g, 90%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·2 (*t*, 3H, J = 7·3 Hz), 2·9 (*q*, 2H, J = 7·3 Hz), 3·05 (*s*, 6H), 6·65 (*d*, 2H, J = 9·2 Hz), 7·9 (*d*, 2H, J = 9·2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 8·8, 30·9, 40·0, 110·6, 124·6, 130·3, 153·3, 199·8.

2.3 *2,2-Dibromo-1-(4-dimethylaminophenyl)-propan-1-one* **11**

Ketone **9** (25 g, 0·14 mol) was dissolved in concentrated H_2SO_4 (150 ml) and bromine (8 ml, 0·16 mol)

was added drop-wise while maintaining the temperature at 0°C. After stirring 6 h the temperature was allowed to warm to room temperature and ice cold water was then added. The precipitate obtained was filtered off and washed with ice cold water to give the dibrominated compound **11** (42·2 g, 90%) pure enough for the next step.

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 2·65 (*s*, 3H), 3·05 (*s*, 6H), 6·60 (*d*, 2H, *J* = 9·2 Hz), 8·3 (*d*, 2H, *J* = 9·2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 38·0, 40·0, 58·8, 110·0, 118·5, 133·8, 153·3, 186·1.

2.4 *2-Bromo-1-(4-dimethylaminophenyl)-propan-1-one* **12**

11 (30 g, 0·09 mol) was dissolved in THF (150 ml) and cooled to 0°C. $(EtO)_2PH{=}O$ (13 ml, 0·1 mol) was then added followed by a solution of NEt_3 (14 ml, 0·1 mol) in THF (80 ml). The temperature was allowed to warm to room temperature under stirring for 6 h and the resulting mixture concentrated under reduced pressure and hydrolysed by ice cold water (300 ml). The resulting precipitate was filtered off and washed with ice cold water to afford the monobrominated compound **12** as a yellow solid (23 g, 90%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·2 (*d*, 3H, *J* = 6·6 Hz), 3·05 (*s*, 6H), 5·2 (*q*, 1H, *J* = 6·6 Hz), 6·6 (*d*, 2H, *J* = 9·2 Hz), 7·85 (*d*, 2H, *J* = 9·2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 20·9, 40·4, 42·1, 111·2, 121·9, 131·5, 154·0, 191·6.

2.5 *1-(4-Dimethylamino-phenyl)-2-fluoro-propan-1-one* **13b**

Crown ether 18C6 (269 mg) and KF (1·8 g, 30 mmol) were dissolved in dry CH_3CN (20 ml). The solution was stirred 30 min. at 90°C prior to addition of bromoketone **12** (15 mmol). The mixture was then stirred for 24 h. After cooling ether was added (50 ml) and the salts were removed by filtration. The organic layer was washed with water (100 ml), dried and concentrated under reduced pressure. The crude material was purified (flash chromatography on silica, PE : EtOAc 90 : 10) to give the fluoro compound **13b** as a solid (16 g, 91%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·65 (*dd*, 3H, *J* = 6·8, 23·9 Hz), 3·05 (*s*, 6H), 5·65 (*dq*, 1H, *J* = 6·8, 49·9 Hz), 6·60 (*d*, 2H, *J* = 8·7 Hz), 7·9 (*d*, 2H, *J* = 8·7 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 18·9 (*d*, *J* = 23·1 Hz), 39·9, 90·5 (*d*, *J* = 178 Hz), 110·7, 121·6, 131·2 (*d*, *J* = 3·8 Hz), 153·7, 194·4 (*d*, *J* = 18·1 Hz).

2.6 *5-(4-Dimethylaminophenyl)-2,4,4-trimethyl-5-oxo-pent-2-enal* **14c**

To a solution of iPr_2NH (freshly distilled, 1·3 ml, 9 mmol) in THF (20 ml) cooled to –20°C was added drop wise nBuLi (5·7 ml, 1·6 M in hexane, 9 mmol). After 30 min stirring, temperature was cooled to –78°C and ketone **13c** (1·44 g, 7·5 mmol) in THF (10 ml) was added. After stirring 1·5 h at –78°C, MeOCH=C(Me)CHO (0·86 g, 7·5 mmol) in THF (5 ml) was added. The temperature was brought to room temperature overnight. A saturated NH_4Cl solution was added (100 ml) and the resulting mixture extracted with ether (3 × 100 ml). The combined organic layers were dried ($MgSO_4$) and concentrated under reduced pressure. Purification (flash chromatography on silica, EtOAc : PE 30 : 70) gave **14c** as a yellow oil (1·5 g, 77%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·45 (*d*, 3H, *J* = 1·3 Hz); 1·51 (*s*, 6H), 3·1 (*s*, 6H), 6·5 (*d*, 2H, *J* = 9·2 Hz), 6·9 (*d*, 2H, *J* = 1·3 Hz), 7·8 (*d*, 2H, *J* = 9·2 Hz), 9·4 (*s*, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 9·4, 27·2, 40·0, 48·0, 110·2, 121·5, 131·6, 139·5, 153·0, 160·1, 195·2, 199·6.

2.7 *5-(4-Dimethylaminophenyl)-4-fluoro-2,4-dimethyl-5-oxo-pent-2-enal* **14b**

Prepared as **14c** from **13b** (7·5 mmol in 10 ml THF), LDA (iPr_2NH, 1·3 ml, 9 mmol; *n*BuLi (5·7 ml, 1·6 M in hexane, 9 mmol in 20 ml THF) and MeOCH=C(Me)CHO (0·86 g, 7·5 mmol, in 5 ml THF) as a yellow oil (1·62 g, 82%).

^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·85 (*d*, 3H, *J* = 22·5 Hz), 1·85 (*t*, 3H, *J* = 1·5 Hz), 3·05 (*s*, 6H), 6·60 (*dd*, 1H, *J* = 1·5, 22·0 Hz), 6·65 (*d*, 2H, *J* = 9·2 Hz), 7·9 (*dd*, 2H, *J* = 1·3, 8·7 Hz), 9·4 (*s*, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 9·5 (*d*, *J* = 4·4 Hz), 23·7 (*d*, *J* = 24·7 Hz), 39·9, 99·9 (*d*, *J* = 183·3 Hz), 110·5, 120·9 (*d*, *J* = 3·8 Hz), 132·5 (*d*, *J* = 7·1 Hz), 139·4 (*d*, *J* = 3·8 Hz), 150·7 (*d*, *J* = 24·7 Hz), 153·7, 193·0 (*d*, *J* = 24·2 Hz), 194·3.

2.8 *7-(4-Dimethylaminophenyl)-4,6,6-trimethyl-7-oxo-hepta-2,4-dienoic acid ethyl ester* **15c**

To a solution of aldehyde **14c** (1·3 g, 5 mmol) in benzene (15 ml) was added $Ph_3P{=}CHCOOEt$

(1·73 g, 5 mmol). After 4 h reflux, benzene was removed under reduced pressure and the crude oil purified (flash chromatography EtOAc : PE 10 : 90) to give a yellow oil. Recrystallisation from CH_2Cl_2/PE gave the ester as a colourless oil (1·44 g, 85%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·35 (*t*, 3H, *J* = 7·1 Hz), 1·40 (*s*, 3H), 1·45 (*d*, 3H, *J* = 0·7 Hz), 3·1 (*s*, 6H), 4·2 (*q*, 1H, *J* = 7·1 Hz), 5·7 (*d*, 1H, *J* = 15·4 Hz), 6·60 (*d*, 2H, *J* = 9·2 Hz), 6·3 (*s*, 1H), 7·3 (*dd*, 1H, *J* = 0·7, 15·4 Hz), 7·9 (*d*, 2H, *J* = 9·2 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 12·5, 14·3, 28·0, 39·8, 47·8, 60·1, 110·1, 116·4, 121·9, 131·8, 133·8, 147·9, 149·5, 152·7, 167·3, 201·2.

2.9 *7-(4-Dimethylaminophenyl)-6-fluoro-4,6-dimethyl-7-oxo-hepta-2,4-dienoic acid ethyl ester* **15b**

Prepared as **15c** from aldehyde **14b** (1·31 g, 5 mmol), Ph_3P=CHCOOEt (1·73 g, 5 mmol) and benzene (15 ml) as a colourless oil (1·42 g, 85%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·28 (*t*, 3H, *J* = 7·1 Hz), 1·75 (*d*, 3H, *J* = 22·7 Hz), 1·85 (*t*, 3H, *J* = 1·25 Hz), 3·05 (*s*, 6H), 4·20 (*d*, 2H, *J* = 7·1 Hz), 5·8 (*d*, 1H, *J* = 15·7 Hz), 6·27 (*d*, 1H, *J* = 20·9 Hz), 6·60 (*d*, 2H, *J* = 9·0 Hz), 7·3 (*d*, 1H, *J* = 15·7 Hz), 7·95 (*dd*, 2H, *J* = 1·1, 9·1); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 12·7 (*d*, *J* = 3·3 Hz), 14·2, 24·4 (*d*, *J* = 25·2 Hz), 39·7, 60·3, 99·1 (*d*, *J* = 179·5 Hz), 110·4, 118·5, 120·8 (d, *J* = 3·3 Hz), 132·4 (*d*, *J* = 5·5 Hz), 135·2 (*d*, *J* = 6·6 Hz), 139·1 (*d*, *J* = 23·7 Hz), 148·3, 153·5, 166·7, 193·9 (*d*, *J* = 24·7 Hz).

2.10 *7-(4-Dimethylaminophenyl)-4,6,6-trimethyl-7-oxo-hepta-2,4-dienoic acid* **16c**

To a solution of ester **15c** (930 mg, 2·97 mmol) in THF : MeOH (5 : 1, 12 ml) was added an aqueous solution of LiOH (2·5 M, 2 ml). After stirring overnight at ambient temperature, the mixture was acidified to pH 1 with HCl (6N). The mixture was then diluted with H_2O (50 ml) and saturated with NaCl and extracted with EtOAc (6 × 50 ml) until no more acid was found in the aqueous layer. The combined organic layers are dried ($MgSO_4$) and concentrated to give the crude acid. Minimum CH_2Cl_2 was then added to dissolve the acid and 100 ml of EP was added to obtain the acid as a white precipitate. After removal of the supernatant, the acid is obtained as a white solid. This purification was repeated twice and give the acid (850 mg, 95%) pure enough for the next step. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·40 (*s*, 6H), 1·45 (*s*, 3H), 3·1 (*s*, 6H), 5·7 (*d*, 1H, *J* = 15·4 Hz), 6·4 (*s*, 1H), 6·60 (*d*, 2H, *J* = 9·2 Hz), 7·4 (*d*, 1H, *J* = 15·4 Hz), 7·9 (*d*, 2H, *J* = 9·0 Hz).

2.11 *7-(4-Dimethylaminophenyl)-4,6,6-trimethyl-7-oxo-hepta-2,4-dienoic acid* **16b**

Prepared as **16c** from ester **15b** (1·13 g, 3·4 mmol), THF : MeOH (5 : 1, 46·4 ml) and LiOH (2·5 M aq., 7·7 ml) as a white solid (986 mg, 95%).

2.12 *7-(4-Dimethylaminophenyl)-4,6,6-trimethyl-7-oxo-hepta-2,4-dienoic acid (2-aminophenyl)-amide* **6c**

To a solution of acid **16c** (157 mg; 0·52 mmol) in dry THF (5 ml) was added 1,2-diaminobenzene (340 mg, 3·12 mol) and EDC (145 mg, 0·68 mmol). After stirring overnight, the solution was concentrated under vacuum. EtOAc was added (50 ml) and the resulting organic layer washed with H_2O (30 ml) and then twice with aqueous 1N NaOH (20 ml). The organic layer was dried ($MgSO_4$) and solvents removed under vacuum. The resulting solid was purified (flash chromatography EtOAc : PE 60 : 40) to give the benzamide **6c** as a pale yellow powder (144 mg, 71%, mp: 136–140°C); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·40 (*s*, 6H), 1·5 (*s*, 3H), 3·0 (*s*, 6H), 3·9 (*s*, 2H), 5·8 (*d*, 1H, *J* = 15·4 Hz), 6·3 (*s*, 1H), 6·60 (*d*, 2H, *J* = 9·0 Hz), 6·8 (*d*, 1H, *J* = 7·5 Hz), 7·0 (*t*, 1H, *J* = 7·5 Hz), 7·2 (*d*, 1H, *J* = 7·5 Hz), 7·4 (*d*, 1H, *J* = 15·4 Hz), 7·5 (*s*, 1H), 7·9 (*d*, 2H, *J* = 9·0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 12·8, 28·0, 39·9, 47·9, 110·1, 118·1, 118·2, 119·5, 121·9, 124·5, 125·0, 127·0, 131·9, 133·6, 140·7, 147·3, 147·6, 152·7, 164·7, 201·6.

2.13 *7-(4-Dimethylaminophenyl)-6-fluoro-4,6-dimethyl-7-oxo-hepta-2,4-dienoic acid (2-aminophenyl) amide* **6b**

Prepared as **6c** from acid **16b** (159 mg, 0·52 mmol), THF (5 ml), 1,2-diaminobenzene (340 mg, 3·12 mmol) and EDC (145 mg, 0·68 mmol) as a pale yellow solid (150 mg, 73%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 1·7 (*m*, 6H), 3·0 (*s*, 6H), 6·0 (*d*, 2H, *J* = 8·8 Hz), 6·04 (*d*, 1H, *J* = 15·3 Hz), 6·3 (*d*, 1H, *J* = 20·3 Hz), 6·8 (*m*, 2H), 7·0 (*t*, 1H, *J* = 7·2 Hz), 7·25–7·35 (*m*, 3H), 8·0 (*d*, 2H, *J* = 8·8 Hz); HRMS

ESI: ($C_{23}H_{26}N_3O_2F$) M^+ calcd: 395·20091, found: 395·2003.

2.14 *7-(4-Dimethylaminophenyl)-4,6,6-trimethyl-7-oxo-hepta-2,4-dienoic acid hydroxyamide* **5c**

To a solution of carboxylic acid (188 mg, 0·625 mmol) in DMF (2 ml) were added TBTU (300 mg, 0·937 mmol) and NEt_3 (0·17 ml, 1·25 mmol). After 2 h NH_2-OTHP was added (110 mg, 0·937 mmol) and stirring continued overnight. The resulting solution was neutralized with $NaHCO_3$ (saturated) and extracted with EtOAc (3 × 50 ml). The organic layers were combined and dried ($MgSO_4$), and the solvent removed under reduced pressure. The protected O-THP derivative was purified (flash chromatography on silica, EP : EtOAc 60 : 40) and dissolved in MeOH (5 ml). Amberlyst was then added (150 mg) and the mixture stirred at ambient temperature until the starting material is completely consumed (for 3 to 6 h). The resin is filtered off and MeOH removed under reduced pressure. Purification (flash chromatography on silica, EtOAc) gave de hydroxamate as a pale pink solid (123 mg, 62%). ^{1}H NMR (300 MHz, CD_3OD) δ ppm: 1·35 (*m*, 9H), 3·1 (*s*, 6H), 5·65 (*d*, 1H, J = 15·2 Hz), 6·35 (*s*, 1H), 7·2 (*m*, 3H), 8·0 (*d*, 2H, J = 9·0 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ ppm: 13·4, 28·5, 45·1, 117·0, 118·6, 132·7, 133·4, 136·0, 147·0, 147·3, 149·9, 166·9, 204·0.

2.15 *7-(4-Dimethylaminophenyl)-6-fluoro-4,6-dimethyl-7-oxo-hepta-2,4-dienoic acid hydroxyamide* **5b**

Prepared as **5c** from **16b** (191 mg, 0·625 mmol), DMF (2 ml), TBTU (300 mg, 0·937 mmol) and NEt_3 (0·17 ml, 1·25 mmol) and NH_2-OTHP (110 mg, 0·937 mmol) as a light red solid (94 mg, 47%). ^{1}H NMR (300 MHz, CD_3OD) δ ppm: 1·70 (*m*, 6H), 3·0 (*s*, 6H), 5·9 (*d*, 1H, J = 15·4 Hz), 6·3 (*d*, 1H, J = 19·6 Hz), 6·66 (*d*, 2H, J = 8·8 Hz), 7·2 (*d*, 1H, J = 15·5 Hz), 7·8 (*d*, 2H, J = 8·8 Hz); ^{13}C NMR (75 MHz, CD_3OD) δ ppm: 13·1 (*d*, J = 3·3 Hz), 25·2 (*d*, J = 25·2 Hz), 40·0, 99·3 (*d*, J = 178 Hz), 111·5, 119·0, 121·5, 133·5 (*d*, J = 4·9 Hz), 137·1 (*d*, J = 7·1 Hz), 139·1 (*d*, J = 23·6 Hz), 145·5, 155·3, 166·1, 196·2 (*d*, J = 23·6 Hz).

2.16 *Cell culture*

The NCI-H661 non-small cell lung cancer cells were grown in RPMI 1640 plus 10% fetal calf serum (InVitrogen) at 37°C and under 5% CO_2.

2.17 *Cell proliferation*

The Cell Proliferation Kit II (XTT; Roche) was used to assess cell proliferation. This assay is based on the cleavage of XTT by metabolic active cells resulting in the production of an orange formazan dye that is quantified by spectrophotometry. Assays were carried out essentially as described by the manufacturer. Briefly, 4×10^3 cells/well were plated in 100 μl of media in a 96-well plate. Cells were grown for 24 h before adding the compound at the indicated concentration. After 48 h treatment, 50 μl of the XTT labelling mixture were added per well. Cells were further incubated for additional 4 h at 37°C before reading the absorbance at 480 nm.

2.18 *Western blot analysis*

5×10^5 cells were treated with the compounds for 5.30 h for histone H4 and tubulin acetylation analysis. Cells were lysed in 200 μl of the electrophoresis loading buffer from Laemmli and sonicated. Protein lysates were resolved by SDS–PAGE, followed by Western blot. Primary antibodies were rabbit polyclonal anti-acetylated histone H4 antibody (1/500; Upstate) or mouse monoclonal anti-α-tubulin antibody (1/2000; Sigma). HRP-conjugated anti-mouse and anti-rabbit secondary antibodies (Amersham) were used at 1/5000 dilution. Detection was performed with ECL (Perkin Elmer).

3. Results and discussion

A general route was thus designed in a convergent way and allowing the potential introduction of several functional groups in the alpha position of the ketone of trichostatic acid. A methyl group was selected to compare the rigid compound **5a** and **6a** with the flexible derivatives **5b**,**c** and **6b**,**c** (figure 2). Substitution of the hydrogen in position 6 was also envisioned with an atom not increasing the steric hindrance around the ketone. The fluorine atom was selected, having an equivalent atom radius compared to the hydrogen but introducing higher electronegativity. Some fluorinated compounds were described as HDACi,[21,22] but fluorinated TSA was not described. Benzamide derivatives like MS-275, although weaker zinc ligands than the hydroxamic acids, generally lead to bioactive molecules and possess higher stability in biological environments. In this respect we prepared both hydroxamic acids and benzamide versions of the target structures.

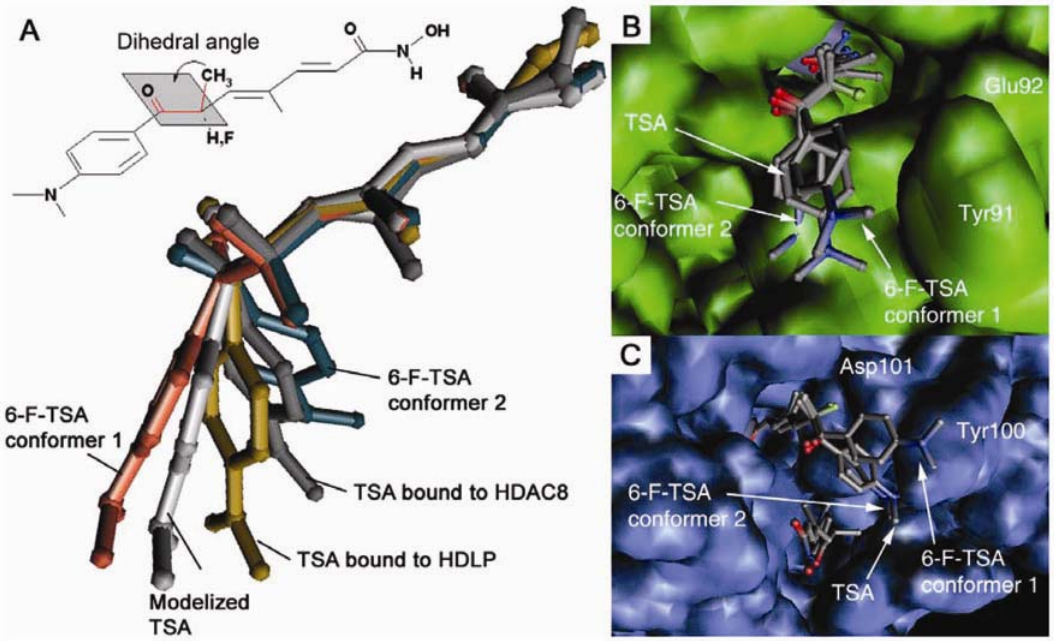


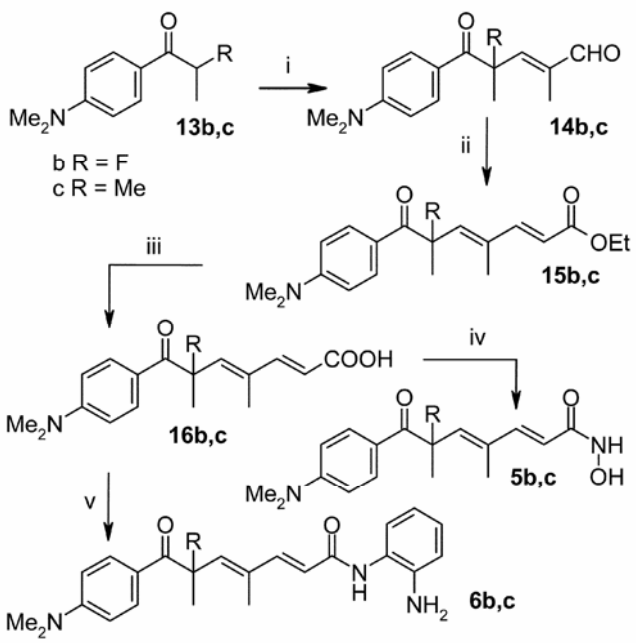
Figure 3. (**A**) Crystallographic structures of TSA bound to HDAC8 and HDLP, compared to modelized TSA and the two stable modelized conformers C1 and C2 of 6-Fluoro-TSA. (**B**) Docking of C1 and C2 in HDLP. (**C**) Docking of C1 and C2 in HDAC8.

Molecular modelling of the fluoro hydroxamate **5b** was performed at the AM1 level. Validation of the method was made by modelling TSA and superimposition with the described structures of TSA[23,24] (figure 3A) based on the overlapping of the hydroxamic acid group, alignment of the butadiene chain, and matching the two methyl groups and the carbonyl function. In trichostatic acid, the four atoms of the propanone system (Ar–C=O–C–CH_3) define a dihedral angle (figure 3A, top). In the several published crystallized HDACs with TSA bound, this dihedral angle is varying to accommodate the observed small differences in HDLP or HDAC8 conformations. We compared these differences with modelled TSA and **5b**. AM1 modelled TSA (figure 3A) showed the highest dihedral angle. Interestingly, analogue **5b** produced two stable conformers C1 (–63·107 kcal/mol) and C2 (–63·348 kcal/mol). In conformer C1, the dihedral angle increased probably in order to manage the electronic repulsions between the fluorine atom and the aromatic ring. This resulted in an aromatic ring plane positioned between the methyl group and the fluorine atom of carbon 6. The dihedral angle decreased in the slightly more stable conformer C2, with the aromatic ring plane positioned between the fluorine atom and the butadiene chain. The docking of the two conformers C1 and C2 of compound **5b** was investigated with the known X-ray structure of TSA (figure 3B, C respectively). In the case of TSA bound to HDLP, fluorinated conformer C1 was found more adequate while C2 produced interactions of the dimethylamino group with the surface of the protein. In the case of TSA bound to HDAC8, interactions with Tyr100 were found higher for conformer C1. Thus conformer C2 appeared more adequate for comparison of fluorinated TSA bound to HDAC8. As X-ray crystallographic data with benzamide derivatives are still missing, modelling of the benzamide versions was not investigated.

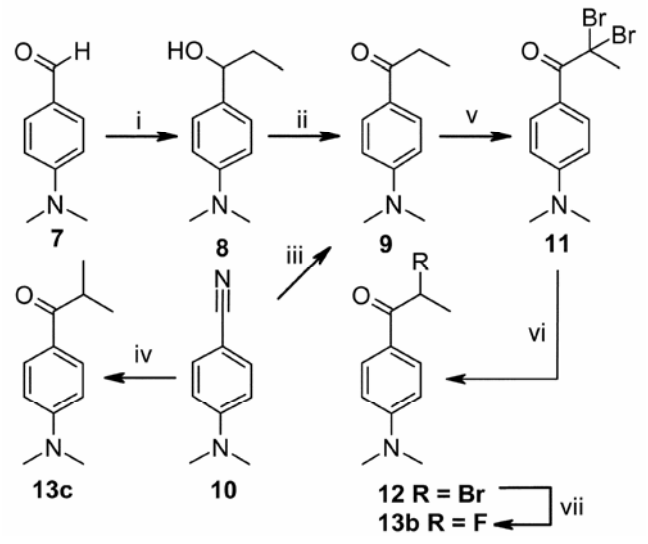
Our synthetic strategy relied on the alkylation of convenient propiophenones by methoxymethacrolein, leading to intermediate precursor of pentadienoic acid. These acids were then converted to hydroxamic acids **5** or to benzamide derivatives **6**. Propiophenones **13** were prepared from the corresponding derivatives **7** or **10** (scheme 1). In a first route, the aldehyde **7** was alkylated (EtMgBr, THF) to give alcohol **8** that under Oppenhauer oxidation gave the key ketone **9**. A more convergent route was then implemented from the nitrile **10**. Direct preparation of ketone **9** was obtained by alkylation of compound **10** with EtMgBr under copper catalysis.[25] The same protocol allowed the preparation of ketone

13c. Direct fluorination of compound **9** was not possible. A two-step strategy was implemented based on the monobromination of **9** and subsequent nucleophilic displacement by a fluorine atom.[26] Unfortunately, bromination of **9** afforded exclusively in high yields the dibromo derivative **11**.[27] According to Diwu and Liu, mono debromination by diethylphophite afforded **12**.[28] The nucleophilic substitution under phase transfer catalysis finally gave the desired fluoro derivative **13b**. Deprotonation of compounds **13b**,**c** (LDA, –78°C) and ketolisation with methoxymethacrolein, followed by acidic work-up, gave propenal derivatives **14b**,**c** (scheme 2). The aldehydes **14b**,**c** were then converted to the conjugated *trans* esters **15b**,**c** by a Wittig elongation. Subsequent hydrolysis of the ester group gave the acids **16b**,**c**. Hydroxamic acid derivatives **5b**,**c** were obtained under TBTU coupling conditions between acids **16b**,**c** and the O-tetrahydropyranyl (O-THP) ether of hydroxylamine, followed by acidic hydrolysis of the intermediate O-THP ether of the hydroxamic acids. The benzamides **6b**,**c** were prepared under EDC coupling conditions between acids **16b**,**c** and the 1,2-diaminobenzene.

New derivatives were tested for their antiproliferative activity against H661 human non-small cell lung cancer cells.[20] After 48 h treatment (figure 4), IC_{50} were determined for 50% inhibition and were compared to those of SAHA, TSA and the reported HDACi **5a** and **6a** (table 1). Compounds **5c** and **6c** were found inactive at the highest tested concentrations (25 μM), probably because they cannot fit in



Scheme 2. (i) LDA, THF, –78°C, MeOCH=C(Me) CHO, 77–82%; (ii) Ph_3P=CHCOOEt, toluene, 85%; (iii) LiOH, MeOH, 95%; (iv) (**a**) H_2N-OTHP, TBTU, DMF, NEt_3; (**b**) CSA, CH_2Cl_2, MeOH, 47–62% (a + b); (v) H_2N-Ph-NH_2, EDC, THF, 71–73%.



Scheme 1. (i) THF, EtMgBr, 98%; (ii) tBuOK, benzophenone, 60%; (iii) THF, EtMgBr, CuBr, 90%; (iv) THF, iPrMgBr, CuBr, 90%; (v) H_2SO_4, Br_2, 100%; (vi) $(EtO)_2POH$, NEt_3, THF, 96%; (vii) KF, 18-C-6, CH_3CN, 95%.

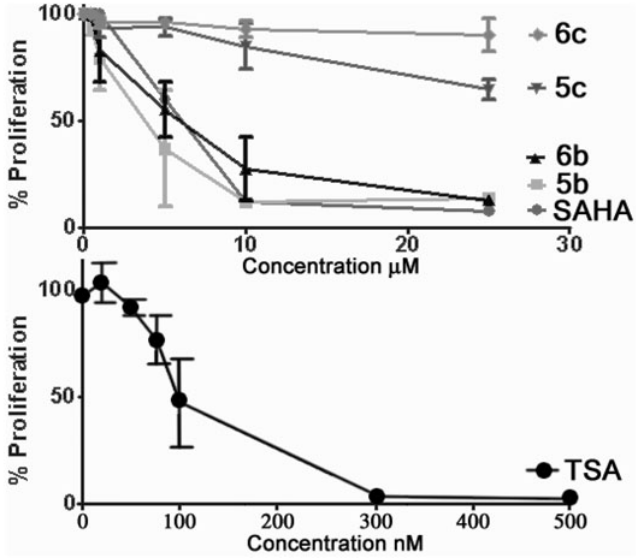


Figure 4. Antiproliferative activity of the indicated compounds after 48 h treatment of NCI-H661 cells. Two independent experiments were done in triplicate. Standard deviation is indicated.

the entry pocket of the active site. This result is explained by the comparison of the indanone derivatives **5a** and **6a** with **5c** and **6c**.

The indanone moiety generated a contraction[20] that compensated for the steric hindrance of the methylene bridge. This is not possible for methylated derivatives **5c** and **6c**, due to the free rotation. The fluorinated derivatives **5b** and **6b** were found active with respective ICs_{50} of 4 and 6 μM, equivalent to SAHA (figure 4), and 40 fold less active than TSA in the NCI-H661 lung cancer cell line. Only HDACi activity of the most active compound **5b** was evaluated and compared to TSA. A 5 μM concentration of compound **5b** gave similar inhibition of HDAC than 300 nM TSA (figure 5). This result showed that HDACs from class I and HDAC6 specific for tubulin are targets of this compound.[29,30]

Vanini *et al* described a tetrapeptide $R^{379}HK_{Ac}K_{Ac}{}^{382}$, related to p53, bound to HDAC8.[31,32] The alpha amino group of $K_{Ac}{}^{382}$ is positioned close to HDAC8 Tyr100 and Asp101, generating hydrogen bounding with the HDAC8 Asp101 residue. An analogous Tyr–Asp pair is found in the bacterial histone deacetylase homologue HDAH,[33] while it is a Tyr–Glu in HDLP. HDAC1 and HDAC2 have a Glu98–Asp99 pair and HDAC3 a Asp92–Asp93 pair.[34] These pairs of residues are a common feature in HDAC and were suggested to be important for the development of selective HDACi. The docking of the two conformers C1 and C2 of compound **5b** (figure 3B, C) resulted in a fluorine atom near these pairs of resi-

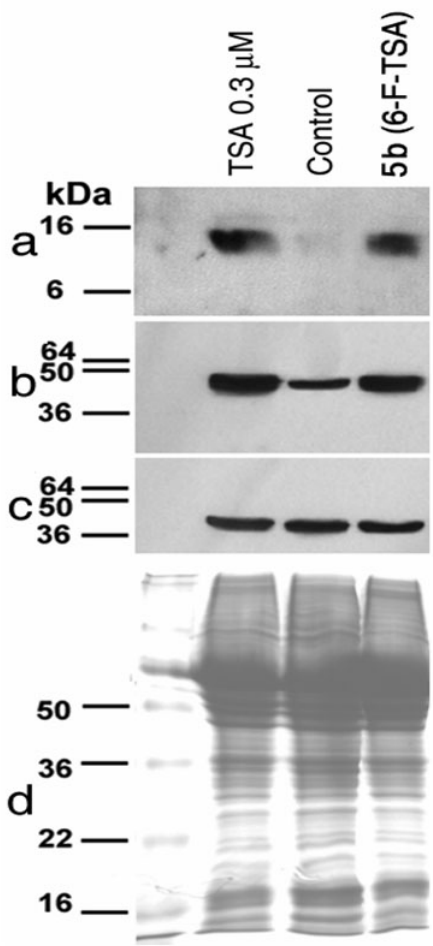
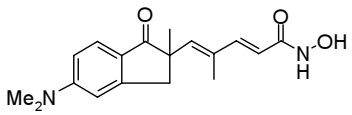
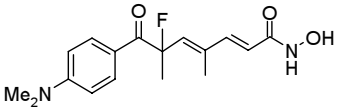
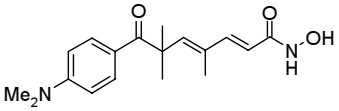
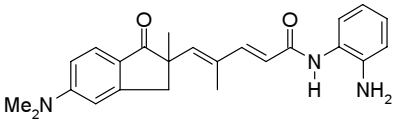
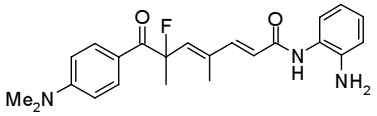
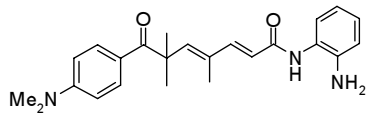


Figure 5. Analysis of histone H4 and tubulin acetylation. NCI-H661 cells were treated for 5 h 30 min with TSA (0·3 μM) and 6-Fluoro-TSA **5b** (5 μM). Control is untreated cells. Western blots are representative of three independent experiments. Molecular weights are indicated in KDa on the left. (**a**) acetylated histone H4; (**b**) acetylated tubulin; (**c**) α-tubulin; (**d**) loading control stained with Coomassie blue.

Table 1. Antiproliferative activities after 48 h treatment of TSA, SAHA, reported and new TSA derivatives on H661 cells.

Compounds		Inhibition IC_{50}, μM
1	TSA	0·1
2	SAHA	5
5a		0·3[20]
5b		4
5c		>25
6a		2·5[20]
6b		6
6c		>25

IC_{50} were determined from the curves of antiproliferative activities (figure 4) at the concentration required for 50% inhibition of cell proliferation. Values are means of two experiments done in triplicate

dues for both HDAC8 and HDLP. As the fluorine atom is an electronegative element, this possibly resulted in unfavourable electronic interaction with the probably anionic form of the terminal carboxylate found at the entry of the active site (Glu92 in HDLP, Asp101 in HDAC8). On the other hand, it is also possible that the introduction of the fluorine atom increased the hydrophobic properties of the molecule, resulting in a lower cell membrane penetration.

4. Conclusion

This work presents a short synthesis of substituted trichostatic acid derivatives allowing the introduction of new functional groups in the alpha position of the ketone group. The methylated derivatives **5c** and **6c** do not show substantial activities while the fluoro derivatives retained antiproliferative activities at micromolar concentrations in our lung cancer cell model. Interestingly, two stable conformers were obtained from modelling for the most active compound **5b**, with the first one suitable for docking to HDLP, while the other was found more suitable for HDAC8. HDAC inhibitory activity of compound **5b** was confirmed. Work is in progress to develop this synthetic strategy for the production of more potent HDACi.

Acknowledgements

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