LC-(TIC/EIC)-MS as Tool in the Analysis of Diastereomeric 3,12-aza-analogues of Deoxycholic Acid

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Dedicated to Professor Alain Krief on the occasion of his 65th birthday

Abstract

The non-conventional Total Ion Current (TIC) detection and corresponding Extracted Ion Current (EIC) chromatogram in LC-MS measurements, have been used for quantitative analysis of reactions involving UV-inactive aza-analogues of deoxycholic acid. LC-(TIC/EIC)-MS experiments allowed for the accurate determination of diastereomeric and overall purity during the development of a new synthetic route for an orthogonally protected dipodal steroid scaffold with defined stereochemistry. Moreover, this technique facilitated adequate monitoring of further scaffold derivatization on solid support. The described methodology offers an attractive solution in cases where conventional HPLC analysis with UV detection fails.

Keywords: LC-MS, TIC/EIC detection, steroids, dipodal scaffold, Alloc deprotection

Introduction

In our quest for new synthetic models of naturally occurring proteins we became interested in the design of dipodal peptidosteroid receptors, based on the attachment of short minimal recognition peptides to steroid scaffold **1**, derived from deoxycholic acid (3α,12α-dihydroxy-5β-cholan-24-oic acid) (Figure 1). The defined geometrical properties and the orthogonal protection of the attachment points are the main features of this scaffold, permitting the design of not only homo-, but also heterodimers with a specific pre-organization. Furthermore, the C24 carboxylic acid provides a natural link for immobilization on a solid support.
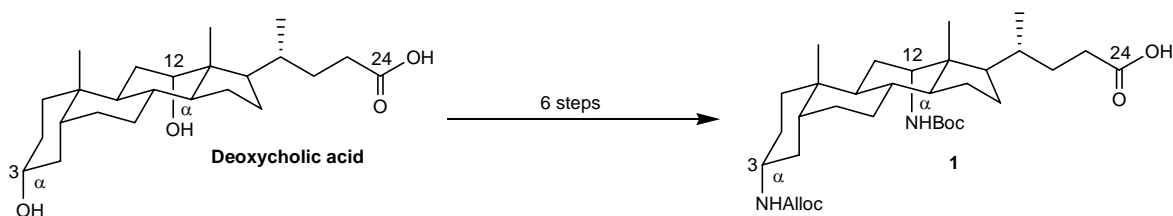
Figure 1. Deoxycholic acid and dipodal steroid scaffold **1**.

To efficiently obtain sufficient quantities of scaffold material in a short time period, we conceived a new synthetic route, applying a very straightforward strategy.[1] Whereas previously published routes for similar scaffolds require a large number of synthetic steps (usually >10),[2] we were able to significantly limit the number of steps to only six. In contrast to the commonly used sequential derivatization of the hydroxyl functions, necessary to guarantee the correct stereochemistry and differentiation of the different positions, we proceeded via the simultaneous transformation of these functions to the corresponding diamine. Further differentiation was based on the difference in steric impediment of the C3 and C12 position in (deoxy)cholic acid (derivatives).[3]

During the development of this short synthetic route we faced various challenges, especially concerning the purification and analysis of the formed diastereomers. We here report in detail on the large-scale, chromatographic separation of these diastereomers and the combination of analytical techniques necessary for the unambiguous determination of purity and identity of the formed/isolated compounds. In particular the on-line use of non-conventional Total Ion Current detection during reversed-phase HPLC analysis and the resulting Extracted Ion Current chromatogram (TIC/EIC) proved invaluable in this context.

Results and Discussion

Scheme 1 depicts the key steps of the optimized synthetic route, which also represent the most challenging ones. In order to significantly lower the amount of synthetic steps, we decided to directly transform deoxycholic acid via the dioximo carboxylic acid **2** into the corresponding diamine **3** (**a** + **b**), applying conditions previously reported by Burrows et al. for reduction of analogous dioximo derivatives with C24 alcohol.[4] A one-pot esterification procedure was necessary to obtain the compound as pure diamino *n*-propyl ester. Being less polar, this compound proved much easier to isolate from the reaction mixture by conventional liquid extraction than its charged diamino carboxylic acid analogue.[1] In this way it is possible to conserve the carboxylic acid (protected as an ester) without the need for additional reduction or protection steps. Conservation of the C24 carboxylic acid moiety allows for later immobilization on solid support via a stable amide bond, circumventing possible problems associated with products immobilized through a more labile ester bond via the C24 alcohol.
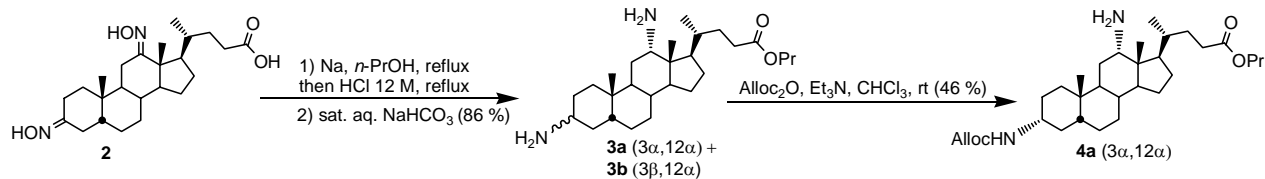
Scheme 1. Key steps of the short synthetic route towards **4a**.[1]

Thin Layer Chromatography (TLC) clearly showed the formation of products with similar Rf values (0.18; 0.27; 0.35; 0.43 in EtOAc:MeOH:NH$_4$OH$_{aq}$ 9:1:1), assumed to be diastereomers formed during the reduction. The major spot (Rf = 0.27) could be isolated by flash chromatography. Separation is efficient provided conditions are rigorously controlled. Flash chromatography using Merck grade 9385 silica gel (60 Angström, 230-400 mesh) was necessary to achieve succesful and reproducible separation. Elution in this large-scale separation should proceed with a somewhat atypical EtOAc:MeOH:NH$_4$OH$_{aq}$ 28:3:1 mixture. In the work published by Burrows et al. formation/isolation of diastereomers was not mentioned and only the isolation of pure 3α,12α-diamino-5β-cholan-24-ol in 62 % yield from reduction of 3,12-dioximo-5β-cholan-24-ol was reported.[4]

Until now, published procedures frequently use ^{1}H NMR to judge the diastereomeric purity of similar compounds.[2b-c,4,5] The α or β orientation of the proton adjacent to the electron withdrawing N-atom results in a specific chemical shift and coupling pattern. In our hands ^{1}H NMR spectroscopy alone proved insufficient to fully assess the diastereomeric purity. The most intense and clearly identifiable signals of the C3-H and C12-H were consistent with the 3βH,12βH geometry, thus the 3αN,12αN products being the main compounds. Chemical shifts (δ) proved very similar to literature data for both the 3β and 12β proton and integrations were consistent. Moreover the shape of both peaks was consistent with coupling patterns, since 3βH is broadened due to large axial-axial couplings, in contrast to 12βH. Table 1 illustrates the satisfying agreement of experimental with literature data.[4]

Table 1. ^{1}H NMR δ (in ppm) comparison for compound **3a** with literature

	Literature[4]	Experiment
3βH	2.71	2.72
12βH	3.13	3.17

However no reliable, reproducible and clearly identifiable signal for the corresponding αH-(βN)–isomers could be located. Furthermore, there was no clear difference between spectra of crude extract and purified fractions.

Therefore, in order to accurately judge the diastereomeric purity of the compounds, we had to rely on HPLC based methods. The complete absence of UV-activity above 214 nm complicated the analysis and no clear peaks could be observed in the chromatograms. However, the hyphenation of reversed-phase HPLC to an Electrospray Ionization Mass Spectrometer (LC-MS) and the resulting Total Ion Current (TIC) chromatogram provided the necessary information. Especially the corresponding Extracted Ion Current (EIC) chromatogram proved invaluable in judging the diastereomeric ratio of the compounds. In the EIC chromatogram only the ions of a particular molecular mass are taken into account, thus (mathematically) extracting specific information (EIC) out of the crude data (TIC). This methodology is in our case justified since the diastereomers can be assumed to ionize equally. In order to reduce tailing of the amines the addition of 0.1 % formic acid to the eluent was necessary to obtain sharp, separated signals.

The data extraction principle using EIC is clearly illustrated in Figure 2. The TIC chromatogram A, obtained from the reduction/esterification reaction mixture (Scheme 1, conversion of **2** to **3a** + **3b**), looks quite messy and interpretation is therefore difficult. Whereas this chromatogram gives no clear information, the corresponding EIC chromatogram B proves very diagnostic in identifying the formation of four diastereomers, as previously suggested by TLC results.
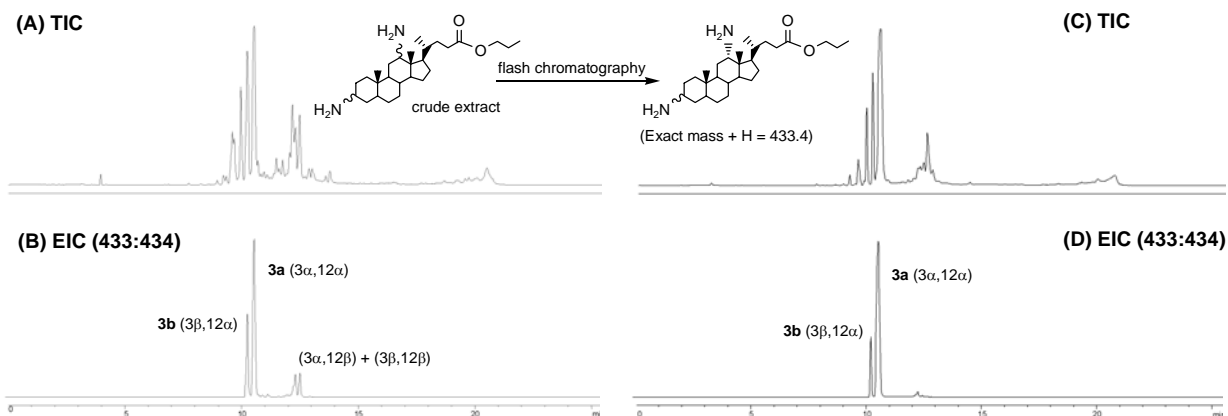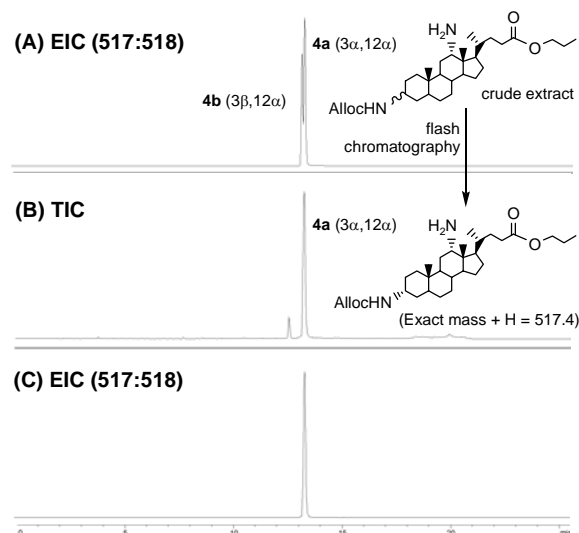

Figure 2. (A) TIC chromatogram of crude reaction extract; (B) Corresponding EIC chromatogram; (C) TIC chromatogram of chromatographically purified mixture; (D) Corresponding EIC chromatogram *(target molecular weight range is shown between brackets).*

Results from literature indicated the 3α,3β/12α-isomers being expected as major compounds and the 3α,3β/12β-isomers as minor compounds.[2b-c,4,5a-b,5d] Surprisingly EIC chromatogram D, corresponding to TIC chromatogram C, clearly shows that the pure, main spot on TLC still consists of the two major compounds, again believed to be the 3β,12α (**3b**) and 3α,12α (**3a**)

isomers (diastereomeric ratio 15:85). Separation of the undesired isomer at this stage proved very labour-intensive and we therefore decided to proceed with C3 protection and separate at a later stage.

After introduction of the Allyloxycarbonyl (Alloc) protecting group[6] (Scheme 1, conversion of **3a** + **3b** to **4a**), the product mixture consequently consisted of the two C3-monoprotected diastereomers **4a** + **4b** (Figure 3, EIC chromatogram A). In contrast to the diamino *n*-propyl ester mixture **3a** + **3b**, the monoprotected derivatives could be completely separated chromatographically, as shown in Figure 3, TIC chromatogram B and corresponding EIC chromatogram C. From these chromatograms it could be concluded that compound **4a** was obtained in a satisfying overall purity (TIC chromatogram B) and an excellent diastereomeric purity (EIC chromatogram C), considering only one flash chromatographic purification is required. Furthermore, after a verification step (see experimental section for details) involving the removal of the C3-NHAlloc protecting group from compound **4a**, LC-(TIC/EIC)-MS (not shown) clearly confirmed the absence of the minor diamino isomer **3b**. Flash chromatographic conditions should again be strictly controlled. Elution with CH_2Cl_2:MeOH 98:2, again using Merck grade 9385 silica gel are prerequisites for successful separation.


Figure 3. (A) EIC chromatogram of crude extract; (B) TIC chromatogram of purified **4a**; (C) Corresponding EIC chromatogram *(target molecular weight range is shown between brackets)*.

In this way the desired C3α-NHAlloc,C12α-NH$_2$ diastereomer **4a** could be isolated and characterized. Although comparison of ^{1}H NMR spectra with literature data[2c,4a,5a-d] confirm the correct identity of the major isomer, LC-(TIC/EIC)-MS experiments were clearly necessary to judge the overall and diastereomeric purity of this compound.

The LC-(TIC/EIC)-MS technique described above proved also very helpful in monitoring further on-bead scaffold derivatization. The development of analytical tools for the accurate

monitoring of reactions on solid support is an important, progressing field in contemporary research. Analysis often has to rely on minute quantities obtained from cleavage of a single bead and sensitive methods based on mass spectrometry are therefore necessary for quality control of solid phase (library) synthesis. In the process of generating dipodal steroidal peptidomimetics on solid support, some preliminary experiments concerning the deprotection of the scaffold on solid support were performed. In one particular case, we tested the sequential deprotection of the C12-NHBoc group and C3-NHAlloc group of the scaffold. The use of a photocleavable linker[7] (Figure 4) makes cleavage of small compound quantities from the support very easy, providing samples immediately ready for further HPLC and MS based analysis.[8]
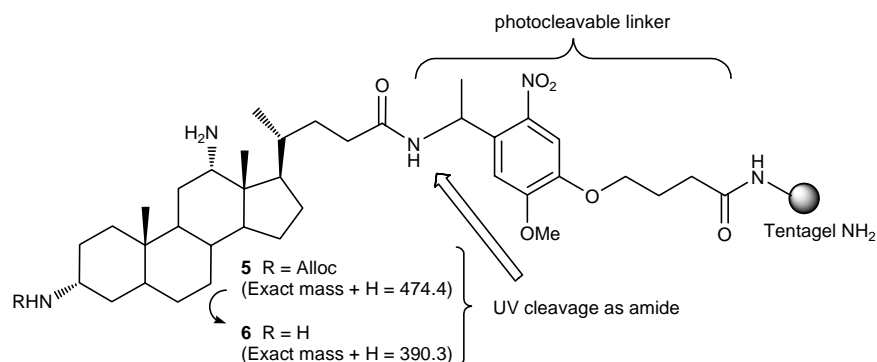
**Figure 4.** Alloc deprotection of solid supported construct **5**.

Although generally used as protecting group in a variety of applications, the Pd-catalyzed Alloc deprotection is often a challenging reaction. Next to incomplete, sluggish reactions, the N-allylation of the free amino group by the liberated allyl cation is a considerable side reaction.[6,9] Until recently, we usually followed reaction progress by photolytic cleavage of a few beads and immediate analysis of the obtained reaction mixture by ESI[+]-MS. From these ESI[+]-MS spectra (Figure 5), some preliminary conclusions can be drawn.
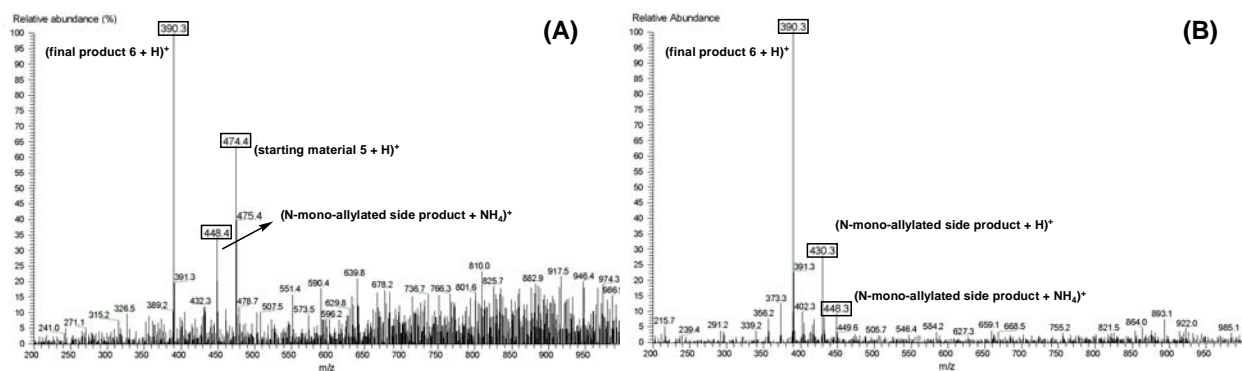
Figure 5. ESI[+]-MS spectra of Alloc deprotection (A) after second treatment with Pd(PPh$_3$)$_4$/Bu$_3$SnH/morpholine; (B) after treatment with Pd(PPh$_3$)$_4$/anilinium p-toluenesulfinate.

The use of Pd(PPh$_3$)$_4$/Bu$_3$SnH with morpholine for 22 hours didn't result in quantitative removal of the Alloc group, even after repeating the reaction. As shown in spectrum A, the sample still consisted of a mixture of Alloc protected starting material **5** and deprotected final product **6**. Moreover, a significant signal of N-(mono)-allylated side product (Exact mass + H = 430.4; Exact mass + NH$_4^+$ = 447.4) was detected. In contrast, the ESI$^+$-MS spectrum after the use of Pd(PPh$_3$)$_4$ with anilinium *p*-toluenesulfinate[9b,10] for only 2 hours showed almost complete deprotection, with no significant increase of N-(mono)-allylated side product (spectrum B). The ionization of the compounds being unpredictable and unreproducible, it was difficult to draw more quantitative conclusions about the progress of the reaction and the extent of the side reactions.

Using the described LC-(TIC/EIC)-MS methodology, more quantitative information was obtained and the reaction could be monitored more adequately (Figure 6).
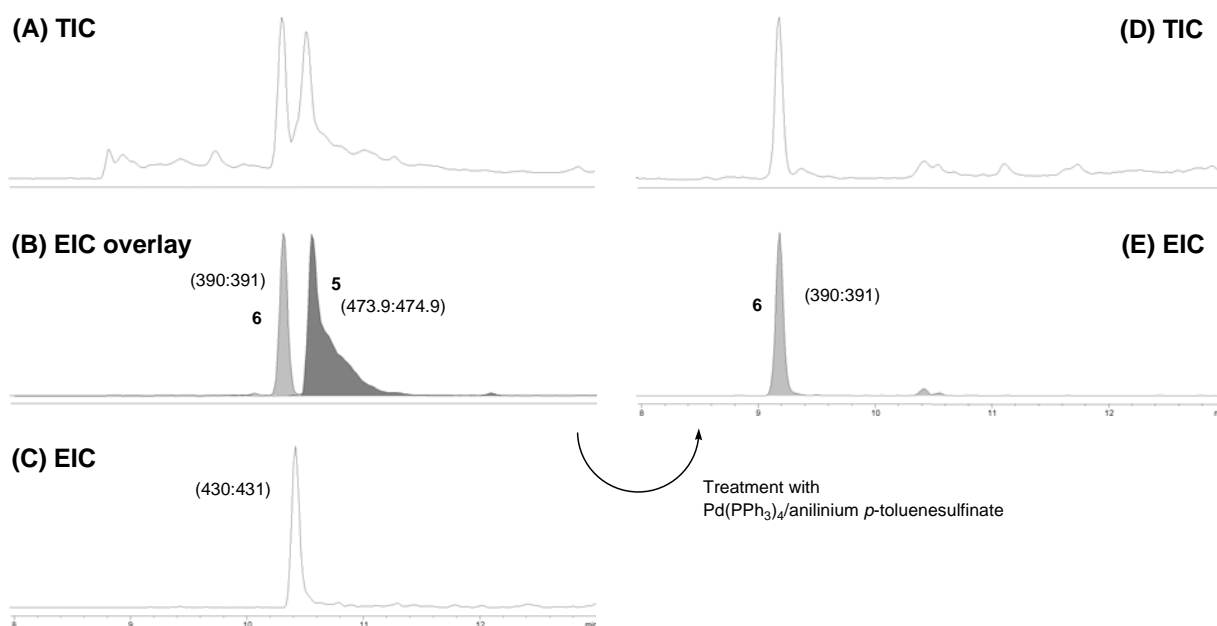
Figure 6. LC-(TIC/EIC)-MS of Alloc deprotection: (A) TIC chromatogram after second treatment with Pd(PPh$_3$)$_4$/Bu$_3$SnH/morpholine; (B) Superimposed EIC chromatograms of Alloc protected starting material **5** and deprotected final compound **6**; (C) EIC chromatogram of N-(mono)-allylated side product; (D) TIC chromatogram after treatment with Pd(PPh$_3$)$_4$/anilinium *p*-toluenesulfinate; (E) EIC chromatogram of deprotected final compound **6** *(target molecular weight range is shown between brackets).*

The two peaks obtained in TIC chromatogram A after twice applying Pd(PPh$_3$)$_4$/Bu$_3$SnH/morpholine were identified as starting material and final product by the corresponding EIC chromatograms, as shown in B, where EIC chromatograms of **5** and **6** are

superimposed. The N-(mono)-allylated side product can be found in between starting material **5** and deprotected compound **6** as derived form EIC chromatogram C.

Results obtained after treatment with Pd(PPh$_3$)$_4$/anilinium *p*-toluenesulfinate clearly showed the disappearance of starting material (TIC chromatogram D and EIC chromatogram E). Moreover, from TIC chromatogram D it could be concluded that only a negligible amount of N-(mono)-allylated side product was formed. These results illustrate the usefulness of LC-(TIC/EIC)-MS for the more accurate monitoring of reactions on solid support and made a significant contribution to the optimization of the Alloc deprotection.

Conclusions

In conclusion, we have demonstrated that the use of the TIC/EIC technique in LC-MS allows for a clear assessment of diastereomeric purity of UV-inactive aza-analogues of deoxycholic acid. To our knowledge, this is the first report on the use of this method in a steroid context. LC-(TIC/EIC)-MS provided valuable data supplementary to other analytical methods such as ^{1}H-NMR and ESI-MS. Both the development of a diastereomerically pure dipodal steroid scaffold for combinatorial chemistry and the adequate monitoring of further scaffold derivatization on solid support were greatly facilitated by this technique. This method could be generally interesting for detailed HPLC analysis of reaction mixtures when working with compounds of low UV-activity.

Experimental Section

General information. All reagents were obtained either from Aldrich or ACROS Organics and were used without prior purification. Reactions were performed under argon atmosphere using HPLC grade solvents (obtained from Sigma-Aldrich or Fisher Scientific). Optical rotations were recorded on a Perkin-Elmer 241 polarimeter at room temperature. Analytical TLC was carried out on glass plates precoated with silica gel (Macherey Nagel, 60F254, 0.25 mm). Compounds were visualized with phosphomolybdic acid (PMA), KMnO$_4$, ninhydrin (2,2-dihydroxy-1,3-indanedione) or chloranil (tetrachloro-1,4-benzoquinone, 2 % solution in DMF). Due to large differences in polarity between most starting materials and reaction products, two different eluent systems were used to follow most reactions on TLC. Flash chromatography was performed on Kieselgel Merck Typ 9385 230-400 mesh, 60 Angström.
NMR spectra were recorded at room temperature on a Bruker Avance-500 spectrometer at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C spectra. CDCl$_3$ (99.8 atom % D) was obtained from Aldrich. Chemical shifts (δ units) are expressed in parts per million (ppm) relative to tetramethylsilane (TMS) and the internal solvent peak was used for calibration. When peak multiplicities are reported, the following abbreviations are used: s, singlet; d, doublet; t, triplet;

m, multiplet; br, broad. Coupling constants (*J* values) are expressed in Hertz (Hz). The Attached Proton Test (APT) technique was used to assign ^{13}C peaks (C, CH, CH$_2$, CH$_3$). Assignment was aided by comparison with literature values for similar compounds.[2d,4,11] Infrared spectra were recorded on a Perkin-Elmer 1600 Series FTIR spectrometer. Bands were quoted in cm^{-1} and the following abbreviations are used: w, weak; m, medium; s, strong; br, broad. Only the diagnostic signals are listed.

ESI-MS spectra were acquired on a quadrupole ion trap LC mass spectrometer (Thermo Finnigan MAT LCQ mass spectrometer) equipped with electrospray ionization. HR-MS values were measured on a Thermo Finnigan MAT95XP-Trap mass spectrometer. Reversed-phase LC-MS was performed on an Agilent 1100 series HPLC, using a Phenomenex Luna C18 (2) (250 mm x 4.6 mm) column hyphenated to an Agilent ESI-single quadrupole MS detector type VL. The following gradient elution was applied:

Time (min)	0	2	17	22	23	26
A (%): H$_2$O + 0.1 % HCOOH	100	100	0	0	100	100
B (%): CH$_3$CN	0	0	100	100	0	0

Preparation of 3α,β-diastereomeric mixture of *n*-propyl 3,12α-diamino-5β-cholan-24-oate (3a + 3b). Dioximo carboxylic acid **2** (17.04 g, 40.71 mmol) was suspended in *n*-PrOH (3.5 L) and heated at reflux under argon. Sodium (56.44 g, 2.46 mol, 60.3 eq) was carefully added portionwise over a period of 3 h. After addition of the last piece of sodium the reaction was heated at reflux overnight to ensure complete reaction of the sodium before quenching.

TLC indicated the complete consumption of starting material (acetone:MeOH 7:3 + trace HOAc; chloranil). A 12 M HCl aqueous solution (210 mL) was added very slowly to the refluxing mixture, leading to the immediate precipitation of NaCl. 10 mL of a 12 M HCl aqueous solution was further added and the suspension was heated at reflux overnight (pH = 1-2).

TLC showed complete conversion (EtOAc:MeOH:NH$_4$OH 28.0-30.0 % aq 9:1:1; chloranil or KMnO$_4$), indicating 4 diastereomers. The mixture was cooled to room temperature and the solvent was co-evaporated with toluene. The off-white residue was transferred to a separation funnel with CHCl$_3$ and a saturated NaHCO$_3$ aqueous solution was added (~1 L aqueous/~1 L CHCl$_3$). The phases were separated and the H$_2$O was further extracted with two portions of CHCl$_3$ (700 and 300 mL). The organic phase was partially concentrated and washed with a saturated NaHCO$_3$ aqueous solution. The organic solvent was further evaporated to obtain an orange oil.

The orange oil was carefully purified by flash chromatography (column: diameter: 8 cm, length: 34 cm; crude oil dissolved in EtOAc, elution with EtOAc:MeOH:NH$_4$OH 28.0-30.0 % aq 28:3:1) to obtain the diamino ester as diastereomeric mixture **3a** + **3b** (diastereomeric ratio 85:15). The yellow oil solidifies upon freezing (15.16 g, 35.04 mmol, 86 %).

Rf = 0.27 (EtOAc:MeOH:NH$_4$OH 28.0-30.0 % aq 9:1:1; chloranil); ^{1}H NMR (500 MHz, CDCl$_3$, only indicative peaks are listed) δ 4.01 (t, 6.5 Hz, 2 H, ester O-CH$_2$), 3.17 (br. s, 1 H, 12 βH), 2.73 (br. s, 1 H, 3 βH), 2.31-2.40 (m, 2 H), 2.18-2.26 (m, 2 H), 2.02 (s, 2 H), 0.99-1.94 (series of m), 0.88-0.99 (m, 12 H), 0.71 (s, 3 H); IR (neat): v_{max} = 3382 (m), 3321 (m), 3188 (m, br), 1732 (s); ESI-MS: m/z (% rel. int.) = 433.0 (100) [M + H]$^+$; HR-MS (ESI) m/z calcd. for C$_{27}$H$_{48}$N$_2$O$_2$ + H 433.3789, found 433.3783.

Preparation of *n*-propyl 3α-(allyloxycarbonylamino),12α-amino-5β-cholan-24-oate (4a).
The diastereomeric mixture **3a** + **3b** (5.07 g, 11.71 mmol) was dissolved in CHCl$_3$ (500 mL) and dry Et$_3$N (3.4 mL, 24.39 mmol, 2.1 eq) was added. The yellow solution was stirred at room temperature, Alloc$_2$O (1.75 mL, 10.54 mmol, 0.9 eq) was added in one portion and the mixture was reacted overnight. After TLC showed completeness of reaction (EtOAc:MeOH:NH$_4$OH 28.0-30.0 % aq 9:1:1 and CH$_2$Cl$_2$:MeOH 9:1; chloranil and ninhydrin), the mixture was transferred to a separation funnel and a saturated NaHCO$_3$ aqueous solution (300 mL) was added. The phases were separated and the aqueous phase was extracted with CHCl$_3$ (300 mL). The combined organic phases were partially concentrated under reduced pressure and washed with a new portion of a saturated NaHCO$_3$ aqueous solution. The organic phase was concentrated under reduced pressure to obtain a viscous yellow oil. The oil was carefully purified by flash chromatography (column: diameter: 4.5 cm, length: 32 cm; crude oil dissolved in CH$_2$Cl$_2$, elution with CH$_2$Cl$_2$:MeOH 98:2) to obtain diastereomerically pure **4a** as a yellow oil (2.80 g, 5.42 mmol, 46 %).

$[\alpha]_D$ = + 96.7 (c = 0.770, EtOH); Rf = 0.66 (CH$_2$Cl$_2$:MeOH 9:1; ninhydrin); ^{1}H NMR (500 MHz, CDCl$_3$) δ 5.86-5.97 (m, 1 H, Alloc), 5.29 (dd, 1.6 Hz; 17.3 Hz, 1 H, Alloc), 5.20 (dd, 1.3 Hz; 10.4 Hz, 1 H, Alloc), 4.59 (d, 6.7 Hz, 1 H, NH), 4.54 (d, 4.4 Hz, 2 H, Alloc), 4.02 (t, 6.7 Hz, 2 H, ester O-CH$_2$), 3.49 (br. s, 1 H, 3 βH), 3.17 (br. s, 1 H, 12 βH), 2.32-2.40 (m, 1 H), 2.18-2.27 (m, 1 H), 0.99-1.91 (series of m, 26 H), 0.90-0.99 (m, 9 H), 0.71 (s, 3 H); ^{13}C NMR (125 MHz, CDCl$_3$) δ 174.2 (COOR), 155.4 (Alloc C=O), 133.0 (Alloc *C*H=CH$_2$) 117.3 (Alloc CH=*C*H$_2$), 65.8 (ester O-CH$_2$), 65.1 (Alloc O-CH$_2$), 54.0 (CH), 50.9 (CH), 47.9 (CH), 47.8 (CH), 46.1 (C), 42.3 (CH), 36.2 (CH), 35.6 (CH$_2$), 35.1 (CH), 34.0 (C), 33.8 (CH$_2$), 33.5 (CH), 31.3 (CH$_2$), 30.9 (CH$_2$), 28.7 (CH$_2$), 28.1 (CH$_2$), 27.6 (CH$_2$), 26.9 (CH$_2$), 26.0 (CH$_2$), 23.7 (CH$_2$), 23.2 (19-CH$_3$), 21.9 (ester CH$_2$), 17.1 (21- CH$_3$), 13.7 (18-CH$_3$), 10.3 (ester CH$_3$); IR (neat): v_{max} = 3336 (w), 1724 (s); ESI-MS: m/z (% rel. int.) = 517.5 (100) [M + H]$^+$; HR-MS (ESI): m/z calcd. for C$_{31}$H$_{52}$N$_2$O$_4$ + H 517.4000, found 517.4020.

Verification experiment (conversion of 4a to 3a). 4a (95.8 mg, 0.185 mmol) was dissolved in dry THF (1.9 mL) and the reaction mixture was stirred at room temperature under argon. Pd(PPh$_3$)$_4$ (4.5 mg, 0.0039 mmol, 0.02 eq) was added and the bright yellow solution was stirred for 5 minutes, followed by the addition of NaBH$_4$ (14.4 mg, 0.38 mmol, 2.1 eq). The mixture was stirred overnight at room temperature.[12]

After TLC check (CH$_2$Cl$_2$:MeOH 9:1 and EtOAc:MeOH:NH$_4$OH 28.0-30.0 % aq 9:1:1; ninhydrin and PMA) the reaction was quenched with a 1 M HCl aqueous solution (15 mL) and the mixture was transferred to a separation funnel. The mixture was made basic with a saturated

NaHCO$_3$ aqueous solution (40 mL) and the product was extracted in CH$_2$Cl$_2$. The organic phase was dried on K$_2$CO$_3$, filtered and concentrated under reduced pressure.

The brownish oil was purified by flash chromatography (EtOAc:MeOH:NH$_4$OH 28.0-30.0 % aq 28:3:1). Although the product looked pure on TLC (EtOAc:MeOH:NH$_4$OH 28.0-30.0 % aq 9:1:1; chloranil or PMA), no clear NMR spectra could be obtained.

Therefore a solid phase extraction was performed: ~20 mg of the product was dissolved in MeOH (2 mL) and transferred into a solid phase reactor. Amberlyst 15 (wet) acidic ion-exchange resin (105.6 mg) was added and the mixture was shaken for 30 minutes. The resin was filtered and washed with CH$_2$Cl$_2$, EtOAc and MeOH. A 7 M NH$_3$ solution in MeOH (2 mL) was added and the reaction mixture was shaken manually, filtered and washed with MeOH. The filtrate was evaporated and dried under high vacuum to obtain 8 mg of diastereomerically pure **3a** as a colourless oil, which proved pure enough by NMR.

Alloc deprotection on solid support (conversion of 5 to 6)

Deprotection with Pd(PPh$_3$)$_4$ /Bu$_3$SnH /morpholine. Resin-bound construct 5 (30.1 mg, 0.11 mmol/g, 0.0033 mmol) was weighed into a 5 ml plastic syringe and swollen in dry CH$_2$Cl$_2$ (500 μl) for 10 minutes under argon. Morpholine (109 μl, 1.24 mmol, 376 eq) and Bu$_3$SnH (37 μl, 0.14 mmol, 42 eq) were added, followed by Pd(PPh$_3$)$_4$ (1.6 mg, 0.0014 mmol, 0.4 eq) and the reactor was flushed with argon. The mixture was agitated for 22 h at room temperature, shielded from light.

The resin was filtered and washed with N,N-dimethylformamide, MeOH and CH$_2$Cl$_2$. An analytical sample (~1 mg) was suspended in CH$_3$CN and irradiated with UV light (365 nm) for 3 h.

When analysis showed incomplete deprotection, the reaction was repeated. Since we still observed incomplete deprotection, we decided to test a different protocol:

Deprotection with Pd(PPh$_3$)$_4$ /anilinium p-toluenesulfinate.[9b,10] The resin was swollen in N-methylpyrrolidinone (204 μl) under argon for 15 minutes. The resin was filtered and anilinium *p*-toluenesulfinate (32.5 mg, 0.14 mmol, 42 eq) was added. The mixture was made homogenic by addition of N-methylpyrrolidinone (170 μl) and Pd(PPh$_3$)$_4$ (1.1 mg, 0.0001 mmol, 0.03 eq) was added. An additional 30 ml N-methylpyrrolidinone was added. The mixture was flushed with argon and shaken for 2 hours at room temperature, shielded from light.

The resin was filtered and washed with dry N,N-dimethylformamide, MeOH and CH$_2$Cl$_2$. Similar to the previous reaction a small sample was cleaved photolytically from the resin and analyzed as described.

Acknowledgements

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