Chapter 2

Synthetic Protocol for AFCS: A Biologically Active Fluorescent Castasterone Analog Conjugated to an Alexa Fluor 647 Dye

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Abstract

Synthetic derivatization of hormonally active brassinosteroids (BRs) can provide useful small molecule tools to probe BR signaling pathways, such as fluorescent analogs. However, most biologically active BRs are not suitable for direct chemical conjugation techniques because their derivatization typically requires extensive synthetic work and chemistry expertise. Here, we describe an operationally simple, two-step procedure to prepare and purify an Alexa Fluor 647-castasterone (AFCS) from commercially available materials. The reported strategy is also amenable to the introduction of various other amine-based labeling groups.

Key words Fluorescent labeling, Brassinosteroids, Castasterone, Steroid hormones, Oxime conjugation

1 Introduction

Fluorescent labeling of biologically relevant molecules is a valuable and classical strategy to probe biological processes [1]. Many reactive fluorophores have been developed and commercialized, such as the very popular fluorescein isothiocyanate, a reactive fluorescent dye that can covalently trap a range of chemical functions. Several commercial kits are now also available to perform standardized labeling of various substrate types, including some tailored for users with minimal experience in synthetic chemistry. However, for typical steroid hormones, most of the standard labeling strategies are hampered by a lack of suitable chemical functions, such as amines. Furthermore, steroids often contain multiple less reactive but similar functions within the same molecule, such as hydroxyls, precluding site-selective derivatization with “general” labeling methods. Moreover, because most functional groups in natural
steroids are often essential for their bioactivity, their derivatization can easily result in loss of the hormonal function.

The plant steroid hormone, brassinolide (BL) (Fig. 1) is a particularly problematic steroid substrate for standard labeling methods. No less than four different but chemically similar hydroxyl groups are present at C-2, C-3, C-22, and C-23 that cannot be readily discriminated by typical chemical reagents. Moreover, all of these hydroxyl functions are directly implicated in the biological activity of BRs [2] and, thus, their derivatization is expected to result in significant loss of hormonal function. Furthermore, because the reactive lactone function at C-6 is an integral part of the BL secosteroid backbone, its derivatization through acylation reactions (lactone opening) also drastically alters the overall topology of the rigid “flat” molecular scaffold and influences its ability to be recognized by steroid receptors. In this regard, the biologically active precursor of BL, castasterone (CS) (Fig. 1) is a more attractive labeling substrate, because the ketone function at C-6 can be selectively modified without a priori risking major disruption of the receptor affinity. Moreover, CS is commercially available in multi-mg amounts at reasonable cost or can be synthesized from stigmasterol with a classical multistep sequence [3–5]. Previously, C-6 derivatives of CS have been shown to be able to retain their biological function as BR hormones [6].

In this chapter, we provide a detailed method for a small-scale derivatization of CS (approximately 1 mg) that should also be applicable for a number of related 6-oxo steroids. The method can be performed by researchers or technicians with minimal experience in synthetic chemistry. The protocol is a two-step derivatization strategy that goes through an oxime-conjugation step to introduce a carboxylic acid moiety into the CS framework that generates CS \( O \)-carboxymethyloxime (CS-CMO) (Fig. 2), followed by an amide coupling to an amine derivative of an Alexa Fluor (AF) 647 dye (AF 647 cadaverine). This strategy has also been employed to incorporate alternative tagging groups, such as

![Chemical structures of the hormonally active brassinosteroids brassinolide (BL) and castasterone (CS)](image)

Fig. 1 Chemical structures of the hormonally active brassinosteroids brassinolide (BL) and castasterone (CS)
a dansylcadaverine [6] or a BODIPY group [7]. Although a wealth of coupling reagents is available to affect an amide-type coupling between an amine and a carboxylic acid, the 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) reagent used here is especially suitable for the envisaged small-scale derivatization, because of its high tolerance for water and operational ease [8].

2 Materials

2.1 Chemicals and Reagents

1. 5–10 mg powder of castasterone (CS) (CAS, 80736-41-0). CS can be prepared from stigmasterol via a number of well-established synthetic routes [3–5], but is also available from different chemical suppliers.

2. 1 mg powder of Alexa Fluor 647 cadaverine, disodium salt (AF 647 cadaverine; molecular mass approximately 1000 g/mol or 1 μmol). It can be purchased in 1-mg batches in individual screw capped plastic tubes and stored at –20 °C.

4. $4\cdot(4,6$-dimethoxy-1,3,5-triazin-2-yl)$-4$-methylmorpholinium chloride (DMT-MM) (CAS: 3945-69-5) This commercially available reagent should not be stored for very long times, because it loses activity over time by decomposition. The quality of the reagent is also known to vary. Fortunately, this problem can usually be solved by simply adding more reagent than required.

5. High-purity $N,N$-diisopropylethylamine (DIPEA) (CAS: 7087-68-5) (redistilled grade, $>99\%$) that can be used without further purification.

6. Solvents: methanol, glacial acetic acid, pyridine, acetonitrile, and chloroform, all at anhydrous high-performance liquid chromatography (HPLC) quality and used without further purification.

2.2 Chromatography Material

1. Standard silica gel for chromatography ($60\,\text{Å}$).

2. C18-reversed phase silica gel for column chromatography (fully end-capped).

3. Standard glass-backed thin layer chromatography (TLC) plates with a conventional binder and fluorescent indicator.

4. Washed and dried sand for laboratory use.

2.3 Chromatography Solutions and Solvent Mixtures

1. LC solvent mixture: A mixture of chloroform (8.5 mL), methanol (1.5 mL), and glacial acetic acid (100 $\mu$L) prepared in a small volumetric cylinder and then transferred to a standard elution tank for TLC, i.e., a wide, flat-bottomed beaker with a cap to avoid evaporation.

2. TLC reference solution of CS: A CS solution in methanol (approximately 1 mg/mL) must be prepared in a small sealable vial or tube as a reference sample for TLC analyses (approximately 20 $\mu$L is enough).

3. A phosphomolybdic acid (PMA) TLC staining solution (10 $\%$ (v/v) in ethanol): 10 $\%$ (w/w) mixture of PMA hydrate (CAS: 51429-74-4) and ethanol (denatured technical grade).

4. A solvent mixture for chromatography elution: A mixture of 50 mL of chloroform, 2.5 mL of anhydrous methanol, and 250 $\mu$L of glacial acetic acid. This eluent is prepared, homogenized, and stored in a volumetric cylinder, sealed with a plug of laboratory tissue paper to avoid evaporation.

5. TLC solvent mixture: A mixture of chloroform (6 mL), methanol (4 mL), and glacial acetic acid (100 $\mu$L), prepared in a small volumetric cylinder and transferred to a standard elution
tank for TLC, i.e., a wide flat-bottomed beaker with a cap to avoid evaporation.

6. A solvent mixture for reversed-phase chromatography elution: A mixture of 33 mL of acetonitrile and 100 mL of a 5 mM aqueous solution of ammonium acetate (NH₄OAc), prepared by dissolving 38.5 mg of NH₄OAc in 100 mL of milli-Q deionized water.

2.4 Equipment and Other Materials

1. Glass Pasteur pipettes (approximately 2 mL internal volume) to transfer organic liquids, such as chloroform.

2. Micropipettes (ranges 1–20, 20–100, 100–500, and 500–2500 μL), suitable for organic solvents, with disposable tips to transfer and measure mixtures and solutions based on water, methanol, and/or acetonitrile.

3. High vacuum pump (down to 0.1 mm Hg).


5. A rotary evaporator.


7. Screw-capped glass reactor vials (1–10 mL volume) and Teflon-coated stirring bars that fit the internal diameter of the vials.

8. Glass fraction collection tubes (1–10 mL)


10. A benchtop centrifuge.

11. A centrifugal concentrator.

12. An analytical balance, accurate to 0.1 mg.

3 Methods

Although the protocol has been written for researchers with minimal experience in organic synthesis, it is recommended to carry out the work in a well-equipped synthetic chemistry laboratory, with access to a fume hood, organic solvent handling and storage facilities, and analytical instruments, with minimal supervision of a trained organic chemist or laboratory technician.

3.1 Synthesis of CS-CMO

1. Weigh out CS (3.8 mg or 8.18 μmol) and CMHA (4.1 mg or 37.5 μmol) as powders (see Note 1) and place them together with a Teflon-coated stirring bar in a thick-walled screw-capped glass vial (volume 2 mL, approximately 0.5 cm internal diameter) (see Note 2). Dissolve the powders in approximately 0.25–0.30 mL of dry pyridine. Handle pyridine only in a well-ventilated fume hood to avoid exposure. Make sure no powder remains
on the sides of the reaction vessel. Immediately after addition of the dry pyridine, close the reaction vessel with a screw cap and stir the resulting mixture overnight at room temperature.

2. Sample the obtained reaction mixture (after the overnight stirring) by removing approximately 2 μL of the reaction mixture (with a micropipette or capillary) from the vessel. Transfer this liquid aliquot to a separate vial or tube. Inside a working fume hood, dry it under a small stream of nitrogen gas to remove most of the pyridine.

3. Redissolve the dried residue in approximately 25 μL of the LC solvent mixture. After homogenization of the resulting dilute solution, transfer a few μL to the baseline of a glass-backed TLC plate (with a capillary or micropipette tip), in a lane next to the applied similar amount of the TLC reference CS solution. Place the TLC plate upright in the elution tank containing the remainder of the LC solvent mixture, and allow it to absorb the eluent from its bottom (see Note 3). When the solvent front reaches the appropriate level, dry the TLC plate and visualize compounds under a UV lamp, by treatment with a PMA staining solution, and by heating (see Note 4). The starting CS material should appear as a spot with a ratio-to-front (Rf) value of approximately 0.53 (Fig. 3). The corresponding oxime CS-CMO appears as a lower running spot with an Rf value of approximately 0.23. Some minor spots may appear at Rf values above 0.53, including the remaining pyridine.

4. When the conversion of the starting CS material to the CS-CMO reaction product is incomplete, i.e., when it is still observed by the TLC analysis (Subheading 3.1, step 3) (Fig. 3), add additional CMHA (approximately 2 mg). Seal the reaction

![Fig. 3 Representation of thin-layer chromatograms (NP silica) for a complete oxime-forming reaction between CS and carboxymethylhydroxylamine (left), and that of an incomplete reaction between CS and carboxymethylhydroxylamine (right). All spots are non-UV fluorescent, except those at a very high Rf (pyridine)
vessel with the screw cap and then heat it to 60 °C for 90 min, with a complete consumption of all starting material as a result. Verify by TLC as described (Subheading 3.1, steps 2 and 3).

5. When a complete conversion to CS-CMO is finally confirmed by TLC analysis (Fig. 3), concentrate the reaction mixture under a small stream of nitrogen gas (see Note 5) and dry the residue on a high-vacuum pump.

6. Plug a glass Pasteur pipette (internal volume approximately 2 mL; see Note 6) with a small piece of cotton and then fill it with 500 mg of dry normal-phase silica gel (approximately 1 mL or half-height). Forcibly, wash the column filled with dry silica gel with 10–20 mL of the solvent mixture for chromatography elution with 1-mL portions. Use a nitrogen line or manual pump (pipette head) to apply pressure to the top of the column in order to remove any air from the silica during this rinsing. Be careful that no additional air is introduced into the silica column by maintaining the solvent line above the top of the silica column at all times (i.e., not pushing air through the silica). Discard the solvent that elutes during this rinsing and packing of the column. When the silica gel is visibly packed, i.e., semi-translucent without small air pockets or “white spots,” allow the solvent line to drop to the top of the silica gel column (without pressure).

7. Dissolve the dried residue (obtained in step 5) in a pointed microcentrifuge tube, or reaction vessel that had been dried in a high vacuum, in approximately 50 μL of the solvent mixture for chromatography elution, and transfer the resulting solution immediately with a pipette to the top of the freshly packed silica gel column (prepared in step 6). From now on, collect the eluate of the column in a rack of small glass tubes (2–5 mL volume). Do not use plastic fraction collection tubes, because most are not compatible with the eluent. Rinse the sample tube containing the dried reaction mixture with approximately 50 μL of solvent, and transfer this solution to the top of the column. Finally, rinse the sides of the Pasteur pipette column with approximately 100 μL of eluent. Then add a few mm of sand to the top of the silica gel column to avoid silica gel “whirling,” before eluting the column by adding larger amounts of solvent (approximately 1-mL portions). Continuously collect fractions of approximately 200–250 μL without letting the solvent line drop below the level of the sand. Elute the column with three times 1 mL of the initial solvent mixture, resulting in the collection of 10–15 fractions. Then, elute twice with 1 mL of the same solvent mixture after enriching it with 2 % (v/v) of methanol (see Note 7). Next, elute the column twice with 1 mL of the used solvent mixture, further enriched with 3 % (v/v) of methanol. At this point (around fraction 25), the oxime product
should start eluting from the column, as can be confirmed later by TLC analysis. Continue to elute four times with 1 mL of the same solvent enriched with another 5 % (v/v) of methanol.

8. Test the fractions collected in step 7 for the presence of CS-CMO by TLC analysis (Fig. 4). Pool the fractions that are positive for the desired compound, and also do not show any other products, because some minor mixed fractions may occur in the early fractions. Concentrate the combined CS-CMO-containing fractions with a rotary evaporator, in a vial or tube that has been carefully weighed beforehand. Redissolve the residue in a small amount of toluene (500 μL) and concentrate it again to dryness. Then, redissolve the residue in acetonitrile (500 μL) and evaporate to dryness three times (see Note 8). Finally, dry the residue under a high vacuum and weigh the vial to determine the amount of obtained product (approximately 5 mg; the maximum theoretical yield is 4.4 mg, i.e., near quantitative yield). The compound can be used without further purification in the next step. Standard analytical data (nuclear magnetic resonance or mass spectroscopy) can be checked and compared to literature data [6] to verify identity and purity.

3.2 Amide Coupling between CS-CMO and Alexa Fluor Cadaverine

1. Remove a 1-mL sealed plastic tube containing 1 mg AF 647 cadaverine disodium salt from the cold storage (−20 °C) and briefly allow it to warm to room temperature before use.

2. Dissolve the dried final compound CS-CMO (approximately 4.5–5.0 mg) (see Subheading 3.1, step 8) in 440 μL of anhydrous methanol (100 μL/mg). This solution will be used as CS-CMO stock solution (see Note 9).

![Fig. 4 Typical thin-layer chromatogram (NP silica) for fractions obtained from flash chromatography of the oxime-forming reaction between CS and carboxymethylhydroxylamine (CHMA), resulting in the CS-CMO compound](image)
3. In a separate sealable vial or tube, prepare a homogenous stock solution of DIPEA in methanol by mixing 20 μL of DIPEA with 1980 mL of anhydrous methanol. This stock solution should be prepared directly before use to avoid or minimize contamination and evaporation and the excess should be discarded. Also weigh out two portions of approximately 1.3 mg of DMT-MM reagent (powder) in small sealable vials.

4. Open the tube containing 1 mg of AF 647 cadaverine disodium salt and fill it with 150 μL of the CS-CMO methanolic solution obtained in step 2 (approximately 2.0–2.7 μmol). After homogenation by taking up and expelling the solution a couple of times in the tube with a micropipette, transfer the resulting bright blue solution to a similar reaction vessel as before (screw-capped 2-mL glass vial of approximately 0.5 cm diameter, see Note 2), equipped with a magnetic stirring bar. Rinse the AF 647 container tube with 20 μL of anhydrous methanol, and transfer this washing solution to the reaction vessel.

5. Add 60 μL of the freshly prepared DIPEA stock solution (approximately 2.7 μmol, see step 3) (see Note 10) to the reaction vessel already containing CS-CMO and AF 647 (see step 4). Next, add one portion of the DMT-MM reagent (1.3 mg, approximately 4 μmol) to the reaction vessel. Make sure all powder is dissolved in the reaction mixture, if needed rinse the sides with 20 μL methanol, before closing the vessel and stirring it magnetically with a Teflon-coated stir bar, for 10 min. At this time, another portion of DMT-MM reagent is added (1.3 mg) and the resulting mixture is stirred for another 20 min at ambient temperature.

6. Remove 1–2 μL of the reaction mixture obtained in step 5 and transfer it directly to the bottom of a normal TLC plate. On the same plate, place a reference sample of both CS-CMO and AF 647 cadaverine in methanol, prepared by adding approximately 20 μL to the rinsed AF container tube of step 4. After running the TLC with the appropriate TLC solvent mixture, the conversion of the AF 647 cadaverine can be observed directly on the TLC plate, without visualization, by the disappearance of a bright blue spot at an Rf value of 0.11. A single bright blue new spot should appear at a significantly higher Rf value of 0.23 (Fig. 5, left) (see Note 3). After development with the PMA solution (see Note 4), some remaining CS-CMO compound, added in excess, can be observed near the top of the TLC plate (Rf 0.76). Upon observation of a complete reaction (see Note 11), concentrate the reaction mixture under a slow stream of nitrogen gas (see Note 5), and dry the residue under a high vacuum.

7. Purify the residue obtained in step 6 by manual reversed-phase chromatography (see Note 12) with the solvent mixture for
reversed-phase chromatography elution. Using this eluent in TLC analysis, the AFCS compound should have an Rf of approximately 0.2 on a reversed-phase silica gel TLC plate, whereas the impurities all have an Rf above 0.8 (Fig. 5, right) (see Note 3). Plug a standard laboratory-ware column (internal diameter 0.5–1 cm and a least 20 mm high) with a piece of cotton wool and fill it with approximately 2 g of slurried C18-reversed-phase silica with the same aqueous eluent. Rinse the column by forcibly washing it with the same eluent (see Note 13) until a good packing is achieved.

8. Dissolve the residue of step 6 in 60 μL of aqueous eluent, and transfer the resulting bright blue homogenous solution to the top of the silica gel column. Rinse the vial with 30 μL of the aqueous eluent, and transfer this washing solution also to the top of the column. Rinse the sides of the column with 100 μL of eluent, and then further elute the column, while collecting fractions of approximately 200 μL, using either glass collection tubes or plastic microcentrifuge tubes. The progress of the chromatographic separation can be observed easily and directly by the appearance of two dark blue bands, one of which moves very rapidly down the column, virtually with the solvent front. This front running band should already elute in the first few fractions. As the second blue band can be observed to move slowly down the column, start collecting smaller and smaller fractions just before the blue band approaches the bottom of the column and starts to elute at a high concentration. Next, collect 100-μL fractions until the eluate is (almost) colorless. Analyze the lightly colored fractions before and after the dark
blue fractions by TLC to check purity. Pool all clean and AFCS-positive fractions, by using minimal amounts of eluent to rinse the fraction tubes.

9. Divide the combined clean AFCS-positive fractions from step 8, and distribute them equally over five microcentrifuge tubes (approximately 350 μL per vial). Next, centrifuge the tubes (g value?), freeze the spun down samples in liquid nitrogen, and then concentrate them under high vacuum with a centrifugal concentrator. Weigh the combined tubes before and after this operation to get an accurate determination of the isolated amount of AFCS: approximately 1.4 mg or 0.285 μg per tube (see Note 14). The identity of the AFCS compound can be confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry.

4 Notes

1. It is important to get an accurate weight for the CS, because it can also be used as the basis for the calculations for reagents in the second step. For the added CMHA, a large excess is used, so its exact weight is less important. A 50 % relative deviation of the stated amount is acceptable and will give the same result.

2. The choice of reaction vessel is quite critical because of the small scale of the reaction. The reaction vessel should be air tight to avoid evaporation of solvent and exposure to moisture/oxygen. Classic round-bottomed flasks with a rubber septum are not suitable, because of the large surface area of the reaction mixture in such a vessel. In the absence of a straight-walled glass pressure tube, a plastic sealed microcentrifuge tube can also be acceptable.

3. The outcome of the TLC analysis can be influenced by the presence of the solvent remaining from the compound application (methanol or pyridine). Therefore, the spots on the TLC plate could be dried for 1 min under a stream of nitrogen gas, and then left to dry for 10 min before transfer to the elution tank. Also make sure the solvent line in the elution tank is well below the line on the TLC plate where compounds are applied.

4. Other common laboratory TLC staining reagents will also give the desired result, such as anisaldehyde- or permanganate-based stains.

5. Alternatively, the reaction mixture can be concentrated with a standard rotary evaporator. On this small scale, however, simple insertion of a Pasteur pipette connected to a source of nitrogen gas halfway in the reaction vessel will effectively and quickly remove all volatiles in a matter of minutes. The evapo-
ration can be helped by gently heating the vessel with your hands. The stream of nitrogen should be as fast as possible without risking splashing of the solvent.

6. A standard laboratory-ware column can also be used, but because of the usually larger internal diameter, more silica gel is required, resulting in a higher loss of compound through irreversible absorption. A Pasteur pipette is most convenient and its small dead volume at the exit can be further reduced by breaking off the tip just below the cotton plug. A disadvantage of the use of a pipette is that the chromatographic separation cannot be halted at any time, but because of the small scale, should not be an important problem. However, all handlings have to be done swiftly without delay to avoid “drying out” of the column.

7. Keeping the solvent mixture for column elution in a 50-mL measuring cylinder allows rapid gradient adjustment by simple addition of the required amount of additional methanol and homogenization of the resulting mixture.

8. This coevaporation with toluene and acetonitrile is absolutely required to remove all residual acetic acid from the compound, because it will also be reactive to the AF 647 cadaverine and will thus severely limit the efficiency of the conjugation reaction.

9. As the CS-CMO compound is not a powder, a stock solution in methanol is the most convenient way to control the quantity of the compound at this scale. The method also does not require accurate weighing of very small amounts, which can be tedious. The unused stock solution can be concentrated and dried again and stored for future use.

10. The addition of DIPEA is usually not required for DMT-MM amide couplings, but is necessary in this case because the commercial AF 647 cadaverine compound is in fact an internal ammonium sulfonate salt that needs to be deprotonated before it can be acylated. To this end, addition of this non-nucleophilic amine in an equimolar ratio to the CS-CMO compound is the most convenient.

11. When some AF 647 starting material can still be seen on the TLC plate, it is most probably due to partial decomposition of the labile DMT-MM reagent, because the amide formation reaction should be fast. In this case, the reaction can be driven to completion by addition of 1–2 mg more DMT-MM reagent and by stirring the resulting mixture for another 30 min. Do not add additional DIPEA.

12. A semi-preparative automated HPLC purification can also be carried out, but the manual chromatography is very easy and convenient to perform because of the bright blue color of both
the desired compound and side products. Manual normal-phase chromatography can also be used to purify AFCS, but the acidic medium can result in partial methanolysis of the AFCS compound during chromatography, as can already be observed as a light blue “tailing” of the AFCS spot, making this technique less suited. Moreover, the separation is very straightforward on reversed-phase silica, because all AF-based contaminants have a significantly shorter retention time and the excess CS elutes much later.

13. Some inconvenience may arise from the fact that the density of (dry) reverse-phase silica gels is lower than that of the solvent mixture. Slurrying the silica gel before loading the column should help, and rinsing with a lot of solvent for a long time under high pressure should give a tightly packed column. This problem is also the reason why performing the manual flash chromatography on a Pasteur pipette (see Note 6), is not a practical option in this case. A wider internal diameter is needed to allow the aqueous slurry to accumulate at the bottom of the column.

14. A more accurate quantification of AFCS can be done on solutions prepared from these tubes by standard colorimetric methods.

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References

Brassinosteroids
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