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Chromatography as an Enabling Technology in Pharmaceutical Process Development: Expedited Multikilogram Preparation of a Candidate HIV Protease Inhibitor

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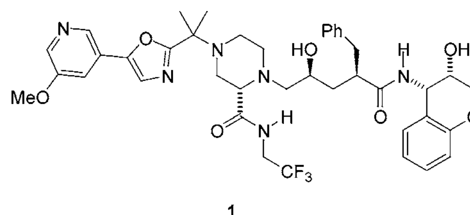
Abstract:

Chromatography plays a vital role in supporting preclinical pharmaceutical development, whether in providing assays for purity determinations, preparative separation of small amounts of intermediates for route selection studies, or purification of bulk drug substances on multikilogram scale. All three approaches are illustrated in the recent development of a candidate HIV protease inhibitor in these laboratories. Chiral supercritical fluid chromatography (SFC) on the hundreds-of-milligrams scale afforded an enantiopure intermediate to facilitate early synthetic studies, HPLC on the tens-of-grams scale provided purified material for use in salt form investigations, and HPLC using a 30 cm column was used to purify 5.6 kg of a key intermediate to provide material for early preclinical evaluations.

Introduction

Two decades after the emergence of HIV/AIDS, this disease remains a major threat to human health.[1] Despite considerable progress, the emergence of resistant viral strains and the limitations of current treatments mean that improved therapies are needed.[2] Candidate HIV protease inhibitors with structural similarity to the commercial Crixivan/indinavir HIV protease inhibitor[3] but with an improved profile in the treatment of resistant strains of HIV in preliminary screening have recently been reported.[4] We now describe the preparation of several kilograms of a related candidate, **1**, using a development strategy in which chromatographic separation plays a key role in the synthesis. The rapid development of a method to provide kilogram amounts of **1** illustrates the vital role that chromatography often plays in modern pharmaceutical process research. In addition to the traditional role as the preferred technique for purity and enantiopurity analysis, chromatography is becoming increasingly utilized for rapid purification of bulk material,[5] especially during the earlier stages in pharmaceutical discovery and development.



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The use of preparative HPLC in support of synthesis has a long history, dating at least to R. B. Woodward's 1973 prediction that preparative HPLC "...will be indispensable in the laboratory of every organic chemist in the very near future".[6] Recent improvements in equipment, materials, and techniques have led to an increasing adoption of the technique in pharmaceutical development, driven largely by the sheer speed and the minimal labor required to develop a preparative chromatographic method.

We herein describe some of the ways in which chromatography has been utilized in the development of **1**, including assaying purity and enantiopurity of intermediates, preparative separation of small amounts of intermediates for route selection studies, and larger-scale purification of bulk drug substances.

Results and Discussion

A number of routes to the preparation **1** were investigated. Several approaches to the enantioselective synthesis of a key aminochromanol precursor have been described.[7] The central feature of the route that was ultimately selected for preparation of **1** involves the coupling of substituted piperazine, **3**, with epoxide, **4**, to give acetonide, **2**, which affords the target, **1**, upon acetonide hydrolysis.

As with the synthesis of any complex target containing multiple stereocenters, the development of a scaleable process for the synthesis of **1** involved the investigation of a number of routes and intermediates, each of which required timely

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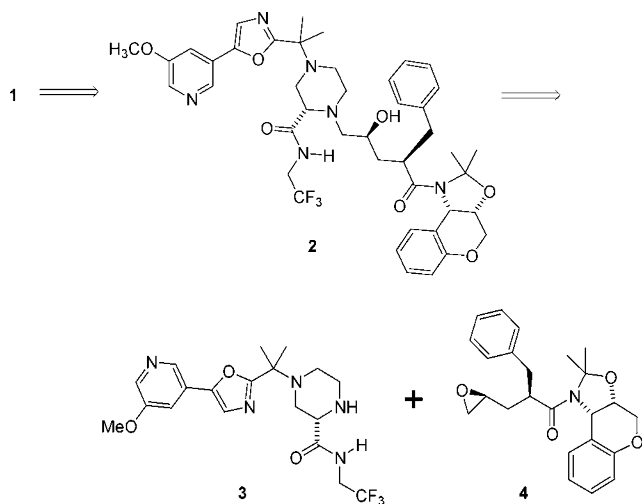

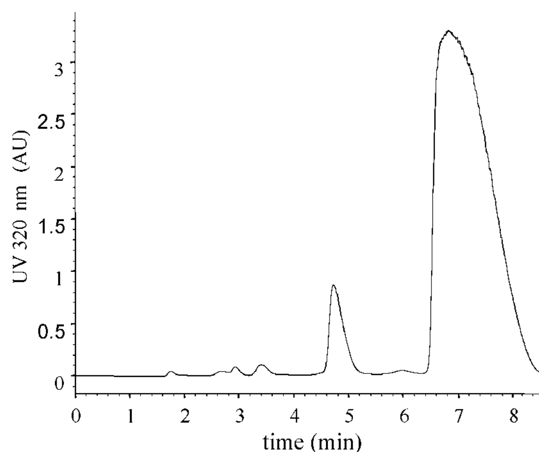

Figure 1. **Semipreparative SFC separation of an enriched mixture of substituted piperazine, 3. Conditions: Chiralpak AS (20 × 250 mm), 12% (25 mM isobutylamine in methanol)/carbon dioxide, 100 bar, 35 °C, 50 mL/min, UV 320 nm, injection of 1 mL @ 60 mg/mL.**

analytical chiral chromatographic support. We employ a standardized approach for developing chromatographic methods for enantiopurity determination for the potentially dozens of different intermediates evaluated during the development of a typical process.[8] A robotic screen of at least 12 different chiral columns using supercritical fluid chromatography (SFC) with a standard gradient of 4−40% methanol in carbon dioxide successfully elutes most pharmaceutical intermediates. For basic compounds that are poorly eluted under these conditions, we employ an identical gradient elution using 25 mM isobutylamine in methanol as the polar modifier. The screening procedure requires only a few minutes to set up, is normally performed overnight, and often affords a method for enantiopurity analysis that can be utilized without any further worker intervention. We have found that this automated chiral method development capability is helpful in expediting route investigation and process research on enantiopure drug candidates, allowing us to rapidly evaluate new synthetic concepts with minimal assay development labor. Furthermore, the use of SFC offers a number of inherent advantages over HPLC, including generally superior efficiency and lower eluent viscosity, which together permit faster separations.

In addition to its great value in analytical determinations of enantiopurity, we have found that SFC is very useful for rapid isolation of small amounts of intermediates or final drug candidates.[9] In addition to the previously mentioned advantage of speed, preparative SFC offers the important advantage over HPLC of significant reductions in waste solvent generation resulting from the replacement of petrochemical hydrocarbon solvents with supercritical carbon dioxide. Selecting a method for semi-preparative SFC separation often requires only a straightforward extension of the results obtained in analytical SFC screening. Thus, it is sometimes possible for a worker to screen a new racemate and resolve a small amount of the individual enantiomers within a day of sample receipt. We routinely use a semi-preparative SFC instrument with a 2-cm i.d. chiral column operating at a flow rate of 50 mL/min to purify up to 20-g quantities of selected intermediates. This timely availability of chromatographically derived enantiopure material allows researchers to rapidly conduct important studies aimed at devising a crystallization, developing a final salt form, or measuring racemization rates or reaction diastereoselectivities. The availability of enantiopure intermediates from preparative SFC allows the synthetic effort on a project to be focused primarily on the key synthetic challenges, and not on how to prepare the small amounts of enantiopure materials that are essential for informed route exploration.

An illustration of the utility of SFC in expediting process research is provided by an example involving the substituted piperazine **3**. Preliminary attempts at the opening of epoxide **4** with piperazine **3** afforded a number of minor impurities in addition to the desired acetonide, **2**. These impurities were subsequently identified and brought under control, but during the initial stages of the project the question arose as to whether the formation of these impurities was related to the low enantiopurity of the substituted piperazine **3** used in the reaction. This question was easily and quickly answered by providing enantiopure **3** using SFC. Initial evaluation of an 80% ee sample of **3** using the standard twelve column screen with methanol as the polar modifier afforded poor peak-shapes, a not uncommon occurrence for basic compounds. Subsequent screening using 25 mM isobutylamine in methanol as the polar modifier showed baseline resolution of the enantiomers using the Chiralpak AS column, with partial resolution being obtained on the Chiralpak AD and Whelko columns. The separation using the Chiralpak AS column was scaled up to a 2-cm i.d. column for semi-preparative SFC separation, where 290 mg of **3** at only 80% ee was upgraded to afford 215 mg of material with >99% ee. A representative chromatogram illustrating this separation is shown in Figure 1.

Similarly, achiral preparative chromatography can be useful for upgrading the purity of intermediates or final products containing unsuitably high levels of offending impurities. Although medium-pressure chromatography on

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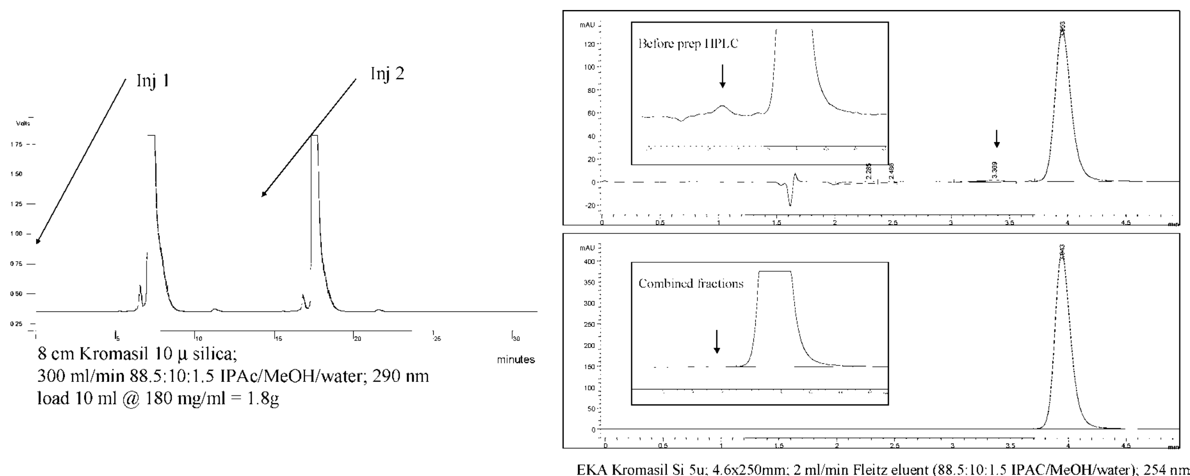


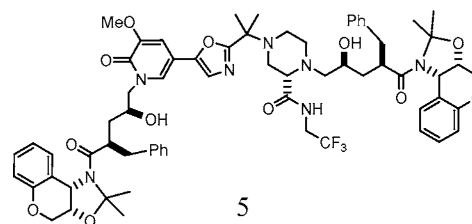
Figure 2. **Preparative HPLC upgrade of purity of 1 to prepare material for use in salt form studies.**

silica has been widely used in organic synthesis for a number of years, recent widespread availability of preparative HPLC equipment has resulted in the increased use of this technique in routine purifications in support of pharmaceutical process research. As a result, preparative HPLC is now often used as a "safety net" to enable the rapid preparation of new compounds for biological evaluation or clinical studies, an important consideration when there is an urgent need to move a drug candidate through the development process with the greatest possible speed.

An illustration of this approach is given by the example in Figure 2. In an early route to the preparation of **1**, the presence of a problematic impurity that was not readily rejected by recrystallization was noted. The presence of impurities in a free base oftentimes hinders the appropriate selection of a final salt form, and when possible, it is advisable to conduct salt-selection studies with material of the highest possible purity. In this instance, preparative HPLC on silica easily provided several grams of highly pure material that enabled the work on the final salt form of the drug candidate to begin well before the complete synthetic route had been developed.

This same HPLC method was subsequently used to purify additional 20- and 15-g batches of **1** generated by different routes, and in each case having a different profile of offending impurities. In each instance, silica HPLC proved highly productive and afforded material with >99% purity. These relatively small amounts of material supplied by preparative HPLC enabled important initial development work to proceed without delay. At this point we considered the possibility of using chromatography for the preparation of more substantial amounts of **1** to support advanced preclinical testing and early clinical evaluations.

Preparative HPLC on multikilogram scale is increasingly used in pharmaceutical development.[10] While relatively nonproductive chromatographic methods may be suitable for smaller-scale separations, a highly productive method is essential for larger-scale implementation. Both high chromatographic selectivity (α) and excellent solubility in the mobile phase are required for outstanding productivity. After consideration of a number of options, we selected the crude acetonide, **2**, formed by coupling of the substituted piperazine, **3**, with epoxide, **4**, to be the most suitable species for larger-scale HPLC purification. We observed the presence of several low-level impurities in the crude material, one of which, the double addition product, **5**, proved especially difficult to reject by crystallization as the pharmaceutically desirable HCl salt. Certainly, impurity rejection with other salts may have been possible, but we chose in this instance to proceed rapidly with the HPLC purification approach, rather than to devote time and material to exploring alternative strategies.



HPLC analysis using the conditions previously developed for the purification of **1** also afforded acceptable purification of crude acetonide, **2**. However, a modified method employing an eluent of 10% methanol in isopropyl acetate afforded better productivity and was used in a pilot separation conducted on 40-g scale using a 6-cm i.d. dynamic axial compression (DAC) column packed with 600 g of silica. Excellent solubility of crude **2** in the eluent was observed, and a feed concentration of 38% was used. Injection of increasing volumes of feed with collection of the central portion of the main peak revealed excellent purity and recovery, even with injection of 40 mL of the 38% feed solution (Figure 3). With the potential for a cycle time of less than 10 min if overlapping injections are used, this corresponds to a productivity of nearly 4 kkd (kilograms of desired component per kilogram of stationary phase per day), suggesting relatively facile access to multikilogram quantities of **2**.

Following satisfactory evaluation at the pilot level, we deemed that purification of **2** on multikilogram scale would be feasible. We opted to carry out the separation using a 30

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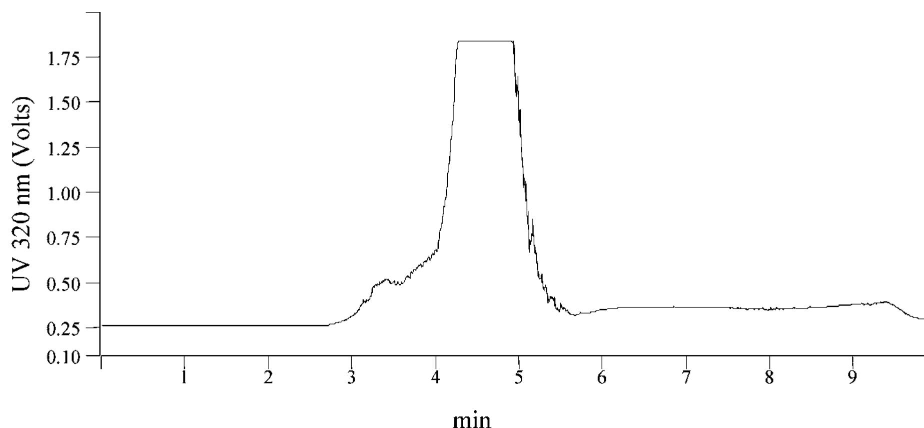

Figure 3. **Pilot study of HPLC purification of 2 using 6-cm i.d. DAC HPLC column. Preparative conditions: 6-cm i.d. ProChrom DAC column packed with 600 g of Amicon grade 631 silica (18 μm, irregular); 90:10 IPAc/MeOH; 300 mL/min; UV 320 nm; injection 40 mL of 38% crude 2 in IPAc. A productivity as great as 4 kkd may be possible with overlapping injections.**

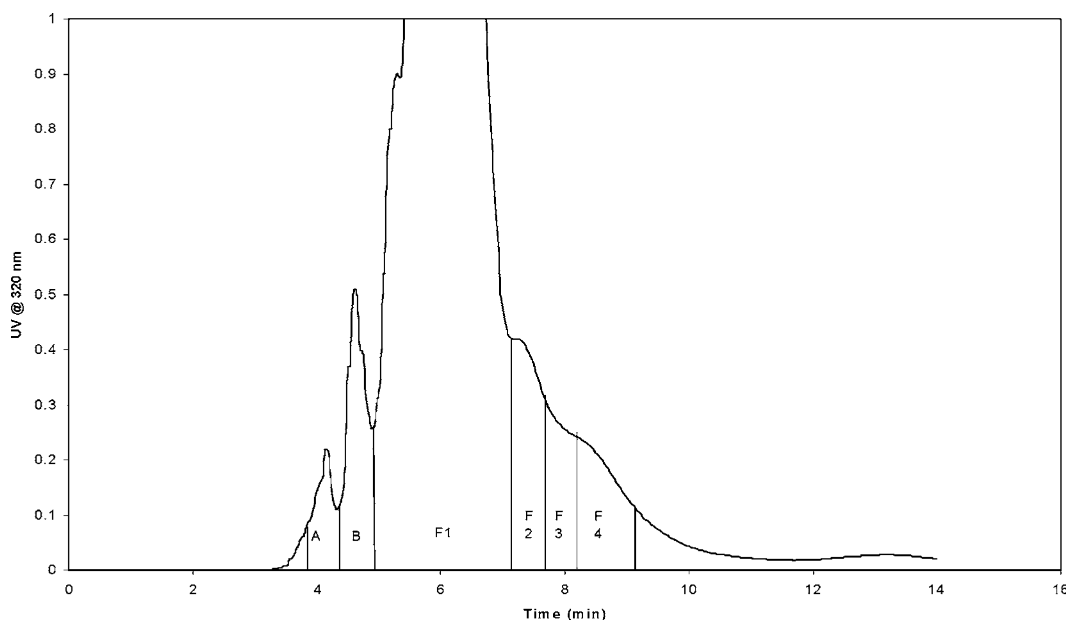

Figure 4. **Large-scale preparative HPLC purification of 2 using 30-cm i.d. DAC HPLC column, showing typical location of fractions collected during the run. Purified 2 was recovered in fraction F1, while bis-adduct 5 was separated in fraction B. Preparative conditions: 30-cm i.d. ProChrom DAC column packed with 14 kg of Amicon Grade 631 silica (18 μm, irregular). 90:10 IPAc/MeOH, 7.5 L/min; UV 320 nm; 0.9 kkd with specific solvent consumption of about 400 L/kg.**

cm i.d. DAC column without overlapping injections. Accordingly, the column was packed with 14 kg of silica and used to purify 15.7 kg of a 35.1 assay wt % solution of crude acetonide, **2**, (total of 5.51 assay kg) in IPAc. The feed material contained about 1% (by LC area) of bis-adduct **5**, which needed to be reduced below 0.5 A%. Crude acetonide **2** was chromatographed in 19 runs, each 16 min long, with an eluent of 10% MeOH in IPAc and a flow rate of 7.5 L/min, with detection at 320 nm. This corresponds to a demonstrated productivity of 1.9 kkd and a specific solvent consumption of about 400 L/kg purified product. A representative chromatogram is illustrated in Figure 4.

Although no further attempts were made to optimize the method, it is evident from the chromatogram in Figure 4 that with overlapping injections, injection cycle time could have been reduced to about 11 min, corresponding to a productivity of 2.7 kkd and a specific solvent consumption of 280 L/kg.

Following HPLC analysis to verify purity, fractions were combined and concentrated, and the solvent was switched to methanol under vacuum in a 500-gal cone-bottomed stainless steel still. The methanol concentrate (42.2 kg) contained 5.65 assay kg of the acetonide, **2**, with bis-adduct impurity **5** reduced to 0.30 area %.

Following successful purification of acetonide **2**, removal of the acetonide protecting group and crystallization of **1** as the HCl salt were required to complete the synthesis. A number of conditions were evaluated for carrying out the required deprotection of acetonide **2**. Trifluoroacetic acid in THF/H_2O afforded no deprotection, even upon heating to 40 °C, while the use of concentrated HCl in IPA gave only slow deprotection. Changing the solvent to methanol significantly shortened the reaction time, but even in methanol, 30 equiv of concentrated HCl were required for complete reaction. However, treatment of acetonide **2** with a solution of gaseous HCl in methanol allowed the reaction to be run

at lower temperature with reduced reaction time, affording the deprotected target **1** as the free base in 90% yield following extractive workup and charcoal treatment.

After deprotection, target molecule **1** was isolated as the pharmaceutically desirable hydrochloride salt by first solvent switching the MeOH/IPAc free base extracts to IPAc, then adding IPA, and heating to 60 °C, followed by addition of a solution of HCl in IPA. Crystallization was induced by the addition of seed crystals (obtained from workup of the earlier pilot-scale chromatographic separation) and then aging for 2 h at 60°C with overnight cooling to room temperature to complete the crystallization. Isolation by filtration afforded 2.71 kg of **1**·HCl as a white solid (needles) in which the offending impurity **5** was reduced to an acceptable 0.27 HPLC area percent. Following analytical testing, this material was delivered for use in preclinical evaluation.

Conclusions

Process research on emerging drug candidates must strike a balance between synthetic quality and speed. On one hand, it is important to produce material quickly, so that preclinical evaluation may proceed with the fastest possible pace. On the other hand, it is important to develop a synthesis which will be suitable for production at larger scale, should the compound survive the intense scrutiny of the drug selection process. In the development of the candidate HIV protease inhibitor described herein, a middle ground approach proved to be the best strategy. We employed a synthesis with real scale-up potential, but used the enabling tool of chromatography to allow fast early-stage production at the multi-kilogram level. As a result, we were able to prepare the several kilograms of investigational compound required for preclinical development with the greatest possible speed.

Experimental Section

General. Details concerning preparation of **2**, **3**, and **4** will be described in a separate publication. Chiral SFC screening was carried out using a pair of Berger Instruments analytical supercritical fluid chromatographs fitted with six-position column selection valves and Agilent model 1100 diode array UV−visible detectors. Chiral HPLC screening was carried out using an Agilent model 1100 HPLC instrument. Loading studies were performed with an Agilent 1100 HPLC instrument fitted with a preparative autosampler and a well plate fraction collector. Semi-preparative SFC purification was carried out using a Berger Instruments MultiGram Preparative SFC Instrument. Intermediate-scale preparative HPLC was carried out using a system containing dual Varian SD-1 pumps (800 mL/min), Varian 215 injector pump 100 mL/min, Varian 320 variable wavelength UV/vis detector, R&S Techologies/Varian LC ReSonator liquid handling module and control software, with a Prochrom/Novasep dynamic axial compression (DAC) column (6 or 8 cm i.d.). Larger-scale preparative HPLC was carried out using a system consisting of a KP3000 pumping skid (Biotage, Charlottesville, VA), consisting of a main eluent pump (capable of 13000 mL/min flow) with a smaller-feed pump with a 3000 mL/min capacity, and a variable-wavelength detector with a full-flow flow cell and a 30-cm i.d. ProChrom DAC column (NovaSep, Inc., Boothwyn, PA).

Preparative SFC Upgrade of enantiopUrity of 3. Chiral SFC screening was carried out using a pair of Berger Instruments analytical supercritical fluid chromatographs fitted with six position column selection valves and Agilent model 1100 diode array UV−visible detectors. Chiral stationary phases evaluated included Chiralpak AD and AS, Chiralcel OD, OJ, OF, and OB (Chiral Technologies), Whelko (Regis Technologies), Chirobiotic V, R and T (Astec), and TBB (Eka-Nobel). An achiral silica column (Kromasil, Eka-Nobel) was also included in the SFC screening system as a means of identifying the presence of achiral impurities. All screening columns were of a standard 25-cm length and 4.6-mm inner diameter. The two systems are run in parallel, and employ a standard gradient method with a flow rate of 1.5 mL/min, an outlet pressure of 200 bar, an oven temperature of 35 °C, UV detection at 215 nm, and a mobile phase of 4% MeOH in CO_2 for 4 min, increasing to 40% MeOH over 18 min with a hold at 40% MeOH for 3 min and a 5 min post time. Initial screening of **3** revealed very poor chromatographic peakshapes; thus, an identical screen utilizing a 25 mM solution of isobutylamine in MeOH as the polar modifier was performed. The results indicated that Chiralpak AS afforded the best separation of the enantiomers of **3**. Semi-preparative SFC enantiopurity upgrade of 290 mg of an enriched mixture of substituted piperazine, **3**, was performed using a Berger Instruments multigram preparative SFC instrument. Conditions: Chiralpak AS (20 mm × 250 mm), 12% (25 mM isobutylamine in methanol)/carbon dioxide, 100 bar, 35−C, 50 mL/min, UV 320 nm, with a typical injection of 1 mL @ 60 mg/mL.

Preparative HPLC Purification of 1. Preparative HPLC upgrade of purity of **1** was carried out using a Varian preparative HPLC system with an 8-cm i.d. ProChrom DAC column packed with Kromasil 10 μm silica operating at a flow rate of 300 mL/min with an eluent of 88.5:10:1.5 IPAc/MeOH/H_2O and using UV detection at 290 nm. A typical injection consisted of 10 mL@180 mg/mL (1.8 g) with a run time of about 12 min. Analytical HPLC assay conditions: Kromasil 5 μm, (4.6 mm × 250 mm); 2 mL/min; 88.5:10:1.5 IPAc/MeOH/H_2O, UV 254 nm.

Pilot-Scale HPLC Purification of 2. Pilot-scale HPLC upgrade of the purity of **2** was carried out using a Varian preparative HPLC system with a 6-cm i.d. NovaSep ProChrom DAC column packed with 600 g of Amicon grade 631 silica (18 μm, irregular); operating at a flow rate of 300 mL/min with an eluent of 90:10 IPAc/MeOH and using UV detection at 320 nm. A typical injection consisted of 40 mL@ 38% crude **2** in IPAc with a run time of about 10 min.

Large-Scale HPLC Purification of 2. Large-scale HPLC upgrade of the purity of **2** was carried out using a Biotage preparative HPLC pumping skid with a 30-cm i.d. NovaSep ProChrom DAC column packed with 14 kg of Amicon grade 631 silica (18 μm, irregular; packed column bed length ca. 42 cm) operating at a flow rate of 7500 mL/min with an eluent of 90:10 IPAc/MeOH and using UV detection at 320 nm. A typical injection of crude feed solution contained about

290 assay g of **2** (800 mL, 35.1 assay wt %), and the run time was 16 min.

Prior to the initial run, the packing integrity of the column was tested by injecting 2 mL of the feed solution into the column, using an eluent flow rate of 7500 mL/min. Plate count of the main peak (product **2**) calculated by the half-height method, suggested an efficiency of about 20000 plates/m.

In the first production run 800 mL of feed solution was injected, and 21 fractions were collected across the main peaks in the chromatogram. On the basis of analysis of these fine fractions, cut points for fraction collection for future runs were established according to the scheme illustrated in Figure 4. A total of about 310 L of combined rich cuts were collected from the 19 production runs.

Following appropriate analysis, the rich cuts were fed into a cone-bottomed stainless steel vessel and concentrated by distillation under 29 in. Hg vacuum to about 35 L, and subsequently solvent switched at approximately constant volume to methanol, producing a total of 42.2 kg of the methanol solution at 13.4 assay wt % and containing a total of 5.65 kg of purified **2**.

Deprotection of Acetonide 2. Combined fractions totaling 25.3 kg of the methanol solution of chromatographically purified acetonide **2** (containing 3.514 assay kg of acetonide, 4.28 mol) was concentrated to a total volume of 13.8 L and then cooled to −3 °C. A solution of gaseous HCl in methanol (5.95 N, 7.2 L, 42.8 mol) was added at a controlled rate such that the internal temperature remained below 0 °C. The resulting homogeneous mixture was then stirred at 0 °C until HPLC assay showed >98.5% deprotection (~12 h). The reaction was quenched by addition of 2.5 N aqueous NaOH (17.0 L, 47.5 mol) at a controlled rate such that the internal temperature remained below 0 °C. Isopropyl acetate (35 L) was then added, the two-phase mixture was warmed to room temperature, and the layers were separated. The isolated organic layer was washed with brine (7.0 L). The organic layer was then treated with Darco G-60 (636 g) and heated at 50 °C for 1 h. After cooling to room temperature the slurry was filtered, and the filter cake washed with isopropyl acetate (3 times, 6 L per wash). The filtrate and washes were combined, and the solution of **1** as the free base was taken forward to the salt formation/crystallization step. By HPLC assay the combined filtrate and washes contained 3.02 kg of **1** (90.4% yield).

Preparation of 1 HCl. The IPAc/MeOH solution of **1** as the free base was concentrated to a total volume of 9.0 L. The concentrate was diluted with IPAc (6.0 L) and then reconcentrated to a total volume of 3 mL/g and the dilution/reconcentration repeated. After the final concentration the solution was diluted with IPAc (9.0 L) and IPA (7.5 L). After heating to 60 °C a solution of HCl in IPA (1.8 L, 0.512 N, from dilution of concentrated HCl with IPA) was added over 10 min. Seed (**1**·HCl, 3.0 g) was then added as a slurry in 200 mL of 1:1 IPAc:IPA. The seed bed was aged for 1 h at 60 °C, and then additional HCl/IPA was added (5.75 L, 0.512 N, from dilution of concentrated HCL with IPA) slowly over 2 h. Upon completion of the second acid addition, the slurry was aged for 2 h at 60 °C and then allowed to cool to room temperature overnight. The solids were collected by filtration, and the cake was washed twice with 1:1 IPAc:IPA (6.0 L each wash). The wet cake was then dried in a vacuum oven (22 in Hg, 40 °C) with a nitrogen sweep. After drying, a total of 2.71 kg of **1**·HCl (85.7%) was obtained as a white powder (needles, mp 205 °C) containing 0.27 LCAP of **5**. Additional analytical testing (HPLC−LCAP, GC, residual solvents, titration, and residue upon ignition) indicated that the isolated **1**·HCl met all purity criteria and was suitable for use in clinical evaluations.

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