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Supercritical fluid chromatography approach for a sustainable manufacture of new stereoisomeric anticancer agent

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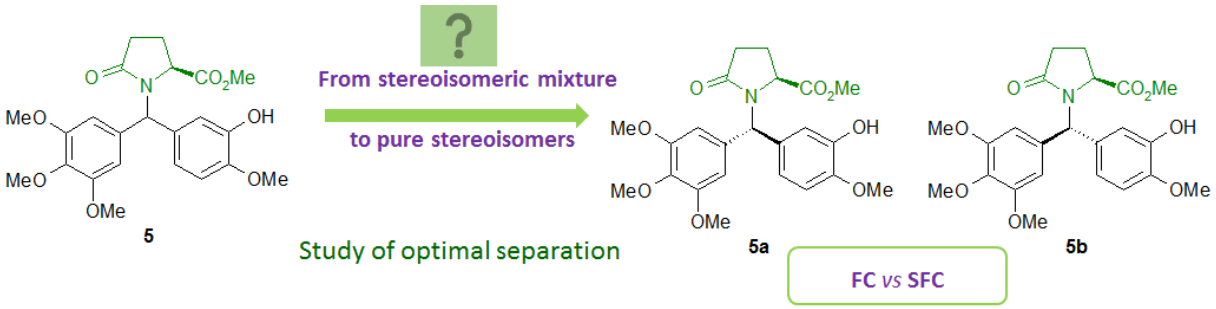
GRAPHICAL ABSTRACT



Highlights

- Stereoisomeric combretastatin A-4 analogue as anticancer agent
- Two different approaches were applied for diastereomeric separation.
- Optimization of percentage of co-solvent, flow-rate and outlet pressure in pSFC.
- Green factors evaluation

Abstract

Two routes aimed at the manufacture of unprecedented stereoisomeric combretastatin A-4 analogue were described: flash chromatography *vs* supercritical fluid chromatography. The latter has many advantages over liquid chromatography and was therefore chosen for the small scale separation of methyl 1-[(3-hydroxy-4-methoxyphenyl) (3,4,5-trimethoxyphenyl)methyl]-5-oxo-L-prolinate **5**, with potential antitumoral activity. After a screening of six different polysaccharide based chiral stationary phases and four co-solvents, the percentage of co-

solvent, the flow-rate and the outlet pressure were optimized through a design of experiments (DoE). The preparation of 50 mg of each stereoisomer was achieved successfully on a Chiralpak AD-H with *iso*propanol as a co-solvent. Productivity (kkd), solvent usage and environmental factor (E Factor) were calculated. Flash chromatography and supercritical fluid chromatography approaches were compared in terms of yield and purity of each stereoisomer manufactured.

Keywords: Diastereoisomers; chirality; productivity; kkd; E factor

Introduction

The development of drugs marketed as single enantiomers is growing continuously in the pharmaceutical business and in academic laboratories alike, as it can be observed from the number of single enantiomers launched in recent years [1]. At the discovery stage, the most important factor is time. The required amount usually ranges between a few milligrams to 50 g and the cost factor as well as the scale-up feasibility are negligible. However, it is important to be able to isolate the single enantiomers in a short period of time in order to rapidly perform the biological tests. Over the past fifty years high performance liquid chromatography (HPLC) has become very popular, particularly for preparative scale. However, faster analysis time demands of the market, together with the necessity of a greener and sustainable way of separation issued by governmental authorities have facilitated a supercritical fluid chromatography (SFC) renew of these last 15 years. Taking advantage of its intrinsic properties [2] this technique became the mainstream one in preparative scale. In SFC, the mobile phase is constituted by, at least, 50% of supercritical carbon dioxide (from T = 31°C and P = 74 bar). The low viscosity and high diffusivity of the mobile phase together with a lower pressure drop permit high flow-rate with a reduced influence on peak efficiency. Carbon dioxide is not polar

enough [3] and most of the time a polar modifier such as alcohol or acetonitrile must be added to elute chiral pharmaceutical compounds. It is worth noting that the addition of a liquid modifier moves conditions to subcritical, however advantages stated previously still exist.

Among the widely known twelve principles of green chemistry stated by Anastas and Warner in 1998 [4] not all, but many, can be directly applied to analytical chemistry: prevent waste, use safer solvents and processes, minimize use of energy, avoid chemical derivatives, analyze in real time to prevent pollution and lastly increase the safety of the operator [5]. Some papers have addressed this topic of green chromatography in practice [6-9]. Nevertheless, three of the principles, called the three Rs: Reduce, Replace, Recycle are considered to be most relevant for greening both the analytical [10-11] and large scale preparative separation technologies [12] and can be applied daily in every laboratory [6]. Based on those trends, SFC has established it-self as a green method, in particular for the small-scale preparative, mainly because of the numerous advantages of CO_2.

Indeed the replacement of hazardous organic solvent by a large amount of carbon dioxide is desirable in an eco-friendly point of view. It is non-toxic, non-flammable, and a renewable resource as the SFC installation uses carbon dioxide that is condensed from the atmosphere (or industrial waste plumes) delivered into the chromatographic device and then returned to the atmosphere (or recycled). It is important to indicate that preparative SFC is not a net generator of carbon dioxide. This gas is a recovered industrial waste byproduct (such as the production of cement, the production of metals or alloys such as iron and steel, and the production of chemicals) or comes from natural processes like beverage fermentation, while incineration of organic solvents results in the net generation of carbon dioxide [13].

From an economical point of view, replacing the mobile phase by large carbon dioxide amount reduces expensive organic solvent volume to be used, and evaporated (energy cost). With regard to HPLC, which consumes large volume of water (in Reversed Phase) needing high

energy costs for distillation, or alkane (in Normal Phase) and organic solvents, SFC is a relevant and economical alternative to both RP and NP-HPLC, offering reduced time and cost of purification. It is worth noting that the total amount of waste generated by HPLC instruments worldwide represents 34 million liters per year [14] and it must be kept in mind that waste removal generates very significant costs. Lastly, in a chemical point of view, after being eluted from the column, CO_2 is removed by decreasing pressure leaving small amount of modifier (of analytical grade), this reduced solvent volume allows higher product concentration and purity. All these reasons made SFC a sustainable chromatography, and a technique of choice for small preparative scale (a few milligrams to a few kilograms) separation. In the willingness to take the direction of a sustainable chromatography, green metrics have emerged in order to evaluate the environmental impact of a separation process: in particular solvent usage (L/g racemate) corresponds to the volume of solvent consumed to purify a known amount of racemate and environmental factor (E-factor) defined as waste to product ratio for any chromatographic procedure, [15] for which an ideal value is zero.

Our research group is interested in developing new anticancer agents, analogues of combretastatin A-4 (**1**) or phenstatin (**2**) with different connectors between the A (3,4,5-trimethoxyphenyl) and B (3'-hydroxy-4'-methoxyphenyl) units (Figure 1). Numerous structural modulations of the ethylenic bridge of (**1**) have been explored in the literature. Besides its low solubility in biological media, combretastatin A-4 (**1**) has the inconvenience of being highly cytotoxic only in the *Z* configuration, *E* compound is significantly less active [16]. However, only few connectors bearing an asymmetric center have been described. For example, isoerianin (**3**) with a 1, 1-ethane bridge exhibited promising antiproliferative activity in the nanomolar range and is regarded as simplified analogue of podophyllotoxin (**4**), potent antimitotic agent [17]. The biological evaluation of both pure enantiomers of isoerianin (**3**) revealed similar antiproliferative activities against HCT-116 cell lines. However, chiral isomers

of other microtubule-interacting agents have displayed significant differences in biological potential in cell growth proliferation [18]. To further extend structure-activity relationships of parent compounds (**1**) and (**2**), we replaced the ethylenic bridge by an unprecedented connector in the structure of compound **5**, leading to a new potential antitumoral combretastatin A-4 analogue (Figure 1). We then focused our efforts on obtaining pure stereoisomers to evaluate separately their potency on tubulin polymerization and on cancer cell growth inhibition.

After some failures in finding optimal chromatographic conditions, the stereoisomeric separation of compound **5** was firstly implemented by flash chromatography on a silica column with an *n*-hexane/2-PrOH 75:25 eluent mixture. The unexpected mediocre results obtained in terms of separation and yields, led us to choose another chromatographic method. Therefore, profiting from its numerous environment advantages, SFC was chosen to separate a methyl 1-[(3'-hydroxy-4'-methoxyphenyl)(3,4,5-trimethoxyphenyl)methyl]-5-oxo-L-prolinate stereoisomeric mixture. The aim of this work is to achieve the semi-preparative separation of this original molecule while evaluating its ecological impact with supercritical fluid chromatography. The type of chiral stationary phase and the nature of organic modifier were chosen after a preliminary screening, the parameters values (percentage of 2-PrOH, flow-rate and outlet pressure) were determined thanks to a design of experiments to obtain the highest resolution. Those conditions were then scaled-up on a preparative chiral column, Chiralpak AD-H. Each method was compared in terms of solvent usage and environmental factors (E Factor), yields, spent time and stereoisomeric purity. The preparation of about 100 mg of stereoisomers was targeted to allow the determination of their pharmacological and toxicological activities.

2. Experimental

2.1 Chemicals

Starting materials were commercially available and used without further purification. The synthetic pathway of the target compounds (Figure 1) started with L-pyroglutamic acid according to the scheme 1 (in Supporting Information). The methanol, ethanol, *iso*propanol, and acetonitrile were HPLC grade and were purchased from VWR (Strasbourg, France). Carbon dioxide (CO_2) with purity of 99.995% was purchased from Air Liquide (Loos, France).

2.2 Sample solutions.

For volume overloading, solutions of samples were prepared in ethanol at 1 mM (0.41 $g.L^{-1}$) and 40 mM (16.54 $g.L^{-1}$). The solutions were always degassed by an ultrasonic bath and filtered on a 0.45 µm PTFE syringe-filter (15 mm diameter) prior to be used.

2.3 Chiral supercritical fluid chromatography apparatus

Stationary phases. The five chiral analytical columns used for the enantiomeric purity verification, were Chiralpak AD-H (tris-3,5-dimethylphenylcarbamate of amylose) and AS-H (tris-(*S*)-1-α-methylbenzylcarbamate of amylose), Chiralcel OD-H (tris-3,5-dimethylphenylcarbamate of cellulose), purchased from Chiral Technologies Europe (Illkirch, France) and Lux™ Amylose-2 (tris-5-chloro-2-methylphenylcarbamate of amylose), Lux™ Cellulose-2 (tris-3-chloro-4-methylphenylcarbamate of cellulose), purchased from Phenomenex (Le Pecq, France). All columns have 250 mm x 4.6 mm i.d. dimensions with 5 µm particle size. The chiral preparative column used for this study was Chiralpak AD-H and has 250 mm x 10 mm i.d. dimensions with 5 µm particle size.

Chromatographic system and conditions for analytical and semi-preparative steps.

The chromatographic system used was an SFC-PICLAB hybrid 10-20 apparatus (PIC Solution, Avignon, France) equipped with an autosampler comprised a 48-vial plate and a 24-vial plate (model Alias, Emmen, Netherlands), three model 40P pumps: two for CO_2 and a third for the modifier (Knauer, Berlin, Germany), a column oven with a Valco ten-position column selection valve, and a Valco six-position solvent switching valve. The proportion of the modifier in the

mobile phase was adjusted by a piston pump. It was then directly added in the CO_2 feeding, and the mixture of the both (modifier and CO_2) was pumped by another piston pump at the total flow rate. The pump head used for pumping the CO_2 was cooled to – 8°C by a cryostat (model Minichiller, Huber, Offenburg, Germany). The injection valve was supplied with 20, 50, 100, 250 or 485 µL sample loops. The system was also composed of a Smartline 2600 diode array detector (DAD) (Knauer, Berlin, Germany) plumbed before the back pressure regulator. The detection wavelength was set at 210 nm. After the detector, the outlet pressure was controlled by a back-pressure regulator (BPR). The outlet regulator tube was heated to 55°C to avoid ice formation during the CO_2 depressurization. The system was controlled and the data were acquired with the SFC PicLab Analytic Online v.3.1.2 software and the data were processed with the Analytic Offline v.3.2.0 software (PIC Solution, Avignon, France). During the separation optimization (not including the design of experiments), the mobile phase was always CO_2-modifier mixtures with the proportion of co-solvent (methanol, ethanol, *iso*propanol and acetonitrile) equal to 20%, flow rate was 4 $mL.min^{-1}$ for all columns. Methanol was used as a needle wash solvent. All analyses were run in isocratic mode. The column oven temperature was 40 °C and the outlet pressure was maintained at 150 bar for all screening experiments.

2.4 Experimental design and data analysis

A central composite circumscribed design (CCC) was applied for optimizing our separation. Three factors were selected to build the design of experiments: outlet pressure (P_{out}), flow-rate of the mobile phase (FR) and percentage of *iso*propanol as co-solvent (2-PrOH). Five levels were set for each factor corresponding to a two-level full factorial design (coded: +1, and -1), superimposed by a replicated center-point (coded 0) and symmetrically arrayed star points (coded: +α and -α). Arrayed star points are located on the corresponding factor axes at a distance of ± 1.68 from the center point (Table S1 in Supporting Information for detailed values of each level). This experimental design was constructed from eight corner points, six axial

points and a center point. Six replicates were performed at the center point in order to assess the goodness of fit. All points were repeated twice. The worksheet of the CCC was composed of 40 (20x2: 1 run and 1 replicate) randomized runs (Table S2 in Supporting Information). Once the experiments were carried out, the investigated responses *i.e.* resolution were input into the MODDE 9 software (Umetrics, Malmö, Sweden) [19]. Then, the data were fit into a quadratic model to evaluate factor effect and two-factor interaction. Models were chosen based on F-test and lack of fit test. ANOVA was also performed to screen critical factors. The response surface plots were then created by the software to examine the main effect and interaction between significant factors. Finally, the optimum combination of all factors was generated by the software *via* the optimiser mode (using the Nelder-Mead method [20]) to achieve maximum resolution.

*2.5 Synthesis of compound **5***

Methyl 1-[(3-hydroxy-4-methoxyphenyl)(3,4,5-trimethoxyphenyl)methyl]-5-oxo-L-prolinate (**5**).

A convergent synthetic pathway was privileged to obtain the targeted compound **5** (Scheme 1, in Supporting Information). A mixture of 11.40 g of (2-methoxy-5-{(3,4,5-trimethoxyphenyl)[(trimethylsilyl)oxy]methyl}phenoxy)(trimethyl)silane (**12**), 7.6 mL of methyl *N*-trimethylsilyl-(L)-pyroglutamate (**8**) and 0.11 mL of triflic acid was heated at 130 °C for 4 hours. After cooling to room temperature, the crude was poured in distilled water and extracted with dichloromethane. The organic phase was recovered, dried over $MgSO_4$ and evaporated *in vacuo*. The resulting oil was purified by flash chromatography on silica. The two key intermediates (**8**) and (**12**) were first prepared by linear synthesis. The strategy started with L-pyroglutamic acid (**6**), renewable raw material from recycled waste beet, available in large quantities and at low cost, which after esterification, then reaction with hexamethyldisilazane in the presence of catalytic amount of saccharine allowed to isolate key intermediate (**8**) in 88%

yield, according to a reported procedure [21]. Bis-silylated intermediate (**12**) was obtained starting from guaiacol (**9**), which after protection of the free phenol group as monochloroacetate (**10**), then Eaton's reagent assisted condensation with 3,4,5-trimethoxybenzoic acid provided the corresponding protected benzophenone in 85% yield. After treatment with sodium acetate in refluxing methanol, phenstatin (**2**) was isolated in 98% yield and further reacted with sodium borohydride to provide benzhydrol (**11**) [22] which was finally silylated by adapting the reported procedure [21] to obtain bis-silylated intermediate (**12**) in quantitative yield. Further condensation of silylated benzhydrol (**12**) with methyl *N*-trimethylsilyl-(L)-pyroglutamate (**8**) was accomplished in the presence of catalytic amount of triflic acid at 130°C, without solvent and allowed to obtain stereoisomeric mixture (**5**) in 85% yield (90% purity). The same reaction conducted at lower temperatures failed to provide the target product.

2.6 Flash chromatography

The silica column was a Redisep prepacked column with 80 g of 15-40 µm SiOH, 230-400 mesh, pore size: 60 angstroms, flow rate 60 mL/min, purchased from Serlabo Technologies, Entraigues-sur-la-Sorgue) with *n*-hexane/*iso*propanol 75/25 as eluent. The single products (**5a**) and (**5b**) were obtained as beige solids in a very low yield: 1 and 1.2% respectively (Figure S1 in Supporting Information for physico-chemical characterization).

3. Results and discussion

3.1 Stereoisomeric separation by flash chromatography on silica phase

The stereoisomeric separation was tried with 80 g of different silica phase (15-40 or 40-60 µm) by using different mixtures of eluents (ethyl acetate/*n*-hexane, ethyl acetate/dichloromethane or *n*-hexane/*iso*propanol, at a flow rate 60 mL/min). After numerous tests, the only condition that allowed separation of diastereoisomers **5a** and **5b** was flash chromatography on silica column (15-40 µm) with *n*-hexane/*iso*propanol as eluent in 75/25 ratio. However, the final yields were

extremely negligible, compound **5a** being isolated in 1% yield and compound **5b** in 1.2% yield, certainly due to the quasi-identical retention factors of studied isomers. Indeed, the only chromatographic condition that permitted the separation by flash chromatography used n-hexane/isopropanol 75/25 as eluting system. The thin layer chromatography (TLC) realized with the same proportion of these eluents gave a beat elongated spot (Rf = 0.3) unlike the other solvent systems tested which gave a single spherical spot for the two isomers, thus preventing their separation. The green and energy accounting of the reaction scheme has thus proved very dark. Indeed, in the final reaction step, 5.585 grams of bis-silylated benzhydrol (**12**), 3.105 g of *N*-silylated derivative (**8**) and 13.125 L of solvents mixture (with 9.850 L of *n*-hexane and 3.275 L of *iso*propanol) were necessary to finally recover 50 mg of each isomer as light beige solids. However, the maximum isomeric purity of compounds **5a** and **5b** was of 96%. This aspect will be detailed later in the 'Stereoisomeric excess' section and compared to the SFC purification method.

3.2 Stereoisomeric separation by supercritical fluid chromatography on chiral stationary phase

Preliminary experiments

Choice of the stationary and mobile phases

As compounds **5a** and **5b** were diastereoisomers, silica and octadecyl stationary phases were firstly tested. Unfortunately, a single peak was observed, highlighting once again the problematic separation of compound **5**, particularly. Consequently, chiral stationary phases were further explored to separate targeted diastereoisomers [23].

A screening was carried out using five polysaccharide-based stationary phases revealing that the Chiralpak AD-H was the most efficient CSP in the current study.

Meanwhile, the choice of the eluent was then undertaken. Four solvents of various polarity were chosen: methanol, acetonitrile, ethanol and *iso*propanol to be tested towards the five columns (results are summarized in Table S3 in Supporting Information). The experiments were realized

at 40°C with an outlet pressure of 150 bar. Compound **5** was eluted under isocratic condition with 80% of CO_2 and a flow-rate equal to 4 mL.min^{-1}. Chiralpak AS-H and Lux Amylose-2 CSPs provided only one peak whereas Chiralpak AD-H and Lux Cellulose-2 led to a separation whatever the modifier was. However the first one has shorter analysis times and was available in preparative dimensions in the lab, thus was chosen, with *iso*propanol giving best resolution.

Multivariate approach

Choice of the experimental factors

After preliminary experiments showing that their variations have an influence, three factors were optimized using the chemometric approach, *i.e.* outlet pressure (P_{out}), flow-rate of the mobile phase (FR) and percentage of *iso*propanol (2-PrOH). Temperature parameter (parameter that might be optimized) was not included in the experimental factors because its effect is complex. Indeed temperature can act in two opposite ways on the retention, at constant pressure an increase of the temperature enhances the coefficient of diffusion and the volatility of the solutes then decreases their retention. In the same time, the density of the carbon dioxide is reduced leading to a rise of the retention phenomenon. One response was selected to optimize the performance of separation: the resolution of the stereoisomers. To estimate the quadratic effects it was necessary to select three levels for each factor. The proportion of modifier (*iso*propanol) ranging from 10 to 30% and flow rate ranging from 2 to 5 mL.min^{-1}. The outlet pressure was ranging from 80 to 200 bar (Table S1 in Supporting Information). For that purpose, a quadratic regression model was applied in order to emphasize possible quadratic effects and interaction effects besides the main ones. The model can be expressed by the following second-order polynomial model:

$$y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{23} X_2 X_3 + \beta_{31} X_3 X_1 + \varepsilon \quad \text{(Eq. 1)}$$

where *y* is the response, the stereoisomeric resolution; β_0 (intercept), β_1, β_2 and β_3 (coefficients for linear effects), β_{11}, β_{22} and β_{33} (coefficients for quadratic effects), β_{12}, β_{23} and β_{31} (coefficients for the interaction effect) and ε (error term) are the different coefficients for the model; X_1, X_2 and X_3 are the three factors (P_{out}, FR and 2-PrOH). To simulate the surface response for each response, a second-order polynomial was chosen (Eq.1).

Validity of the model

The validity of the model was assessed by the fit plots in the DoE software. The four performance indicators summarizing these plots are goodness of fit (R^2), goodness of prediction (Q^2), model validity and repeatability. R^2, which depicted the quality of fit, must be higher than 0.8 for a good regression of the data. A better indication is given by Q^2, a parameter which estimates the predictive power of the model. With a value higher than 0.5, the model is predictive. An excellent model arises high values for both R^2 and Q^2 and preferably not separated by more than 0.2-0.3. In our study, the R^2 and Q^2 values are respectively equal to 0.808 and 0.741 for resolution, indicating a correct power of prediction. The model validity means the adequacy of the model. If the value is higher than 0.25, the model is valid, our one is equal to 0.703. Finally, the repeatability reflects the variability of the repeated center point. The repeatability error is lower if the value is higher, our repeatability value is equal to 0.869. These latter values together with R^2 and Q^2 indicate the suitability of our model.

Effects of the three parameters on resolution

The three parameters were then analyzed and particularly the relative effect of each factor and factor interaction. Three linear terms are significant: P_{out}, FR, and 2-PrOH (*p-values* < 0.05). Based on the absolute values of each coefficient, the flow-rate has the most important effect whereas the outlet pressure and the percentage of *iso*propanol influence the separation in a lesser way. The higher the percentage (or the flow-rate), the lower the resolution is. Besides the linear terms, one interaction term is involved in separation process, indicating that an interaction

occurs between the outlet pressure and the flow-rate factors for this compound, on this stationary phase. Among the squared terms, 2-PrOH² was found to be also significant, revealing that the *iso*propanol dependence is not linear. The response surface plot (Figure S2 in Supporting Information) allows a better visualization of the results and is described, according to the second-order polynomial model, by the following equation:

$$Rs = 1.476 - 1.502\ FR + 0.120\ P_{out} - 0.639\ 2\text{-PrOH} - 0.031\ FR\ P_{out} + 0.165\ 2\text{-PrOH}^2 \quad \text{(Eq. 2)}$$

Optimal conditions at analytical scale

One of the numerous possibilities of the DoE software is to further optimize the factors by implementing the optimizer mode, which uses the downhill simplex method (see experimental section), to obtain accurate value leading to the best resolution. As a result, the three parameters were fixed: an outlet pressure equal to 200 bar, 10% of *iso*propanol as a *co*-solvent and a flow-rate equal to 3.8 mL.min^{-1}. In those conditions retention time of the first (t_{R1}) and second peak (t_{R2}) were equal to 5.70 and 9.50 minutes respectively and resolution was equal to 6.32 experimentally (Figure S3 in Supporting Information), whereas, theoretically, the resolution value was expected to be equal to 6.15.

3.3 Small-scale preparative separation

In those defined conditions, 1 mM (0.41 g.L^{-1}) and 40 mM (16.54 g.L^{-1}) ethanolic solutions were injected with a 20, 50 or 100 µL sample loop, at 40° C on an analytical column. Injection of our concentrated ethanolic solution with 20, 50 and 100 µL sample loops led to resolution respectively equal to 4.27, 2.36 and 1.55. Afterwards, the conditions were transposed to a preparative column in order to inject larger volumes while keeping a resolution.

On this column, with 10 mm of internal diameter, the injection of 485 µL of 40 mM ethanolic solution (corresponding to 8.02 mg injected) led to a resolution of 2.20. Following the same linear velocity as used on the analytical column, a flow-rate of approximatively 18 mL.min^{-1}

should be used to obtain same retention times and resolution (through the calculation of the transposition factor K, Eq.3).

$$K = \frac{(id_{prep}^2 \times L_{prep})}{(id_{ana}^2 \times L_{ana})} \quad \text{(Eq. 3)}$$

However the two SFC pumps can only deliver 8 mL.min^{-1} each, therefore a flow-rate of 15 mL.min^{-1} was tested leading to an important pressure drop. The flow-rate was finally elevated to 11 mL.min^{-1}, and the analysis time of the injection was rather important, equal to 60 minutes. Therefore the percentage of *iso*propanol was increased to 15% to short the analysis time. This latter, even shortened to 50 minutes was quite long, but a correct resolution equal to 2.20 was maintained in order to collect the chemical impurities present between the two peaks (Figure 2). However, thanks to stacked injections *i.e* a second injection was made when the first enantiomer was detected and so on, the cycle time was shorter. The cycle time was then reduced to 25 minutes, and the productivity increased to 50%. By this way 100 mg of stereoisomeric mixture were separated in 13 injections, corresponding to 5 hours and 50 minutes (Figure 2). Each isomer was thus isolated in 96.5% yield.

3.4 Productivity and environmental aspects of separation

It must be kept in mind that the viability and success of a chromatographic process to achieve the industrial scale depends essentially on the right choice of the operating conditions leading to high productivity rate. This key metric can be considered through either productivity or solvent usage. The ecological consequences of the purification are assessed through environmental factor.

Productivity is defined as the amount of product separated per time unit and amount of stationary phase, and expressed in this work in kg racemate per kg CSP per day (kkd) [15]. It can also be expressed as kg enantiomer per kg CSP per day (kkd) [13]. Productivity value has, among others, a relationship to the solubility of the sample and low kkd may arise from poor solubility. Solvent usage (L/g racemate) corresponds to the volume of solvent consumed to

purify a known amount of racemate. In the context of the search of eco-friendly chromatography, green metrics are necessary. Environmental factor is defined as waste to product ratio for any chromatographic procedure [15]. For preparative separation, the amount of waste is dictated by the solvent utilized for the purification. A higher E Factor procedure generates more waste and has a greater environmental impact. In the case of compound **5**, productivity, solvent usage and environmental factor were equal to 0.038 kkd, 5.35 mL 2-PrOH/1 mg mixture and 27.60, respectively.

The chromatographic separation process requires 3850 mL of mobile phase for the 13 injections corresponding to 3272.5 mL of recyclable CO_2 and 577.5 mL of *iso*propanol to separate 100 mg of mixture. Therefore, 5.775 L of *iso*propanol are necessary to separate one gram of compound **5**.

3.5 Comparison of the preparative performance of the two approaches

The performances of each methodology were summarized in Table 1. Each stereoisomer collected in a short fraction of *iso*propanol after depressurization of the CO_2, is evaporated under dryness and then solubilized into ethanol to a sufficient concentration (1 mM) to evaluate their stereoisomeric purity. These verifications were carried-out on the analytical column Chiralpak AD-H, in conditions chosen to allow the complete resolution of the two stereoisomers in the shortest analysis time. For compound **5**, the stereoisomeric excess of **5a**-P2(AD-H) and **5b**-P1(AD-H) was equal to 98.72% and 99.49% respectively (based on their respective area, as their response factors were verified to be identical). The overlayed chromatograms of each pure stereoisomer and mixture **5** were represented on the Figure 3, with 30% of 2-PrOH as modifier. This percentage of *co*-solvent allows shorter retention times while keeping a great resolution between diastereoisomers.

However, the isomeric excess for **5a** and **5b** isolated by flash chromatography, and afterward determined by SFC in the same conditions as described above, was of 95.1 and 96%, respectively (Figure 4).

We were confronted with an uncommon diastereoisomers mixture, not separable nor in Normal Phase neither in Reversed Phase liquid chromatography. This separation was essential to establish the preliminary biological profiles of the single isomers and consequently guide future pharmacomodulations on this new microtubule-interacting agents. In this context, SFC was a green way to achieve this goal, needing less than 6 liters of *iso*propanol to separate 1 gram of compound **5** while the flash chromatography needed almost 100 L of toxic *n*-hexane and 32.75 L of *iso*propanol, reducing the economic cost, energy and time necessary to evaporate this volume of solvent. In addition, isomers are obtained more rapidly in higher yield and isomeric excess with SFC than with FC: 96.5% *vs* 1% yield and 98.72% *vs* 95.10% respectively in 5.83 hours *vs* 35 hours for compound **5a** for instance. Thus, this method surely reduces the environmental impact of the generation of these stereoisomers when compared to other process currently available.

4. *In-vitro* biological evaluation

In terms of medicinal chemistry, we have discovered unprecedented analogues of combretastatin A-4 (**1**) and of phenstatin (**2**) bearing a different connector between the two aromatic rings A and B. The effect of synthesized pyroglutamic derivatives **5a** and **5b** on the inhibition of tubulin polymerization *in-vitro* was investigated relative to reference phenstatin (**2**), along with a DMSO control. The biological screening revealed that diastereoisomers behave differently, compound **5b** being the best tubulin polymerization inhibitor (100% inhibition at 100 µM) while compound **5a** inhibited 65% of the protein at the same concentration. An IC_{50} value has been determined for the best isomer and revealed a very promising value of 3.29 ± 0.45 µM, identical to that of parent phenstatin (**2**) (IC_{50} = 3.43 ± 0.5 µM). The National Cancer Institute (NCI) selected only isomer **5a** for initial biological screening on the NCI-60 cancer cell lines panel at a single dose of 10 µM and further progressed in the 5-dose in vitro 60-cell-lines screen in order to evaluate the GI_{50} values (Table S4 in

Supporting Information). The best cell growth inhibitions were registered on KM12 colon cancer cell lines (GI_{50} = 581 nM), HOP-92 non-small cell lung cancer cells (GI_{50} = 509 nM) and on MDA-MB-435 melanoma cell lines (GI_{50} = 246 nM). These results confirm the pharmacological potential of this new family of compounds and the importance of ideal stereoisomeric separation to compare the biological profiles of isomers and enrich structure-activity relationships.

Conclusion

For enantiomeric preparative separation, it is widely known that SFC, on chiral stationary phases, is a much better technique than the flash chromatography and superior ecologically. In the current work, we were faced with a particular mixture of diastereoisomers, which should be normally, easily separable by conventional flash chromatography on silica stationary phase. Nevertheless, the separation was almost 100 times more effective and 6 times faster through SFC compared to classical liquid chromatography method, highlighting the importance of the SFC also in the separation of special diastereoisomers practically inseparable by other common methods. This issue is essential in order to produce sufficient quantities of active molecules for their *in vivo* evaluation.

Additional chemical and biological efforts are now necessary to reinforce the biological potential and open research for new pyroglutamic derivatives with promising antitumoral activity.

Acknowledgement

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Figure captions

Figure 1. Structure of combretastatin A-4 (CA-4) (**1**), phenstatin (**2**), isoerianin (**3**), podophyllotoxin (**4**) and of target antitumoral agents **5a** and **5b**.

Figure 2. Semi-preparative scale chromatogram with overlapped automated injections of compound **5**, 40 mM in SFC on Chiralpak AD-H (250 x 10 mm; 5 µm); 485 µL volume injected CO_2/2-PrOH 85/15 *v:v*, 11 mL.min^{-1}; 200 bar outlet pressure; 40°C, in diode array detection at λ=220 nm.

Figure 3. Overlayed chromatograms of compound **5** and **5a**-P2(AD-H) and **5b**-P1(AD-H) stereoisomers obtained after semi-preparative separation in supercritical fluid chromatography, on Chiralpak AD-H: CO_2/2-PrOH 70/30 v:v; 3.5 mL.min^{-1}; outlet pressure 140 bar; 40°C in diode array detection at λ=220 nm.

Figure 4. Overlayed chromatograms of compound **5** and **5a**-P2(AD-H) and **5b**-P1(AD-H) stereoisomers obtained after flash chromatography, on Chiralpak AD-H: CO_2/2-PrOH 70/30 v:v; 3.5 mL.min^{-1}; outlet pressure 140 bar; 40°C in diode array detection at λ=220 nm.

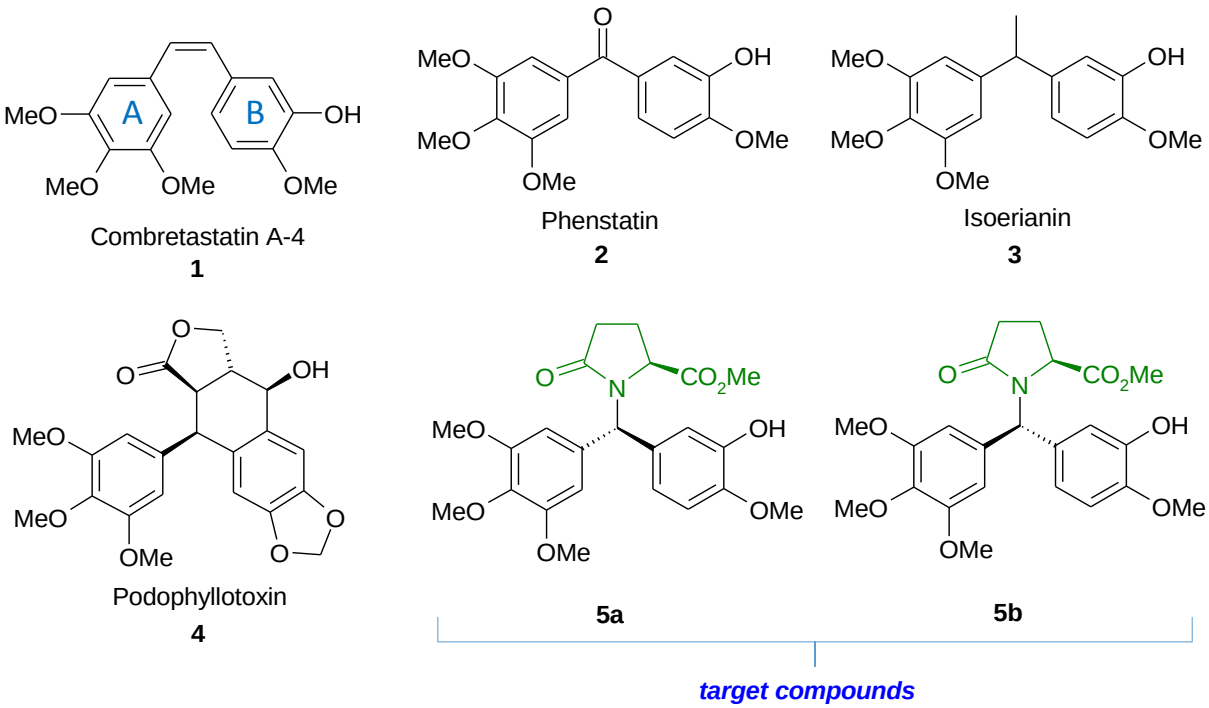


Figure 1

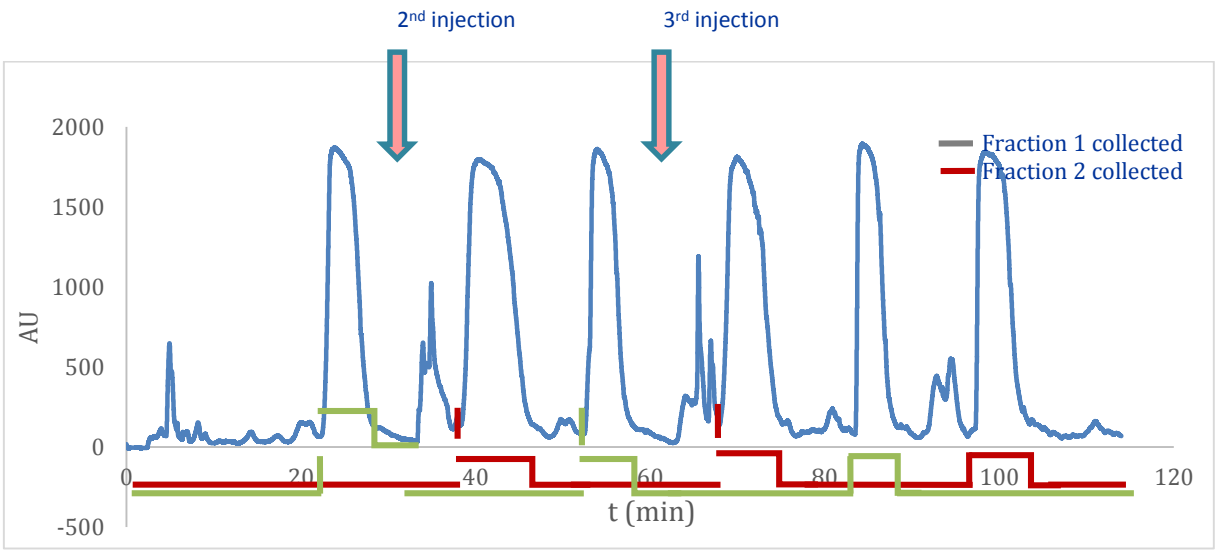


Figure 2

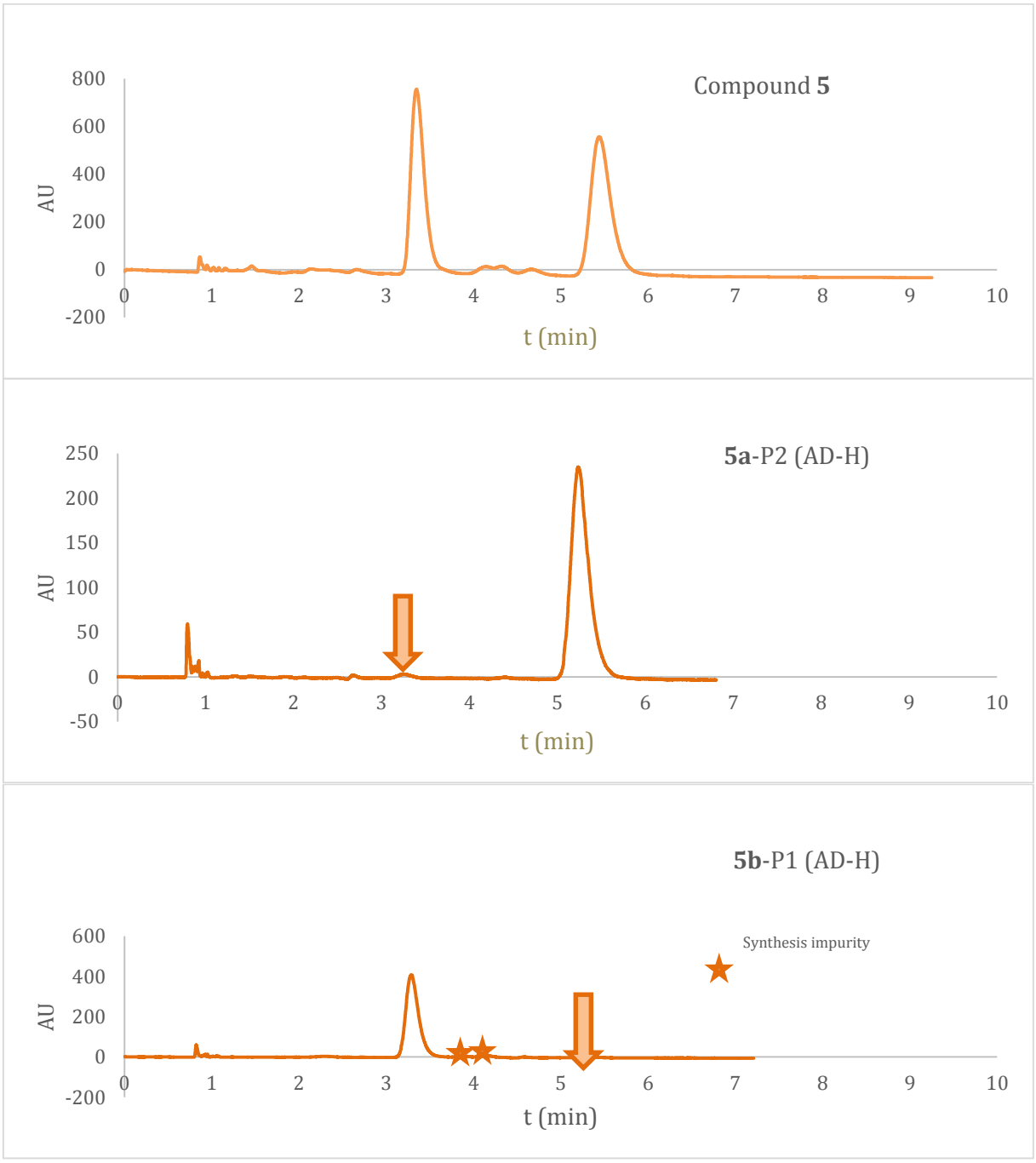


Figure 3

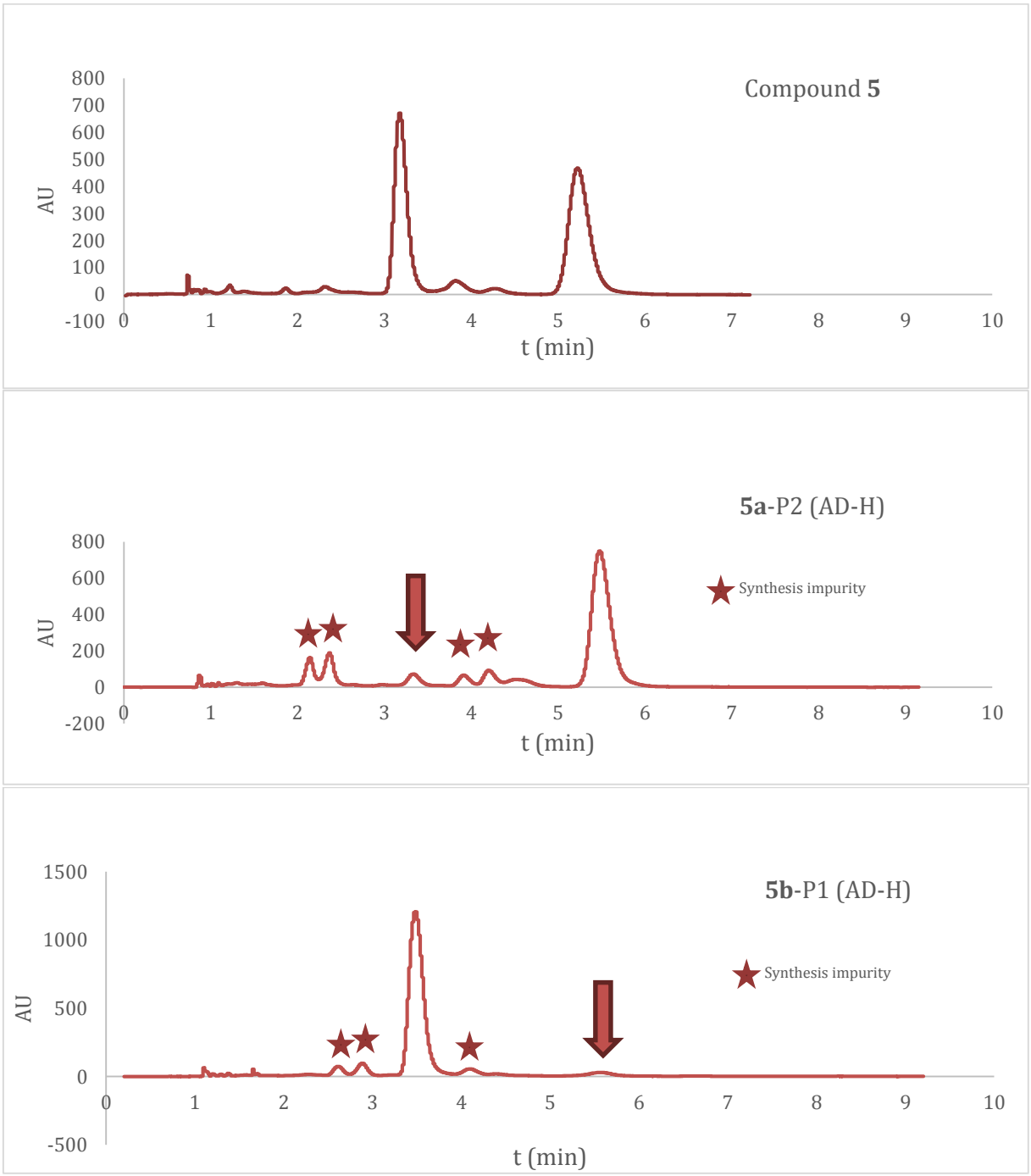
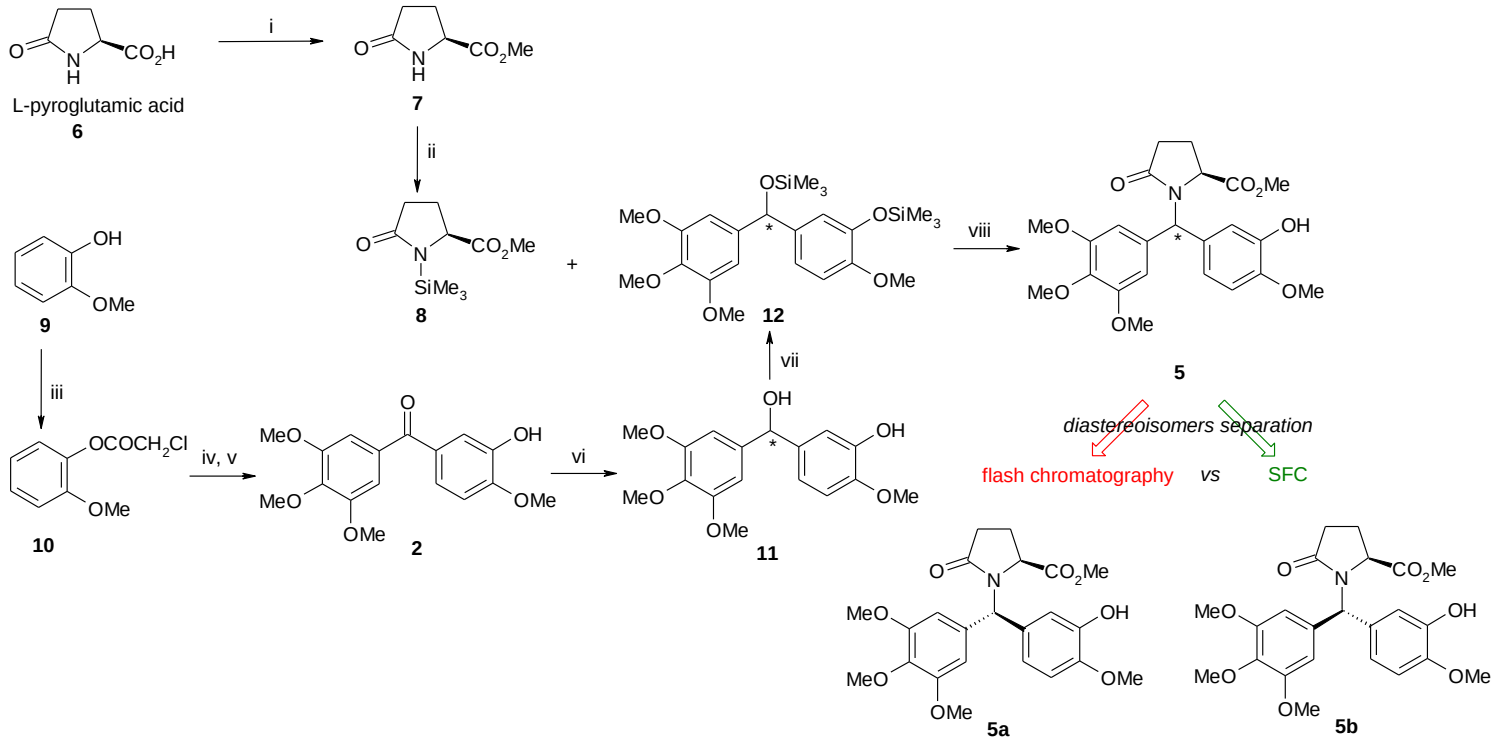


Figure 4

Scheme caption

Scheme S1. *Reagents and conditions*: (i) CH_3SO_3H, azeotrope MeOH/$CHCl_3$/H_2O, reflux, quantitative yield; (ii) hexamethyldisilazane (HMDS) (1.5 equiv), saccharine (0.05 equiv), 130°C, 2h, 88% yield; (iii) monochloroacetic acid chloride (1.44 equiv), 135°C, 5h, 85% yield; (iv) 3,4,5-trimethoxybenzoic acid (1.5 equiv), Eaton's reagent (4 equiv), 60°C, 5h, 85% yield; (v) $AcONa{\cdot}3H_2O$ (4.5 equiv), MeOH, reflux, 2h, 98% yield; (vi) $NaBH_4$ (2.2 equiv), EtOH/H_2O, rt, 4h, 97% yield; (vii) hexamethyldisilazane (HMDS) (3 equiv), saccharine (0.05 equiv), chlorotrimethylsilane (0.38 equiv), 130°C, 1h, quantitative yield; (viii) triflic acid (0.037 equiv), 130°C, 2h, 85% yield for diastereoisomers mixture.



Scheme 1

Table 1. Preparative performances of the two approaches

	Flash chromatography	***Supercritical fluid chromatography***
Separation of 100 mg of compound **5**		
Time:	35 hours	5 hours and 50 minutes
Volume of organic solvents consumed:	*n*-hexane 9.850 L *iso*propanol 3.275 L	recyclable CO_2 3.272 L *iso*propanol 0.577 L
Yields:	1.00% (**5a**) 1.20% (**5b**)	96.50% (**5a**) 96.50% (**5b**)
Isomeric excess:	95.10% (**5a**) 96.00% (**5b**)	98.72% (**5a**) 99.49% (**5b**)
Chemical purity:	presence of impurities	presence of very few impurities