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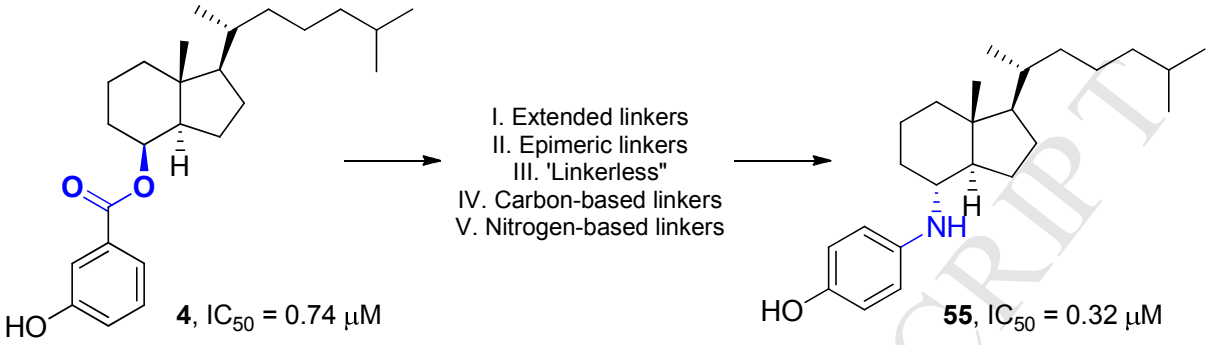
Graphical Abstract:



4, IC_{50} = 0.74 μM

55, IC_{50} = 0.32 μM

Vitamin D3 Analogues that Contain Modified A- and Seco-B-Rings as Hedgehog Pathway Inhibitors.

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ABSTRACT

The hedgehog (Hh) signaling pathway is a developmental signaling pathway that has been implicated as a target for anti-cancer drug development in a variety of human malignancies. Several natural and synthetic vitamin D-based *seco*-steroids have been identified as potent inhibitors of Hh signaling with chemotherapeutic potential. These include the previously characterized analogue **4**, which contains the northern CD-ring/side chain region of vitamin D3 (VD3) linked to an aromatic A-ring mimic through an ester bond. To further explore structure-activity relationships for this class of VD3-based Hh pathway inhibitors, we have designed, synthesized and evaluated several series of compounds that modify the length, composition, and stereochemical orientation of the ester linker. These studies have identified compounds **54** and **55**, which contain an amine linker and an aromatic A-ring incorporating a *para*-phenol, as new lead compounds with enhanced potency against the Hh pathway (IC_{50} values = 0.40 and 0.32 μM, respectively).

1. Introduction

Small molecule mediated inhibition of an inappropriately activated hedgehog (Hh) signaling pathway is being intensely pursued across academia and industry as a selective anti-cancer strategy for a number of human cancers [1]. Mutations in the Hh pathway have been linked to the development of basal cell carcinoma (BCC) and medulloblastoma (MB) and improper Hh signaling may contribute to the growth and progression of other human cancers [2-4]. Hh inhibition as a therapeutic strategy has been clinically validated with the FDA approval of Vismodegib (GDC-0449, **1**, Figure 1) for the treatment of locally advanced BCC [5]. Treatment of an MB patient with **1** demonstrated initial positive results in clinical trials; however, relapse occurred [6]. A point mutation in the binding site of Vismodegib on key pathway component and signal transducer Smoothened (Smo; D473H) was identified as the cause of relapse, rendering the patient insensitive to further treatment with **1 [7]**. In addition, a recent study reported that 21% of patients receiving continuous treatment of **1** for BCC developed regrowth of at least one BCC, suggesting resistance in BCC patients could also be a potential concern [8]. Finally, due to adverse side effects, over half (54%) of basal cell nevus syndrome (BCNS) patients receiving **1** to treat BCCs withdrew from the study voluntarily [9]. These continued drawbacks of Vismodegib underscore the need for further studies to identify novel potent and selective small molecule antagonists of the Hh signaling pathway.

The *seco*-steroid vitamin D3 (VD3, cholecalciferol, **2**, Figure 1), has been identified as a non-selective inhibitor of Hh signaling [10-12]. Although it does not bind to the vitamin D receptor, VD3 induces activation of canonical VDR signaling in Hh-dependent cell culture and murine models of BCC [11-12]. This observation suggests that VD3 is readily converted in vitro and in vivo to chemical structures that can bind and activate vitamin D signaling. Prolonged VDR

activation can result in detrimental side effects; therefore, the development of VD3-based analogues as Hh pathway inhibitors must decouple Hh antagonism and VDR activation. Our preliminary structure-activity relationship (SAR) studies for VD3 identified Grundmann's alcohol, **3**, as the pharmacophore for VD3-mediated inhibition of Hh signaling and also served as a starting point for developing selective Hh pathway inhibitors based on the VD3 scaffold [13]. These studies revealed that **3** was comparable to VD3 in its ability to inhibit Hh signaling without activating canonical VDR signaling. Analogue **3** was subsequently utilized as a handle to develop a series of VD3 analogues in which the natural A-ring was replaced with an aromatic mimic tethered to the CD-ring/side chain region of VD3 through an ester linker [14-15]. The aromatic A-ring was designed to mimic the natural cyclohexanol A-ring of VD3 and analogue **4** was identified as a potent and selective inhibitor of pathway signaling in multiple Hh-dependent cell lines (IC_{50} values = 0.74 – 5.2 μM).

Herein, we report the results of our current progress in the design, synthesis, and evaluation of next generation VD3-based Hh pathway inhibitors utilizing analogue **4** as our lead compound. These analogues contain modifications to the ester linkage as a means to (1) probe spatial parameters in the *seco*-B and A-ring regions, (2) evaluate chemical linkages anticipated to provide additional or enhanced binding interactions, and (3) replace the ester with tethers that are predicted to demonstrate improved metabolic stability.

2. Results and Discussion

2.1. Chemistry.

Using analogue **4** as our lead scaffold, we sought to develop several series of analogues that maintain the natural CD-ring/side chain region of VD3 and incorporate an aromatic A-ring

mimic, while altering the ester bond (linker region or *seco*-B-ring). These compounds were designed to explore optimal linker length between the CD- and A-ring regions, probe the stereochemical aspects of the linker region, and determine to what extent the ester linkage is responsible for potent Hh inhibition for these analogues. Finally, while our previous studies suggested the ester of **4** remains intact in vitro and is required for optimal activity of the scaffold, this moiety is generally considered to be a metabolic liability in vivo and we sought to explore chemical linkers with enhanced stability.

Analogue design and synthesis first focused on generating a series of compounds that address how augmenting the linker length and rigidity of analogue **4** affect its ability to inhibit Hh signaling. Synthesis of this series began with conversion of the commercially available carboxylic acids to the corresponding methyl esters using standard conditions followed by protection of the phenol as either the benzyl or methoxymethyl ether (Scheme 1) [14]. Base-catalyzed hydrolysis of the methyl esters afforded the carboxylic acids (**6**, **10**-**12**, **16**-**18**) for direct coupling to **3** using standard esterification conditions (Scheme 2) [14]. Following formation of esters **19** – **22**, removal of the benzyl-protecting group was accomplished by palladium-catalyzed reduction. This strategy was selected as a straightforward route to access analogues **24** – **26** from commercially available coumaric acids **7** – **9**, respectively, through the simultaneous debenzylation and hydrogenation of the alkene in the α,β-unsaturated esters. This provided ester-linked analogues with a more flexible linker region relative to analogues **30** – **32**. Removal of the methoxymethyl moiety from ester-linked analogues **27** – **29** was accomplished using camphorsulfonic acid (±CSA) to afford the intact α,β-unsaturated esters **30** – **32**.

Lead analogue **4** was obtained from optically pure **3**, which is the sole CD-ring/side chain isomer obtained from the one-pot ozonolysis/reduction of VD3 [16]. To determine whether this stereochemical orientation is required for activity of our scaffold, we sought to prepare the epimer of analogue **4**. Our initial synthetic plan followed a previously disclosed procedure to access key intermediate **37** (Scheme 3A) [17]. In addition to providing **37**, this strategy would also yield several truncated CD-ring/side chain region analogues whose Hh modulation potential would be evaluated as part of our ongoing structure-activity analysis for VD3. Formation of the thermodynamic silyl enol ether **34** from Grundmann's ketone (**33**) was followed by phenylselenium addition and peracid (*m*-CPBA) treatment of the phenylselenyl-hydrindane to afford enone **35**. Luche reduction selectively afforded **36** as the alcohol with the desired C-8 inversion. Following the reported procedure of allylic alcohol **36**, hydrogenation was carried out in the presence of tris(triphenylphosphine)rhodium chloride (Wilkinson's catalyst). While **37** was generated through this route, the overall yield from **33** was low due to the unanticipated formation of several byproducts in steps 2 through 5 and the incomplete reduction of **36** with up to stoichiometric amounts of Wilkinson's catalyst.

A recent report detailed conditions for the preparation of a secondary alcohol structurally similar to **37** directly from a ketone related to **33** [18]. This conversion was accomplished through the sodium metal reduction of the corresponding ketone in the presence of an acidic resin in good yield. We anticipated that **33** would react similarly in the acid-promoted radical reduction of the carbonyl to afford alcohol **37**. Upon employment of these conditions (Scheme 3B), **33** was readily consumed, generating multiple products (observed by TLC analysis), one of which matched the polarity (R_f = 0.4, 6:1 Hex:EtOAc) and ^{1}H NMR of **37** prepared in the initial route. Intermediate **38** was isolated as the only other pure compound from this mixture (R_f =

0.45, 6:1 Hex:EtOAC; **37** and **38** were formed in ~3:1 ratio, respectively). The previous report detailed the use of Amberlite IR118 as the acidic resin and 20 equivalents of sodium metal to afford the desired alcohol as the sole product isolated in 81% yield [18]. However, since this resin was no longer commercially available from the supplier, we replaced the IR118 for the comparable acidic resin, Amberlite IR120. While clearly a suitable resin to generate **37** and a superior route to that utilized in Scheme 3A, it is possible that this change could account for the multiple products observed in the reaction. Further analysis of conditions may be required to achieve the same degree of selectivity for this substrate with this resin. We anticipated that the byproducts resulting from the reaction with **33** also involved epimerization at the α-carbon(s) as previously described and isolation of **38** demonstrated. Nonetheless, the one-step reaction from **33** employing Amberlite IR120 and sodium afforded **37** in 45% yield, an improvement over the initial route. Analogue **40** (*epi*-**4**) was then readily prepared via esterification with 3-(benzyloxy)-benzoic acid (**39**) followed by palladium-catalyzed removal of the benzyl protection group (Scheme 4, **40**) [14]. Chemical structure configurations were verified by comparisons to literature reports and assignment of chemical shifts from ^{1}H NMR analysis, in particular the characteristic methyl signal (singlet) at the C- and D-rings fusion and 1,3- interactions (C-18 and C-8) of the hydrindane system [17-19].

With these modified esters in hand, our focus shifted towards the design and synthesis of analogues with altered chemical compositions in the linker region between the CD- and A-ring motifs. Direct connectivity between these regions and the incorporation of carbon- or nitrogen-based (amine, amide) chemical linkers would address analogue generation of more drug-like and metabolically more stable groups relative to the ester linkage. The latter subclass would also

address exploring the effects of how varying the hydrogen bonding capacity of this region affected Hh inhibition of the scaffold.

First, we prepared a 'linkerless' series—analogues incorporating the A-ring moiety of **4** as a direct appendage to the C-ring at C-8. The initial step in synthesizing this series was the addition of an in situ generated aryllithium species (from 3-methoxymethyl-bromophenol) to Grundmann's ketone **33** (Scheme 5). NMR analysis of **41** indicated a single compound was formed and isolated from the reaction. We anticipated that **41** had the *S*-configuration according to literature precedent for an analogous organolithium addition to **33** [20-21]. Removal of the methoxymethyl protecting group afforded analogue **42**. Intermediate **41** was also dehydrated with Burgess' reagent (methyl *N*-(triethylammoniosulfonyl)carbamate) to afford olefin **43** according to previous protocols [22]. A single isomer was anticipated based on a published study on a similar scaffold, but NMR analysis indicated that two diastereomers, inseparable by flash or thin-layer chromatography, were isolated in approximately a 3:2 ratio (major isomer shown). Intermediate **43** was deprotected to afford **44**, also isolated and evaluated for activity as a 3:2 mixture. Finally, saturation of the C-8-C-9 olefin was accomplished through palladium-catalyzed hydrogenation to afford **45** as a mixture of inseparable diastereomers (~4:1). Mixture **45** was deprotected to yield **46**, which was also isolated as a 4:1 mixture (major isomer shown) and inseparable by standard column chromatography. The mixtures of **44** and **46** were directly evaluated for their anti-Hh effects without further separation.

Our next analogues concentrated on incorporating one, two, or three atom tethers between the phenolic A-ring mimic and the CD-ring/side chain region (Schemes 6 through 8). A two-step homologation was performed on Grundmann's ketone (**33**) to yield aldehyde **48** (via enol ether **47**) with a high degree of diastereomeric purity (Scheme 6A) [23-24]. The stereochemistry at C-

8 was established based on the previous preparation and characterization of **48** and **48**-type compounds via a modified procedure [25]. A number of reports describe the synthesis of **48** [25-26]; however, we found that ylide generation using sodium *tert*-butoxide and (methoxymethyl)triphenylphosphonium chloride (phosphonium salt) and subsequent addition to **33** to generate **47** was high yielding and reproducible. A portion of **48** was reduced to provide **49**, a homologated version of **37**, which extends the hydroxyl functionality further from the C-ring [27]. Installation of the phenolic A-ring was carried out as described in Scheme 5 above. Here, the *in situ* generated aryllithium species was added to **48** forming a mixture of products, which were directly subjected to PDC-facilitated oxidation. Intermediate **50** was easily separated as the least polar component in the mixture and subsequent removal of the methoxymethyl protecting group afforded the one carbon extended linker analogue **51** as a single isomer.

As previously discussed, an aim of these linker-focused modification studies was to evaluate analogues that incorporated groups with the potential to access additional strong bonding interactions (e.g. hydrogen-bonding) with a possible target(s). Thus, a series of nitrogen-based linker analogues was designed and synthesized (Schemes 7 and 8). Specifically, amine- and amide-linkages were used to attach the aromatic A-ring motif to **3**. First, reductive amination of **33** using mono-substituted aminophenols to yield amine-linked analogues following a literature protocol was performed [28]. The stereoisomers isolated from the conditions employed were assigned by comparative ^{1}H NMR analysis. Interestingly, whereas a single isomer was isolated from reactions between 2- or 3-aminophenol and **33**, when 4-aminophenol was utilized, both diastereomers were isolated. We accounted for this observation based on the reaction scale for each respective analogue. The preparations of **52** and **53** were run on a reduced scale (one-third to one-half) relative to synthesis of **54** (which also yielded **55**). Analogues **54** and **55** combined

to yield 50% of the amine-linked products in a 4:1 ratio. Based on this ratio, it is possible that a small quantity of epimer was generated from the syntheses of **52** and **53**, but at such a minute quantity that it went undetected during purification.

As a direct comparison to lead compound **4**, analogue **59** was designed to replace the ester with an amide bond. Oxime **56** was formed in excellent yield from the condensation of hydroxylamine and **33** in pyridine (Scheme 7B) [19]. Reduction of the oxime to amine **57** was accomplished by a slightly modified literature procedure [29]. In one pot, **56** was sequentially refluxed with lithium aluminum hydride followed by zinc in acetic acid. This route afforded mainly amine **57**, major C-8 isomer shown in Scheme 7, matching the stereochemistry of **3** (~6:1). Standard conditions were employed to couple **57** with 3-(benzyloxy)-benzoic acid to afford **58**, which was subsequently deprotected to yield amide **59** [30]. ^{1}H NMR analysis of **59** also indicated an approximately 6:1 mixture of C-8 epimers (major isomer shown in Scheme 7B), which was directly evaluated without further purification.

The final series of analogues prepared for analysis utilized an enamide linker to extend the distance between the A-ring and CD-ring/side chain region while also maintaining the coplanar nature of the natural VD3 scaffold. Triethyl phosphonoacetate was coupled to **33** via a Horner-Wadsworth-Emmons olefination to yield α, β-unsaturated ester **60** [31]. A series of enamide-linked analogues were directly prepared through the trimethylaluminum mediated coupling of aniline substituents with ester **60** [32]. This series included an unsubstitued (**61**), *ortho*-, *meta*-, and *para*-monosubstituted phenolic enamides (**62**, **65**, and **66**). In addition, allylic alcohol **67** was generated from the lithium aluminum hydride reduction of ester **60**. Fully reduced linker-region alcohol **69** was prepared as a single isomer by Pd-catalyzed reduction of the alkene in **60** followed by lithium aluminum hydride reduction of ester **68**. Together with alcohol **49**,

analogues **67** and **69** were prepared for evaluation as one- and two-carbon analogue extensions of **3** and **37**, respectively.

2.2. Biological Evaluation

Each analogue was initially assessed for its ability to inhibit Hh signaling by evaluating its effects on endogenous Gli1 mRNA levels at a single concentration (5 μM) in C3H10T1/2 cells, an Hh-dependent mouse embryonic fibroblast (MEF) cell line. Gli1 is a well-characterized target gene of Hh signaling that is robustly up-regulated in this cell line following activation of Hh signaling with exogenous Hh ligand or pathway agonist(s) [33]. Concomitant administration of a pathway inhibitor prevents Gli1 mRNA over-expression. For these studies, we utilized a standard oxysterol cocktail (20α-hydroxycholesterol and 22(*S*)-hydroxycholesterol, 5 μM each) to activate Hh pathway signaling as previously described [11]. In addition, selectivity for Hh pathway inhibition compared to canonical VDR activation was determined in the same cellular system by monitoring up-regulation of Cyp24a1 mRNA, a well-characterized target gene of VDR [12,34].

SAR studies using this approach for earlier analogue generations [11,13-15] established that structures constructed from ester-linked Grundmann's alcohol (**3**) and hydrophilic aromatic A-ring substituents could more potently and selectively inhibit Hh signaling compared to VD3 (**2**). Initially, analogues with extended ester linkers relative to lead compound **4** were prepared and screened to assess the optimal distance between the CD-ring/side chain and aromatic A-ring regions (Table 1). Analogue **23**, which incorporates an additional methylene in the linker region, demonstrated potency and selectivity comparable to **4**, suggesting this region of the binding site is amenable to a three atom linker. Analogues **24**-**26** and **30**-**32** also contain the ester linker, but are extended an additional methylene (from **23**) to incorporate a four atom linker region between the CD- and A-rings. In addition, evaluating the saturated (**24**-**26**) and unsaturated (**30**-**32**)

analogues allows for a direct comparison of the flexibility/rigidity requirements in this region of the scaffold. Each of these four atom esters demonstrated reduced inhibition of Hh signaling compared to both **4** and **23**, suggesting that more than three atoms in this region is detrimental to the anti-Hh effects of the ester linked analogues. In addition, the saturated analogues (Gli1 expression = 15-47%), were more active than the corresponding unsaturated analogues (Gli1 expression = 57-94%). These results are most likely due to the increased flexibility of the saturated analogues allowing the hydroxyl moiety easier access to key binding interactions. It is interesting to note that in our previous SAR studies the 2-phenolic equivalent of **4** was inactive [15] whereas the corresponding four atom ester **24** regains moderate Hh inhibition. Finally, each of these extended ester analogues demonstrated significantly reduced VDR activation when compared to VD3.

The second subclass of linker region analogues focused on evaluation of epimeric structures of **3** and **4**, specifically at C-8 of the VD3 scaffold (Table 2). Ketone **33** and enone **35** were equipotent at inhibiting Hh signaling (Gli1 expression = 66% and 63%, respectively) and both were slightly less active than **3** (Gli1 expression = 46%). Epimeric alcohols **36** and **37** were significantly less active than **3**, while analogue **38**, which was recovered from the radical-promoted reduction of **33**, demonstrated Hh inhibition and selectivity comparable to **3**. Analogue **40**, which was prepared specifically to evaluate whether inversion of the ester attachment at C-8 affected activity was significantly less active than lead compound **4** (Gli expression = 30% and 1%, respectively). Finally, none of these isomeric analogues demonstrated significant activation of VDR.

Our next analogue series was designed to shorten the linker region by directly appending the 3-phenolic A-ring mimic to the CD-ring region or incorporating a one carbon-based linker

between the two regions (Table 3). The "linkerless" series of analogues (**42**, **44**, **46**) demonstrated moderate potency against the Hh signaling pathway (Gli1 expression = 39-47%) and did not activate VDR. These data indicate that the absence of a linker region acting as chemical spacer between the CD-ring and aromatic A-ring produces analogues with diminished anti-Hh properties. The related one carbon linker series demonstrated a wide-range of Hh inhibitory activity. Analogue **51**, which contains a ketone-based linker and inverted stereochemistry compared with lead compound **4**, demonstrated potent down-regulation of Gli1 mRNA (11% relative to fully activated OHC controls) in an Hh-selective manner. This data suggests that the 3-hydroxyl substituent in **51** is more suitably oriented to generate improved binding interactions when compared with the inverted ester of analogue **40** or the directly appended A-ring in analogue **46**.

With respect to intermediate structures that did not include an aromatic A-ring mimic, **60** was inactive, which was not unexpected based on the previous analysis of compounds lacking or masking hydrophilic group(s) in the A- and *seco*-B-ring regions. Analogue **49**, a one-carbon extension of **37**, was moderately active (Gli1 expression = 50%) which correlated well with the activity demonstrated by Grundmann's alcohol (**3**). An additional carbon between the CD-ring region and the primary hydroxyl (**69**) provided an analogue that completely abolished Hh signaling. Interestingly, constraining the primary hydroxyl such that it is coplanar with the CD-ring region (**67**) was detrimental to its activity, suggesting that the flexibility of the saturated linker is important for activity. Of these analogues, only **67** and **69**, the latter which demonstrated the highest level of Hh inhibition, activated VDR at a modest level (28 and 18.6-fold increase, respectively).

The preliminary screening results for analogues containing a nitrogen-based linker are shown in Table 4. These compounds represent the first series to incorporate a hydrogen-bonding moiety in the linker region. Each of the analogues that included an amine-based linker (**52**-**55**) demonstrated potent inhibition of Hh signaling. The 3-phenol analogue (**53**) was the least active of this series, highlighting the fact that modification of the linker length affects the orientation of the aromatic A-ring in the binding pocket. Intermediate oxime **55** was also evaluated, but demonstrated no anti-Hh activity. In addition, each of the amine-linked analogues up-regulated Cyp24A1 at a modest level, similar to data from previously characterized analogues that completely abrogate Gli1 expression. Analogue **59**, the amide equivalent of lead compound **4**, also maintained potent Hh inhibition. Interestingly, up-regulation of Cyp24A1 by **59** was 3-fold less than **4** and the amine-linked analogues.

The series of enamide linked analogues also demonstrated the ability to down-regulate Hh signaling (Table 4). Analogue **61**, which does not contain any functionality on the aromatic A-ring, demonstrated modest Hh signaling inhibition. This was in direct contrast to our previous results from the original ester-linked analogue series in which a hydrophilic moiety on the A-ring was required for Hh inhibition [14-15]. This result suggests that extending the atoms in the linker region can make key interactions within the analogue binding site. Each of the other enamide analogues (**62** and **65**-**66**) were potent inhibitors of Hh signaling with modest activation of VDR.

Upon completion of the preliminary compound analysis in C3H10T1/2 cells at single concentrations (5 μM), analogues that demonstrated potent anti-Hh signaling activity were selected for further evaluation. In general, analogues that reduce Gli1 mRNA expression to levels less than 20% of the OHC-activated control were defined as potent inhibitors of Hh signaling and compounds fitting this criterion were evaluated for their ability to inhibit Hh

signaling in a concentration-dependent fashion. These follow-up studies were performed in both C3H10T1/2 cells and ASZ (murine BCC) cells (Table 5). Each of the compounds chosen for further evaluation demonstrated the ability to down-regulate Gli1 mRNA expression in both cell lines in a concentration-dependent manner with a range of analogue activity (IC_{50} values = 0.32 - 3.9 μM in C3H10T1/2 and 0.57 – 6.7 μM in ASZ). While the majority of analogues inhibited Hh signaling at a level comparable to lead compound **4**, analogues **54** and **55** were significantly more active. With IC_{50} values in the low to mid-nanomolar range (C3H10T1/2 = 0.40 and 0.32 μM and ASZ = 0.67 and 0.57 μM, respectively), these compounds represent the most potent inhibitors of Hh signaling based on the VD3 scaffold identified to date.

Of additional interest, evaluation of this series revealed analogues whose ability to modulate Hh signaling correlated well between C3H10T1/2 and ASZ cell lines. Previously, no such activity correlation was observed for our VD3-based analogues, but was only determined for the parent VD3 or Hh signaling inhibitors known to function through direct binding to Smo (**1** and cyclopamine) [1]. Lead compound **4** and additional VD3 analogues have demonstrated significantly reduced activity in the ASZ cell line. None of these VD3 analogues demonstrated the capacity to displace a tight-binding fluorescent VDR ligand from full length human VDR, matching previously screened VD3-based synthetic analogues (up to 100 μM) [11,13-15]. These data taken together with the comparatively modest effects on Cyp24a1 mRNA up-regulation seen in C3H10T1/2 and ASZ cells (relative to VD3 and calcitriol) suggests the VD3 analogues with an aromatic A-ring exhibit Hh signaling inhibition through mechanism(s) distinct from VDR.

3. Conclusion

Our continuing efforts to discover potent and selective VD3-based Hh signaling inhibitors directed the design, synthesis, and biological evaluation studies of a series of linker-modified analogues. In particular, with **4** as a lead scaffold, analogues were rationally designed to maintain the CD-ring/side chain region of VD3 and the aromatic A-ring of **4**, while altering the tether between these two regions. Several parameters for this region were explored in these studies, including exploration of linker length and rigidity, orientation of the hydroxyl or phenolic moiety, and incorporation of additional hydrogen-bonding groups within the linker region. The spatial range available from C-8 of **3** allowed us to manipulate presentation of the active substituents in the A and *seco*-B-ring regions, effectively changing the pharmacophore spatial positioning by varying the chemical attachments between the two distinct segments (CD-rings and A-ring mimic). This strategy allowed us to more thoroughly probe positional and chemical modifications of the pharmacophore in this region of lead analogue **4**. Analysis for this linker-modified analogue series indicates that this strategy was successful, as demonstrated by the identification of selective VD3-based inhibitors of Hh signaling with increased potency. Future analogue development will explore the chemical space around the C-ring and further linker modifications are planned, along with mechanistic and *in vivo* studies addressing how VD3 and synthetic analogues exhibit Hh modulation.

4. Experimental Section

4.1. General Information.

VD3 used for chemical synthesis was purchased from HBCChem, Inc. Other chemicals were purchased from Sigma-Aldrich or Fisher Scientific. ACS or HPLC grade methanol, acetone, and tetrahydrofuran were purchased from Fisher Scientific. Anhydrous DCM (low water, <50 ppm water) was purchased from BrandNu Laboratories, Inc. (J.T. Baker solvent). Column chromotagraphy was performed using silica gel purchased from Sorbtech (Sorbent Technologies). NMR data was performed on a Bruker AVANCE 500 or 400 MHz spectrometer and analysis with MestReNova version 8.0.0. HRMS data was analyzed at the Mass Spectrometry Facility at the University of Connecticut, performed by Dr. You-Jun Fu. FT-IR analysis was performed on a Bruker Alpha Platinum ATR instrument using OPUS software (v 7.2).

4.2. Synthesis of extended ester linked analogues.

4.2.1 General procedure for methylation of acetic/coumaric acids.

Thionyl chloride (18.3 mmol) was added dropwise to a solution of phenylacetic or coumaric acid (**5**, **7 – 9**; 6.1 mmol) in methanol (15 mL) at 0°C. The reaction mixture was stirred overnight and allowed to warm to room temperature. After 24 h, ice was slowly added with vigorous stirring, followed by a solution of sodium bicarbonate (80 mL; aqueous, saturated). The reaction mixture was diluted with EtOAc (100mL) and extracted (2 X 100mL). The combined organics were dried over Na_2SO_4, filtered, and concentrated. The crude methyl ester protected coumarates were obtained in good yields (65-85%) and purity was satisfactory (^{1}H NMR) to move directly to the next step. Characterization of intermediates was consistent with that previously reported [35-36].

4.2.2. General procedure for benzyl protection of phenols.

Protection of the phenols with benzyl bromide (yields 75-90%) followed by saponification of the methyl ester (yields 55-80%) were as detailed previously [14-15,37-39].

4.2.3. General procedure for methoxymethyl protection of phenols.

Sodium hydride (60% mineral oil dispersion; 5.1 mmoL) was added portion-wise with vigorous stirring to a solution of methyl ester coumarate (5.1 mmoL) in THF (30 mL) at 0°C. After completion of the NaH addition, the mixture was stirred an additional 2 h at 0°C. Chloromethyl methyl ether (6.63 mmoL; technical grade, 90%) was added dropwise to this mixture, which was then allowed to warm to room temperature over 16 h. After this time, water (15 mL) was added to the solution, followed by saturated ammonium chloride (60mL) and the aqueous layer was washed with ethyl acetate (3 X 100 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. Column chromatography (SiO_2) on the crude residue afforded MOM-protected methyl ester coumarates in good yields (60-90%). Spectral analysis matched previous reports [40]. Carboxylic acids were prepared by saponification of methyl esters as described previously [15,41-43].

*4.2.4. 3-methoxymethyl propenoic acid (**17**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 11.81 (bs, 1H), 8.15 (d, J = 16.1 Hz, 1H), 7.57 (m, 1H), 7.35 (m, 1H), 7.18 (m, 1H), 7.03 (d, J = 16.1 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 172.5, 156.1, 142.2, 131.9, 128.7, 123.7, 121.9, 117.7, 114.8, 94.5, 56.3. DART-HRMS: *m/z* calcd. for $C_{11}H_{13}O_4$: 209.0814 $[MH]^+$. Found: 209.0815.

4.2.5. General procedures for esterification and deprotection of ester-linked analogues. Esterification of **3** and protected acids was performed as described previously (yields 60-90%)

[14-15]. Palladium hydroxide (10%) reduction/deprotection under an atmosphere of hydrogen in MeOH:THF (2:1) was performed as detailed previously (yields 50-80%) [14-15]. Removal of the methoxymethyl protecting group with ±CSA (2 equivalents) in anhydrous MeOH (2 mL) for 24-36 h was done at room temperature as previously described [44]. The reaction was diluted in ethyl acetate (50 mL) and washed with saturated $NaHCO_3$ (1 X 50 mL). The organic layer was collected, dried over $MgSO_4$, filtered, and concentrated. Column chromatography (SiO_2, various gradients of Hex:EtOAc) afforded ester linked analogues **23**-**26** and **30**-**32** as clear oils (65-90%).

*4.2.5.1. 3-benzyloxy phenyl acetate-VD3 ester (**19**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44 (m, 2H), 7.39 (m, 2H), 7.33 (m, 1H), 7.23 (m, 1H), 6.93 (m, 1H), 6.88 (m, 2H), 5.16 (m, 1H), 5.07 (s, 2H), 3.59 (s, 2H), 1.97 (m, 1H), 1.80 (m, 2H), 1.60 (m, 1H), 1.52 (m, 2H), 1.41 (m, 5H), 1.32 (m, 3H), 1.27 (m, 2H), 1.21 (m, 2H), 1.14 (m, 4H), 1.05 (m, 1H), 0.99 (m, 1H), 0.88 (m, 10H), 0.73 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.2, 158.8, 136.9, 135.8, 129.4, 128.5, 127.8, 127.3, 121.9, 115.8, 113.4, 77.2, 72.0, 69.8, 56.4, 51.3, 42.1, 41.8, 39.9, 39.4, 35.9, 35.3, 30.4, 29.6, 27.9, 27.0, 23.7, 22.7, 22.5, 22.5, 18.5, 17.8, 12.8. DART-HRMS: *m/z* calcd. for $C_{33}H_{46}O_3$: 490.3447 $[M]^+$. Found: 490.3461.

*4.2.5.2. 2-benzyloxy coumarate-VD3 ester (**20**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.06 (d, *J* = 16.1 Hz, 1H), 7.56 (m, 1H), 7.45 (m, 2H), 7.39 (m, 2H), 7.33 (m, 2H), 6.98 (m, 2H), 6.48 (d, *J* = 16.2 Hz, 1H), 5.26 (m, 1H), 5.12 (s, 2H), 1.99 (m, 1H), 1.89 (m, 1H), 1.79 (m, 1H), 1.71 (m, 1H), 1.53 (m, 2H), 1.45 (m, 4H), 1.33 (m, 4H), 1.25 (m, 1H), 1.15 (m, 5H), 1.06 (m, 1H), 0.98 (m, 1H), 0.90 (d, *J* = 7.1 Hz, 3H), 0.88 (d, *J* = 6.5 Hz, 3H), 0.81 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.1, 157.2, 139.1, 136.4, 131.2, 128.6, 128.4, 128.0, 127.6, 123.8, 121.0, 119.4, 112.4, 71.3, 70.4, 56.5, 51.5, 41.9, 40.0, 39.5, 35.9,

35.3, 30.6, 28.0, 27.1, 23.7, 22.8, 22.6, 22.5, 18.5, 18.0, 13.1. DART-HRMS: *m/z* calcd. for $C_{34}H_{47}O_3$: 503.3525 $[MH]^+$. Found: 503.3510.

*4.2.5.3. 3-benzyloxy coumarate-VD3 ester (**21**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (d, *J* = 16.0 Hz, 1H), 7.45 (m, 2H), 7.40 (m, 2H), 7.35 (m, 1H), 7.31 (m, 1H), 7.15 (m, 2H), 7.01 (dd, *J* = 8.2, 2.4 Hz, 1H), 6.42 (d, *J* = 15.9 Hz, 1H), 5.31 (m, 1H), 5.10 (s, 2H), 2.07 (m, 1H), 1.94 (m, 2H), 1.82 (m, 2H), 1.52 (m, 5H), 1.39 (m, 4H), 1.26 (m, 2H), 1.16 (m, 4H), 1.03 (m, 1H), 1.00 (s, 3H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.90 (d, *J* = 2.1 Hz, 3H), 0.89 (d, *J* = 2.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.6, 159.0, 143.9, 136.6, 135.9, 129.8, 128.5, 128.0, 127.4, 120.9, 119.2, 116.6, 114.0, 71.6, 70.0, 56.4, 51.5, 41.9, 39.9, 39.4, 35.9, 35.3, 30.5, 27.9, 27.0, 23.7, 22.7, 22.6, 22.5, 18.5, 17.9, 13.2. DART-HRMS: *m/z* calcd. for $C_{34}H_{46}O_3$: 502.3447 $[M]^+$. Found: 502.3468.

*4.2.5.4. 4-benzyloxy coumarate-VD3 ester (**22**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (d, *J* = 16.0 Hz, 1H), 7.48 (d, *J* = 8.7 Hz, 1H), 7.41 (m, 4H), 7.34 (1H), 6.98 (d, *J* = 8.6 Hz, 1H), 6.31 (d, *J* = 15.9 Hz, 1H), 5.29 (m, 1H), 5.10 (s, 2H), 2.05 (m, 1H), 1.92 (m, 1H), 1.81 (m, 2H), 1.50 (m, 5H), 1.38 (m, 4H), 1.23 (m, 2H), 1.13 (m, 4H), 1.02 (m, 1H), 0.98 (s, 3H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.89 (d, *J* = 2.1 Hz, 3H), 0.88 (d, *J* = 2.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.0, 160.3, 143.7, 136.4, 129.6, 128.6, 128.0, 127.5, 127.4, 116.5, 115.1, 71.3, 70.0, 56.4, 51.5, 41.9, 40.0, 39.4, 35.9, 35.3, 30.6, 27.9, 27.1, 23.7, 22.7, 22.6, 22.5, 18.5, 18.0, 13.2. DART-HRMS: *m/z* calcd. for $C_{34}H_{47}O_3$: 503.3525 $[MH]^+$. Found: 503.3510.

*4.2.5.5. 3-hydroxy phenylacetate-VD3 ester (**23**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.16 (m, 1H), 6.81 (m, 1H), 6.76 (m, 1H), 6.72 (m, 1H), 5.47 (s, 1H), 5.14 (m, 1H), 3.56 (s, 2H), 1.96 (m, 1H), 1.78 (m, 2H), 1.59 (m, 1H), 1.51 (m, 1H), 1.40 (m, 4H), 1.31 (m, 3H), 1.25 (m, 2H), 1.19 (m, 1H), 1.12 (m, 5H), 1.04 (m, 1H), 0.97 (m, 1H), 0.86 (m, 10H), 0.73 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 171.7, 155.8, 135.8, 129.6, 121.5, 116.2, 114.0, 77.2, 72.3, 56.4, 51.3, 41.9, 41.8, 39.9, 39.4, 35.9, 35.3, 30.4, 28.0, 27.0, 23.7, 22.8, 22.5, 22.5, 18.5, 17.8, 12.8. IR(neat) *v*max 3374, 2947, 2930, 2867, 1703, 1602, 1590, 1488, 1366, 1231, 1152, 762, 690. DART-HRMS: *m/z* calcd. for $C_{26}H_{40}O_3$: 400.2977 $[M]^+$. Found: 400.2976.

*4.2.5.6. 2-hydroxy hydrocoumarate-VD3 ester (**24**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.17 (s, 1H), 7.10 (m, 2H), 6.87 (m, 2H), 5.17 (m, 1H), 2.90 (t, *J* = 6.3 Hz, 2H), 2.71 (t, *J* = 6.4 Hz, 2H), 1.98 (m, 1H), 1.78 (m, 2H), 1.55 (m, 5H), 1.40 (m, 5H), 1.27 (m, 3H), 1.13 (m, 4H), 1.04 (m, 1H), 0.87 (m, 9H), 0.79 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 175.4, 154.3, 130.4, 127.9, 127.4, 120.7, 117.3, 72.8, 56.3, 51.2, 41.8, 39.8, 39.4, 35.9, 35.6, 35.3, 30.4, 29.7, 28.0, 27.0, 24.7, 23.7, 22.8, 22.6, 22.5, 18.5, 17.8, 12.9. IR(neat) *v*max 3428, 2962, 2919, 2871, 2849, 1714, 1691, 1588, 1477, 1446, 1290, 1215, 1162, 1102, 1072, 945, 881, 755, 681. DART-HRMS: (a) *m/z* calcd. for $C_{27}H_{43}O_3$: 415.3212 $[MH]^+$. Found: 415.3217; (b) *m/z* calcd. for $C_{27}H_{42}O_3NH_4$: 432.3478 $[MNH_4]^+$. Found: 432.3489.

*4.2.5.7. 3-hydroxy hydrocoumarate-VD3 ester (**25**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.14 (m, 1H), 6.75 (m, 1H), 6.69 (m, 2H), 5.91 (s, 1H), 5.17 (m, 1H), 2.91 (t, *J* = 7.8 Hz, 2H), 2.63 (t, *J* = 7.8 Hz, 2H), 1.99 (m, 1H), 1.79 (m, 2H), 1.62 (m, 1H), 1.51 (m, 1H), 1.41 (m, 4H), 1.39 (m, 3H), 1.10 (m, 8H), 0.90 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 2.2 Hz, 3H), 0.86 (d, *J* = 2.2 Hz, 3H), 0.84 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.2, 155.9, 142.1, 129.6, 120.3, 115.2, 113.2, 71.9, 56.4, 51.2, 41.8, 39.9, 39.4, 36.2, 35.9, 35.3, 30.9, 30.4,

27.9, 27.0, 23.7, 22.7, 22.5, 18.5, 17.8, 13.0. IR(neat) *v*max 3320, 2947, 2847, 1730, 1701, 1589, 1452, 1391, 1351, 1332, 1231, 1146, 1061, 1032, 997, 875, 777. DART-HRMS: (a) *m/z* calcd. for $C_{27}H_{41}O_3$: 413.3055 $[M-H]^+$. Found: 413.3014; (b) *m/z* calcd. for $C_{27}H_{42}O_3NH_4$: 432.3478 $[MNH_4]^+$. Found: 432.3443.

*4.2.5.8. 4-hydroxy hydrocoumarate-VD3 ester (**26**).*

^{1}H NMR (500 MHz, $CDCl_3$) 7.05 (d, *J* = 8.1 Hz, 2H), 6.75 (d, *J* = 8.0 Hz, 1H), 5.78 (s, 1H), 5.15 (m, 1H), 2.89 (t, *J* = 7.8 Hz, 2H), 2.60 (t, *J* = 7.7 Hz, 2H), 1.99 (m, 1H), 1.78 (m, 2H), 1.63 (m, 1H), 1.51 (m, 1H), 1.41 (m, 4H), 1.33 (m, 3H), 1.23 (m, 2H), 1.13 (m, 4H), 1.06 (m, 1H), 0.99 (m, 1H), 0.87 (m, 12H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.2, 154.2, 132.2, 129.2, 115.3, 71.8, 56.4, 51.2, 41.8, 39.9, 39.4, 36.6, 35.9, 35.3, 30.5, 30.2, 27.9, 27.0, 23.7, 22.7, 22.5, 18.5, 17.8, 13.0. IR(neat) *v*max 3509, 2958, 2928, 2857, 1730, 1644, 1523, 1471, 1443, 13738, 1246, 1130, 1110, 1079, 1033, 956, 883, 773, 666. DART-HRMS: (a) *m/z* calcd. for $C_{27}H_{41}O_3$: 413.3055 $[M-H]^+$. Found: 413.3024; (b) *m/z* calcd. for $C_{27}H_{42}O_3NH_4$: 432.3478 $[MNH_4]^+$. Found: 432.3443.

*4.2.5.9. 2-methoxymethyl coumarate-VD3 ester (**27**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (d, *J* = 16.0 Hz, 1H), 7.47 (d, *J* = 8.7 Hz, 2H), 7.04 (d, *J* = 8.7 Hz, 2H), 6.30 (d, *J* = 16.0 Hz, 1H), 5.28 (m, 1H), 5.20 (s, 2H), 3.48 (s, 3H), 2.05 (m, 1H), 1.92 (m, 1H), 1.80 (m, 2H), 1.50 (m, 5H), 1.36 (m, 4H), 1.24 (m, 4H), 1.12 (m, 4H), 1.03 (m, 1H), 0.97 (s, 3H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.87 (d, *J* = 2.0 Hz, 3H), 0.86 (d, *J* = 2.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.0, 158.8, 143.7, 129.5, 128.3, 116.9, 116.4, 94.2, 71.4, 56.4, 56.1, 51.5, 41.9, 40.0, 39.4, 35.9, 35.4, 30.6, 27.9, 27.1, 23.7, 22.7, 22.6, 22.5, 18.5, 18.0, 13.2. DART-HRMS: *m/z* calcd. for $C_{29}H_{45}O_4$: 457.3317 $[MH]^+$. Found: 457.3319.

*4.2.5.10. 3-methoxymethyl coumarate-VD3 ester (**28**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (d, *J* = 16.1 Hz, 1H), 7.56 (d, *J* = 7.8 Hz, 1H), 7.32 (m, 1H), 7.14 (d, *J* = 8.5 Hz, 1H), 7.01 (m, 1H), 6.47 (d, *J* = 16.1 Hz, 1H), 5.29 (m, 1H), 5.25 (s, 2H), 3.50 (s, 3H), 2.05 (m, 1H), 1.93 (m, 1H), 1.80 (m, 2H), 1.50 (m, 5H), 1.36 (m, 4H), 1.24 (m, 4H), 1.13 (m, 4H), 1.02 (m, 1H), 0.99 (s, 3H), 0.93 (d, *J* = 6.4 Hz, 3H), 0.87 (d, *J* = 6.5 Hz, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.1, 155.7, 139.2, 131.3, 128.0, 124.2, 121.8, 119.3, 114.7, 94.5, 71.4, 56.5, 56.2, 51.5, 41.9, 40.0, 39.4, 35.9, 35.4, 30.6, 27.9, 27.1, 23.7, 22.7, 22.6, 22.5, 18.6, 18.0, 13.1. DART-HRMS: *m/z* calcd. for $C_{29}H_{45}O_4$: 457.3317 $[MH]^+$. Found: 457.3325.

*4.2.5.11. 4-methoxymethyl coumarate-VD3 ester (**29**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.07 (d, *J* = 16.2 Hz, 1H), 7.55 (m, 1H), 7.32 (m, 1H), 7.14 (d, *J* = 8.3 Hz, 1H), 7.02 (m, 1H), 6.46 (d, *J* = 16.2 Hz, 1H), 5.29 (m, 1H), 5.25 (s, 2H), 3.50 (s, 3H), 2.06 (m, 1H), 1.93 (m, 1H), 1.81 (m, 2H), 1.51 (m, 5H), 1.38 (m, 4H), 1.24 (m, 2H), 1.12 (m, 4H), 1.01 (m, 1H), 0.98 (s, 3H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.87 (d, *J* = 1.9 Hz, 3H), 0.86 (d, *J* = 1.9 Hz, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.1, 155.7, 139.2, 131.3, 128.0, 124.2, 121.8, 119.3, 114.7, 94.5, 71.4, 56.5, 56.2, 51.5, 41.9, 40.0, 39.5, 35.9, 35.4, 30.6, 28.0, 27.1, 23.7, 22.8, 22.6, 22.5, 18.6, 18.0, 13.1. DART-HRMS: *m/z* calcd. for $C_{29}H_{45}O_4$: 457.3317 $[MH]^+$. Found: 457.3333.

*4.2.5.12. 2-hydroxycoumarate-VD3 ester (**30**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.62 (d, *J* = 16.0 Hz, 1H), 7.26 (m, 1H), 7.10 (d, *J* = 7.7 Hz, 1H), 7.05 (s, 1H), 6.89 (m, 1H), 6.40 (d, *J* = 16.0 Hz, 1H), 5.72 (bs, 1H), 5.30 (m, 1H), 2.05 (m, 1H), 1.93 (m, 1H), 1.80 (m, 2H), 1.51 (m, 5H), 1.37 (m, 4H), 1.23 (m, 3H), 1.13 (m, 4H), 1.01 (m, 1H), 0.96 (s, 3H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 1.4 Hz, 3H), 0.87 (d, *J* = 1.4 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.1, 156.2, 144.2, 135.9, 130.0, 120.6, 119.0, 117.4, 114.5, 72.0,

56.4, 51.5, 41.9, 39.9, 39.4, 35.9, 35.4, 30.5, 27.9, 27.0, 23.7, 22.8, 22.6, 22.5, 18.5, 17.9, 13.2. IR(neat) *v*max 3435, 2928, 2867, 2850, 1694, 1681, 1636, 1597, 1448, 1219, 1188, 1155, 1131, 1062, 1034, 977, 848, 782, 672. DART-HRMS: *m/z* calcd. for $C_{27}H_{41}O_3$: 413.3056 $[MH]^+$. Found: 413.3071.

*4.2.5.13. 3-hydroxycoumarate-VD3 ester (**31**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.61 (d, *J* = 15.8 Hz, 1H), 7.43 (m, 1H), 6.84 (m, 1H), 6.29 (d, *J* = 16.0 Hz, 1H), 5.58 (s, 1H), 5.28 (m, 1H), 2.05 (m, 1H), 1.91 (m, 1H), 1.79 (m, 2H), 1.64 (m, 1H), 1.50 (m, 5H), 1.37 (m, 4H), 1.23 (m, 4H), 1.12 (m, 4H), 1.01 (m, 1H), 0.96 (s, 3H), 0.92 (d, *J* = 6.3 Hz, 3H), 0.87 (d, *J* = 2.6 Hz, 3H), 0.86 (d, *J* = 2.1 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.4, 157.7, 144.0, 129.9, 127.2, 116.2, 115.8, 71.6, 56.4, 51.5, 41.9, 40.0, 39.4, 35.9, 35.4, 30.6, 29.7, 28.0, 27.1, 23.7, 22.8, 22.6, 22.5, 18.6, 18.0, 13.2. IR(neat) *v*max 3313, 2953, 2869, 1688, 1632, 1587, 1515, 1236, 1170, 1153, 1063, 832. DART-HRMS: *m/z* calcd. for $C_{27}H_{41}O_3$: 413.3056 $[MH]^+$. Found: 413.3075.

*4.2.5.14. 4-hydroxycoumarate-VD3 ester (**32**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.02 (d, *J* = 16.2 Hz, 1H), 7.48 (m, 1H), 7.23 (m, 1H), 6.92 (m, 1H), 6.85 (d, *J* = 8.0 Hz, 1H), 6.62 (d, *J* = 16.2 Hz, 1H), 5.32 (m, 1H), 2.05 (m, 1H), 1.94 (m, 1H), 1.81 (m, 2H), 1.50 (m, 5H), 1. 38 (m, 4H), 1.24 (m, 4H), 1.12 (m, 4H), 1.01 (m, 1H), 0.98 (s, 3H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.87 (d, *J* = 2.0 Hz, 3H), 0.86 (d, *J* = 2.0 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.1, 155.2, 140.0, 131.2, 129.1, 121.8, 120.7, 119.1, 116.3, 71.8, 56.5, 51.5, 41.9, 40.0, 39.4, 35.9, 35.4, 30.6, 29.6, 28.0, 27.1, 23.7, 22.8, 22.7, 22.5, 18.5, 18.0, 13.2. IR(neat) *v*max 3320, 2946, 2928, 2865, 1674, 1602, 1365, 1304, 1274, 1217, 1153, 1108, 1092, 935, 867, 749. DART-HRMS: *m/z* calcd. for $C_{27}H_{41}O_3$: 413.3056 $[MH]^+$. Found: 413.3077.

*4.3. Synthesis of inverted este-linked analogue of **4**.*

*4.3.1. Initial synthesis of epi-Grundmann's alcohol (**37**).*

The initial series of synthetic steps to prepare **37** (Scheme 3A) was as previously described [17]. The characterization of intermediates prepared via this route that were evaluated for Hh activity was also as described.

*4.3.2. Direct reduction of Grundmann's ketone to provide **37**.*

The reduction of **33** was performed according to the procedure by Blakemore et. al., with the only modification being exchanging Amberlite IR120 (dried at 0.1 mmHg and 50ºC, 12 h) for the Amberlite IR118 resin used in the original published protocol [18]. Following completion of the sodium metal addition, the reaction mixture was quenched cautiously with water (dropwise addition, 1 mL; 20 mL additional water). The mixture was then filtered and washed accordingly. The first two chromatographic attempts yielded a mixture of alcohols (100% Hex to 10% EtOAc in Hex) and after a third attempt (on 800 mg material; 100% Hex to 6% EtOAc in Hex), **37** (45%) and **38** (15%) were isolated as clear oils.

*4.3.3. epi-Grundmann's alcohol (**37**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.57 (m, 1H), 1.98 (m, 1H), 1.86 (m, 2H), 1.79 (m, 1H), 1.60 (m, 2H), 1.52 (m, 1H), 1.32 (m, 6H), 1.11 (m, 7H), 1.00 (m, 1H), 0.90 (d, *J* = 6.4 Hz, 3H), 0.86 (d, *J* = 6.9 Hz, 6H), 0.67 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 71.1, 57.2, 56.6, 44.6, 39.4, 39.2, 36.1, 35.9, 35.3, 27.9, 27.9, 23.8, 23.4, 22.7, 22.5, 21.8, 18.5, 11.9. IR(neat) *ν*max 3335, 2969, 2932, 2882, 1466, 1407, 1378, 1340, 1305, 1159, 1127, 1107, 950, 816. DART-HRMS: a) *m/z* calcd. for $C_{18}H_{34}ONH_4$: 284.2953 $[M+NH_4]^+$. Found: 284.2959. b) *m/z* calcd. for $C_{18}H_{33}$: 249.2582 $[M-OH]^+$. Found: 249.2561.

*4.3.4. α-epi-Grundmann's alcohol (**38**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.89 (m, 1H), 1.97 (m, 1H), 1.84 (m, 1H), 1.58 (m, 8H), 1.44 (m, 3H), 1.30 (m, 5H), 1.12 (m, 4H), 0.99 (s, 3H), 0.96 (m, 1H), 0.86 (m, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 70.0, 55.7, 51.8, 44.8, 39.5, 36.4, 34.0, 33.4, 29.8, 27.9, 25.3, 24.5, 23.2, 22.7, 22.5, 20.7, 20.3, 19.8. IR(neat) *ν*max 3315, 2995, 2950, 2926, 2867, 1459, 1384, 1349, 1266, 1047, 1009, 991, 860, 731. DART-HRMS: a) *m/z* calcd. for $C_{18}H_{34}ONH_4$: 284.2953 $[M+NH_4]^+$. Found: 284.2957. b) *m/z* calcd. for $C_{18}H_{33}$: 249.2582 $[M-OH]^+$. Found: 249.2559.

*4.3.5. 3-benzyloxy benzoate-epi-VD3 ester (**39**).*

Esterification and benzyl deprotection to afford **40** as a clear oil was as described previously (65%) [14-15]. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.64 (m, 1H), 7.45 (m, 1H), 7.39 (m, 1H), 7.33 (m, 1H), 7.15 (m, 1H), 5.11 (s, 2H), 5.04 (m, 1H), 2.17 (m, 1H), 1.95 (m, 1H), 1.86 (m, 1H), 1.69 (m, 3H), 1.56 (m, 2H), 1.41 (m, 1H), 1.30 (m, 8H), 1.14 (m, 4H), 1.02 (m, 1H), 0.94 (d, *J* = 6.4 Hz, 3H), 0.88 (d, *J* = 1.8 Hz, 3H), 0.87 (d, *J* = 2.1 Hz, 3H), 0.80 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.0, 158.6, 136.6, 132.3, 129.2, 128.6, 128.0, 127.5, 122.1, 119.6, 115.3, 77.2, 74.9, 70.1, 56.5, 54.3, 45.0, 39.4, 39.1, 36.1, 35.4, 32.3, 28.0, 27.7, 23.8, 23.7, 22.7, 22.5, 21.6, 18.6, 11.9. DART-HRMS: a) *m/z* calcd. for $C_{32}H_{45}O_3$: 477.3369 $[MH]^+$. Found: 477.3384. b) *m/z* calcd. for $C_{32}H_{44}O_3NH_4$: 494.3634 $[M+NH_4]^+$. Found: 494.3652.

*4.3.6. 3-hydroxy benzoate-epi-VD3 ester (**40,** epi-4).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58 (m, 1H), 7.54 (s, 1H), 7.29 (m, 1H), 7.04 (m, 1H), 5.54 (bs, 1H), 5.04 (m, 1H), 2.16 (m, 1H), 1.94 (m, 1H), 1.85 (m, 1H), 1.68 (m, 3H), 1.55 (m, 2H), 1.32 (m, 9H), 1.13 (m, 4H), 1.02 (m, 1H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 2.2 Hz, 3H), 0.86 (d, *J* = 2.2 Hz, 3H), 0.78 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.2, 155.7, 132.3, 129.5, 121.8, 119.8, 116.2, 75.2, 56.5, 54.3, 45.0, 39.4, 39.1, 36.1, 35.4, 32.2, 28.0, 27.7, 23.8, 23.7, 22.8, 22.5,

21.6, 18.6, 11.9. IR(neat) *v*max 3406, 2950, 2925, 2867, 1716, 1687, 1603, 1588, 1454, 1287, 1218, 1102, 1072, 973, 962, 755, 681. DART-HRMS: a) *m/z* calcd. for $C_{25}H_{39}O_3$: 387.2899 $[MH]^+$. Found: 387.2894. b) *m/z* calcd. for $C_{25}H_{38}O_3NH_4$: 404.3165 $[M+NH_4]^+$. Found: 404.3158.

4.4. Synthesis of 'linkerless' analogues.

*4.4.1. 3-methoxymethyl phenyl-Grundmann's alcohol (**41**).*

3-(MOM)-bromophenol [45] had been prepared by the procedure described above for the MOM-protection of phenols. 3-(MOM)-bromophenol (2.16 mmoL) was dissolved in THF (20 mL) and cooled to -78° C. A solution of *n*-BuLi in hexanes (2.05 mmoL) was added slowly to the aryl bromide and stirred for 1 h at this temperature and then warmed to 0° C for 15 minutes. A solution of **33** (1.2 mmoL) in THF (10 mL) was then added to the reaction mixture and stirred for 12 h. The reaction was quenched by the addition of saturated ammonium chloride (50 mL) and washed with DCM (3 X 80 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated. The crude residue was purified using column chromatography (SiO_2, 100% Hex to 5% EtOAc in Hex) and **41** was isolated as a clear oil in 89% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.23 (m, 1H), 7.13 (m, 1H), 7.06 (m, 1H), 6.89 (m, 1H), 5.18 (s, 2H), 3.49 (s, 3H), 2.08 (m, 1H), 1.97 (m, 1H), 1.73 (m, 3H), 1.55 (m, 3H), 1.41 (m, 1H), 1.33 (m, 2H), 1.27 (m, 1H), 1.22 (m, 2H), 1.13 (m, 5H), 1.03 (s, 3H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.87 (d, *J* = 2.3 Hz, 3H), 0.86 (d, *J* = 2.3 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 157.2, 150.9, 128.9, 118.1, 113.5, 113.1, 94.5, 76.2, 56.8, 56.1, 55.9, 42.9, 40.7, 40.2, 39.5, 35.8, 35.2, 27.9, 26.6, 23.7, 22.7, 22.5, 20.0, 19.4, 18.4, 13.2. DART-HRMS: a) *m/z* calcd. for $C_{26}H_{41}O_2$: 385.3107 $[M-OH]^+$. Found: 385.3078. b) *m/z* calcd. for $C_{26}H_{42}O_3NH_4$: 420.3478 $[MNH_4]^+$. Found: 420.3468. c) *m/z* calcd. for $C_{26}H_{42}O_3$: 402.3134 $[M]^+$. Found: 402.3129.

*4.4.2. .3-phenol-Grundmann's alcohol (**42**).*

Removal of the methoxymethyl protecting group to afford phenol **42** as a clear oil in excellent yield (85%) was as described above. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.15 (m, 1H), 6.77 (m, 1H), 6.67 (m, 1H), 6.65 (m, 1H), 4.52 (m, 1H), 2.41 (m, 2H), 2.07 (m, 3H), 1.77 (m, 3H), 1.51 (m, 2H), 1.35 (m, 4H), 1.13 (m, 5H), 0.99 (s, 3H), 0.98 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 1.5 Hz, 3H), 0.86 (d, *J* = 1.5 Hz, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 154.9, 145.8, 145.7, 128.8, 127.4, 120.5, 114.7, 112.7, 55.7, 43.7, 39.5, 37.6, 35.8, 34.8, 30.1, 28.0, 27.2, 27.0, 23.7, 22.7, 22.5, 19.8, 18.9, 18.5. IR(neat) *v*max 3375, 2951, 2924, 2853, 1716, 1580, 1485, 1463, 1445, 1367, 1278, 1181, 1089, 1024, 904, 866, 781, 699. DART-HRMS: a) *m/z* calcd. for $C_{24}H_{37}O$: 341.2844 $[M\text{-}OH]^+$. Found: 341.2828.

*4.4.3. 3-methoxymethyl phenyl-(dehydro)VD3 (**43**).*

Tertiary alcohol **41** (0.25 mmoL) was dissolved in anhydrous benzene (8 mL) and stirred at RT for 1 h in the presence of methyl *N*-(triethylammoniosulfonyl)carbamate (Burgess reagent, 0.67 mmoL). The reaction mixture was heated to reflux for 2 h. After this time, the reaction was cooled to RT, diluted with diethyl ether (75 mL) and washed sequentially with saturated sodium bicarbonate (80 mL) and saturated sodium chloride (100 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated. Column chromatography (SiO_2, 100% Hex to 4% EtOAc in Hex) afforded **43** as a 3:2 mixture of isomers (**43**, 75%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.19 (m, 1H), 6.88 (m, 4H), 5.66 (m, 1H), 5.18 (s, 3H), 3.50 (s, 4H), 2.59 (m, 1H), 2.45 (m, 1H), 2.28 (m, 2H), 2.09 (m, 2H), 1.94 (m, 1H), 1.80 (m, 2H), 1.53 (m, 3H), 1.37 (m, 7H), 1.13 (m, 7H), 1.00 (m, 6H), 0.89 (m, 9H), 0.78 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.8, 145.8, 145.4, 144.5, 140.2, 128.6, 127.5, 125.1, 121.5, 120.6, 115.8, 114.9, 113.8, 113.5, 94.5, 55.9, 55.7, 54.4, 49.9, 43.7, 42.6, 39.5, 37.6, 36.1, 36.0, 35.8, 34.8, 30.1, 28.3, 28.0, 27.2, 27.0, 25.0, 24.3, 23.8, 23.7,

22.8, 22.5, 19.8, 18.9, 18.8, 18.5, 11.2. DART-HRMS: a) *m/z* calcd. for $C_{26}H_{41}O_2$: 385.3107 $[MH]^+$. Found: 385.3088. b) *m/z* calcd. for $C_{26}H_{40}O_2NH_4$: 402.3372 $[MNH_4]^+$. Found: 402.3372.

4.4.4. 3-phenol-(dehydro)VD3 (44).

Removal of the methoxymethyl protecting to afford phenol **44** as a clear oil in excellent yield (75%) was as described above. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.15 (m, 1H), 6.77 (m, 1H), 6.67 (m, 2H), 5.64 (m, ~ 0.6H), 4.80 (s, 1H), 2.55 (m, 1H), 2.43 (m, 1H), 2.26 (m, 1H), 2.07 (m, 2H), 1.92 (m, 1H), 1.76 (m, 2H), 1.41 (m, 8H), 1.15 (m, 4H), 1.05 (m, 1H), 1.00 (m, 3H), 0.88 (m, 6H), 0.77 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 154.9, 145.8, 145.7, 144.8, 140.1, 128.8, 127.4, 125.1, 120.5, 119.6, 114.7, 113.9, 112.9, 112.6, 55.7, 54.5, 49.9, 43.7, 42.6, 39.5, 37.6, 36.2, 36.1, 35.9, 35.8, 34.8, 30.1, 28.3, 28.0, 27.2, 27.0, 25.0, 24.3, 23.9, 23.7, 22.8, 22.5, 19.8, 18.9, 18.8, 18.5, 11.2. IR(neat) *ν*max 3361, 3186, 2951, 2928, 2867, 2490, 2383, 1657, 1628, 1600, 1581, 1487, 1465, 1377, 1365, 1292, 1183, 965, 865, 782, 769, 733, 698. DART-HRMS: a) *m/z* calcd. for $C_{24}H_{37}O$: 341.2844 $[MH]^+$. Found: 341.2839.

*4.4.5. 3-methoxymethyl phenyl-VD3 (**45**).*

4:1. To **43** (0.21 mmoL) was added Pd/C (10%, 10 mg), followed by EtOAc (10 mL). The reaction mixture was purged with hydrogen then left to stir under a hydrogen atmosphere for 24 h. The mixture was filtered through celite, washed with EtOAc, and concentrated. Intermediate **45** was isolated by column chromatography (SiO_2, 100% Hex to 6% EtOAc in Hex) as a clear oil in 68% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.17 (m, 1H), 7.01 (s, 1H), 6.98 (m, 1H), 6.85 (m, 2H), 5.17 (m, 3H), 3.50 (s, ~ 1.5H), 3.49 (s, ~ 3H), 3.17 (m, 1H), 2.93 (m, ~ 0.25H), 2.29 (m, 1H), 2.04 (m, 2H), 1.82 (m, 2H), 1.62 (m, 10H), 1.33 (m, 8H), 1.12 (m, 8H), 0.98 (m, 2H), 0.87 (m, 14H), 0.42 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.6, 146.0, 128.1, 122.9, 117.7, 115.6, 112.7, 94.5, 56.8, 56.8, 55.8, 51.9, 42.5, 40.9, 40.1, 39.5, 36.0, 35.4, 29.7, 27.9, 27.1, 25.5, 23.8,

22.8, 22.5, 20.5, 18.8, 12.2. DART-HRMS: a) *m/z* calcd. for $C_{26}H_{43}O_2$: 387.3263 $[MH]^+$. Found: 387.3261. b) *m/z* calcd. for $C_{26}H_{42}O_2NH_4$: 404.3529 $[MNH_4]^+$. Found: 404.3524.

*4.4.6. 3-phenol-epi-VD3 (**46**).*

Removal of the methoxymethyl protecting to afford phenol **44** as a clear oil in good yield (60%) was as described above. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.14 (m, ~ 0.3H), 7.11 (m, 1H), 6.90 (m, 1H), 6.81 (m, 1H), 6.76 (m, ~ 0.4H), 6.66 (m, ~ 0.7H), 6.62 (m, 1H), 4.78 (s, 1H), 3.13 (m, 1H), 2.89 (m, ~0.25H), 2.27 (m, 1H), 2.05 (m, 1H), 1.99 (m, 1H), 1.81 (m, 1H), 1.68 (m, 5H), 1.53 (m, 4H), 1.29 (m, 7H), 1.12 (m, 7H), 0.99 (m, 1H), 0.94 (m, 1H), 0.87 (m, 12H), 0.41 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 154.6, 146.4, 129.0, 128.4, 121.8, 119.9, 116.2, 114.3, 111.9, 56.8, 51.9, 43.2, 42.4, 40.9, 40.0, 39.5, 36.0, 35.4, 33.9, 33.3, 29.6, 27.9, 27.0, 25.6, 23.8, 22.7, 22.5, 22.5, 20.4, 18.8, 12.2. IR(neat) *ν*max 3326, 2948, 2927, 2865, 1611, 1586, 1489, 1455, 1374, 1216, 1185, 1127, 873, 780, 757, 742, 708, 695. DART-HRMS: a) *m/z* calcd. for $C_{24}H_{39}O$: 343.3001 $[MH]^+$. Found: 343.3015. b) *m/z* calcd. for $C_{24}H_{38}ONH_4$: 360.3266 $[MNH_4]^+$. Found: 360.3248.

4.5. Synthesis of one carbon-linked analogues.

*4.5.1. Methylvinyl-Grundmann's ether (**47**).*

A mixture of methoxymethyl triphenylphosphonium chloride (6 mmoL) in THF (30 mL) was cooled to 0° C. With vigorous stirring, sodium *t*-butoxide (5.7 mmoL) was added. The heterogeneous mixture immediately changed color to yellow and on to deeper yellow and finally deep red at 0° C. After 30 minutes, a solution of **33** (1.5 mmoL) in THF (10 mL) was added to the above mixture. TLC indicated consumption of **33** after 20 minutes. Water (80 mL) was added and the mixture and was washed with ethyl acetate (3 X 80 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. The crude material was purified using

column chromatography (SiO_2, 100% Hex to 2% EtOAc in Hex) to yield **47** in 89% yield as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.84 (m, 1H), 3.43 (s, 3H), 1.97 (m, 3H), 1.77 (m, 4H), 1.52 (m, 3H), 1.42 (m, 3H), 1.33 (m, 3H), 1.25 (m, 1H), 1.19 (m, 5H), 1.01 (m, 1H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.87 (d, *J* = 6.6 Hz, 3H), 0.86 (d, *J* = 6.6 Hz, 3H), 0.68 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.9, 118.2, 59.3, 56.0, 52.1, 44.4, 40.4, 39.5, 36.2, 28.0, 27.9, 25.1, 23.8, 22.8, 22.5, 22.4, 21.9, 18.8, 11.6. DART-HRMS: *m/z* calcd. for $C_{20}H_{37}O$: 293.2844 $[MH]^+$. Found: 293.2849.

*4.5.2. epi-Grundmann's aldehyde (**48**).*

To **47** was added methanol (10 mL) and 3N HCl (5 mL) and the solution was refluxed for 12 h.[24] The acid was neutralized by the addition of 2M NaOH and the aqueous fraction washed with ethyl acetate (3 X 50 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. The crude material was purified using column chromatography (SiO_2, 100% Hex to 2% EtOAc in Hex) to yield **48** in 75% yield as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.55 (m, 1H), 2.30 (m, 1H), 1.99 (m, 1H), 1.86 (m, 1H), 1.78 (m, 1H), 1.66 (m, 2H), 1.53 (m, 2H), 1.30 (m, 8H), 1.12 (m, 5H), 1.02 (m, 1H), 0.93 (d, *J* = 6.5 Hz, 3H), 0.87 (d, *J* = 2.1 Hz, 3H), 0.86 (d, *J* = 2.0 Hz, 3H), 0.71 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 205.1, 55.4, 50.6, 49.4, 42.7, 39.4, 39.4, 36.1, 35.6, 28.0, 27.8, 25.7, 24.2, 23.7, 22.8, 22.5, 20.7, 18.7, 11.6. DART-HRMS: *m/z* calcd. for $C_{19}H_{35}O$: 279.2688 $[MH]^+$. Found: 279.2684.

*4.5.3. epi-Grundmann's methanol (**49**).*

Analogue **49** was prepared by dissolving a portion of **48** (0.34 mmoL) in DCM:MeOH (8 mL), cooling the solution to 0° C, and adding $NaBH_4$ (0.48 mmoL; in 3 equal portions). The reaction was stirred for 15 minutes at 0° C, warmed to room temperature for 30 minutes (**48** was consumed) and was then quenched by addition of 1N HCl. This mixture was washed with ethyl

acetate (3 X 50 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. The crude material was purified using column chromatography (SiO_2, 100% Hex to 8% EtOAc in Hex) to yield **49** in 94% yield as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.60 (m, 1H), 3.36 (m, 1H), 1.95 (m, 1H), 1.81 (m, 2H), 1.55 (m, 5H), 1.34 (m, 3H), 1.23 (m, 2H), 1.00 (m, 8H), 0.91 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 2.2 Hz, 3H), 0.86 (d, *J* = 2.2 Hz, 3H), 0.68 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 67.4, 56.1, 51.9, 43.0, 40.0, 39.5, 39.3, 36.2, 35.7, 29.6, 28.0, 27.9, 24.4, 23.8, 22.8, 22.5, 21.7, 18.7, 11.7. IR(neat) *ν*max 3320, 2922, 2865, 1465, 1379, 1365, 1332, 1308, 1267, 1224, 1203, 1166, 1122, 1049, 1027, 997, 969, 857, 820. DART-HRMS: a) *m/z* calcd. for $C_{19}H_{35}O$: 279.2688 $[MH]^+$. Found: 279.2681.

*4.5.4. 3-methoxymethyl phenyl-epi-VD3 ketone (**50**).*

Intermediate **50** was prepared by the procedure detailed above for **41**: following lithium-halogen exchange between 3-(MOM)-bromophenol (2.0 mmoL) and *n*-BuLi solution in THF (1.96 mmoL) at -78° C, a solution of **48** (1 mmoL) in THF (10 mL) was added. The mixture was stirred for 18 h warming to RT. The reaction was quenched and extracted following the same procedure and the crude residue (oil) was immediately redissolved in DCM (10 mL) and PDC (1.8 mmoL) was added. This mixture was stirred at room temperature for 16 h and the reaction mixture was filtered over a celite pad and rinsed with copious amounts of DCM. The DCM was concentrated and purified using column chromatography (SiO_2, 100% Hex to 3% EtOAc in Hex) to yield **50** in 25% yield (over two steps). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (m, 2H), 7.36 (m, 1H), 7.23 (m, 1H), 5.22 (s, 2H), 3.49 (s, 3H), 3.39 (m, 1H), 2.01 (m, 1H), 1.81 (m, 2H), 1.62 (m, 3H), 1.50 (m, 2H), 1.35 (m, 5H), 1.15 (m, 6H), 1.00 (m, 3H), 0.94 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 2.2 Hz, 3H), 0.85 (d, *J* = 2.2 Hz, 3H), 0.81 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 203.4, 157.4, 139.0, 129.5, 121.7, 120.6, 115.8, 94.5, 56.1, 55.5, 51.4, 44.6, 42.8, 39.5, 39.4, 36.1, 35.7,

30.4, 29.7, 28.0, 27.6, 24.5, 23.8, 22.8, 22.5, 21.7, 18.7, 11.8. DART-HRMS: a) *m/z* calcd. for $C_{27}H_{43}O_3$: 415.3212 $[MH]^+$. Found: 415.3178.

*4.5.5. 3-phenol epi-VD3 ketone (**51**).*

Removal of the methoxymethyl protecting to afford phenol **51** as a clear oil in good yield (75%) was as described above. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 (m, 1H), 7.48 (m, 1H), 7.33 (m, 1H), 7.05 (m, 1H), 5.44 (s, 1H), 3.39 (m, 1H), 3.29 (m, ~ 0.11H), 2.01 (m, 1H), 1.83 (m, 2H), 1.63 (m, 3H), 1.50 (m, 3H), 1.36 (m, 4H), 1.18 (m, 7H), 1.00 (m, 3H), 0.94 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 6.4 Hz, 6H), 0.80 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 206.7, 204.0, 156.0, 139.0, 129.7, 120.8, 120.1, 114.7, 55.4, 51.4, 44.6, 42.7, 39.5, 39.4, 36.1, 35.7, 30.5, 27.9, 27.6, 24.5, 23.7, 22.7, 22.5, 21.7, 18.7, 11.8. IR(neat) *ν*max 3435, 3388, 2950, 2928, 2867, 1667, 1653, 1594, 1449, 1376, 1188, 1078, 1041, 997, 794, 771, 608. DART-HRMS: *m/z* calcd. for $C_{25}H_{39}O_2$: 371.2950 $[MH]^+$. Found: 371.2932.

4.6. Synthesis of amine and amide-linked analogues.

*4.6.1. 2-phenol-VD3 amine (**52**).*

Reductive amination of **33** with 2-aminophenol was described previously to afford **52** as a clear oil in good yield (60%).[28] ^{1}H NMR (500 MHz, $CDCl_3$ + a drop of conc. H_2SO_4) δ 8.89 (bs, 1H), 8.00 (bs, 1H), 7.31 (m, 1H), 7.03 (m, 2H), 6.76 (m, 1H), 3.71 (m, 1H), 2.07 (m, 1H), 1.90 (m, 4H), 1.70 (m, 2H), 1.51 (m, 4H), 1.34 (m, 5H), 1.12 (m, 3H), 1.02 (s, 3H), 0.90 (d, *J* = 6.3 Hz, 3H), 0.87 (d, *J* = 2.2 Hz, 3H), 0.86 (d, *J* = 2.2 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 143.8, 137.4, 119.6, 115.2, 113.1, 109.1, 56.2, 51.1, 50.3, 41.5, 39.7, 38.9, 35.4, 34.7, 30.6, 30.1, 27.3, 26.6, 23.1, 22.6, 22.6, 22.3, 18.3, 17.4, 13.7. IR(neat) *ν*max 3237, 3057, 2928,, 2866, 1607, 1520,

1467, 1445, 1372, 1334, 1271, 1175, 1157, 1100, 1073, 1039, 732. ESI-HRMS: *m/z* calcd. for $C_{24}H_{40}NO$: 358.3110 $[MH]^+$. Found: 358.3106.

*4.6.2. 3-phenol-VD3 amine (**53**).*

Reductive amination of **33** with 3-aminophenol was described previously to afford **53** as a clear oil in good yield (80%).[28] ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.98 (m, 1H), 6.15 (m, 1H), 6.10 (m, 1H), 6.06 (m, 1H), 4.78 (bs, 1H), 3.68 (m, 1H), 1.98 (m, 2H), 1.85 (m, 1H), 1.63 (m, 3H), 1.50 (m, 4H), 1.32 (m, 10H), 1.15 (m, 6H), 0.99 (m, 2H), 0.94 (s, 3H), 0.89 (m, 9H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.7, 150.0, 130.0, 105.8, 103.3, 99.1, 56.8, 51.6, 51.1, 42.1, 40.2, 39.4, 35.9, 35.3, 31.5, 30.5, 28.0, 27.0, 23.7, 23.1, 22.7, 22.6, 22.5, 18.5, 17.8, 14.1, 13.5. IR(neat) *ν*max 3388, 2986, 2866, 1614, 1506, 1464, 1371, 1333, 1272, 1179, 1155, 1077, 977, 828, 757,686. ESI-HRMS: *m/z* calcd. for $C_{24}H_{40}NO$: 358.3110 $[MH]^+$. Found: 358.3115.

*4.6.3. Modified reductive amination procedure (**54** and **55**).*

Slightly modifying the cited protocol [28], ketone (**33**, 0.6 mmoL) and 4-aminophenol (0.8 mmoL) were dissolved in 1,2-dichloroethane (8 mL) at RT. Sodium triacetoxyborohydride (1.6 mmoL) was added followed by acetic acid (1.2 mmoL) and the mixture stirred for 16 h. Based on the presence of **33** after this time, the reaction was refluxed for 5 h. Saturated sodium bicarbonate (20 mL) was added to the reaction mixture followed by diethyl ether (60 mL). The aqueous layer was washed with diethyl ether (3 X 60 mL) and the organic layers combined, dried over $MgSO_4$, filtered, and concentrated. Crude residue was purified by column chromatography (SiO_2, 100% Hex to 25% EtOAc in Hex) affording recovery of the starting ketone **33** (45%), **54** (40%), and **55** (10%).

*4.6.4. 4-phenol-VD3 amine (**54**).*

^{1}H NMR (500 MHz, DMSO) δ 8.34 (s, 1H), 6.48 (m, 4H), 4.13 (m, 1H), 3.48 (m, 1H), 1.93 (m, 1H), 1.76 (m, 3H), 1.51 (m, 4H), 1.37 (m, 7H), 1.13 (m, 7H), 1.00 (m, 2H), 0.90 (m, 6H), 0.87 (d, *J* = 2.3 Hz, 3H), 0.86 (d, *J* = 2.3 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 148.0, 142.1, 115.4, 113.6, 56.1, 51.8, 51.5, 41.6, 40.0, 38.9, 35.4, 34.7, 30.7, 27.3, 26.8, 23.1, 23.0, 22.6, 22.3, 18.3, 17.5, 13.2. IR(neat) *v*max 3441, 3161, 2927, 2866, 1610, 1512, 1466, 1241, 1192, 976, 795, 691. ESI-HRMS: *m/z* calcd. for $C_{24}H_{40}NO$: 358.3110 $[MH]^+$. Found: 358.3107.

*4.6.5. 4-phenol-epi-VD3 amine (**55**).*

^{1}H NMR (500 MHz, DMSO) δ 8.30 (bs, 1H), 6.51 (m, 2H), 6.41 (m, 2H), 4.33 (m, 1H), 2.83 (m, 1H), 1.95 (m, 1H), 1.87 (m, 2H), 1.81 (m, 1H), 1.66 (m, 1H), 1.54 (m, 2H), 1.47 (m, 2H), 1.37 (m, 4H), 1.20 (m, 6H), 1.02 (m, 1H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.89 (s, 3H), 0.88 (d, *J* = 2.1 Hz, 3H), 0.86 (d, *J* = 2.2 Hz, 3H). ^{13}C NMR (126 MHz, DMSO doped with a drop of CDCl3) δ 126.4, 118.2, 115.8, 115.5, 56.1, 55.2, 45.1, 44.9, 44.6, 38.9, 35.9, 35.8, 34.9, 34.7, 34.4, 29.0, 27.4, 27.0, 24.2, 24.0, 22.6, 22.3, 19.2, 19.1. IR(neat) *v*max 3441, 3161, 2927, 2866, 1610, 1512, 1466, 1241, 1192, 976, 795, 691. DART-HRMS: *m/z* calcd. for $C_{24}H_{40}NO$: 358.3110 $[MH]^+$. Found: 358.3102.

*4.6.6. Grundmann's-oxime (**56**) [19].*

Ketone **33** (2.72 mmol) was dissolved in 4 mL of pyridine. Hydroxylamine hydrochloride (9.42 mmol) was then added with stirring and was left to stir at RT for 12h and the mixture was diluted with H_2O (10 mL) and benzene (10 mL). The mixture was washed with H_2O followed by saturated $CuSO_4$, and concentrated. Column chromatography (SiO_2, 100% Hex to 10% EtOAc in Hex) yielded oxime **56** (98%). ^{1}H NMR (500MHz, $CDCl_3$) δ 9.41 (bs, 1H) 3.27 (m, 1H), 2.09 (m, 2H), 1.93 (m, 1H), 1.78 (m, 1H), 1.67 (m, 4H), 1.54 (m, 1H), 1.35 (m, 5H), 1.28 (s, 1H), 1.16 (m, 3H), 1.05 (m, 1H), 0.96 (d, *J* = 6.0 Hz,3H), 0.89 (d, *J* = 6.1 Hz, 6H), 0.67 (s, 3H). ^{13}C

NMR (126 MHz, $CDCl_3$) δ 160.5, 56.1, 54.2, 46.6, 39.6, 39.5, 36.2, 36.0, 28.1, 28.0, 24.0, 23.9, 22.9, 22.7, 21.9, 20.8, 18.9, 12.4. HRMS-ESI: *m/z* calcd. for $C_{18}H_{34}NO$: 280.2640 $[MH]^+$. Found: 280.2647.

*4.6.7. 3-benzyloxy phenyl-VD3 amide (**58**).*

Oxime **56** (1.36 mmol) was dissolved in anhydrous THF (10 mL) and cooled to 0°C. $LiAlH_4$ (1.0M; 9.50 mL, 9.52 mmol) was added drop-wise to the solution. Once all $LiAlH_4$ was added, the reaction was refluxed for 12 h. The mixture was then worked up according to the Fieser method: the reaction was cooled to 0°C, water (30 mL) was added, followed by 75 mL of 15% aqueous NaOH, and then 200 mL water. The mixture was warmed to RT, stirred for 0.5 h, and filtered over a celite pad. Following this protocol, the solution was washed with EtOAc (3 X 100 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated. The crude mixture was then dissolved in glacial acetic acid (12 mL) and zinc powder (3 mmol) was added. The mixture was refluxed until the starting material was completely consumed (5h, byTLC). The mixture was cooled to 0°C, quenched with saturated $NaHCO_3$, washed with EtOAc (2 X 50 mL), and concentrated. Crude amine (**57**) was used directly in the next step. EDCI (0.689 mmol), DMAP (0.123 mmol) and 3-(benzyloxy)benzoic acid (0.679 mmol) were dissolved in 5 mL anhydrous CH_2Cl_2 and stirred for 20 min. Crude **57** (2.23 mmoL) in 3 mL anhydrous CH_2Cl_2 was added to the reaction mixture and stirred at RT for 16 h. Column chromatography (SiO_2, 100% Hex to 5% EtOAc in Hex) afforded **58** in 30% (~6:1) yield. ^1H NMR (500MHz, $CDCl_3$) δ 7.44 (m, 5H), 7.36 (m, 2H), 7.12 (m,1H), 6.31 (m, 1H), 5.14 (s, 2H), 4.46 (m, 1H), 2.06 (m, 1H), 1.97 (m, 1H), 1.91 (m, 1H), 1.62 (m, 6H), 1.39 (m, 4H), 1.26 (m, 4H), 1.16 (m, 4H), 1.03 (m, 1H), 0.95 (m, 6H), 0.90 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.9, 159.2, 136.9, 136.8, 129.8, 128.8, 128.3, 127.8, 127.7, 118.9, 118.4, 113.4, 70.4, 56.8,

51.2, 47.9, 42.3, 39.9, 39.7, 36.1, 35.5, 31.2, 29.9, 28.2, 27.2, 23.9, 23.1, 23.0, 22.7, 18.7, 18.2, 13.9. HRMS-ESI: *m/z* calcd. for $C_{32}H_{46}NO_2$: 476.3528 $[MH]^+$. Found: 476.351.

*4.6.8. 3-phenol-VD3 amide (**59**).*

To a solution of protected amide **58** (0.116 mmol) in MeOH:THF (3:1, 12 mL) under argon was added Pd/C (10%, 50 mg). The vessel was purged with H_2 and the mixture stirred for 12 h. The mixture was filtered through celite, rinsed with copious EtOAc (100 mL), and the filtrate was concentrated. Amide **59** was isolated by column chromatography (SiO_2, 100% Hex to 30% EtOAc in Hex) in 67% yield (~6:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.79 (s, 1H), 7.10 (m, 1H), 7.05 (m, 1H), 6.49 (m, 1H), 4.46 (s, 1H), 2.06 (m, 1H), 1.97 (m, 1H), 1.90 (m, 1H), 1.62 (m, 5H) 1.56 (m, 2H), 1.38 (m, 5H), 1.16 (m, 5H), 1.03 (m, 1H), 0.97 (s, 3H), 0.94 (d, *J* = 6.1 Hz, 3H), 0.90 (d, *J* = 6.4 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.6, 157.7, 136.0, 129.9, 119.3, 116.9, 115.6, 56.8, 51.1, 48.3, 42.3, 39.8, 39.7, 36.1, 35.5, 31.1, 28.2, 27.2, 23.9, 23.1, 23.0, 22.7, 18.7, 18.2, 13.9. IR(neat) *ν*max 3338, 2953, 1652, 1585, 1522, 1450, 1258, 1277, 1239,1146, 938, 809. HRMS-ESI: *m/z* calcd. for $C_{25}H_{40}NO_2$: 386.3059 $[MH]^+$. Found: 386.3037.

4.7. Synthesis of enamide analogues.

*4.7.1. Ethyl-VD3 ene-ester (**60**) [31].*

To a solution of triethyl phosphonoacetate (9 mmol) in THF (30 mL) at 0° C was added KHMDS (9 mmoL in toluene, 10 mL) dropwise. The mixture was stirred for 4 h at 0° C, at which time a solution of **33** (1.5 mmol) in THF (10 mL) was added and the mixture warmed to RT and stirred for 16 h. The mixture was diluted with saturated ammonium chloride (80 mL) and washed with EtOAc (3 X 100 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. Column chromatography (SiO_2, 100% Hex to 3% EtOAc in Hex) afforded ene-

ester **60** (76%), which matched the spectral data reported. ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.12 (q, *J* = 7.1 Hz, 2H), 2.31 (m, 2H), 1.83 (m, 6H), 1.52 (m, 5H), 1.34 (m, 5H), 1.16 (m, 6H), 1.00 (m, 2H), 0.91 (d, *J* = 6.6 Hz, 3H), 0.90 (d, *J* = 6.6 Hz, 3H), 0.85 (m, 9H), 0.70 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.5, 61.4, 60.0, 56.3, 55.2, 50.5, 43.1, 40.4, 39.9, 39.5, 39.4, 36.4, 36.1, 35.7, 35.7, 34.3, 34.0, 32.8, 28.0, 27.9, 27.5, 24.3, 24.1, 23.7, 22.7, 22.5, 22.5, 21.9, 21.2, 20.9, 19.1, 18.6, 14.2, 11.7. DART-HRMS: a) *m/z* calcd. for $C_{22}H_{41}O_2$: 337.3107 $[MH]^+$. Found: 337.3133. b) *m/z* calcd. for $C_{22}H_{40}O_2NH_4$: 354.3372 $[M+NH_4]^+$. Found: 354.3378.

4.7.2. General procedure for synthesis of enamides.

To a solution of aniline or monosubstituted aminophenol [46-47] (2.0 mmol) in anhydrous DCM (10 mL) at 0°C was added trimethylaluminum (1.0 mL, 2M solution in toluene). After stirring for 2 h at 0°C, the aniline-$AlMe_3$ complex was added to a mixture of **60** (1.0 mmoL) and K_2CO_3 (5 mmoL) in DCM (5 mL) at 0°C. The reaction mixture was stirred 6-24 h at room temperature (for the reaction with 2-aminophenol, reaction was refluxed for 8 h following 12 h at room temperature) after which time they were quenched by the addition of saturated sodium bicarbonate and washed with DCM (3 X 80 mL). The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated. Column chromatography (SiO_2, 100% Hex to 8% EtOAc in HEx) afforded **61**-**64** as clear oils in modest to excellent yields (30-82%). For enamides requiring *tert*-butyldimethyl silyl deprotection (**63** or **64**), the standard procedure utilizing ±CSA (2 eq) described above was utilized. Column chromatography (SiO_2, 100% Hex to 12% EtOAc in Hex) of the crude material afforded phenolic enamides **65** or **66** as clear oils in good yield (62-70%).

*4.7.2.1. Phenyl-VD3 enamide (**61**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (m, 2H), 7.50 (m, 1H), 7.28 (m, 2H), 7.06 (m, 1H), 5.72 (m, ~ 0.02 H), 5.52 (s, 1H), 3.94 (m, 1H), 2.09 (m, 1H), 2.02 (m, 1H), 1.91 (m, 1H), 1.72 (m, 2H),

1.64 (m, 1H), 1.52 (m, 3H), 1.34 (m, 6H), 1.14 (m, 3H), 1.02 (m, 1H), 0.94 (d, *J* = 6.0 Hz, 3H), 0.89 (dd, *J* = 6.5, 2.4 Hz, 6H), 0.59 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.3, 159.9, 138.4, 128.7, 123.7, 119.6, 114.8, 56.7, 56.6, 46.8, 40.1, 39.4, 36.0, 35.9, 29.5, 27.9, 27.4, 23.8, 23.7, 22.7, 22.5, 22.1, 18.7, 12.0. IR(neat) *ν*max 3263, 2950, 2926, 2867, 2847, 1661, 1637, 1596, 1541, 1498, 1441, 1299, 1254, 1187, 903,748, 692. ESI-HRMS: *m/z* calcd. for $C_{26}H_{40}NO_2$: 398.3059 $[MH]^+$. Found: 398.3063.

*4.7.2.2. 2-phenol-VD3 enamide (**62**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.35 (s, 1H), 7.35 (s, 1H), 7.11 (m, 1H), 7.03 (m, 1H), 6.92 (m, 1H), 6.84 (m, 1H), 5.57 (s, 1H), 3.93 (m, 1H), 2.15 (m, 1H), 2.04 (m, 1H), 1.93 (m, 1H), 1.77 (m, 2H), 1.56 (m, 6H), 1.37 (m, 7H), 1.25 (m, 2H), 1.14 (m, 4H), 1.02 (m, 2H), 0.94 (d, *J* = 5.4 Hz, 3H), 0.88 (d, *J* = 2.6 Hz, 3H), 0.87 (d, *J* = 2.5 Hz, 3H), 0.61 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.9, 163.4, 149.3, 127.0, 125.9, 122.1, 120.2, 112.9, 109.0, 56.9, 56.7, 47.3, 40.1, 39.4, 36.0, 35.9, 29.7, 28.0, 27.4, 23.9, 23.8, 22.8, 22.5, 22.3, 18.8, 12.1. IR(neat) *ν*max 3331, 2969, 2931, 2882, 1466, 1407, 1378, 1340, 1305, 1159, 1127, 1106, 950, 815. ESI-HRMS: *m/z* calcd. for $C_{26}H_{40}NO_2$: 398.3059 $[MH]^+$. Found: 398.3040.

*4.7.2.3. 3-(dimethyl-tert-butoxide)-phenyl-VD3 enamide (**63**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.30 (m, 1H), 7.20 (m, 1H), 7.11 (m, 2H), 6.55 (m, 1H), 5.68 (m, ~ 0.07H), 5.48 (s, 1H), 3.91 (m, 1H), 2.09 (m, 1H), 2.01 (m, 1H), 1.90 (m, 1H), 1.73 (m, 2H), 1.64 (m, 1H), 1.51 (m, 3H), 1.34 (m, 6H), 1.14 (m, 3H), 1.03 (m, 1H), 0.97 (s, 9H), 0.93 (d, *J* = 6.0 Hz, 3H), 0.88 (dd, *J* = 6.6, 2.4 Hz, 6H), 0.59 (s, 3H), 0.2 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.1, 159.9, 156.0, 139.4, 129.3, 115.4, 114.9, 112.4, 111.5, 56.7, 56.6, 46.8, 40.1, 39.4, 36.0, 35.9, 29.4, 27.9, 27.4, 25.6, 23.8, 23.7, 22.7, 22.5, 22.2, 18.7, 18.1, 12.0, -4.4. DART-HRMS: *m/z* calcd. for $C_{32}H_{54}NO_2Si$: 512.3924 $[MH]^+$. Found: 512.3911.

*4.7.2.4. 4-(dimethyl-tert-butoxide)-phenyl-VD3 enamide (**64**).*

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 (d, *J* = 8.3 Hz, 2H), 7.31 (m, 1H), 6.79 (d, *J* = 8.8 Hz, 2H), 5.69 (s, ~ 0.08H), 5.49 (s, 1H), 3.96 (m, 1H), 2.11 (m, 1H), 2.04 (m, 1H), 1.93 (m, 1H), 1.74 (m, 2H), 1.66 (m, 1H), 1.54 (m, 3H), 1.37 (m, 6H), 1.17 (m, 3H), 1.05 (m, 1H), 1.00 (s, 9H), 0.96 (d, *J* = 6.0 Hz, 3H), 0.91 (d, *J* = 2.4 Hz, 3H), 0.89 (d, *J* = 2.4 Hz, 3H), 0.62 (s, 3H), 0.20 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.1, 159.4, 151.9, 132.0, 121.2, 120.1, 114.8, 56.7, 56.6, 46.8, 40.1, 39.4, 36.0, 35.9, 29.4, 27.9, 27.4, 25.6, 23.8, 23.7, 22.7, 22.5, 22.2, 18.7, 18.1, 12.0, -4.4. DART-HRMS: *m/z* calcd. for $C_{32}H_{54}NO_2Si$: 512.3924 $[MH]^+$. Found: 512.3951.

*4.7.2.5. 3-phenol-VD3 enamide (**65**).*

^{1}H NMR [500 MHz, MeOD:$CDCl_3$ (4:1)] δ 7.22 (m, 1H), 7.10 (m, 1H), 7.00 (m, 1H), 6.54 (m, 1H), 5.87 (m, ~ 0.14 H), 5.69 (s, 1H), 3.90 (m, 1H), 3.34 (s, 1H), 2.16 (m, 1H), 2.06 (m, 1H), 1.96 (m, 1H), 1.75 (m, 2H), 1.66 (m, 1H), 1. 57 (m, 3H), 1.40 (m, 6H), 1.18 (m, 3H), 1.07 (m, 2H), 0.99 (d, *J* = 5.8 Hz, 3H), 0.91 (d, *J* = 6.6, 2.1 Hz, 6H), 0.66 (s, 3H). ^{13}C NMR [126 MHz, MeOD:$CDCl_3$ (4:1)] δ 168.6, 161.2, 159.4, 142.0, 131.1, 117.0, 113.2, 112.6, 109.1, 58.8, 58.7, 48.7, 42.2, 41.4, 38.0, 38.0, 31.3, 29.9, 29.3, 25.7, 25.6, 24.0, 24.0, 23.8, 20.2, 13.3. IR(neat) *ν*max 3363, 3186, 2949, 2926, 2868, 2490, 2383, 1657, 1628, 1600, 1589, 1543, 1492, 1471, 1450, 1427, 1380, 1357, 1321, 1292, 1250, 1229, 1211, 1163, 1148, 1019, 995, 965, 769, 685. DART-HRMS: *m/z* calcd. for $C_{26}H_{40}NO_2$: 398.3059 $[MH]^+$. Found: 398.3063.

*4.7.2.6. 4-phenol-VD3 enamide (**66**).*

^{1}H NMR (500 MHz, MeOD) δ 7.38 (d, *J* = 8.8 Hz, 2H), 6.76 (d, *J* = 8.9 Hz, 2H), 5.84 (s, ~ 0.14), 5.66 (s, 1H), 3.91 (m, 1H), 2.16 (m, 1H), 2.07 (m, 1H), 1.97 (m, 1H), 1.76 (m, 2H), 1.68 (m, 1H), 1.58 (m, 4H), 1.41 (m, 6H), 1.19 (m, 3H), 1.08 (m, 1H), 0.99 (d, *J* = 5.9 Hz, 3H), 0.92

(d, *J* = 2.1 Hz, 3H), 0.90 (d, *J* = 2.1 Hz, 3H), 0.67 (s, 3H). ^{13}C NMR (126 MHz, MeOD) δ 168.4, 160.5, 155.6, 132.6, 123.9, 116.9, 116.8, 58.7, 58.6, 48.6, 42.2, 41.3, 37.9, 37.9, 31.2, 29.8, 29.2, 25.6, 25.5, 24.0, 23.7, 20.1, 13.3. IR(neat) *v*max 3260, 2950, 2930, 2868, 2847, 2432, 1654, 1626, 1605, 1514, 1449, 1414, 1378, 1303, 1235, 1105, 1021, 948, 860. ESI-HRMS: a) *m/z* calcd. for $C_{26}H_{40}NO_2$: 398.3059 $[MH]^+$. Found: 398.3056.

*4.7.2.7. VD3 allyl alcohol (**67**) [48].*

To a solution of ester **60** (120 mg, 0.36 mmol) in THF (8.0 mL) at 0° C, $LiAlH_4$ (1M, 1.08 mmol) was added. After 30 min at 0° C, the mixture was warmed to reflux for 4 h. After cooling to RT, water (20 mL) was added followed by 1N HCl (40 mL) and the mixture was washed with EtOAc (3 X 80 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. Column chromatography (SiO_2, 100% Hex to 20% EtOAc in Hex) of the crude residue afforded **67** as a clear oil (80%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.39 (m, ~ 0.08H), 5.20 (m, 1H), 4.19 (d, *J* = 7.0 Hz, 2H), 2.61 (m, 1H), 1.99 (m, 1H), 1.93 (m, 1H), 1.87 (m, 1H), 1.63 (m, 2H), 1.48 (m, 5H), 1.31 (m, 6H), 1.12 (m, 3H), 1.00 (m, 1H), 0.91 (d, *J* = 6.5 Hz, 3H), 0.86 (d, *J* = 6.5 Hz, 6H), 0.54 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 143.6, 119.1, 58.6, 56.5, 55.6, 45.2, 40.3, 39.4, 36.1, 36.0, 28.6, 27.9, 27.5, 23.8, 23.4, 22.7, 22.5, 22.1, 18.8, 11.8. IR(neat) *v*max 3326, 2949, 2926, 2867, 1668, 1466, 1377, 1366, 1084, 995, 737. DART-HRMS: a) *m/z* calcd. for $C_{20}H_{35}$: 275.2739 $[M\text{-}OH]^+$. Found: 275.2725. b) *m/z* calcd. for $C_{20}H_{35}O$: 291.2698 $[M\text{-}H]^+$. Found: 291.2700.

*4.7.2.8. Ethyl-VD3 (ethyl)ester (**68**).*

Ester **60** (0.3 mmol) was subjected to Pd/C (10%, 20mg) hydrogenation under an hydrogen atmosphere as described previously for compound **45**. Filtration of the crude mixture (after 36 h) was followed by concentration and column chromatography (SiO_2, 100% Hex to 2% EtOAc in

Hex) to afford **68** (90%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.12 (q, *J* = 7.1 Hz, 2H), 2.31 (m, 2H), 1.83 (m, 6H), 1.52 (m, 5H), 1.34 (m, 5H), 1.16 (m, 6H), 1.00 (m, 2H), 0.91 (d, *J* = 6.6 Hz, 3H), 0.90 (d, *J* = 6.6 Hz, 3H), 0.85 (m, 9H), 0.70 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 173.5, 61.4, 60.0, 56.3, 55.2, 50.5, 43.1, 40.4, 39.9, 39.5, 39.4, 36.4, 36.1, 35.7, 35.7, 34.3, 34.0, 32.8, 28.0, 27.9, 27.5, 24.3, 24.1, 23.7, 22.7, 22.5, 22.5, 21.9, 21.2, 20.9, 19.1, 18.6, 14.2, 11.7. DART-HRMS: a) *m/z* calcd. for $C_{22}H_{41}O_2$: 337.3107 $[MH]^+$. Found: 337.3133. b) *m/z* calcd. for $C_{22}H_{40}O_2NH_4$: 354.3372 $[M+NH_4]^+$. Found: 354.3378.

*4.7.2.9. epi-VD3 ethanol (**69**).*

Alcohol **69** was isolated in 70% yield from **68** according to the procedure described above for **60**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.67 (m, 2H), 1.93 (m, 1H), 1.78 (m, 2H), 1.66 (m, 3H), 1.56 (m, 1H), 1.49 (m, 5H), 1.34 (m, 4H), 0.98 (m, 2H), 0.91 (d, *J* = 6.5 Hz, 3H), 0.87 (d, *J* = 2.3 Hz, 3H), 0.86 (d, *J* = 2.2 Hz, 3H), 0.76 (m, 1H), 0.67 (s, 3H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 61.0, 56.4, 55.6, 43.0, 40.1, 39.5, 38.0, 36.2, 35.7, 33.2, 32.5, 28.0, 27.6, 24.7, 23.8, 22.8, 22.5, 22.1, 18.7, 11.8. IR(neat) *v*max 3300, 2949, 2926, 2868, 1446, 1374, 1346, 1301, 1182, 1088, 1061, 1042, 997, 868. DART-HRMS: a) *m/z* calcd. for $C_{20}H_{37}$: 277.2895 $[M-OH]^+$. Found: 277.2915.

4.8. Biological Assay Protocols.

4.8.1. General Information.

Protocols for general cell culture, qPCR (Hh pathway and VDR), and VDR binding assays were previously described [11,13-15]. Data was analyzed using GraphPad Prism 5 and reported values represent mean ± SEM for at least two separate experiments performed in triplicate. RNA expression analysis in C3H10T1/2 cells was performed in either 60mm dishes (500,000 cells plated; TRIzol RNA isolation protocol) or in 24-well tissue culture treated plates (50,000 cells

plated; Cells-to-C_T protocol, TaqMan Gene Expression Kit). Analysis in ASZ001 cells were done in either 35mm dishes (300,000 cells plated; TRIzol) or in 24-well tissue culture treated plates (60,000 cells plated; Cells-to-Ct). RNA isolation, cDNA synthesis, and qPCR analysis of target genes were performed following the manufacturer's protocol specified procedures.

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Supporting Information. ^{1}H and ^{13}C NMR spectra for all new intermediates and final analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations

Hh: Hedgehog; Gli: glioblastoma associated oncogene; Cyp24a1: 1,25-dihydroxyvitamin D3 24-hydroxylase; VD3: vitamin D3; VDR: vitamin D receptor.

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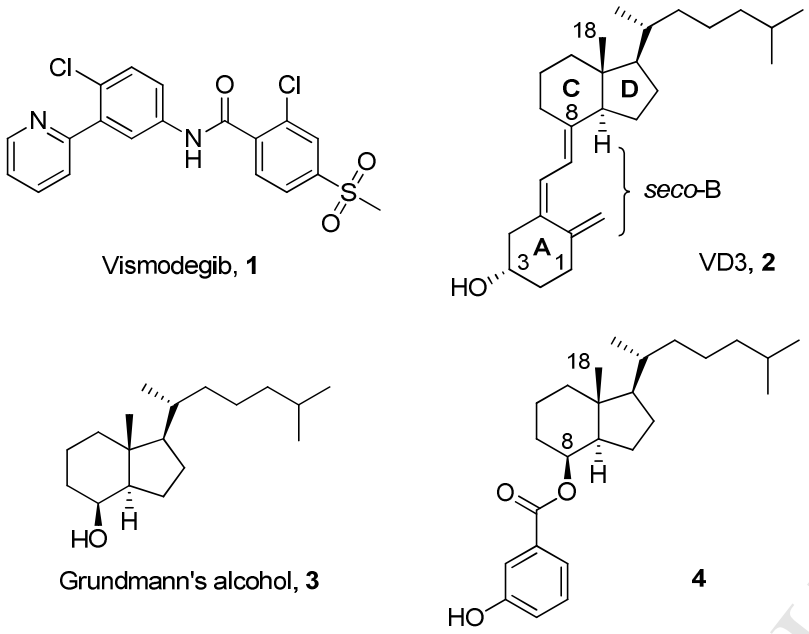
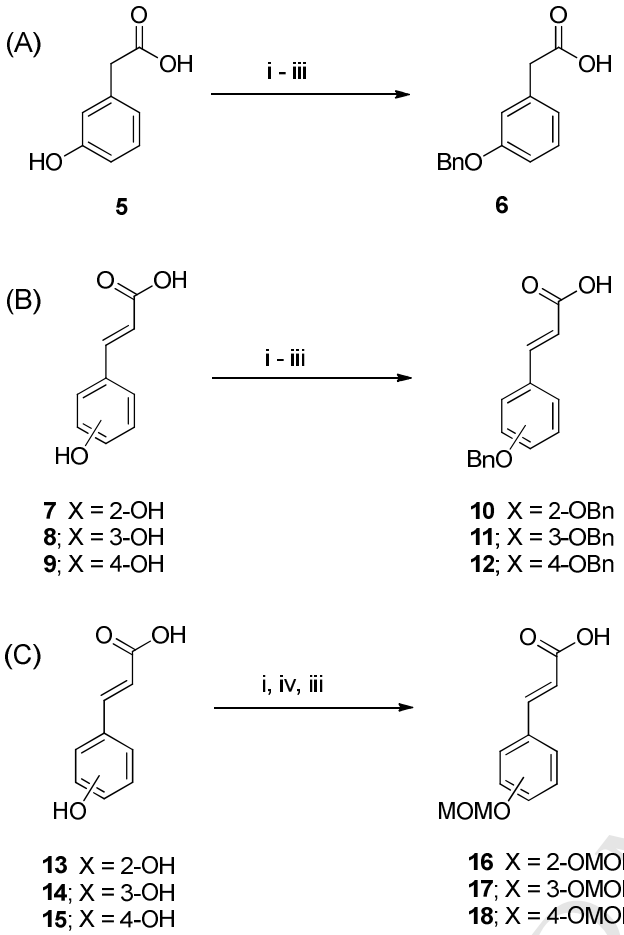
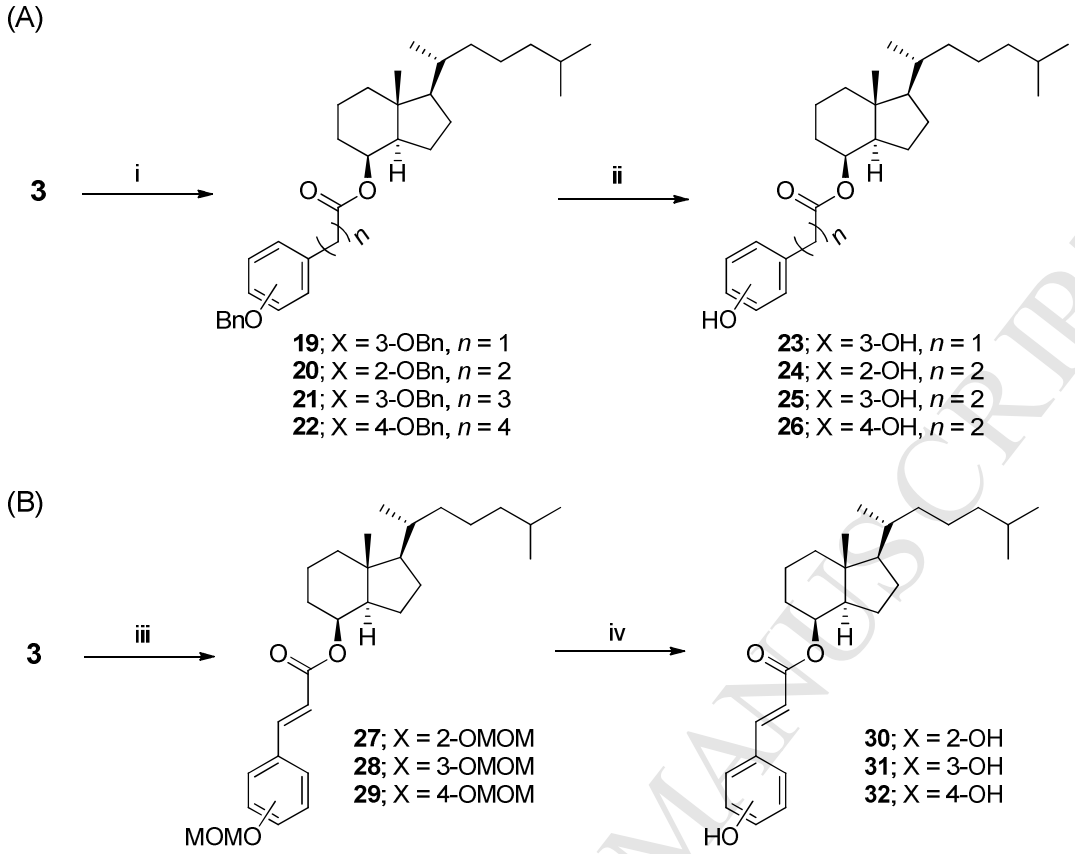


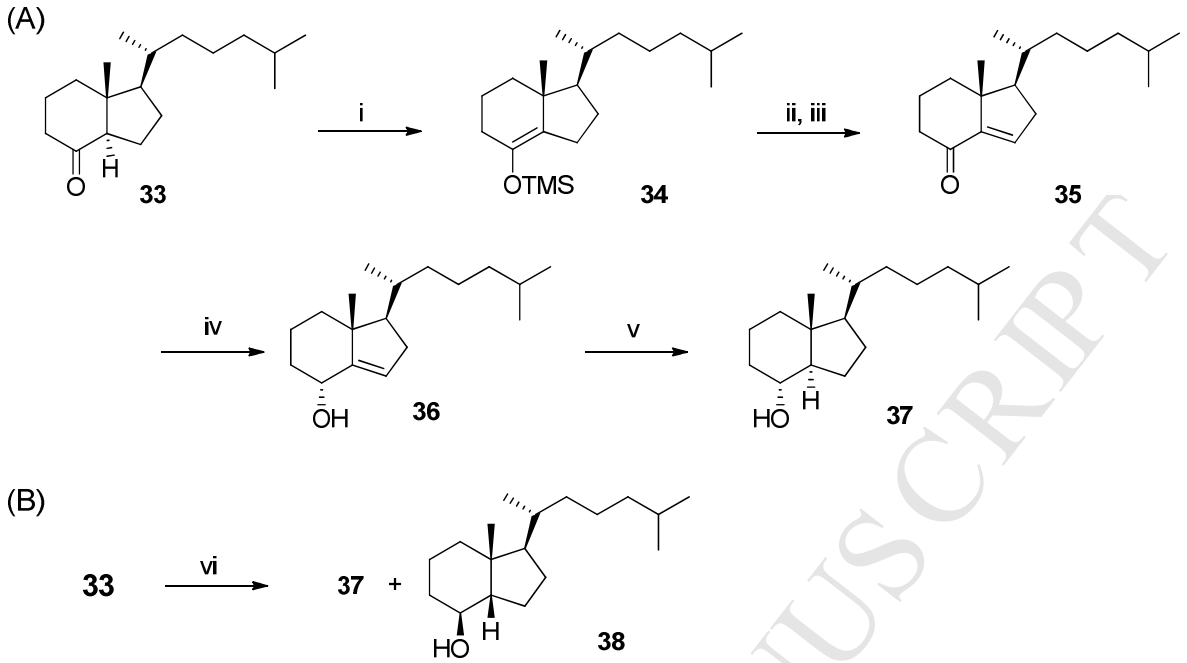
Figure 1. Hh pathway inhibitors



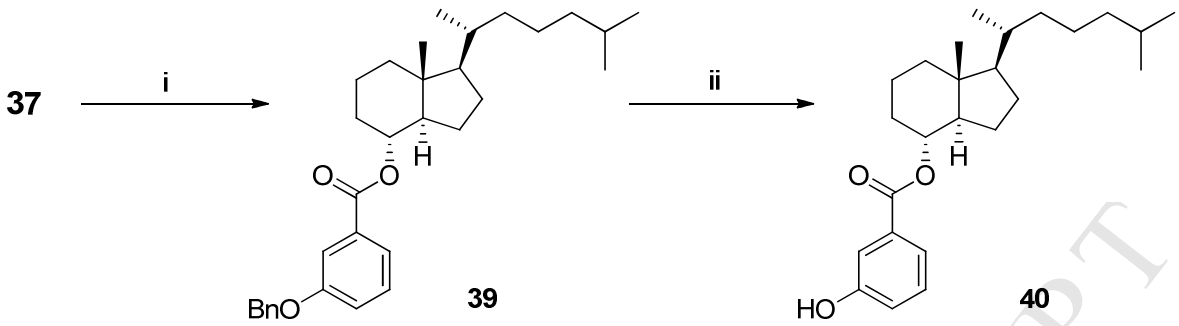
Scheme 1. Protection strategies for aromatic A-ring mimics. Reagents and conditions: (i) $SOCl_2$, MeOH, 0 ºC; (ii) BnBr, K_2CO_3, acetone; (iii) KOH (aq., 20%), THF; (iv) MOMCl, NaH, THF, 0 ºC.



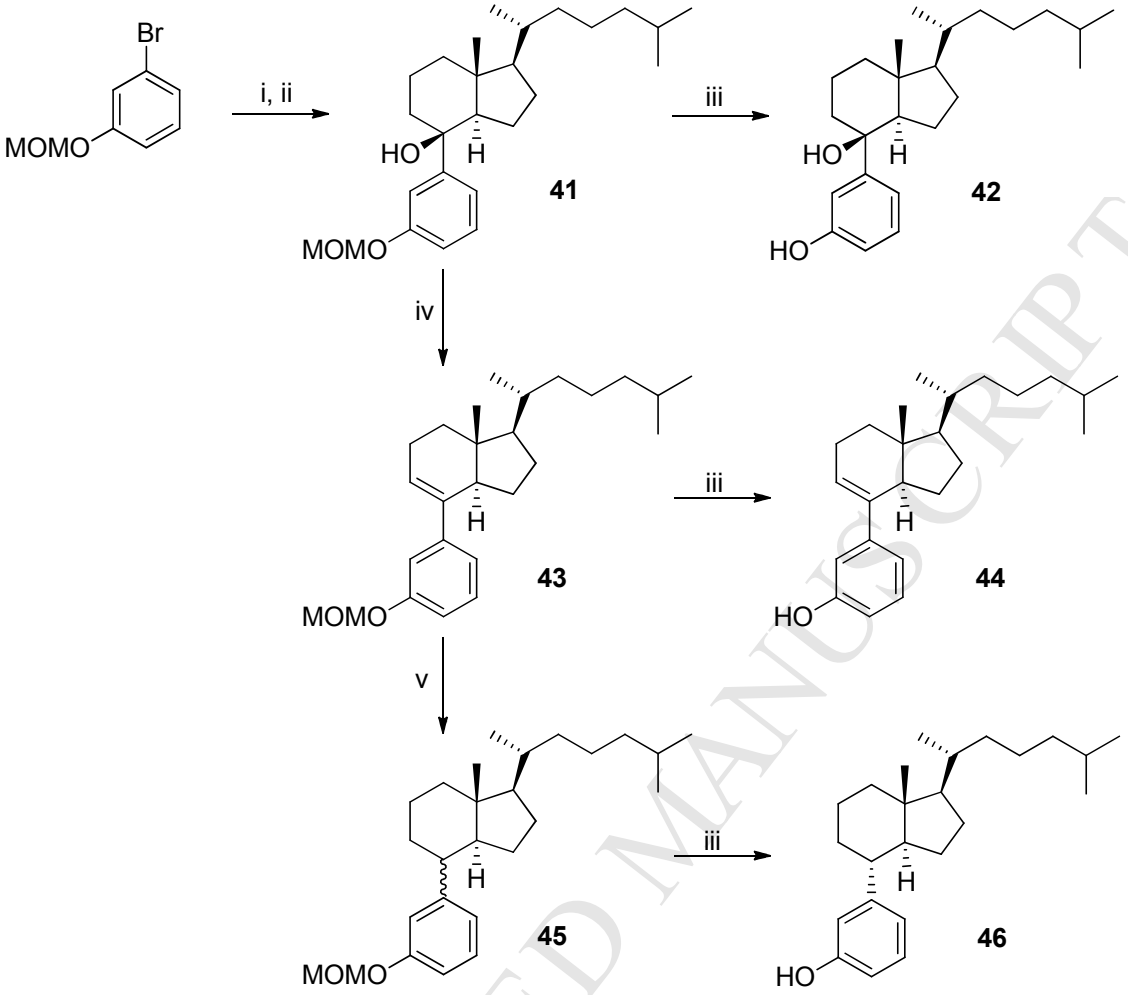
Scheme 2. Preparation of saturated (A) and α,β-unsaturated (B) extended ester-linked analogues: Subclass I. Reagents and conditions: (i) DCC, DMAP, **6**, **10** – **12**, DCM; (ii) $Pd(OH)_2$ (10%), H_2, MeOH:THF (2:1); (iii) DCC, DMAP, **16** – **18**, DCM; (iv) ±CSA, MeOH.



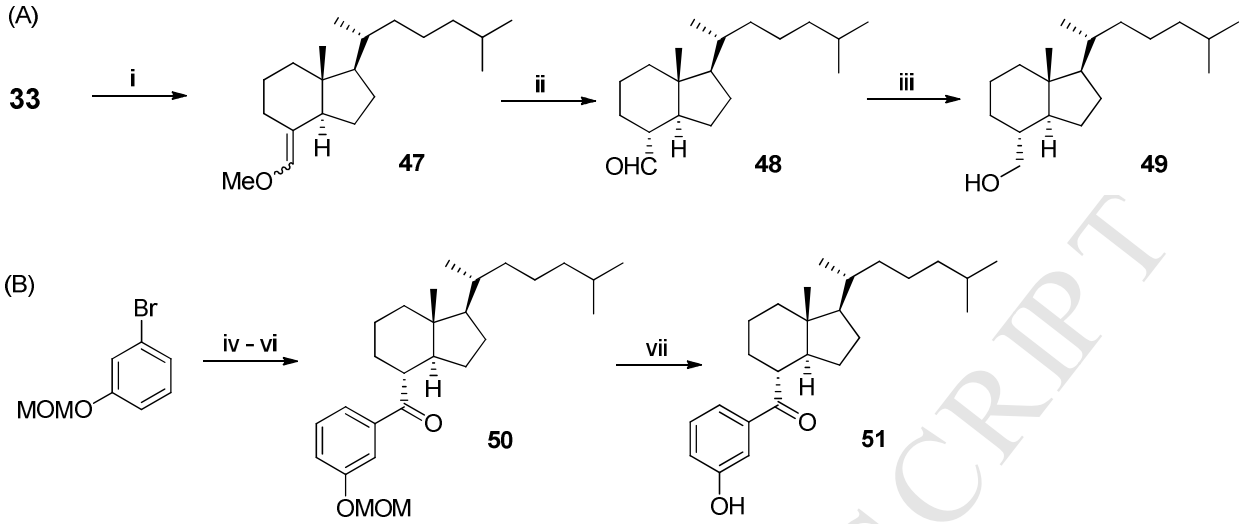
Scheme 3. Strategies for the preparation of epimeric analogues of 3: Subclass II. Reagents and conditions: (i) HMDS, LiI, TMSCl, DCM; (ii) PhSeCl, pyridine, THF; (iii) *m*-CPBA (80%), DCM; (iv) $NaBH_4$, $CeCl_3$, MeOH; (v) $Rh(Ph_3P)_3Cl$, H_2, benzene; (vi) Na, Amberlite IR120, Et_2O:EtOH (9:1), 0 ºC.



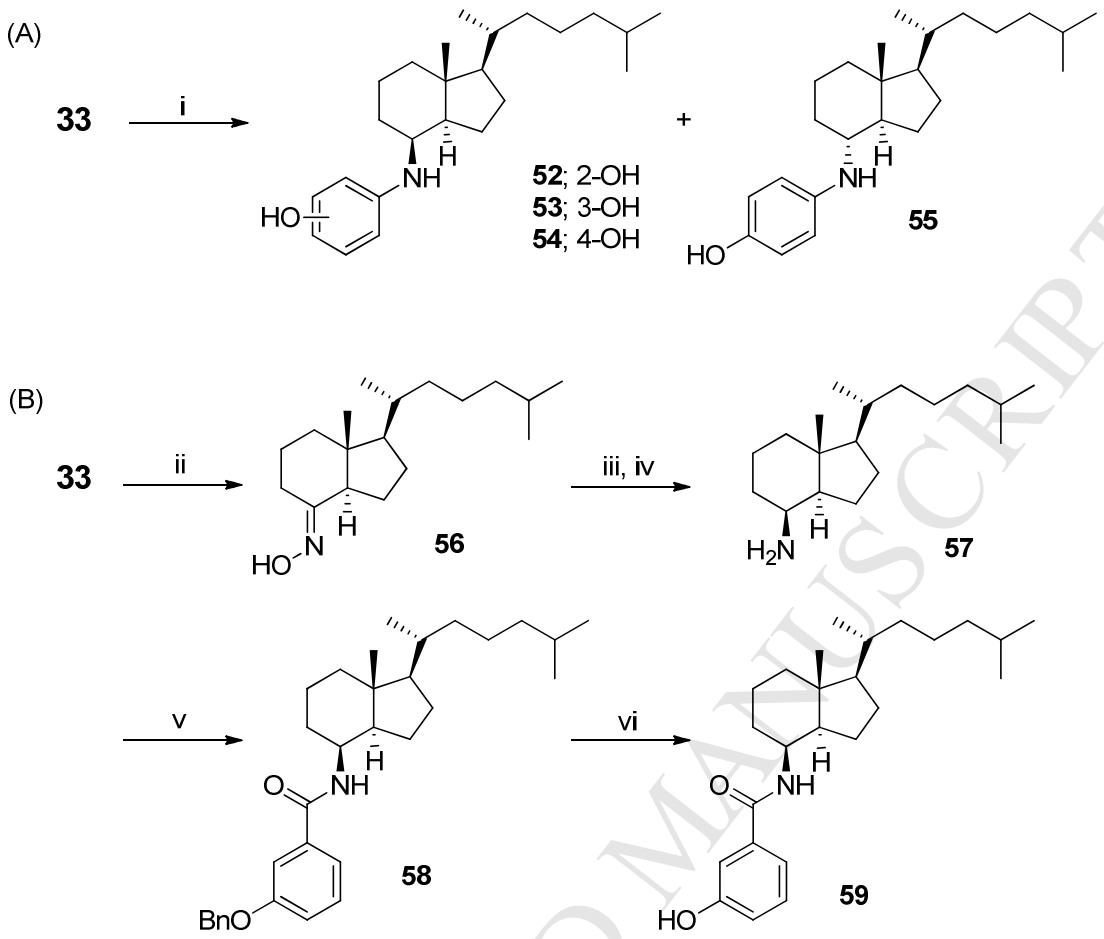
Scheme 4. Preparation of **40** (epi-**4**). Reagents and conditions: (i) DCC, DMAP, 3-benzyloxy-benzoic acid, DCM; (ii) $Pd(OH)_2$ (10%), H_2, MeOH:THF (2:1).



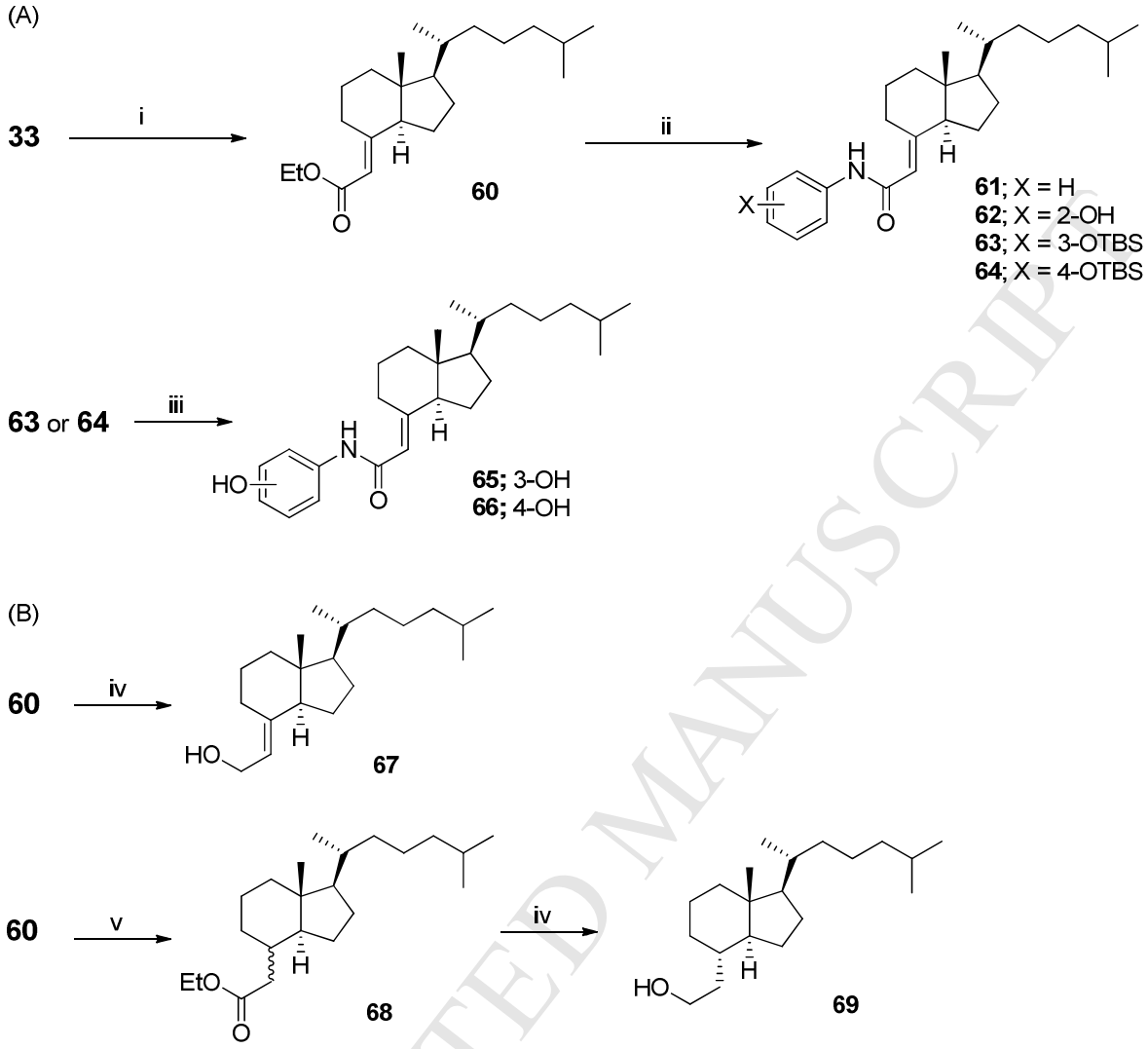
Scheme 5. Preparation of "linkerless" analogues: Subclass III. Reagents and conditions: (i) *n*-BuLi, THF, -78 ºC; (ii) **33**; (iii) ±CSA, MeOH; (iv) Burgess reagent, benzene, *reflux;* (v) Pd/C (10%), H_2, EtOAc.



Scheme 6. Preparation of carbon-based linker analogues: Subclass IV. Reagents and conditions: (i) $[Ph_3PCH_2OMe]Cl$, NaO^tBu, THF, 0 ºC; (ii) 3N HCl, THF, *reflux*, (iii) $NaBH_4$, DCM:MeOH (2:1); (iv) *n*-BuLi, THF, -78 ºC; (v) **48**; (vi) PDC, DCM; (vii) ±CSA, MeOH.



Scheme 7. Preparation of amine- and amide-based linker analogues: Subclass V. Reagents and conditions: (i) aminophenol, $NaBH(OAc)_3$, AcOH, 1,2-dichloroethane; (ii) NH_2OH, pyridine; (iii) $LiAlH_4$, THF, *reflux*; (iv) Zn, AcOH, *reflux*; (v) EDCI, DMAP, 3-benzyloxy-benzoic acid, DCM; (vi) Pd/C (10%), H_2, MeOH:THF (3:1).



Scheme 8. Preparation of enamide and extended hydroxyl analogues: Subclass VI. Reagents and conditions: (i) $EtO_2P(O)CH_2CO_2Et$, KHMDS, THF:toluene (4:1), 0 ºC; (ii) $AlMe_3$, aminophenol, K_2CO_3, DCM, 0 ºC; (iii) ±CSA, MeOH; (iv) $LiAlH_4$, THF, 0 ºC; (v) Pd/C (10%), H_2, EtOAc.

Table 1. Hh inhibition for extended ester-linked, phenolic analogues.

Analogue[a]	Gli1 mRNA (%)[b]	Cyp24A1 mRNA[c]
DMSO	1.0	1.0
OHCs	100	---
VD3	35.7 ± 0.3	8336 ± 38
2	46.4 ± 3.5	3.6 ± 0.5
4	1.9 ± 0.5	22.2 ± 4.7
23	1.8 ± 0.1	24 ± 4.1
24	47.4 ± 0.4	10.3 ± 2.9
25	15.3 ± 10	23 ± 8.2
26	20.1 ± 15	21 ± 10
30	94.2 ± 23	3.3 ± 1.2
31	57.2 ± 1.1	4.9 ± 2.0
32	62.1 ± 15	8.3 ± 1.7

[a]All analogues evaluated at 5 μM and 24 h.
[b]Values represent % Gli1 mRNA expression relative to OHC control (set as 100%).
[c]Values represent up-regulation of Cyp24A1 mRNA normalized to DMSO (set to 1.0).

Table 2. Hh inhibition for isomeric analogues of **3** and **4**.

Analogue[a]	Gli1 mRNA (%)[b]	Cyp24A1 mRNA[c]
3	46.4 ± 3.5	3.6 ± 0.5
33	66.1 ± 0.9	3.0 ± 0.4
35	63.4 ± 2.2	1.2 ± 0.1
36	90.1 ± 1.7	1.1 ± 0.1
37	74.0 ± 6.8	1.8 ± 0.3
38	48.7 ± 18	1.5 ± 0.6
40	30.6 ± 3.1	3.0 ± 0.5

[a]All analogues evaluated at 5 μM and 24 h.
[b]Values represent % Gli1 mRNA expression relative to OHC control (set as 100%).
[c]Values represent up-regulation of Cyp24A1 mRNA normalized to DMSO (set to 1.0).

Table 3. Hh inhibition for 'linkerless' and one carbon linker analogues.

Analogue[a]	Gli1 mRNA (%)[b]	Cyp24A1 mRNA[c]
42	39.5 ± 1.5	2.0 ± 0.8
44	37.2 ± 1.8	7.1 ± 1
46	47.9 ± 6.2	3.8 ± 1
49	55.1 ± 7.5	2.4 ± 0.1
51	11.0 ± 5.0	4.4 ± 0.5
60	107 ± 9.1	1.3 ± 0.5
67	15.1 ± 7.0	28 ± 10
69	2.8 ± 0.6	18.6 ± 2.4

[a]All analogues evaluated at 5 μM and 24 h.
[b]Values represent % Gli1 mRNA expression relative to OHC control (set as 100%).
[c]Values represent up-regulation of Cyp24A1 mRNA normalized to DMSO (set to 1.0).

Table 4. Hh inhibition for nitrogen-based linker analogues.

Analogue[a]	Gli1 mRNA (%)[b]	Cyp24A1 mRNA[c]
52	2.0 ± 0.2	11.6 ± 1.5
53	11.6 ± 0.01	17.0 ± 3.9
54	0.7 ± 0.2	45.3 ± 19
55	1.3 ± 0.6	20.6 ± 3.6
56	86.1 ± 6.2	2.6 ± 0.9
59	2.5 ± 0.1	7.0 ± 0.9
61	41.7 ± 16	3.5 ± 0.1
62	1.1 ± 0.5	5.6 ± 1.6
65	12.1 ± 0.6	8.9 ± 0.4
66	1.8 ± 0.4	26 ± 10

[a]All analogues evaluated at 5 μM and 24 h.
[b]Values represent % Gli1 mRNA expression relative to OHC control (set as 100%).
[c]Values represent up-regulation of Cyp24A1 mRNA normalized to DMSO (set to 1.0).

Table 5. Secondary Hh inhibition and selectivity analysis for potent analogues.

Analogue	C3H10T1/2	ASZ		VDR Binding[c]
	Gli1[a]	Gli1[a]	Cyp24A1[b]	
VD3	4.1 ± 0.3	2.1 ± 0.1	764 ± 24	>100
4	0.74 ± 0.1	5.2 ± 0.2	1.9 ± 0.1	>100
23	0.79 ± 0.003	6.7 ± 1.0	3.5 ± 1.9	>100
25	2.6 ± 0.3	2.3 ± 0.6	7.6 ± 0.05	>100
40	3.9 ± 0.8	6.3 ± 1.1	1.6 ± 0.2	>100
51	1.8 ± 0.4	5.8 ± 0.7	7.8 ± 2	>100
52	1.2 ± 0.03	1.1 ± 0.1	4.4 ± 0.9	>100
53	1.0 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	>100
54	0.40 ± 0.02	0.67 ± 0.2	2.7 ± 0.7	>100
55	0.32 ± 0.06	0.57 ± 0.2	3.0 ± 0.8	>100
59	0.98 ± 0.04	1.8 ± 0.3	1.8 ± 0.2	>100
62	1.5 ± 0.3	1.7 ± 0.3	14.0 ± 4.0	>100
65	1.0 ± 0.2	1.7 ± 0.3	4.5 ± 0.2	>100
66	1.2 ± 0.4	1.3 ± 0.1	4.4 ± 1.4	>100
69	2.4 ± 0.5	7.1 ± 1.0	1.3 ± 0.4	>100

[a]IC_{50} values (μM) represent the Mean ± SEM of at least two separate experiments performed in triplicate.
[b]Values represent Cyp24A1 expression relative to DMSO control (48 h time points: value for VD3 at 2.5 μM treatments; values for all analogues at 5 μM treatments).
[c]Values represent binding affinity (μM) for VD3 and VD3 analogues from at least two separate experiments. Calcitriol (concentration response) is used as a positive control for VDR binding.

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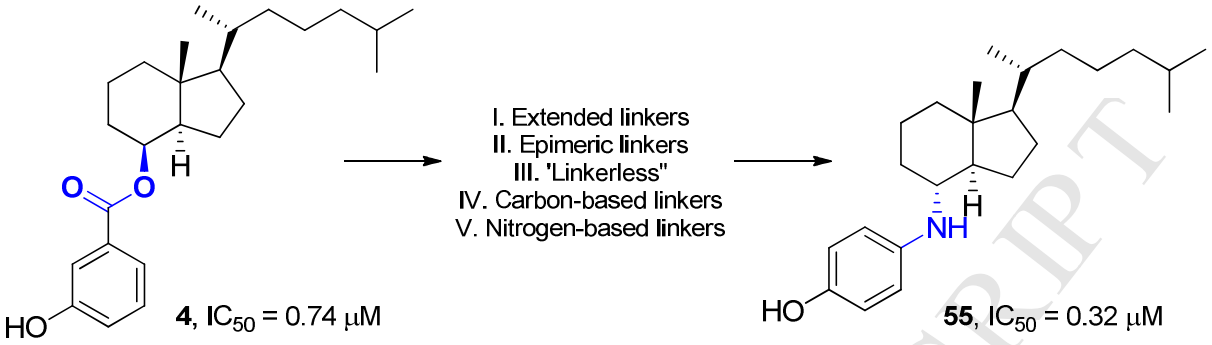

Highlights

- The Hedgehog (Hh) signaling pathway is an important anti-cancer drug target.
- Vitamin D3 has been identified as an Hh pathway inhibitor.
- Vitamin D3 analogues that modify the A-ring and seco-B-ring were evaluated.
- Several of these analogues are potent and selective inhibitors of Hh signaling.

Vitamin D3 Analogues that Contain Modified A- and Seco-B-Rings as Hedgehog Pathway Inhibitors.

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Supporting Information – Biological Assay Protocols, 1H and 13C NMR Spectra

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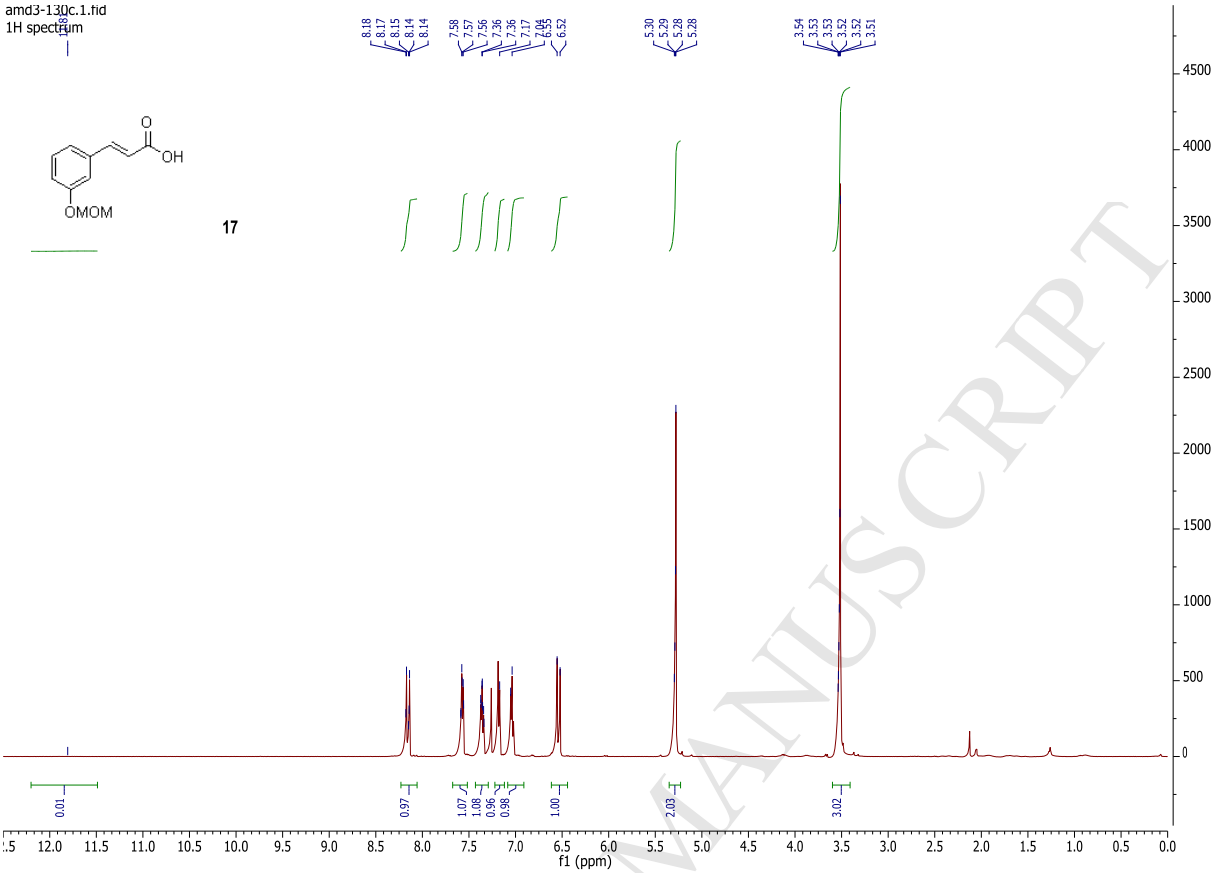
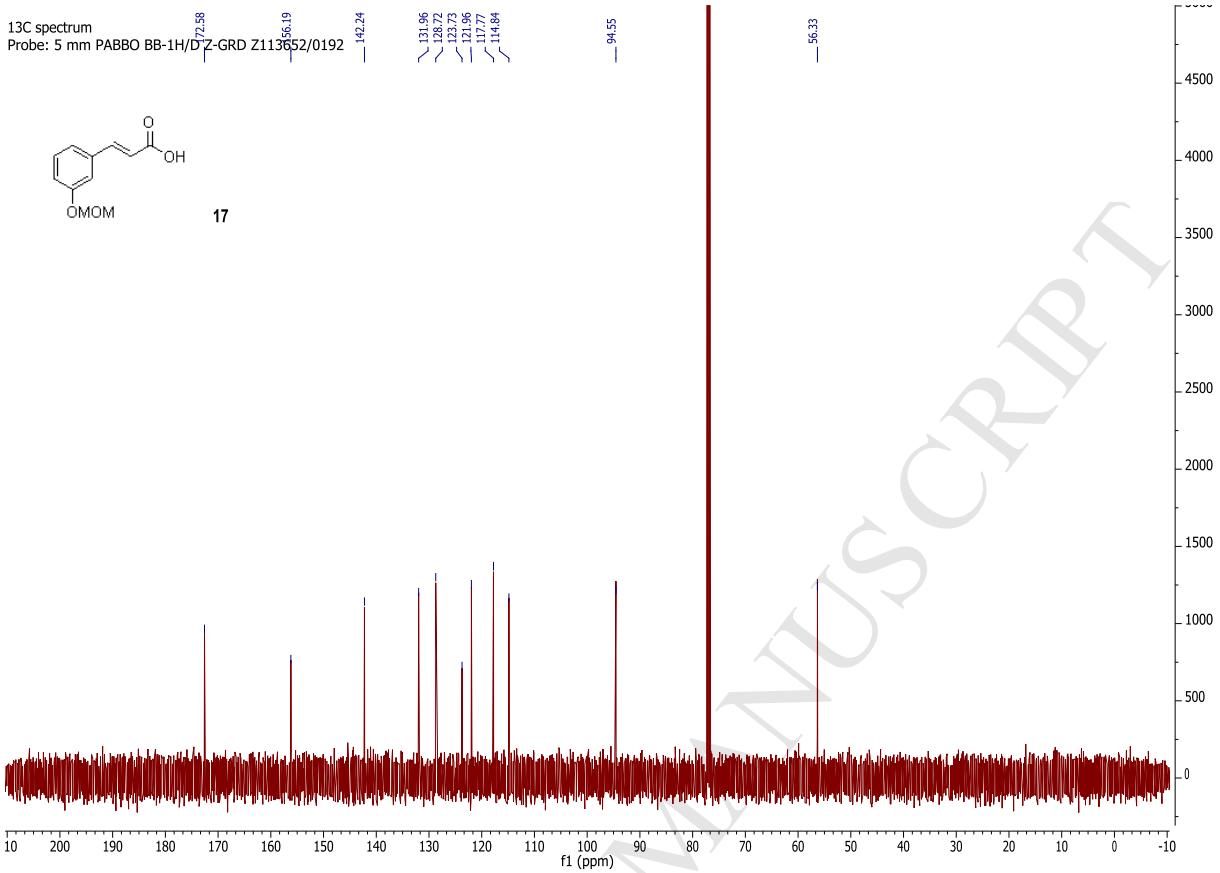
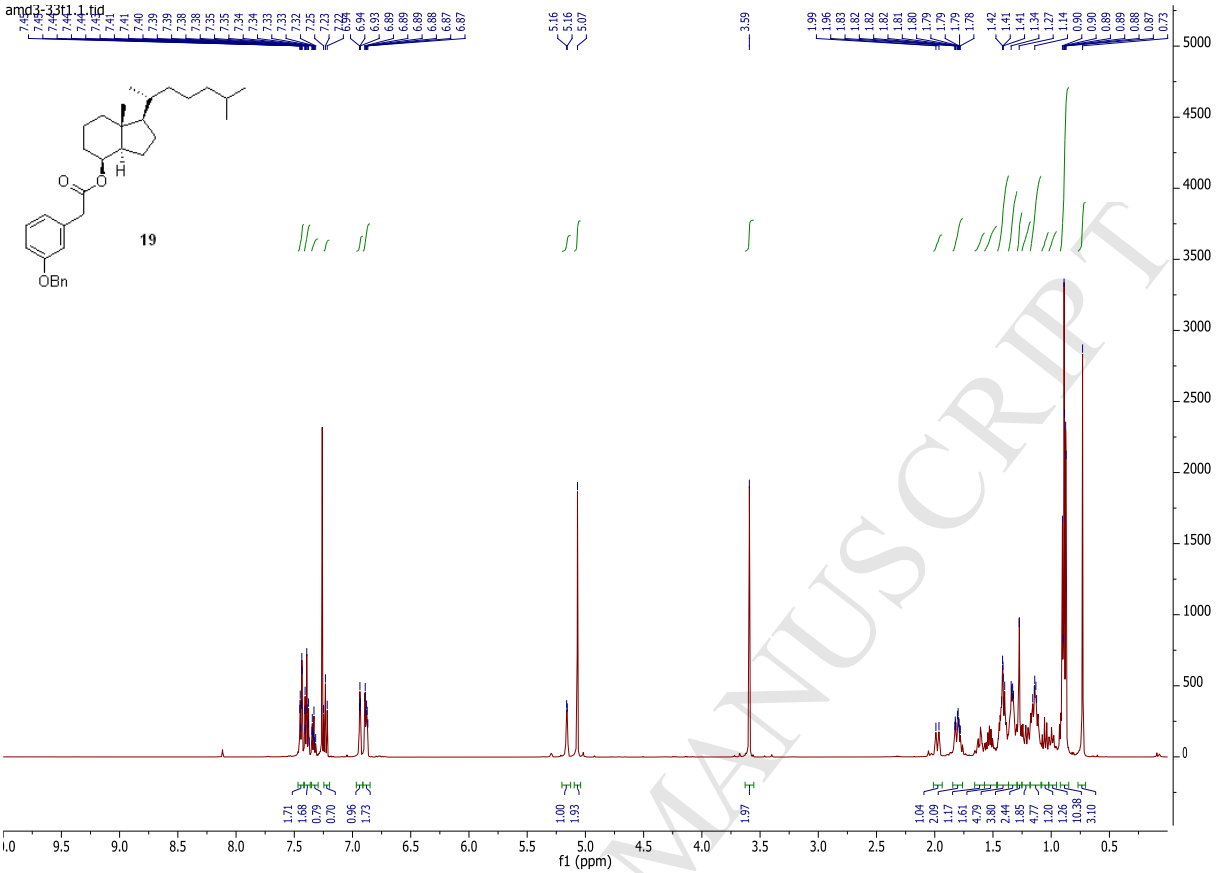
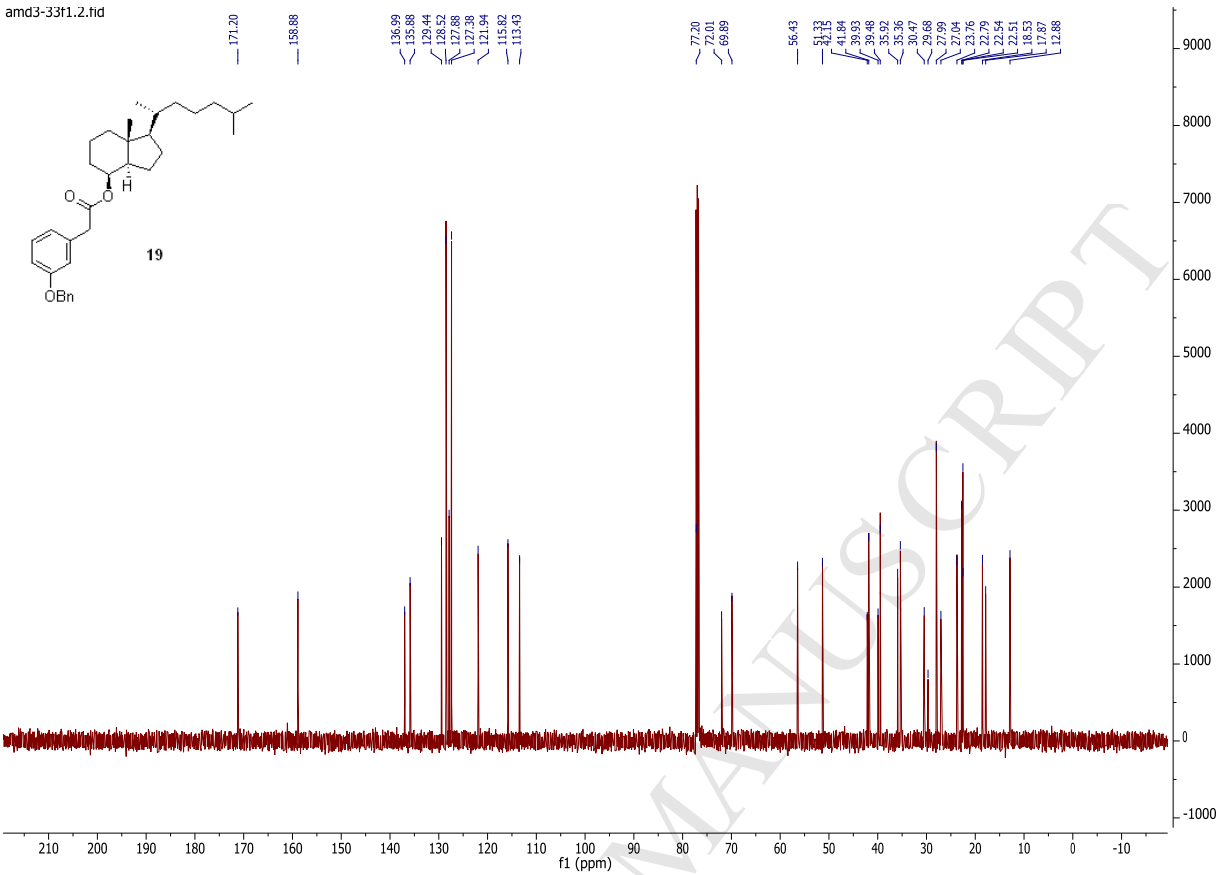
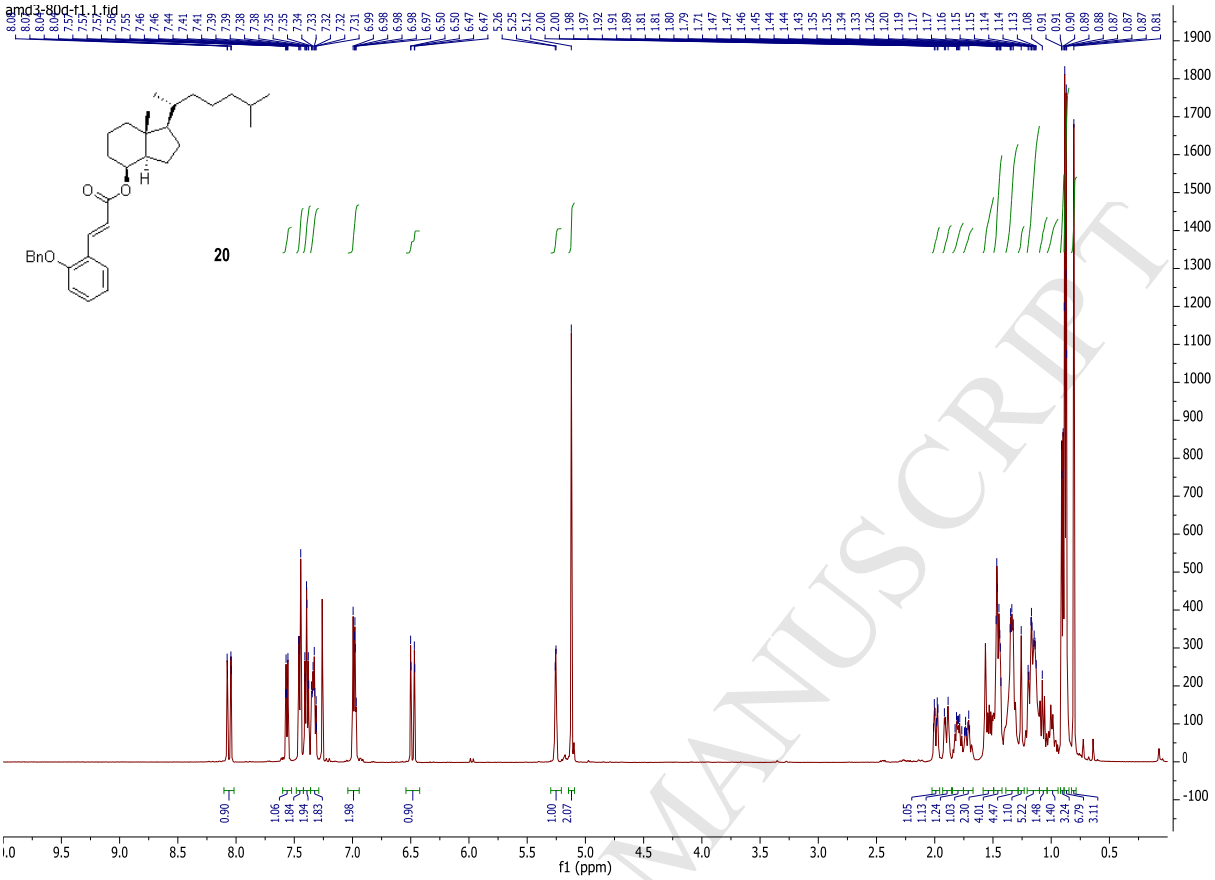
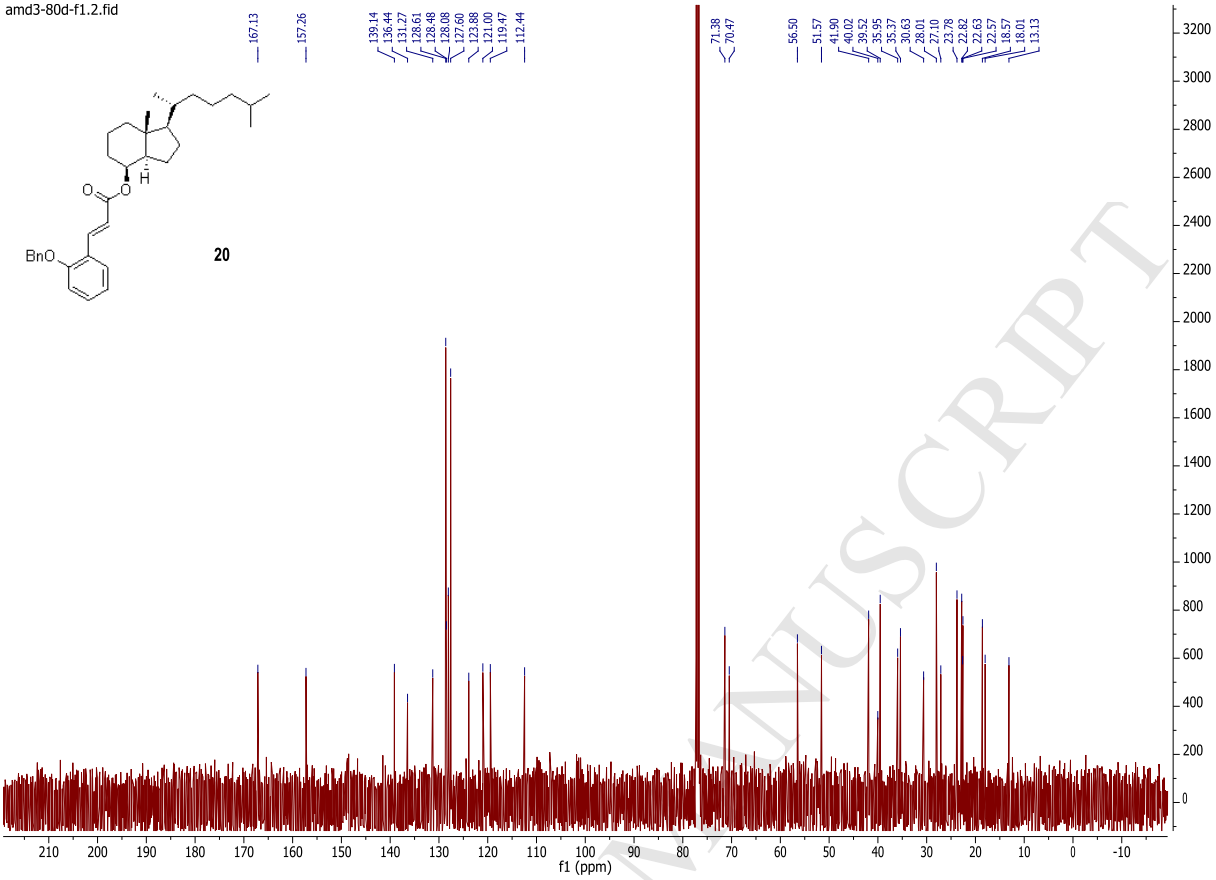


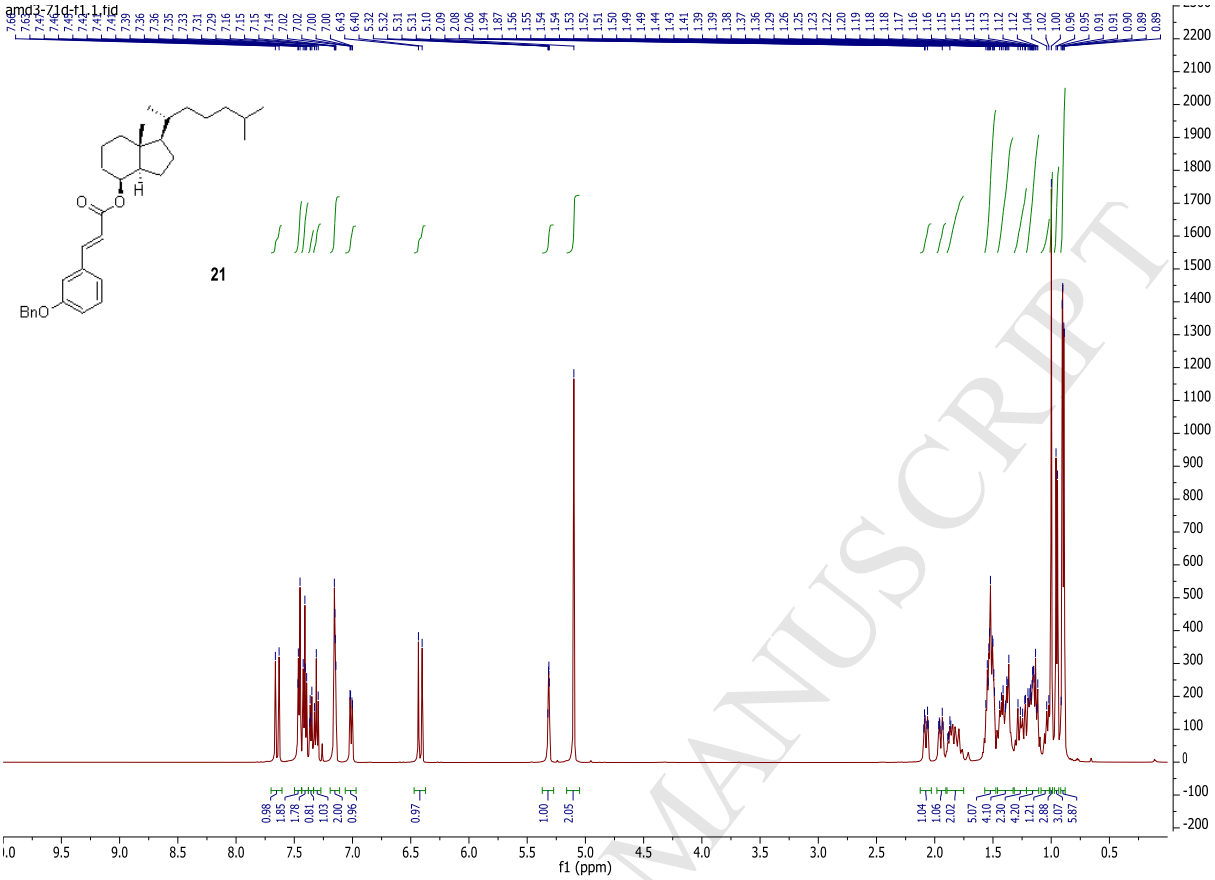


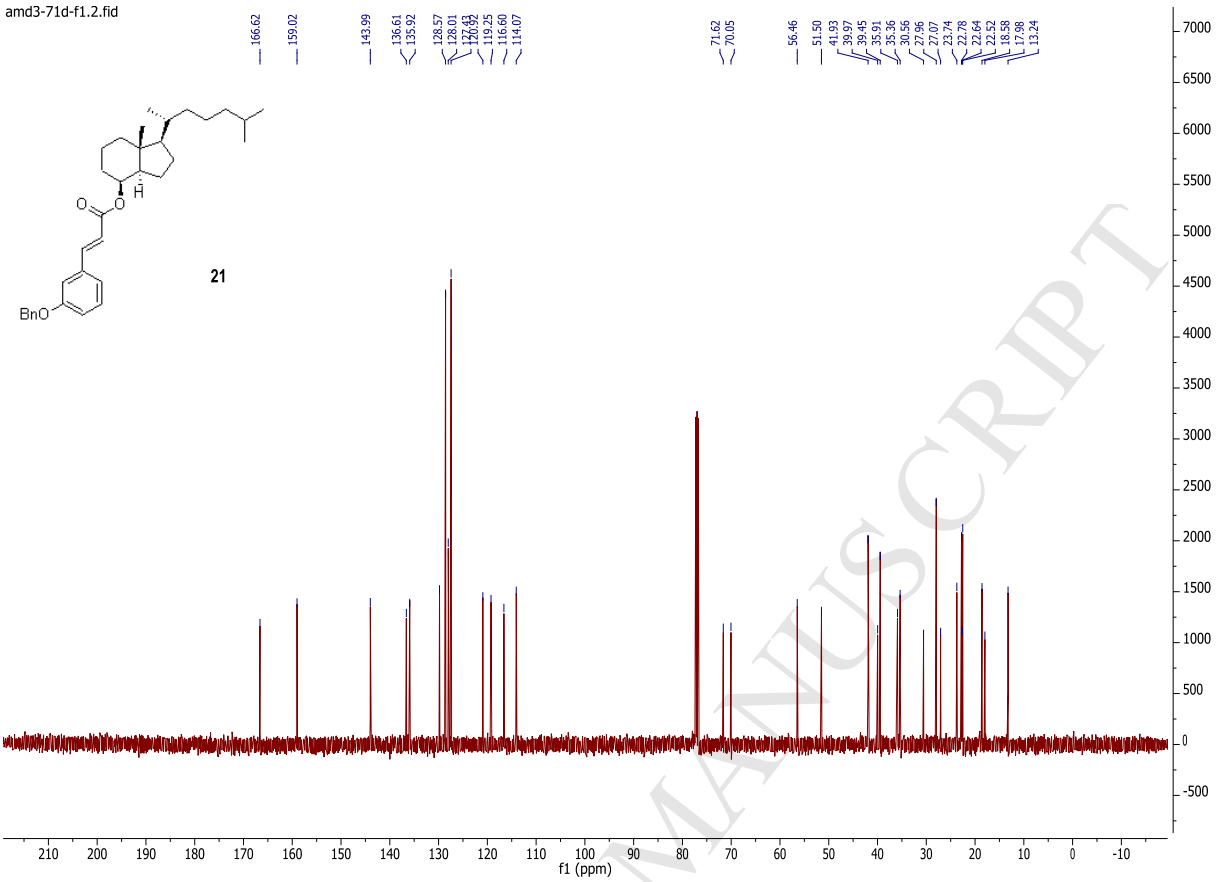


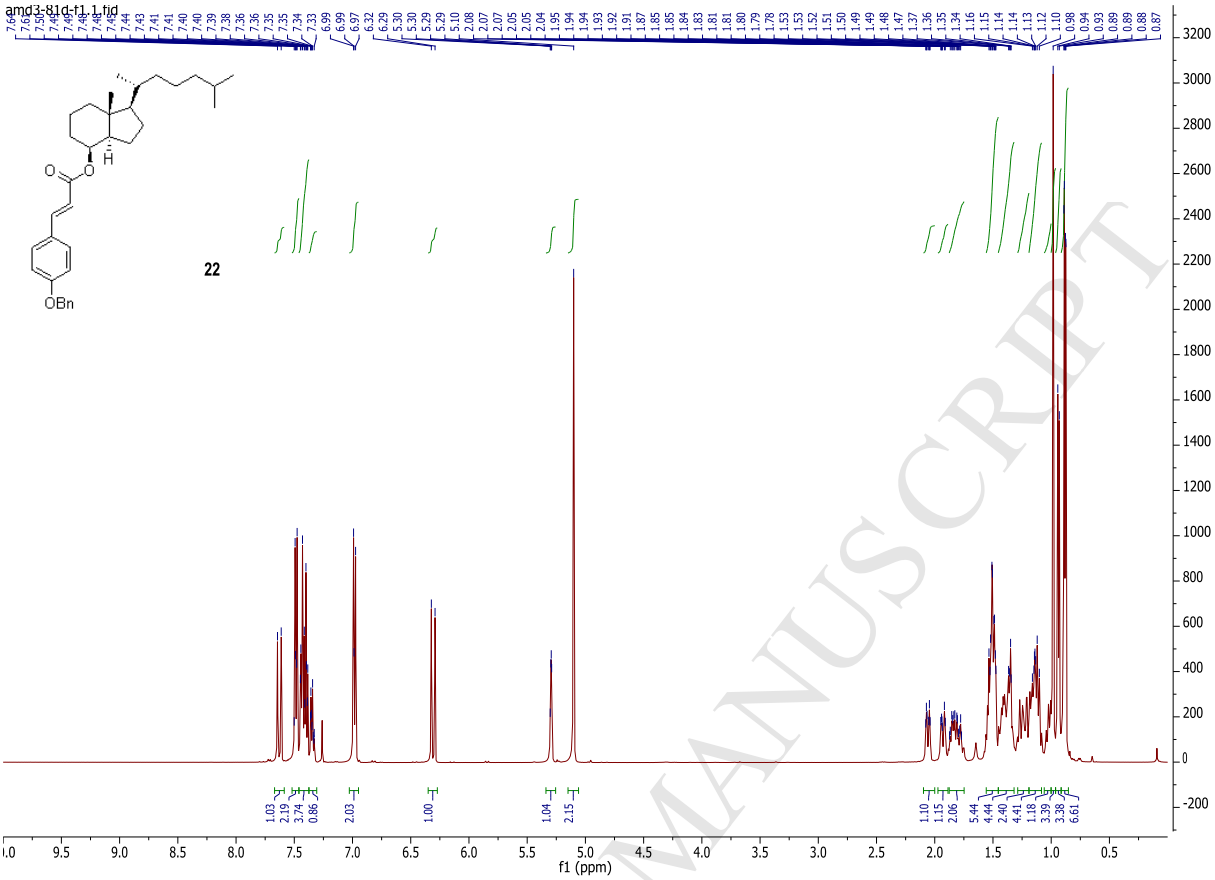


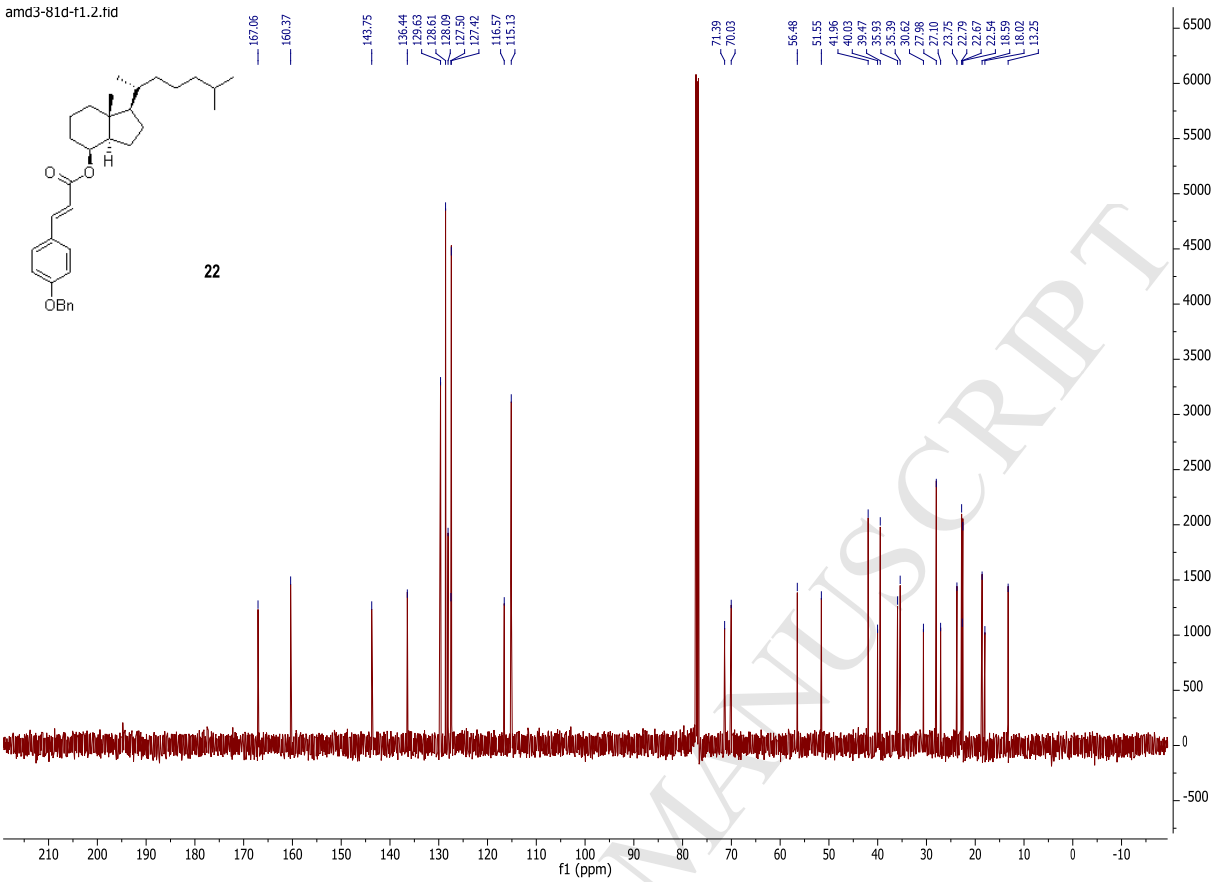


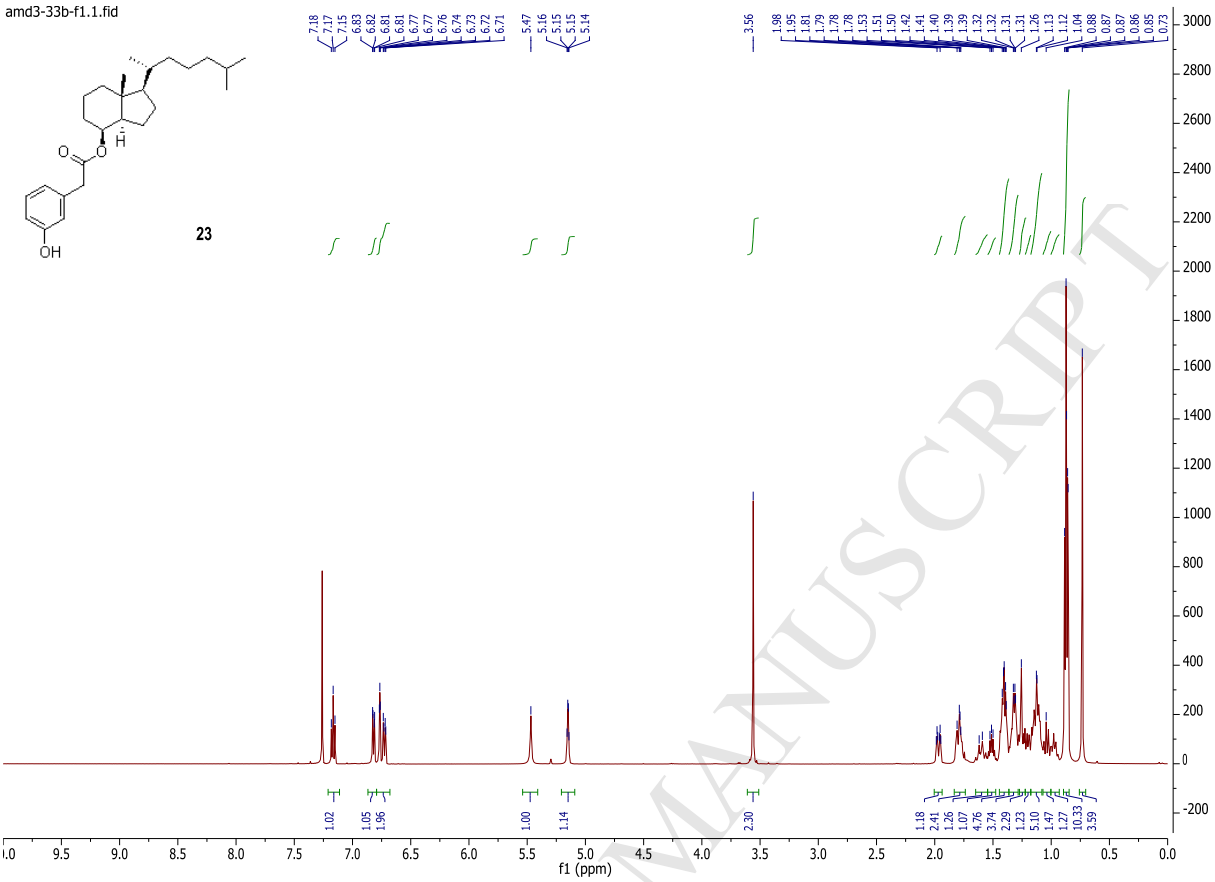


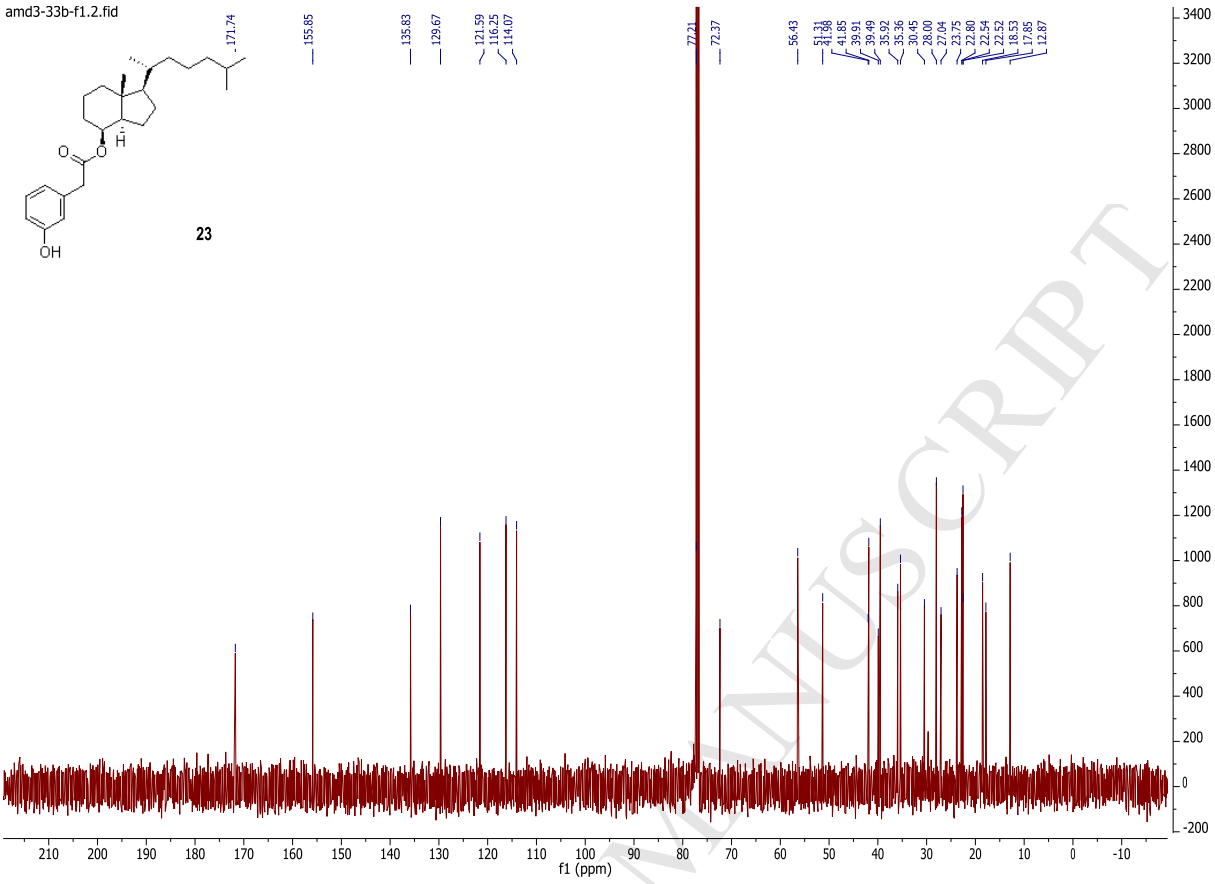


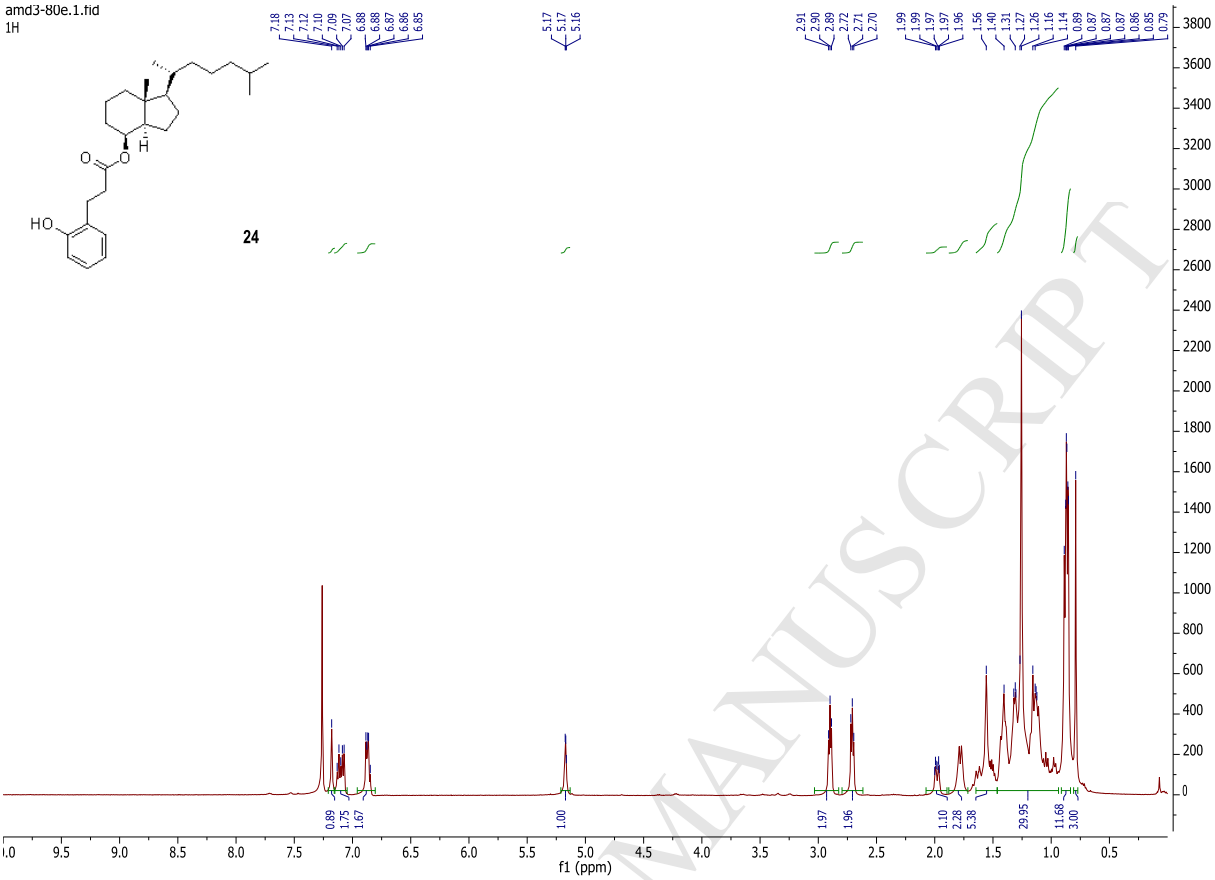


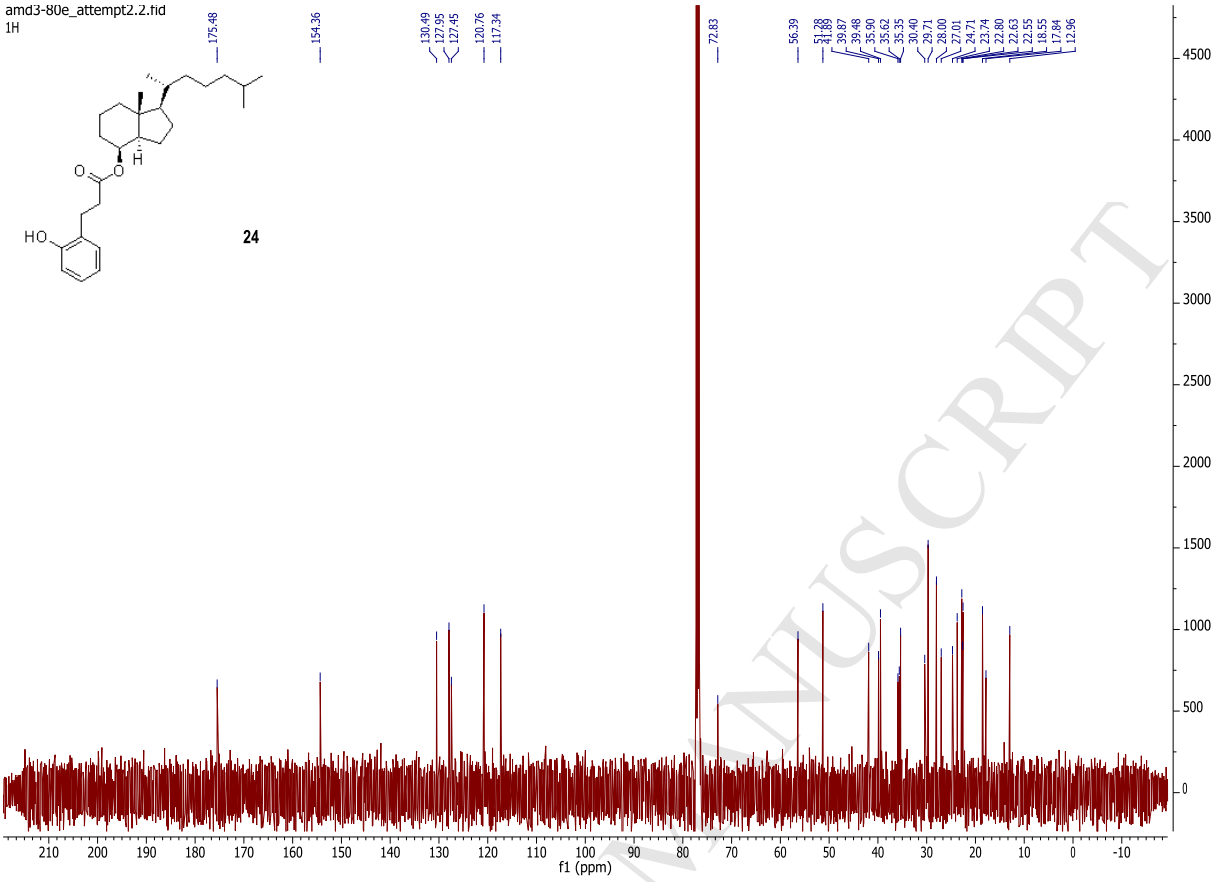


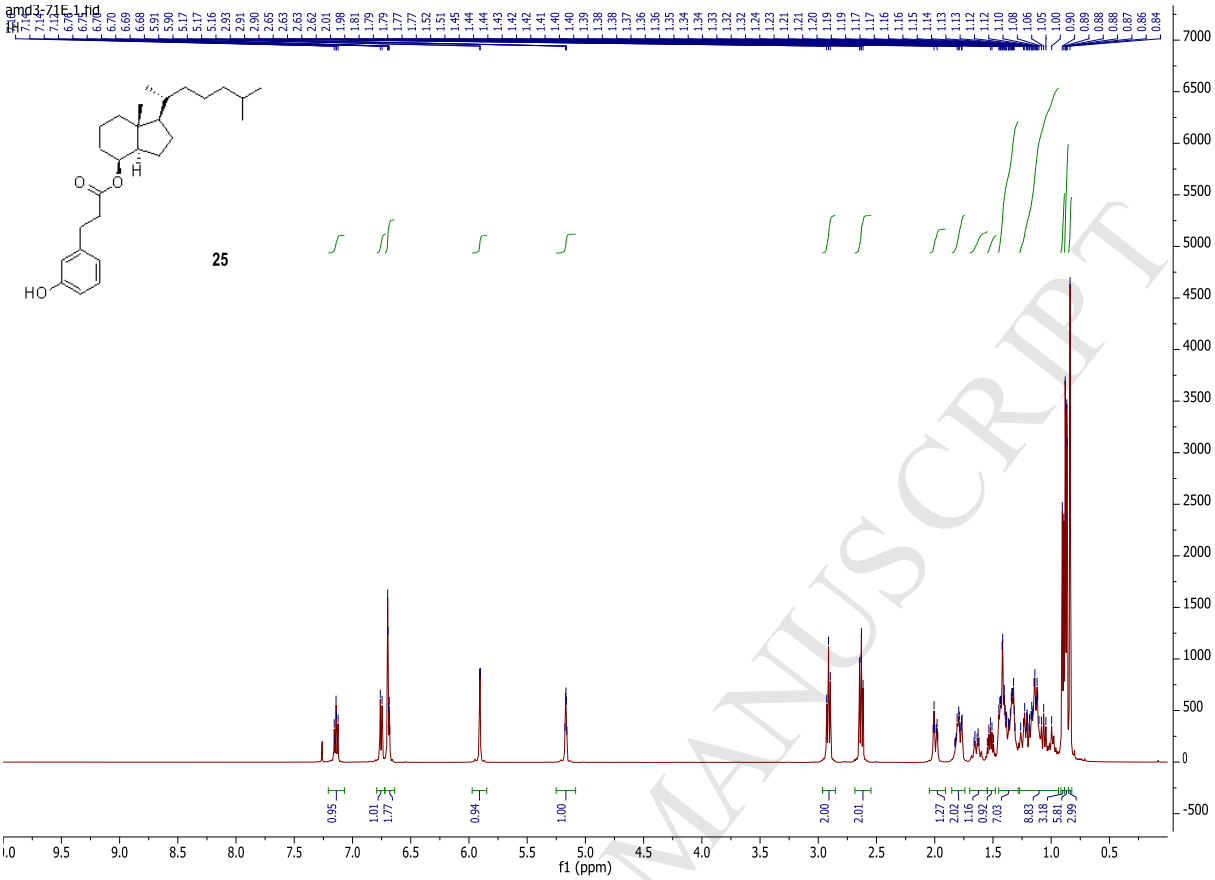


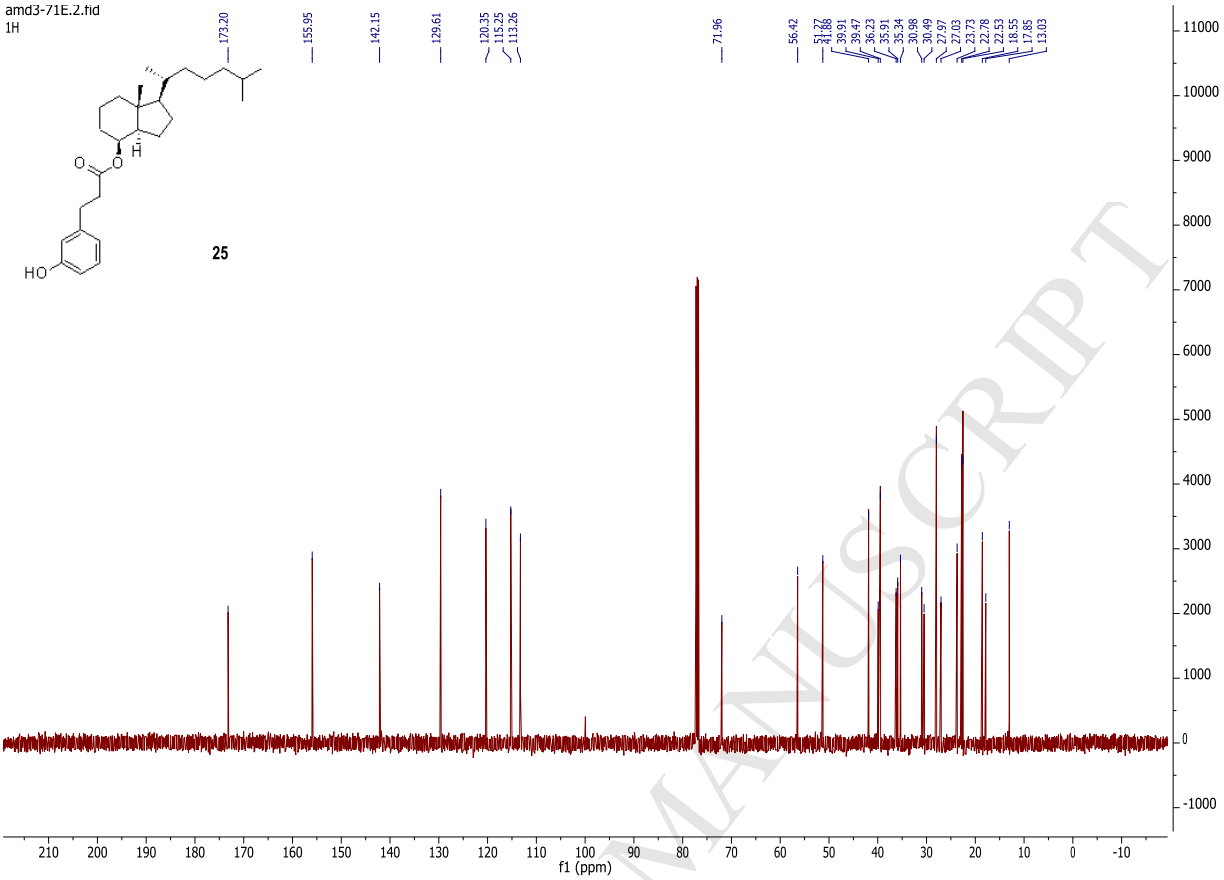


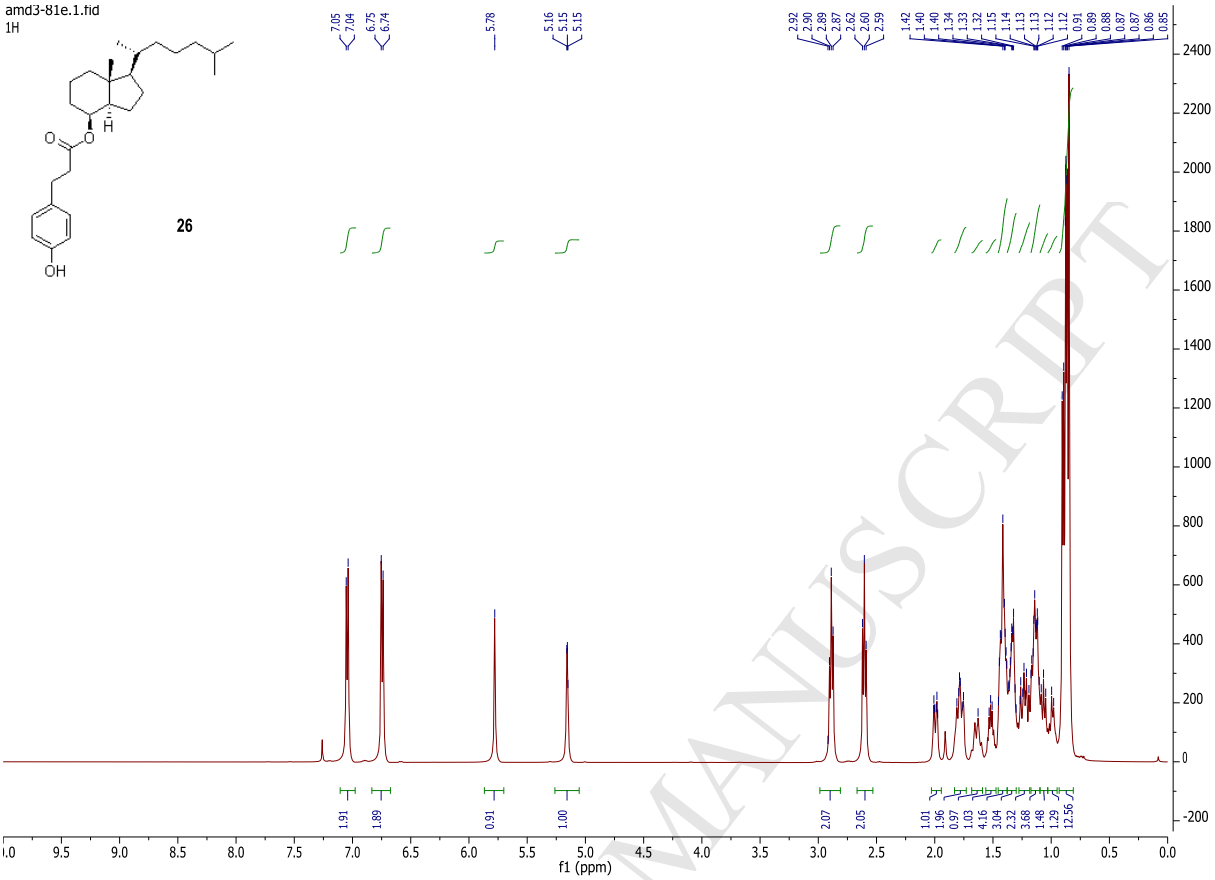


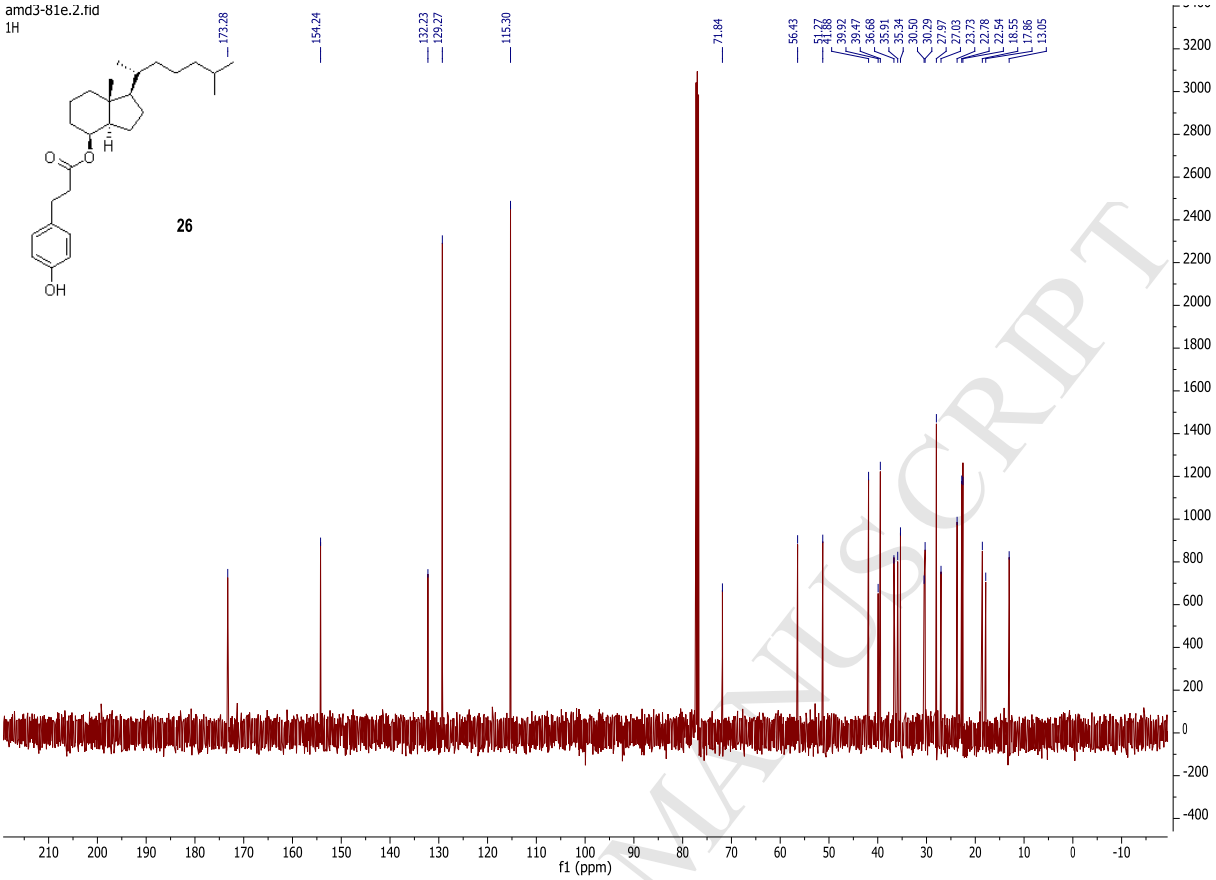


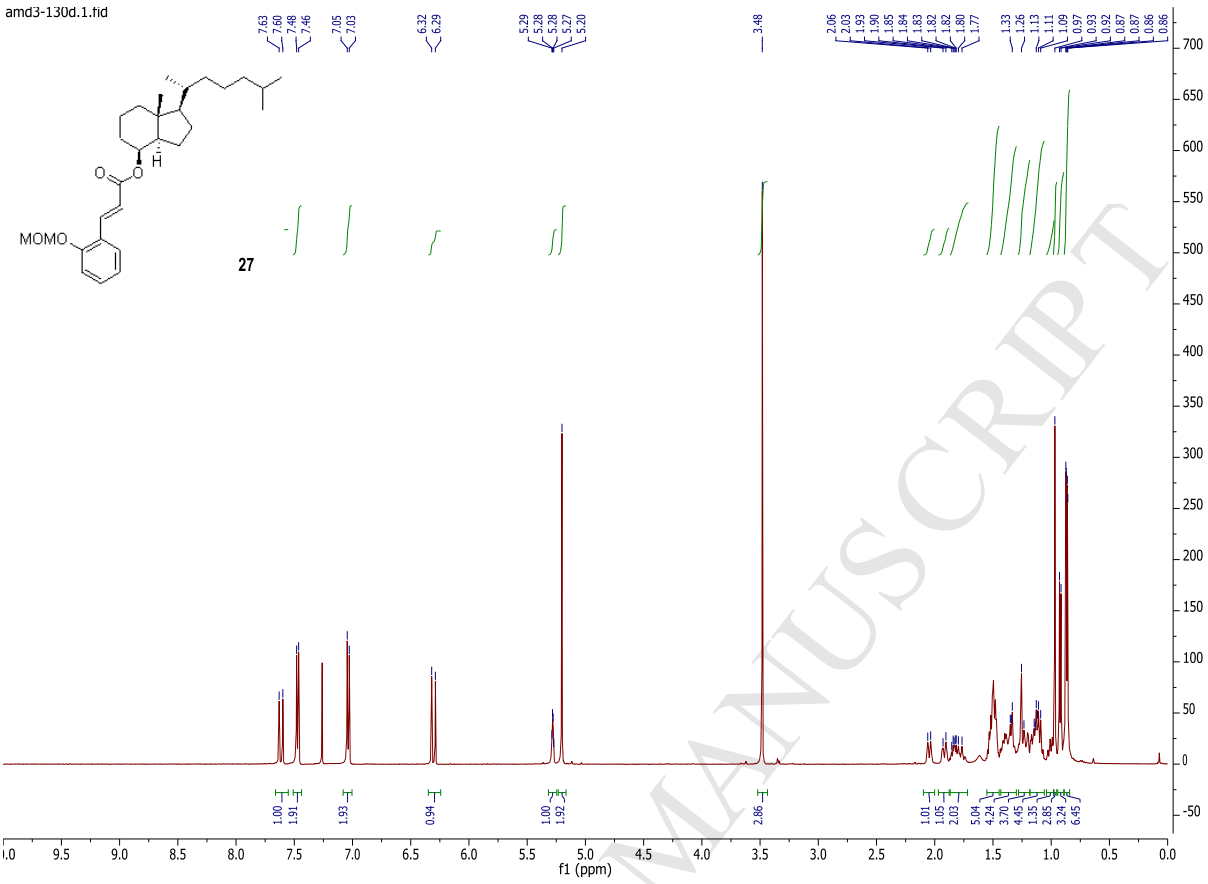


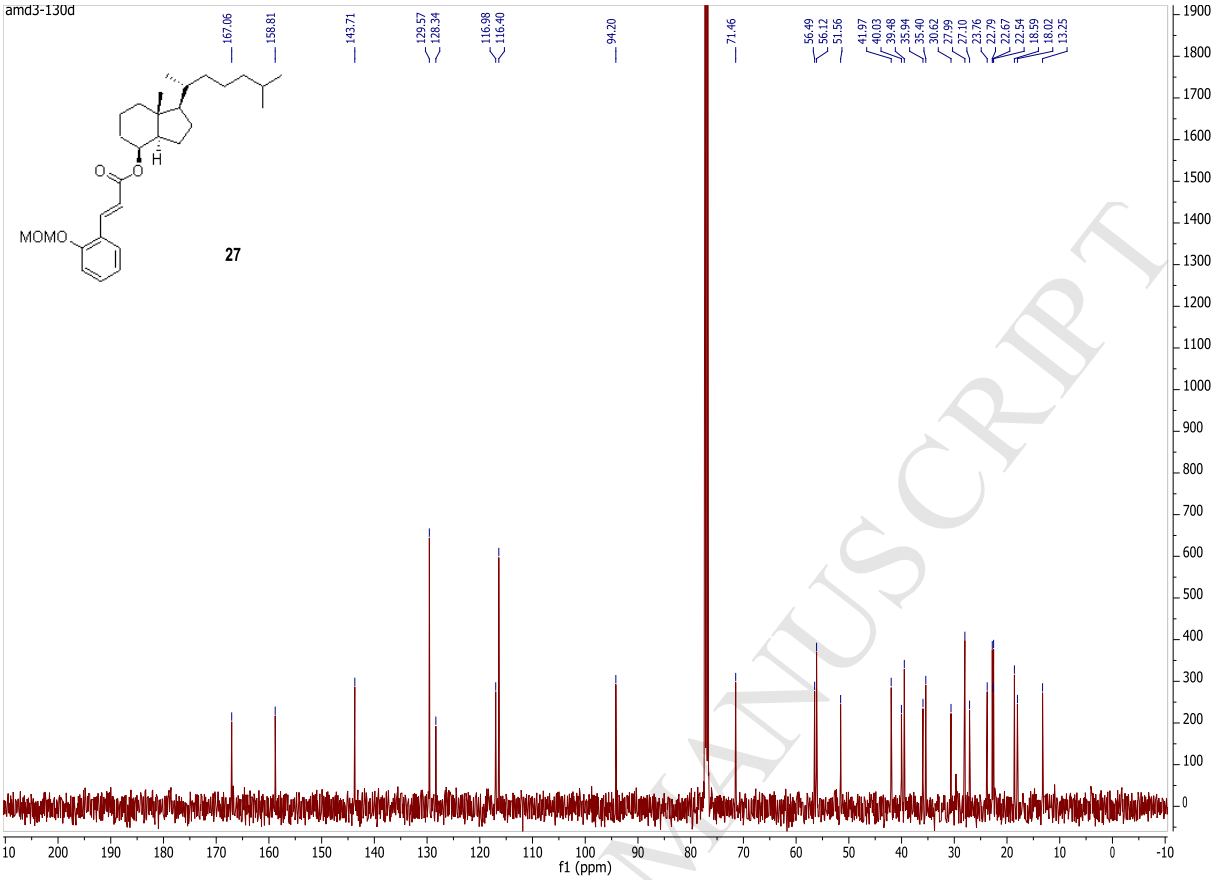


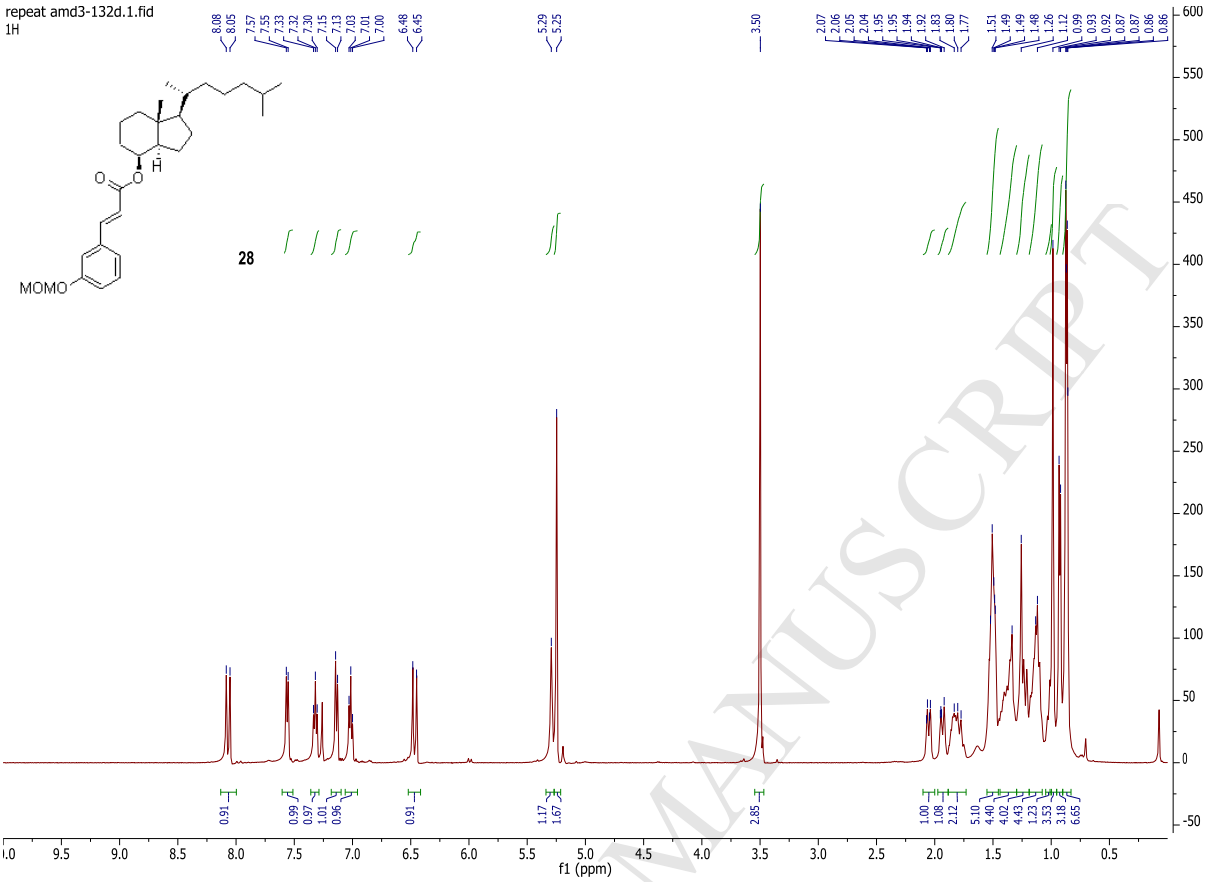


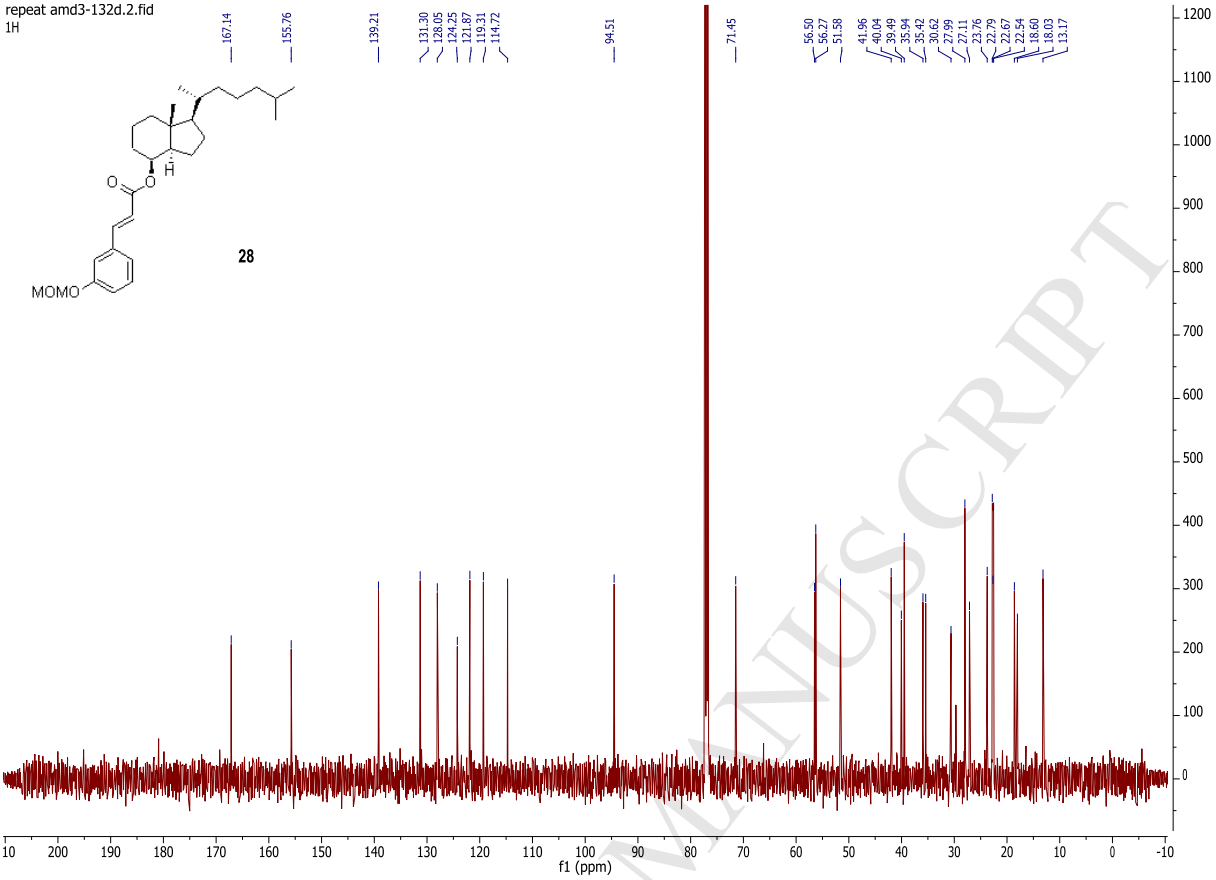


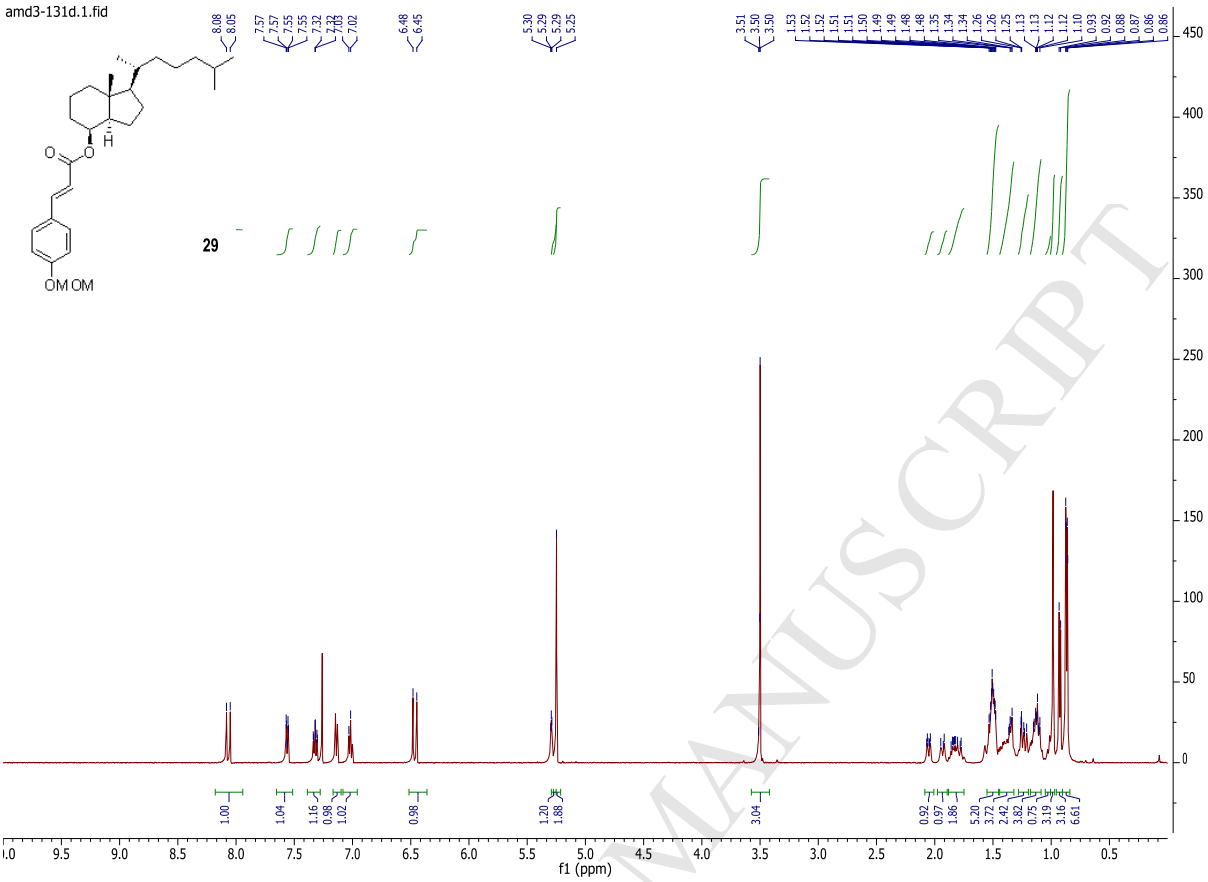


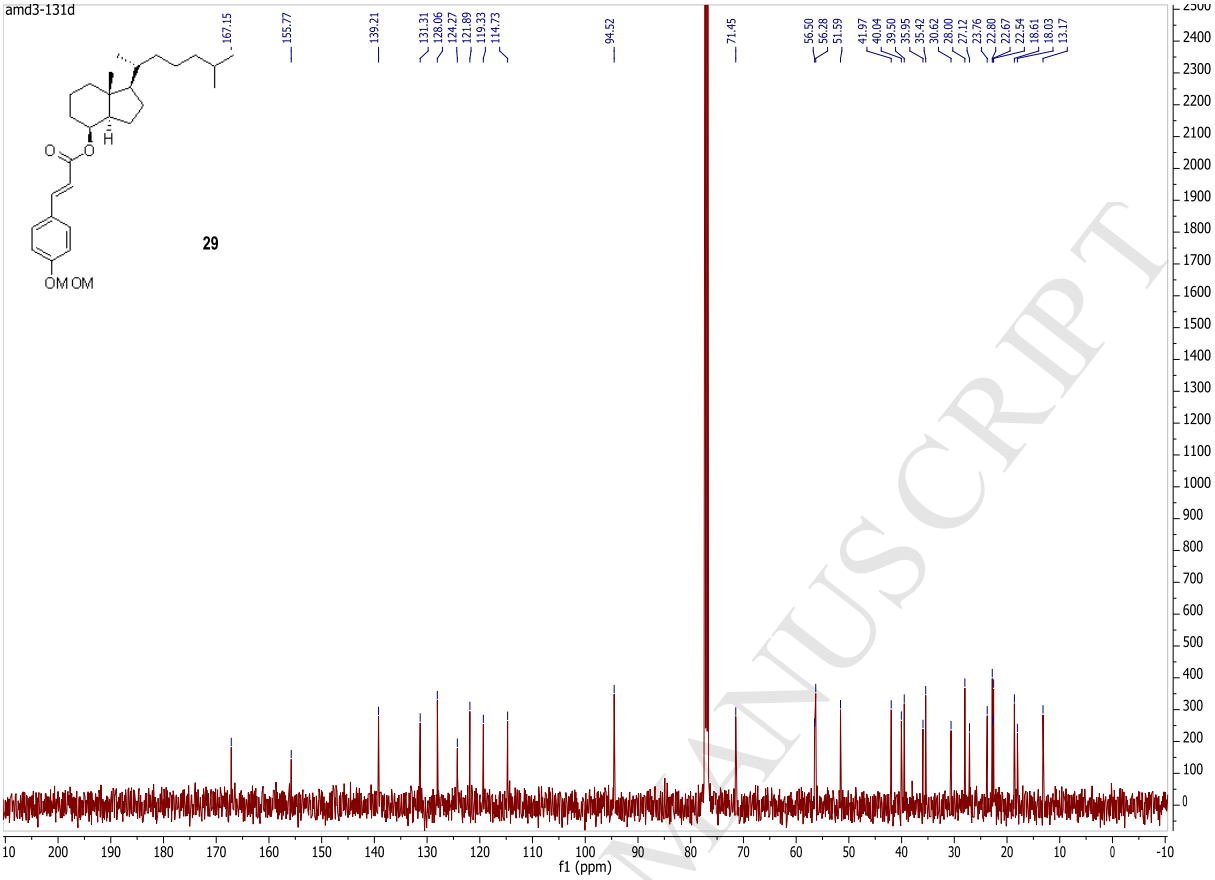


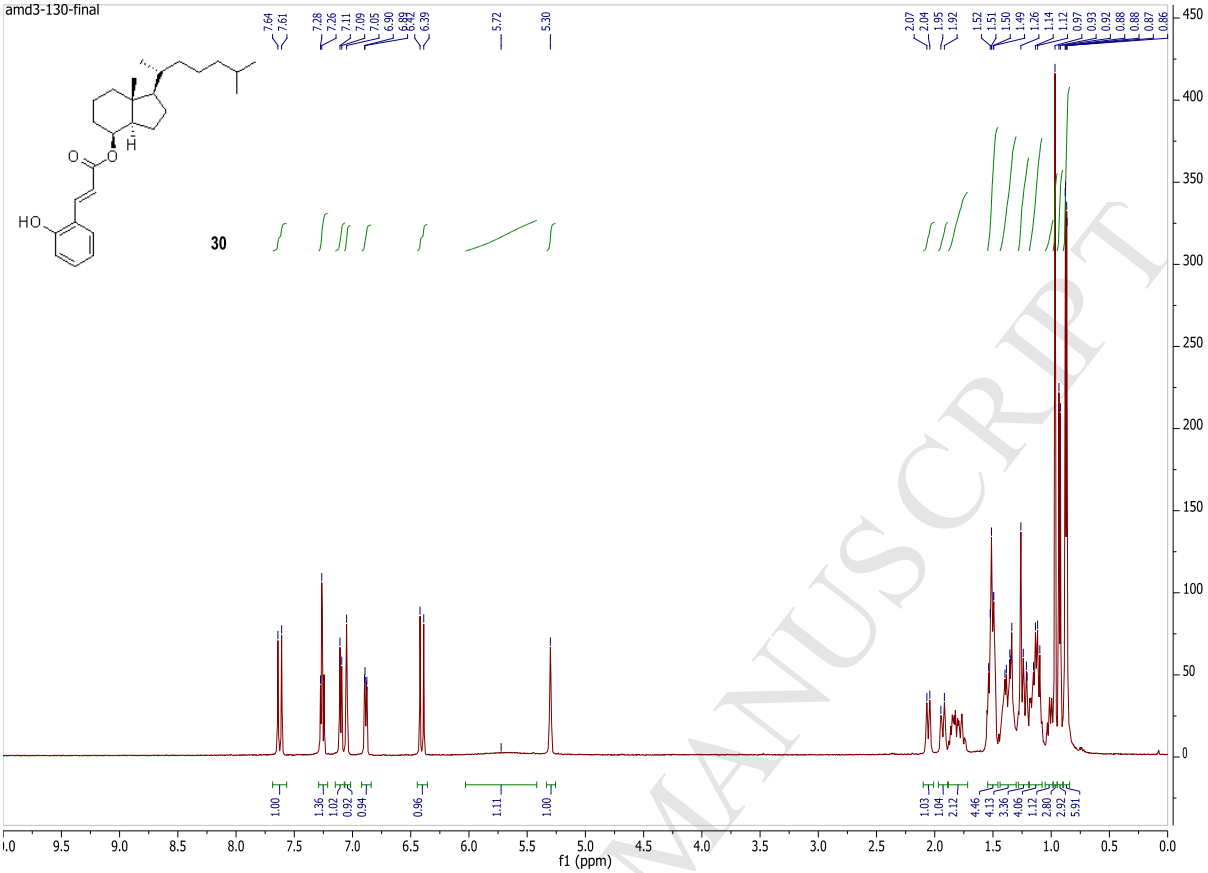


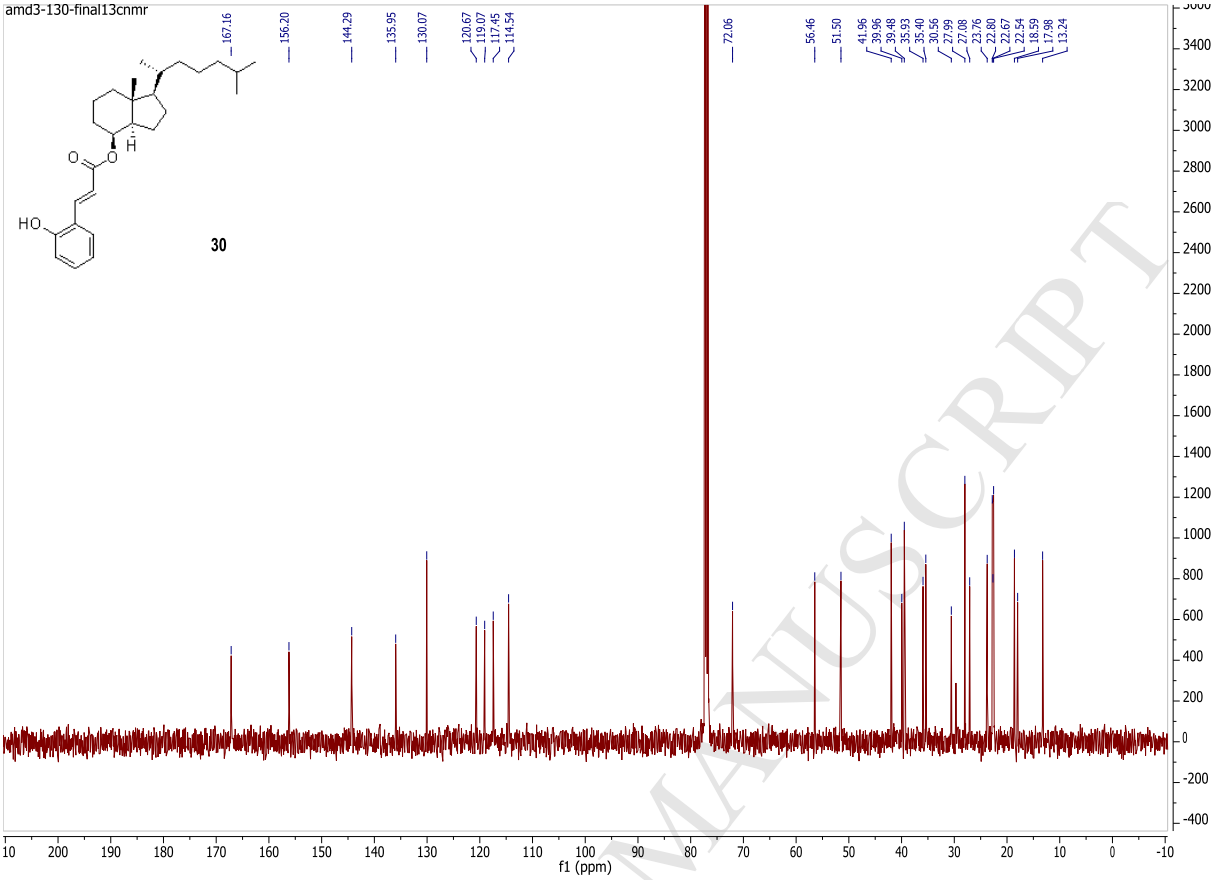


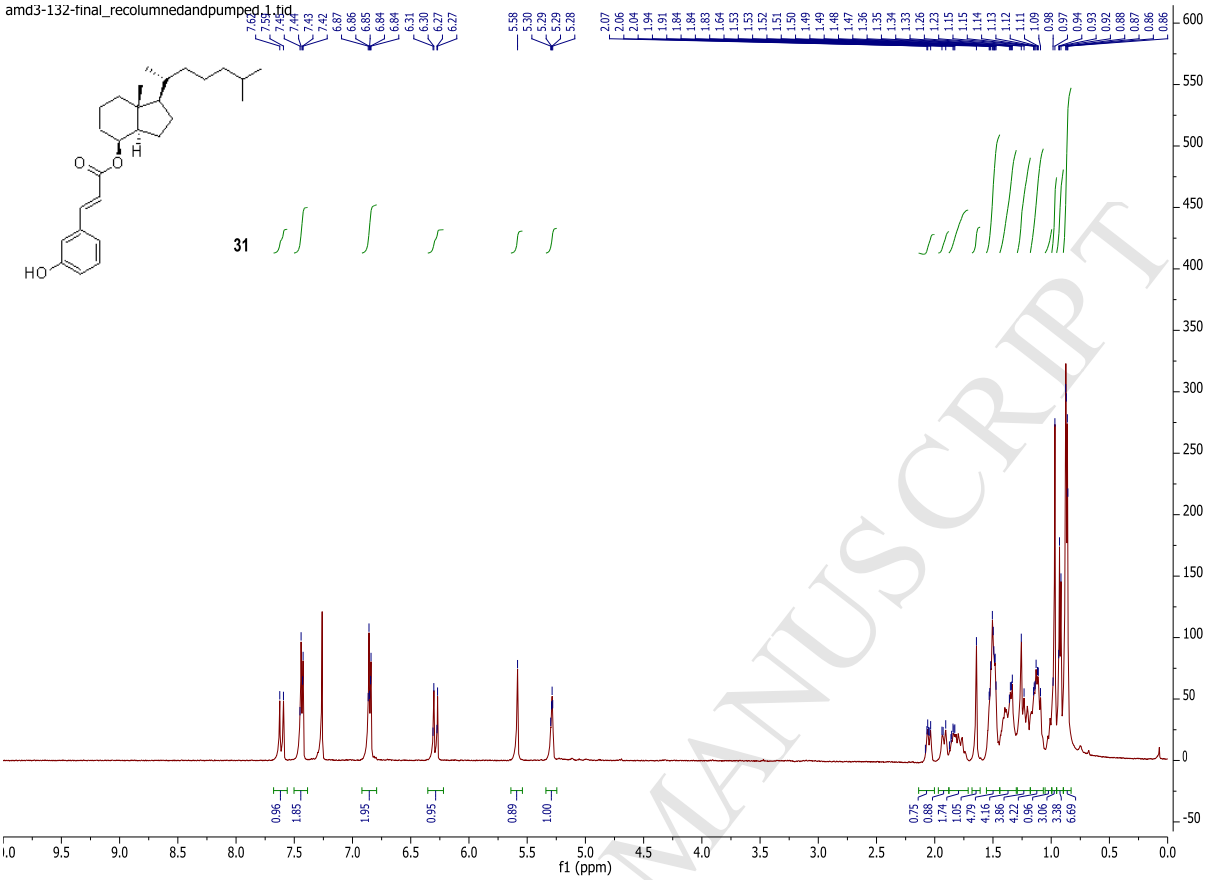


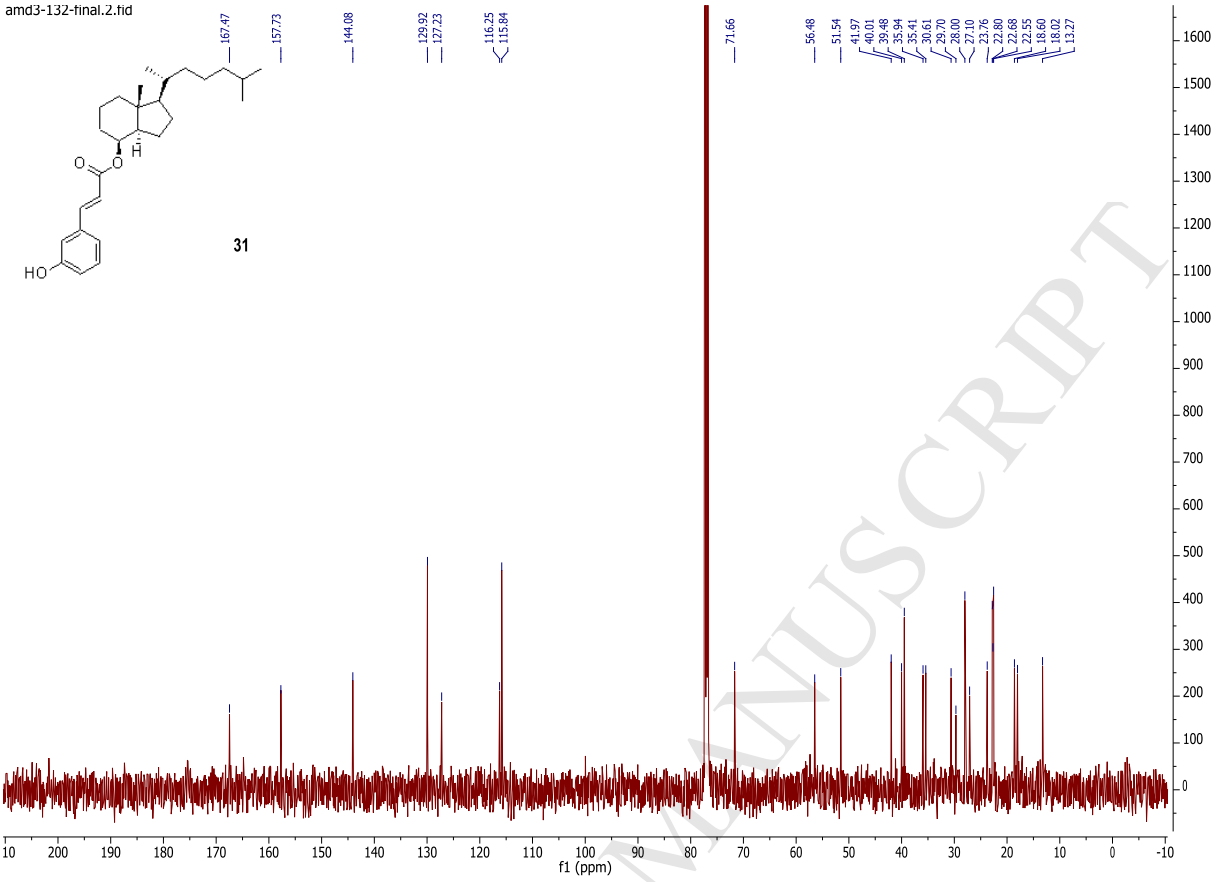


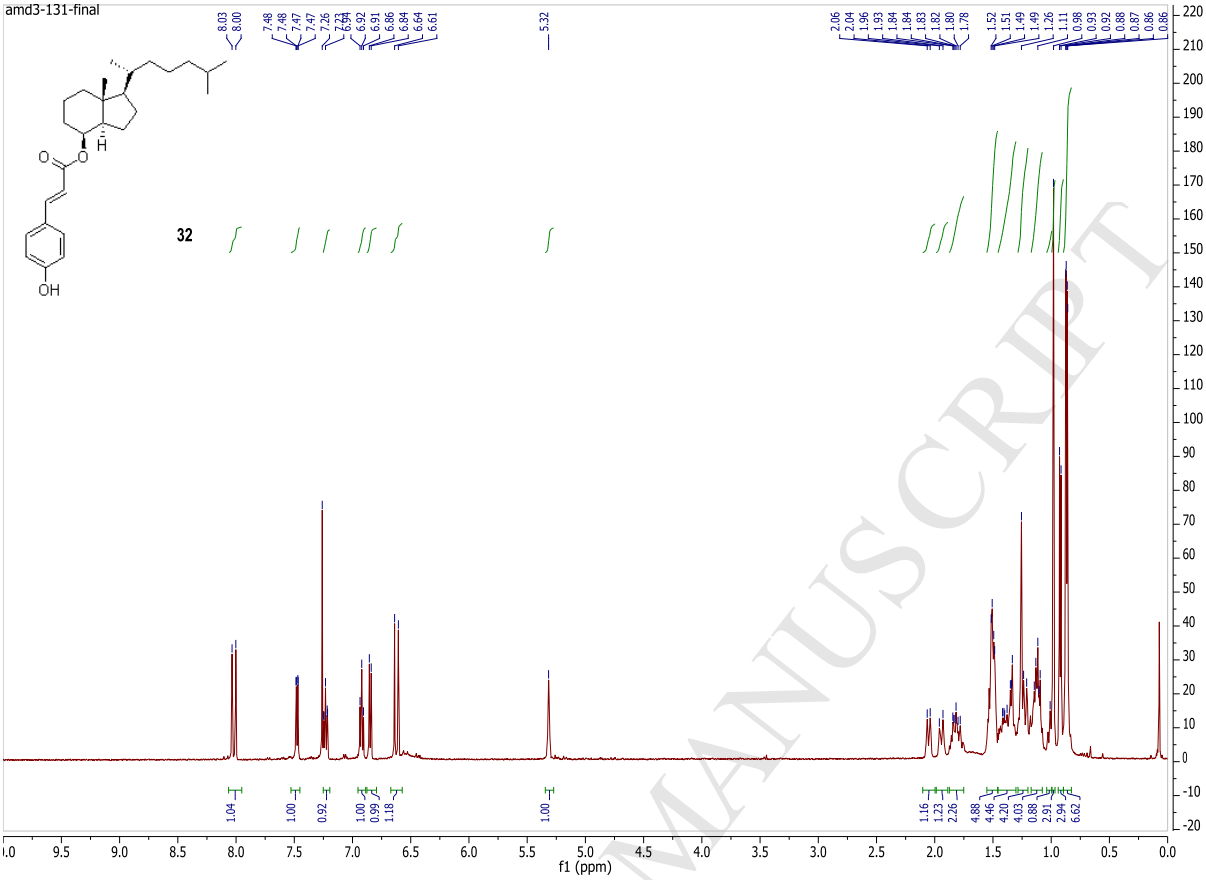


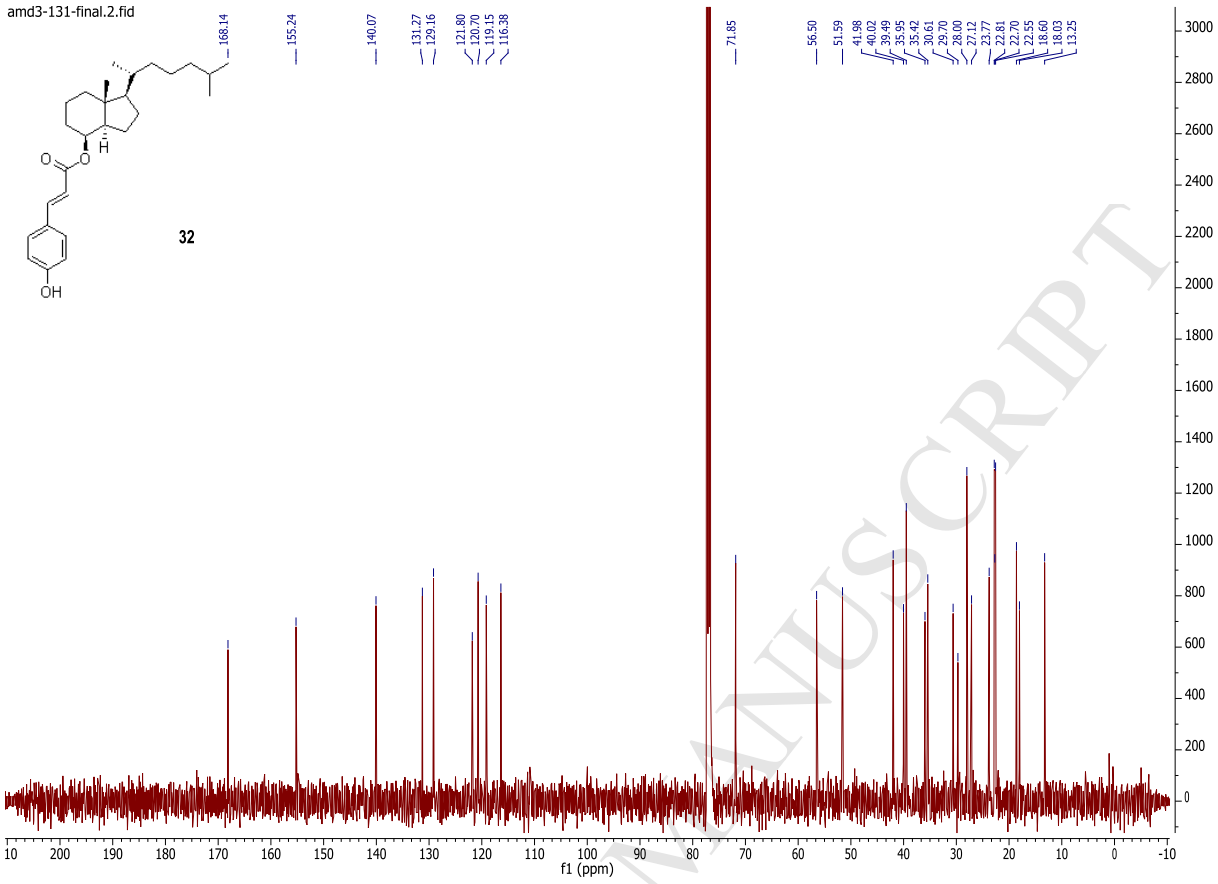


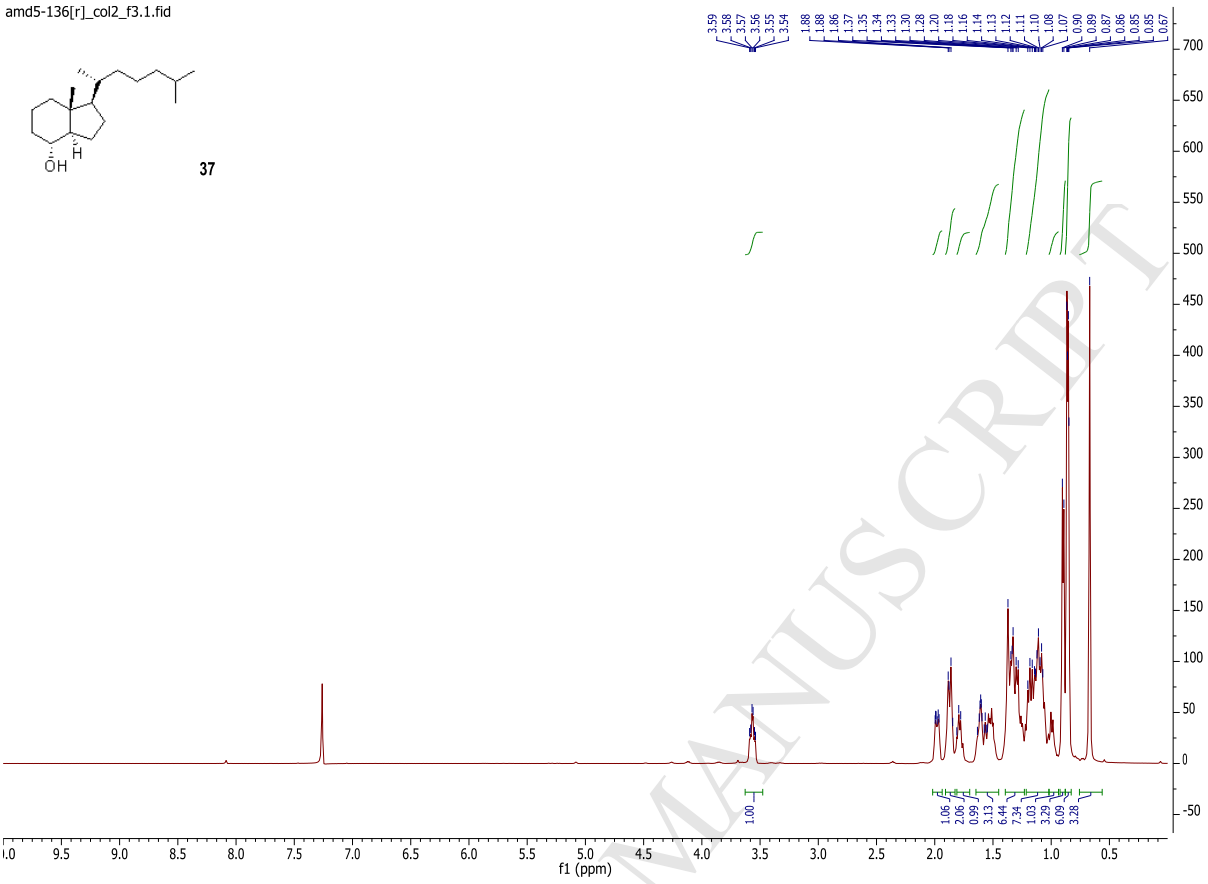


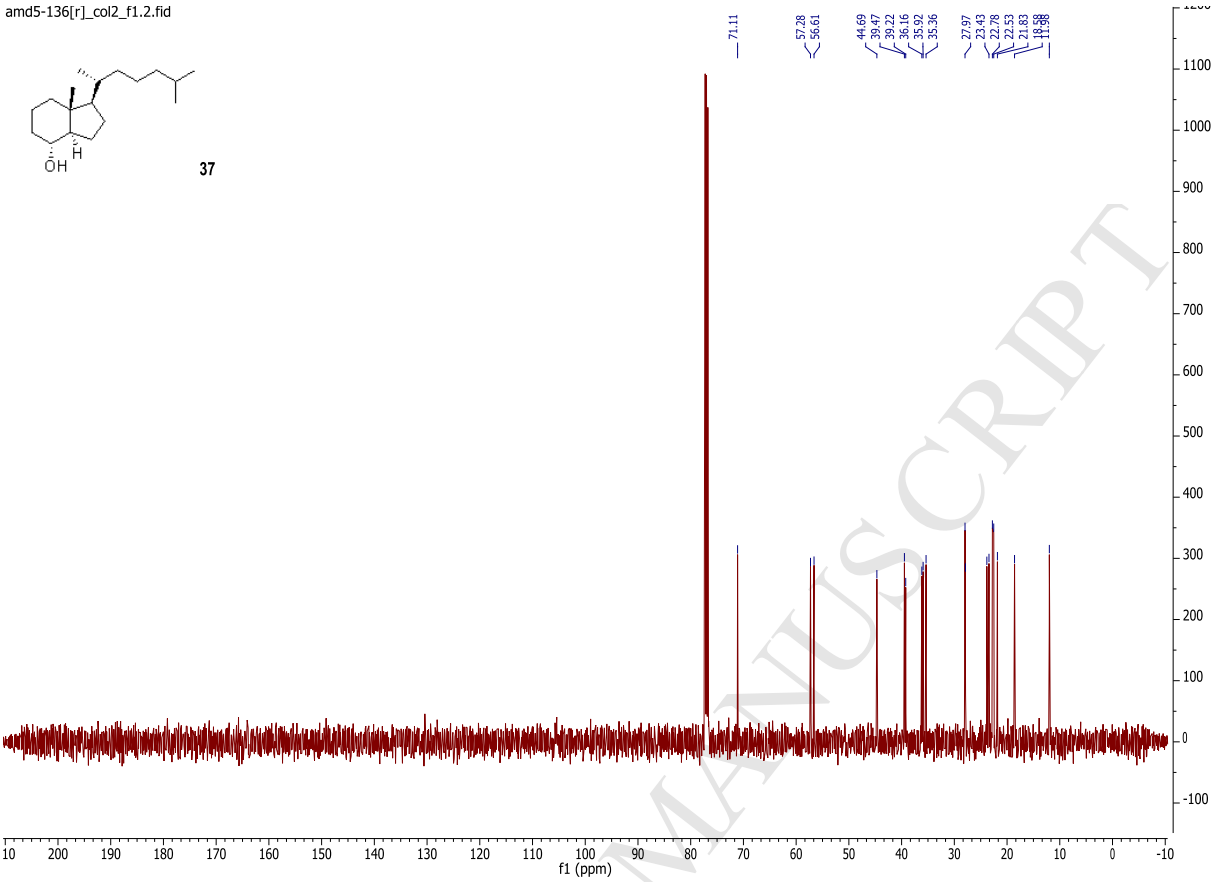


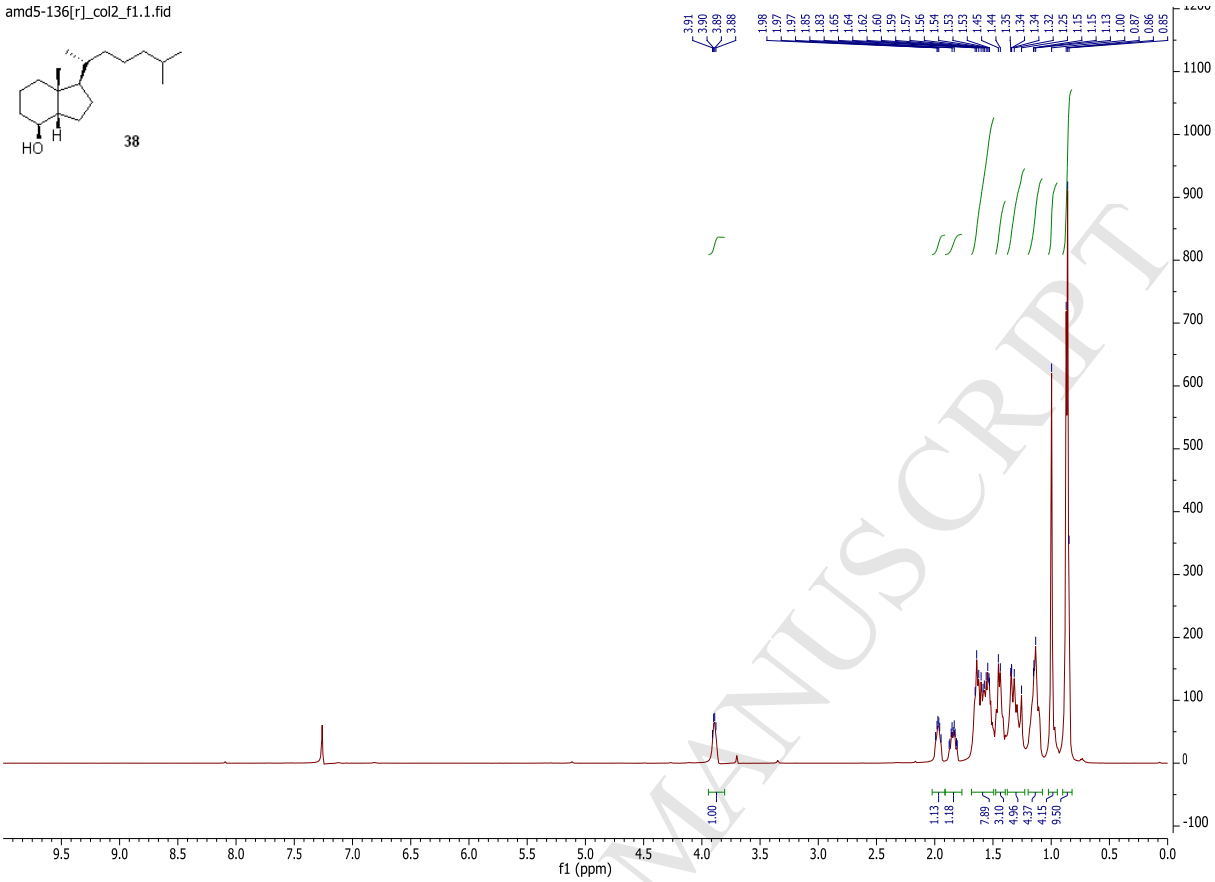


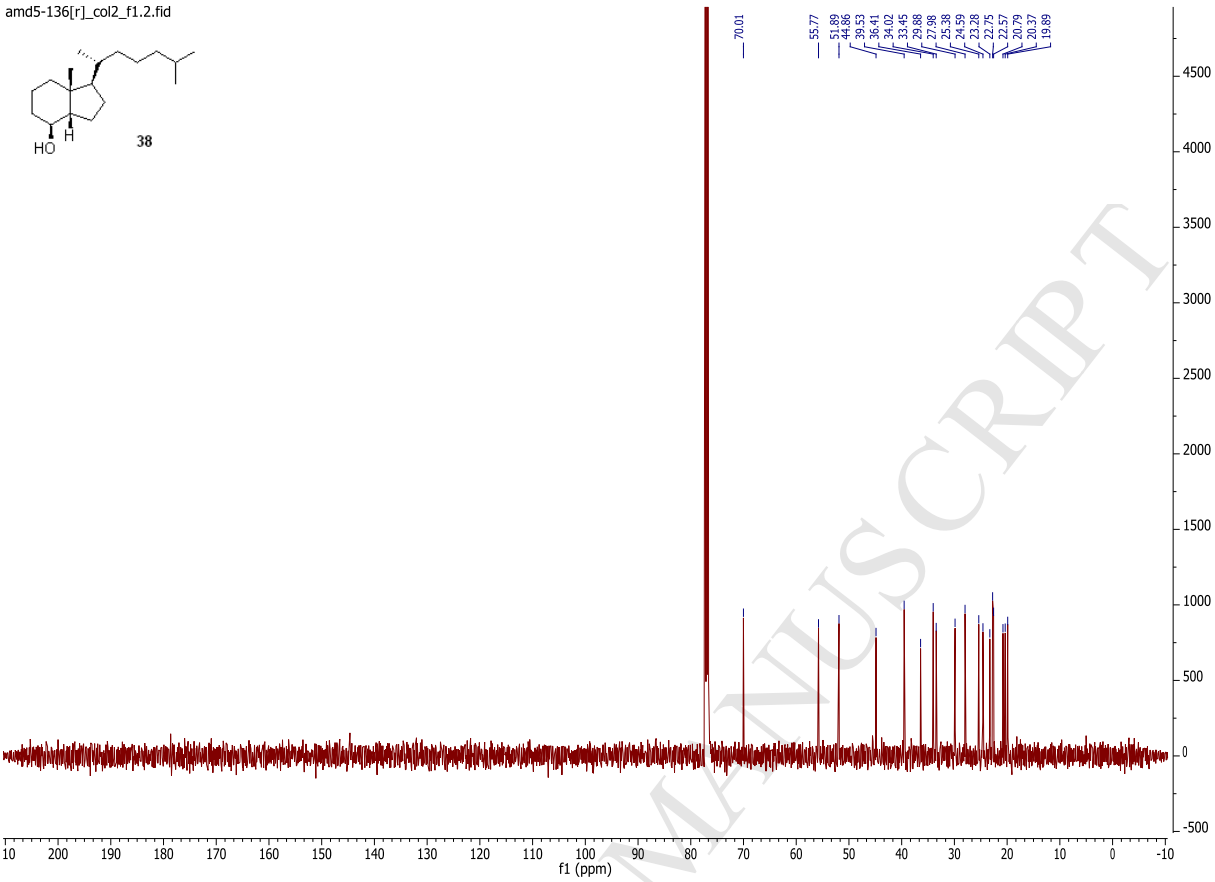


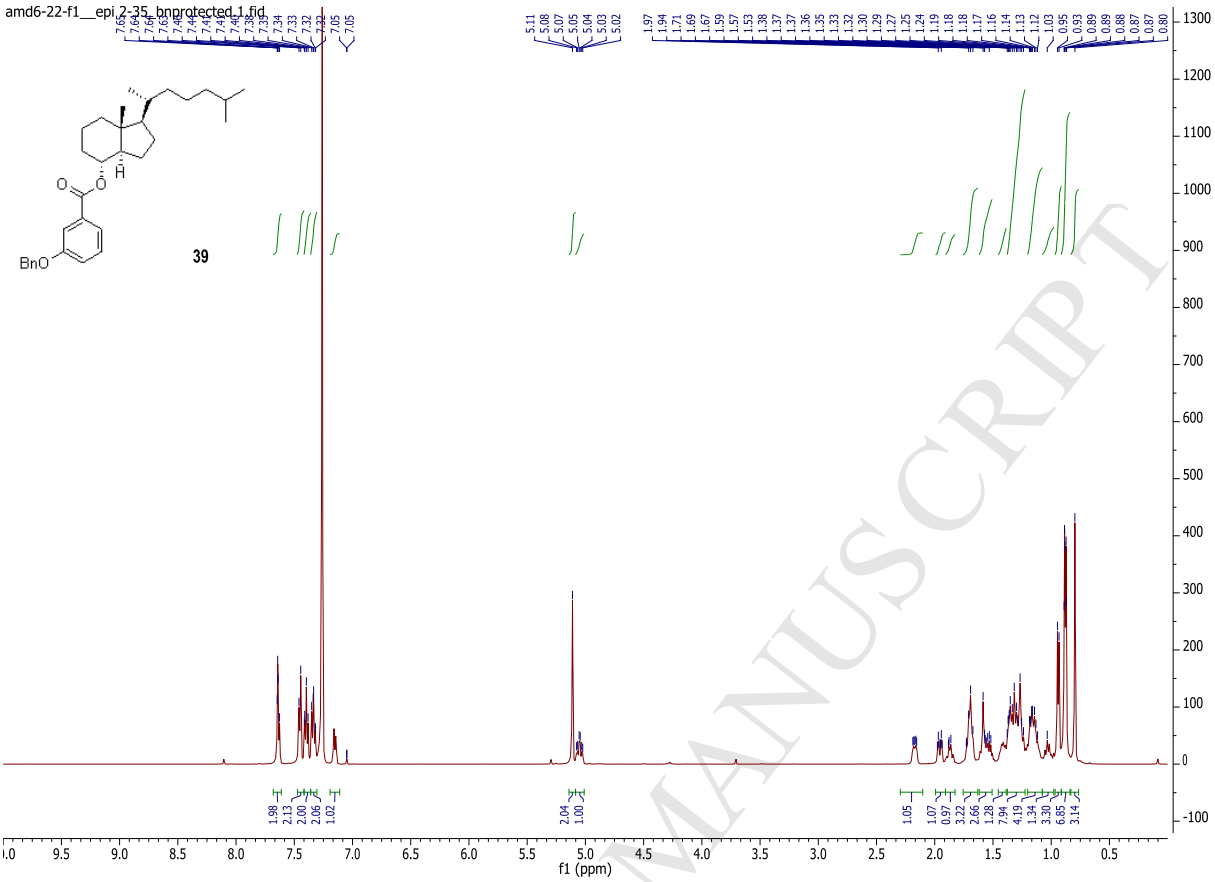


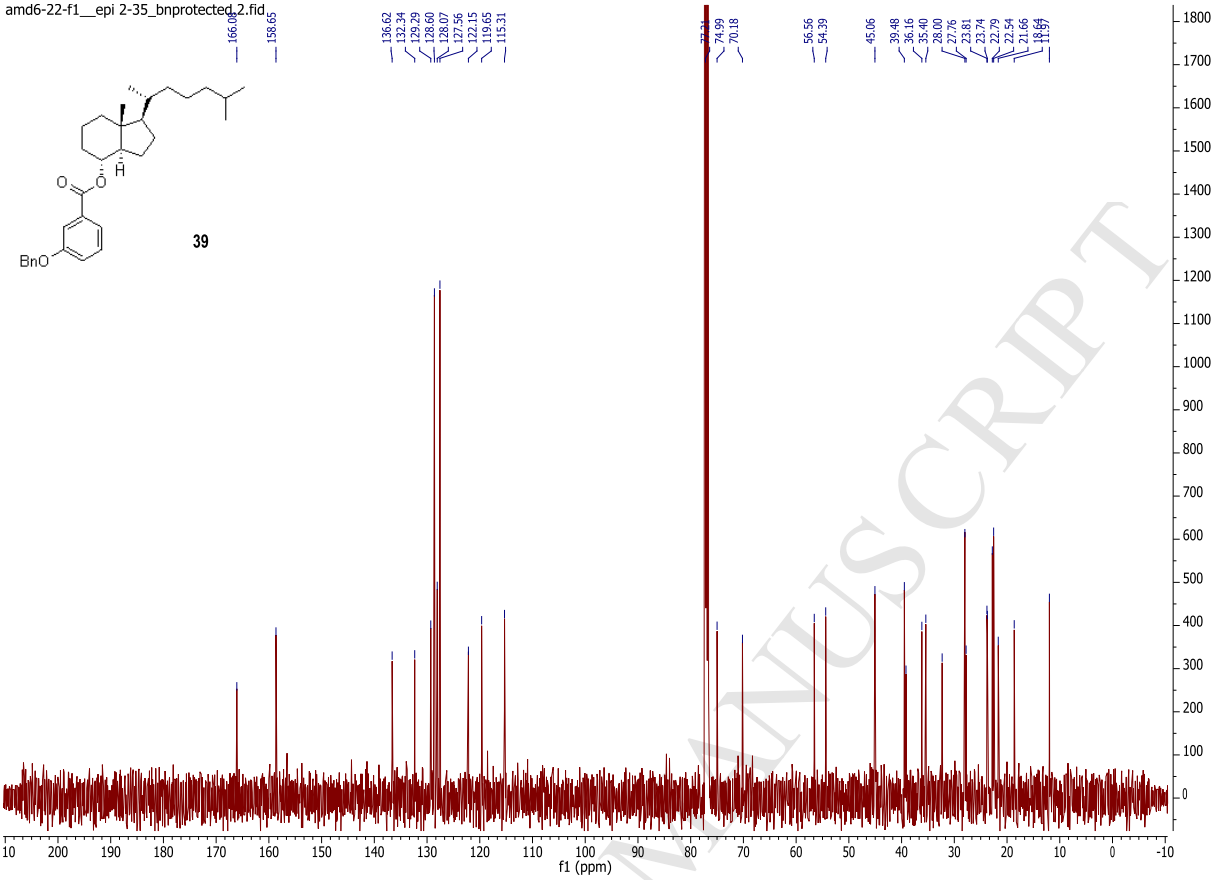


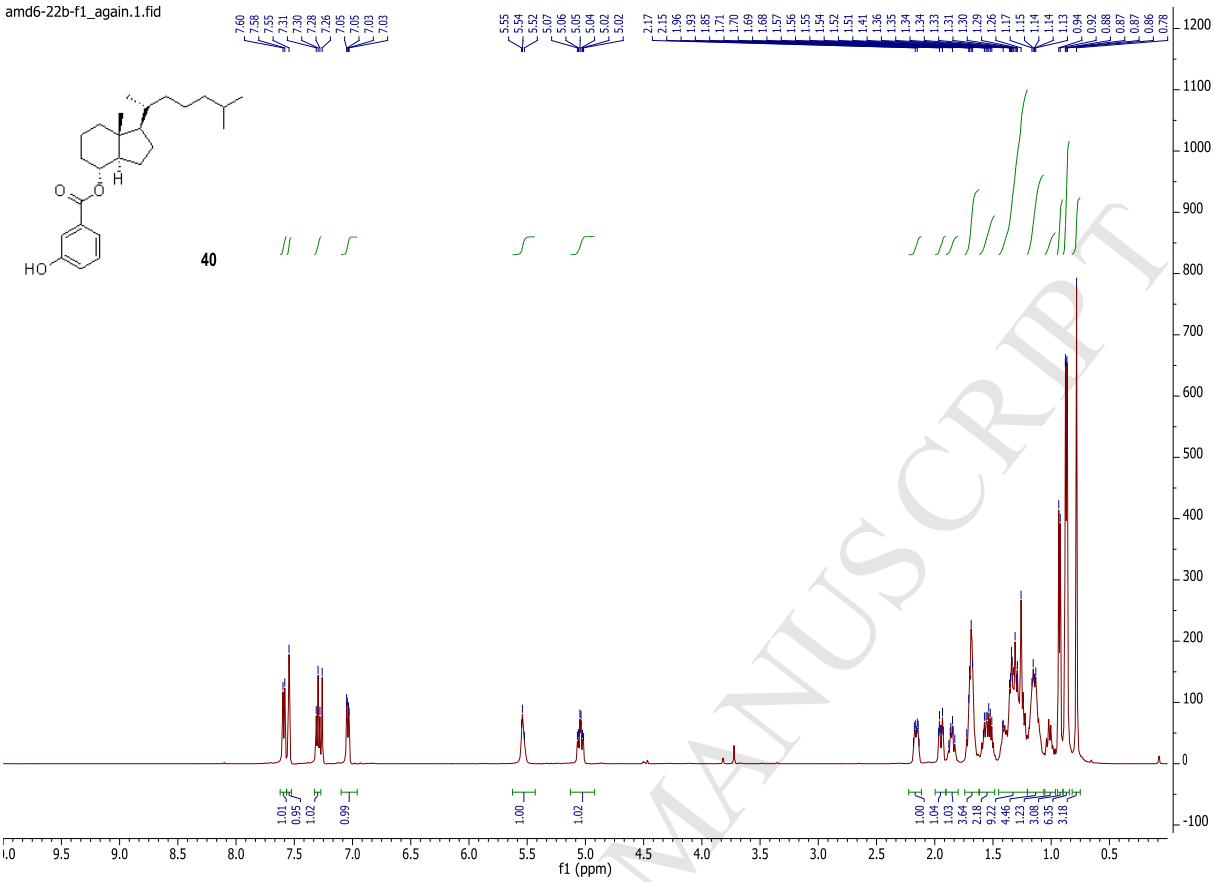


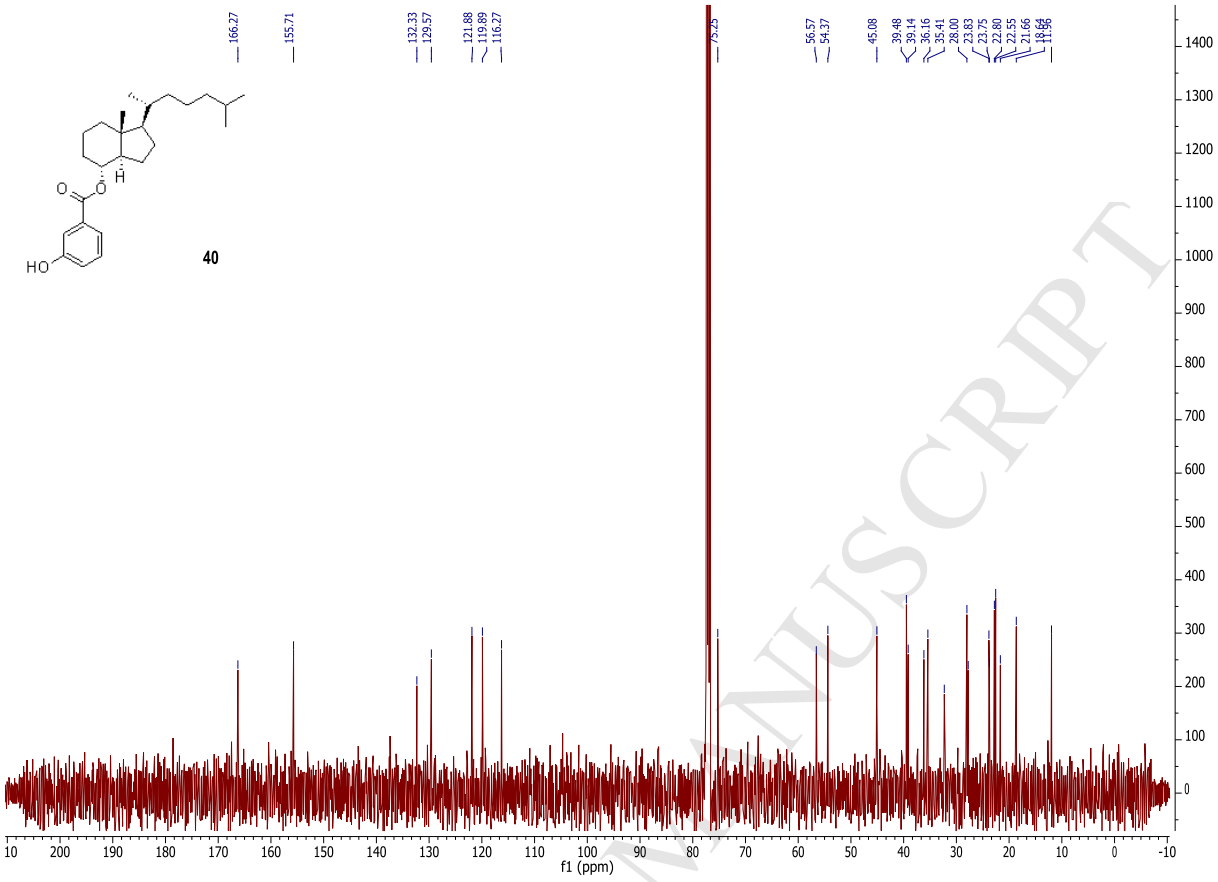


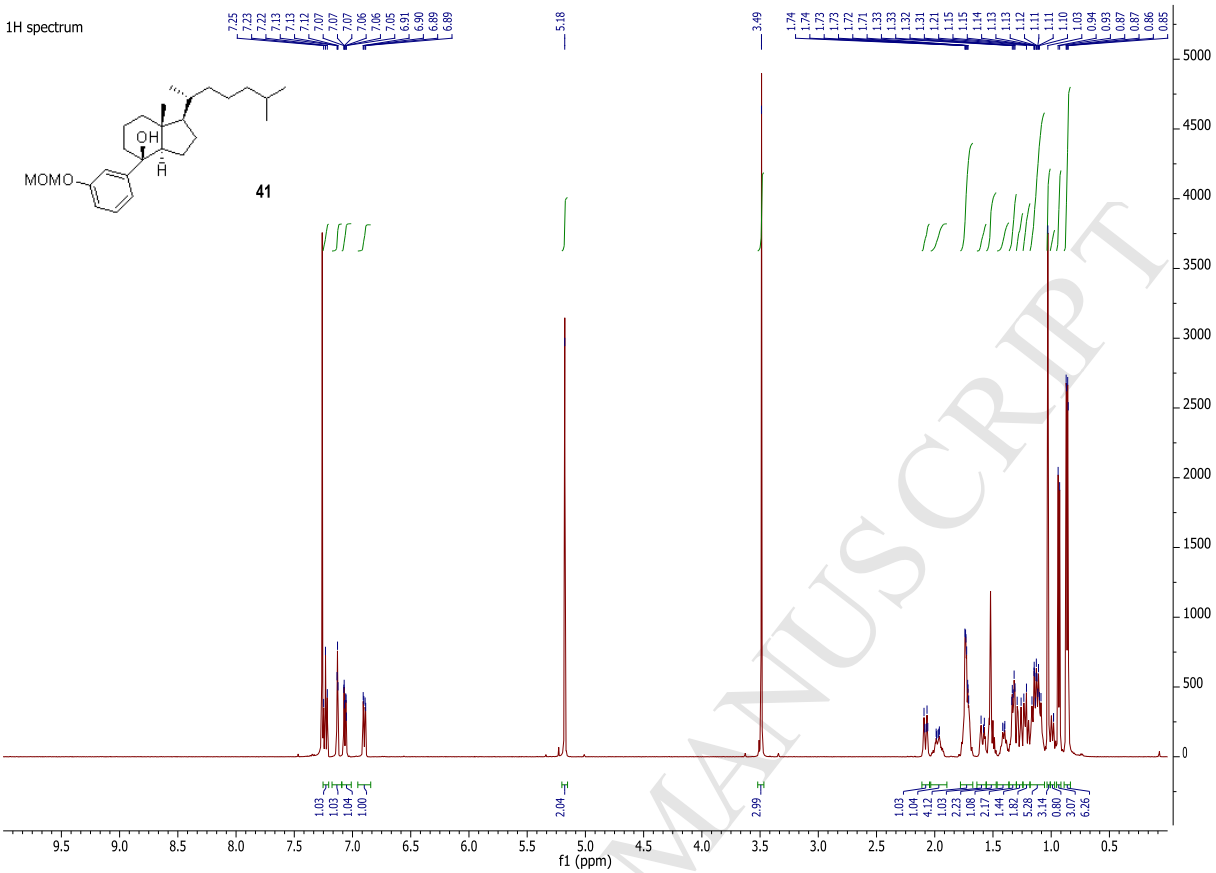


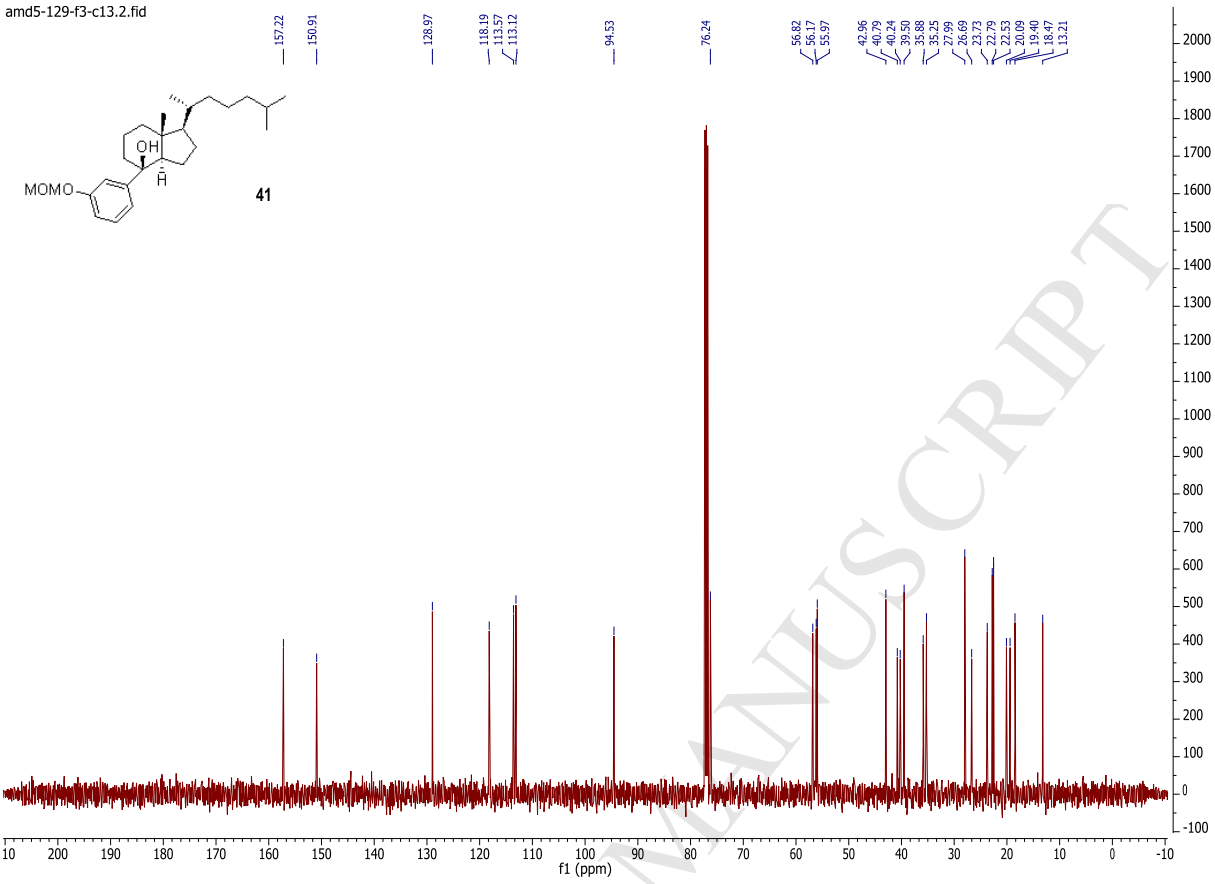


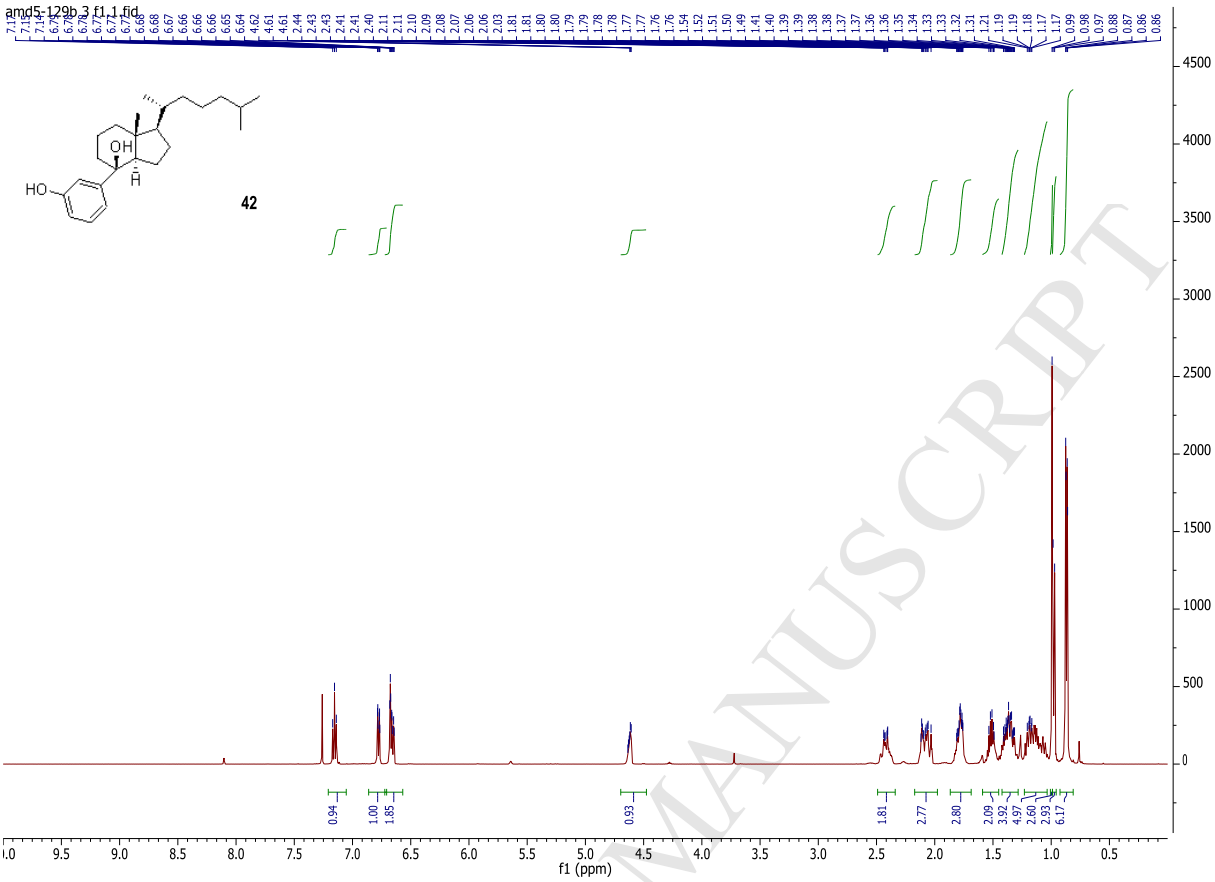


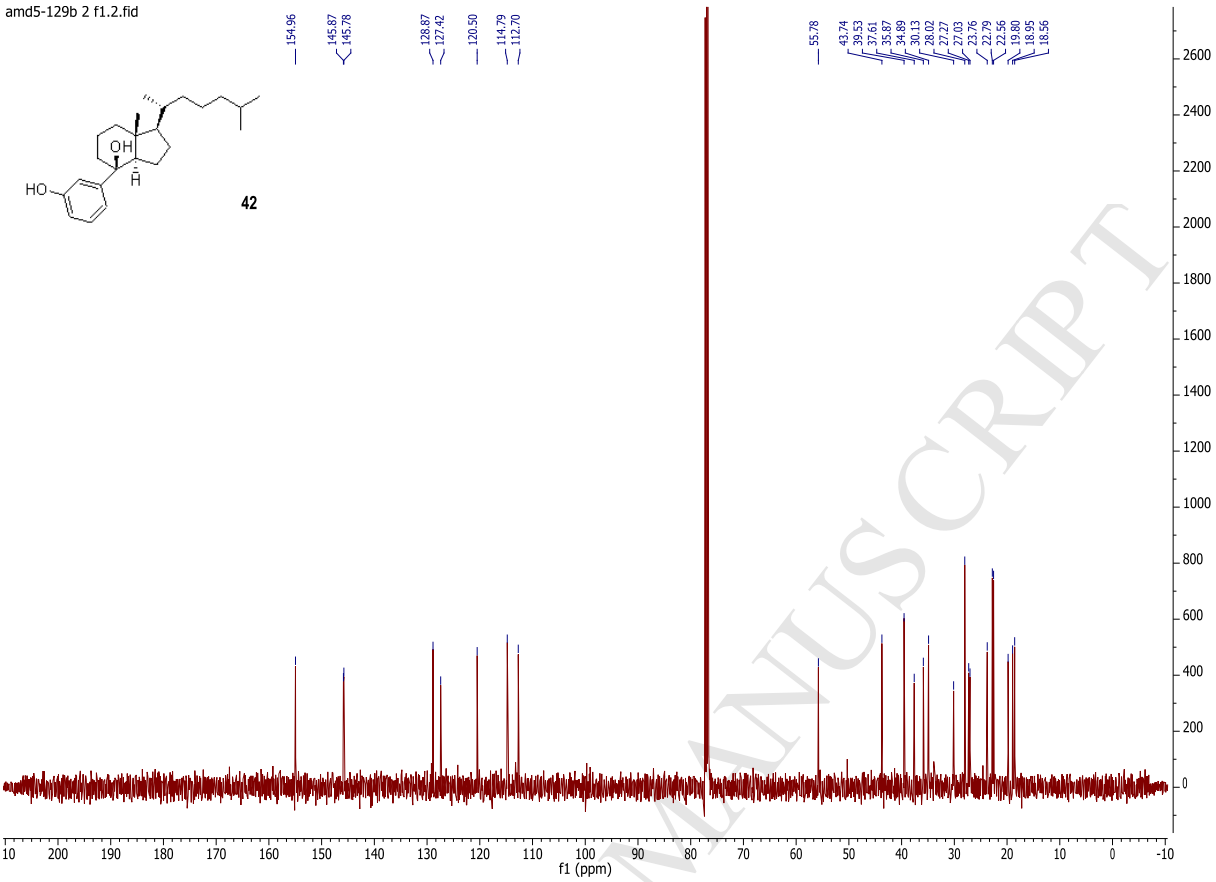


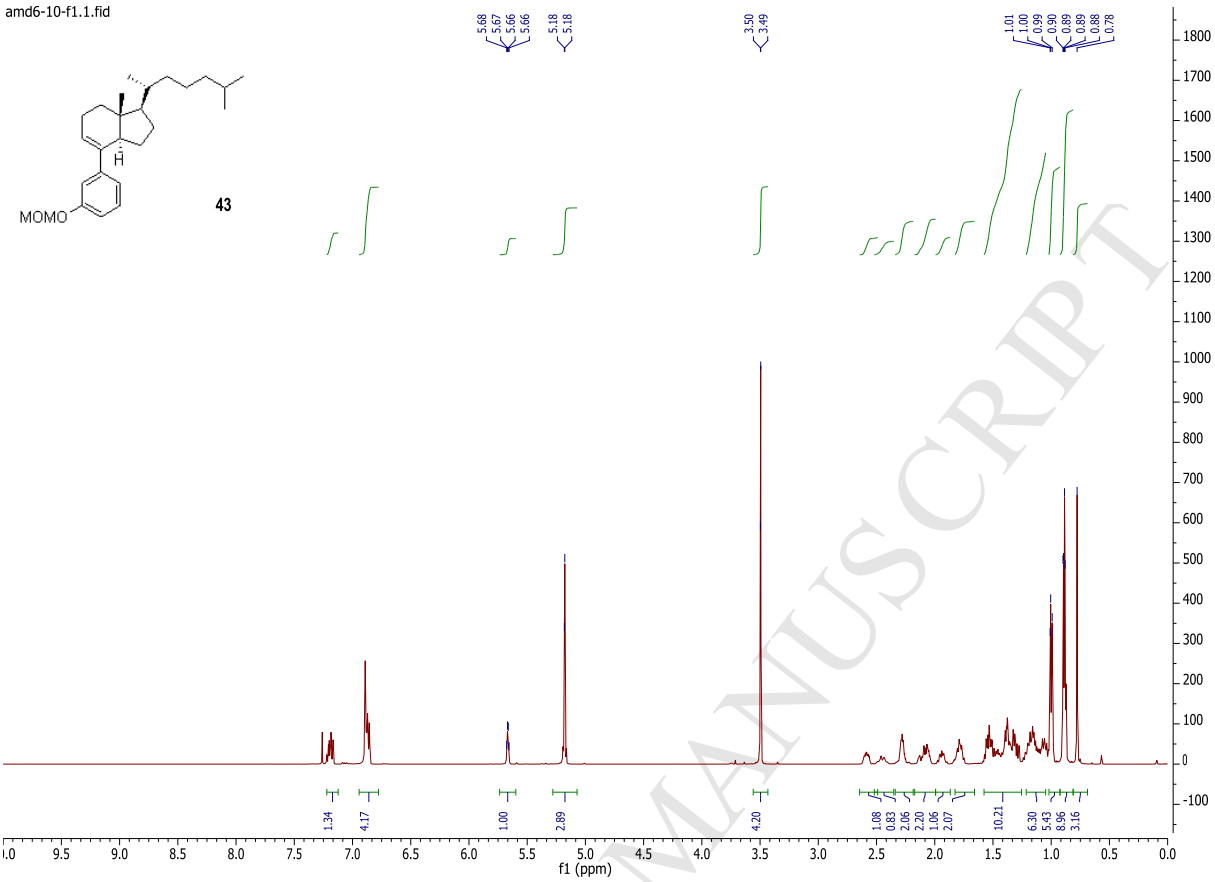


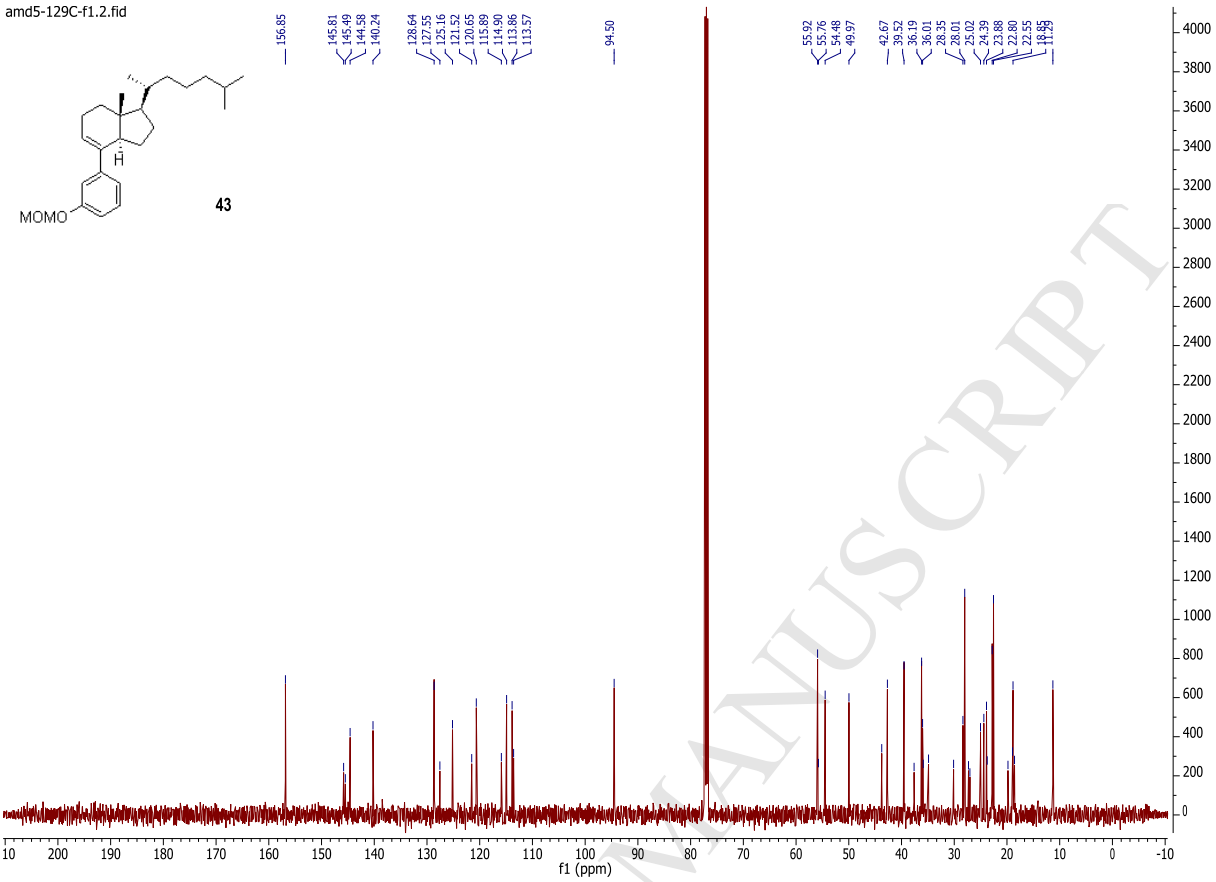


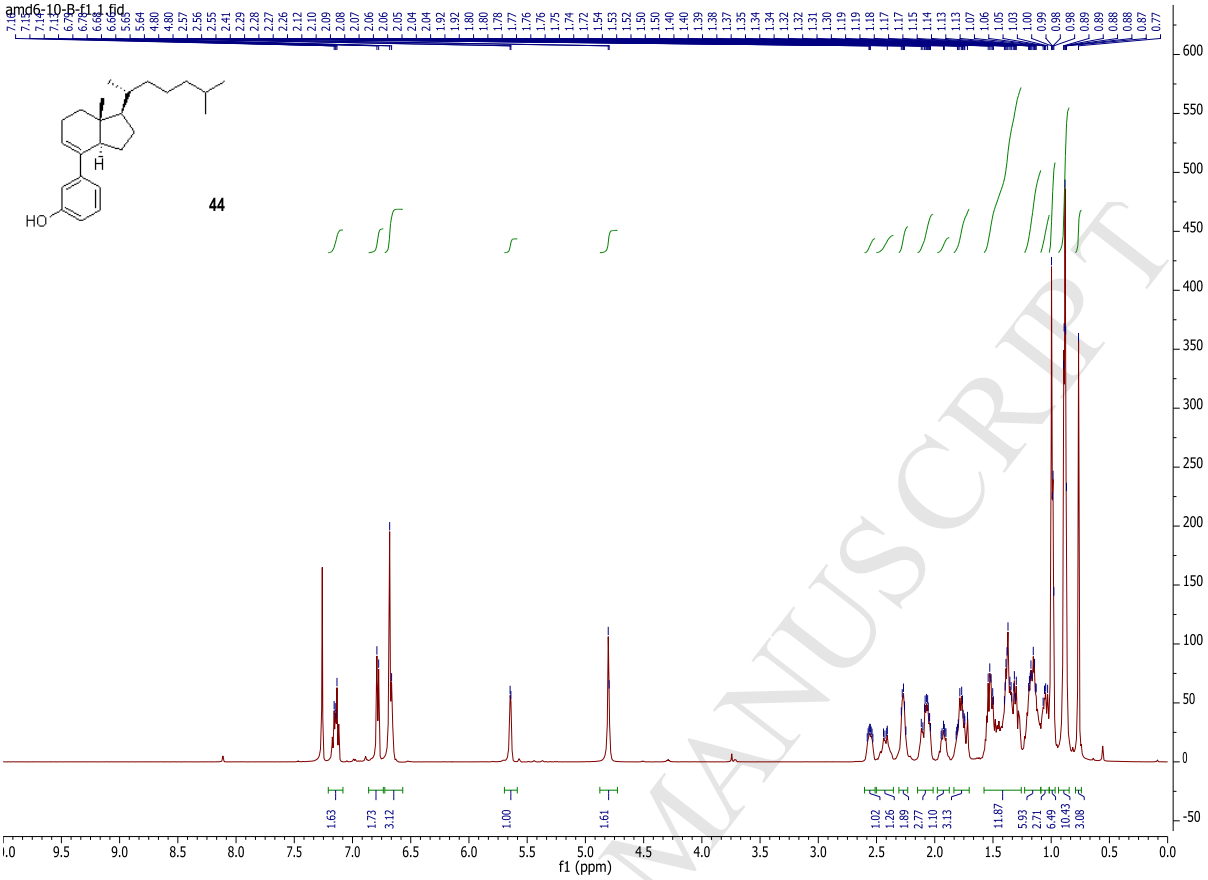


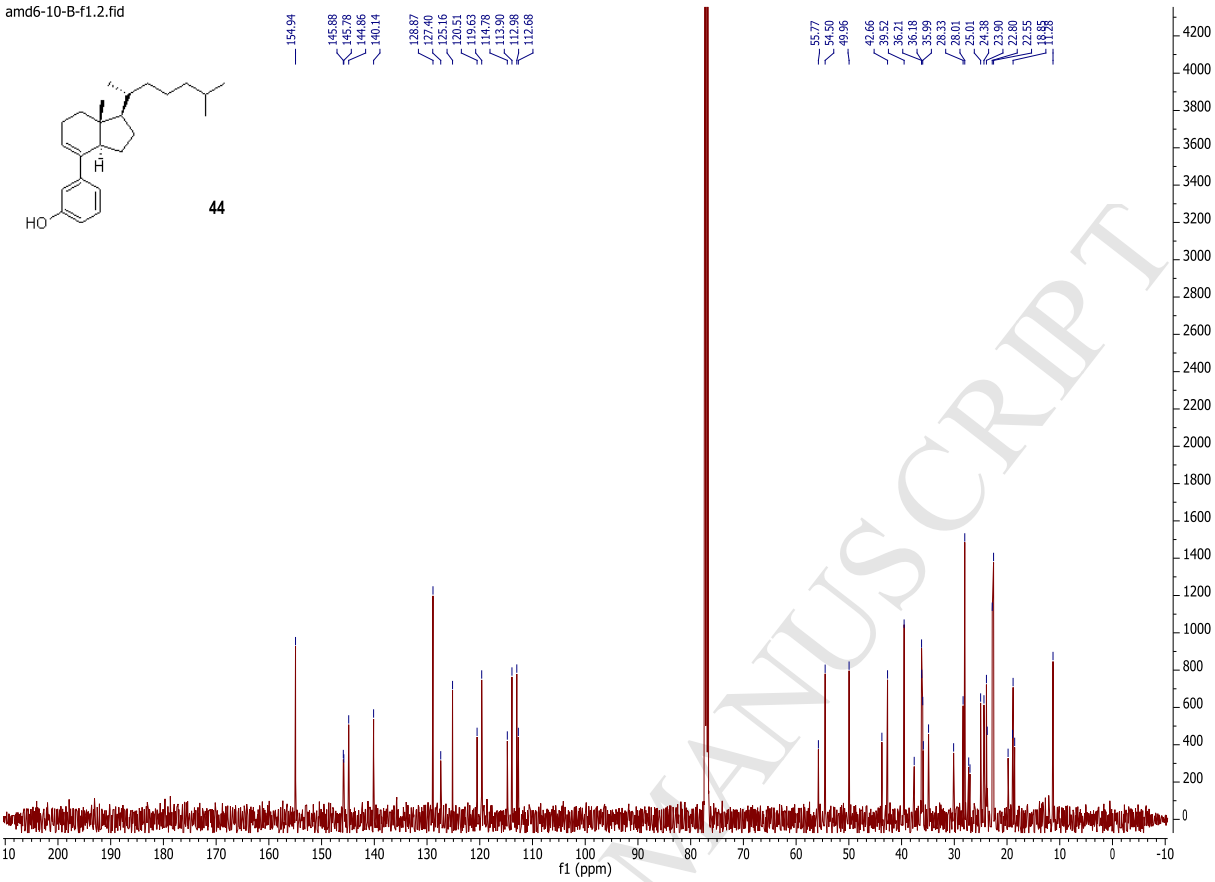


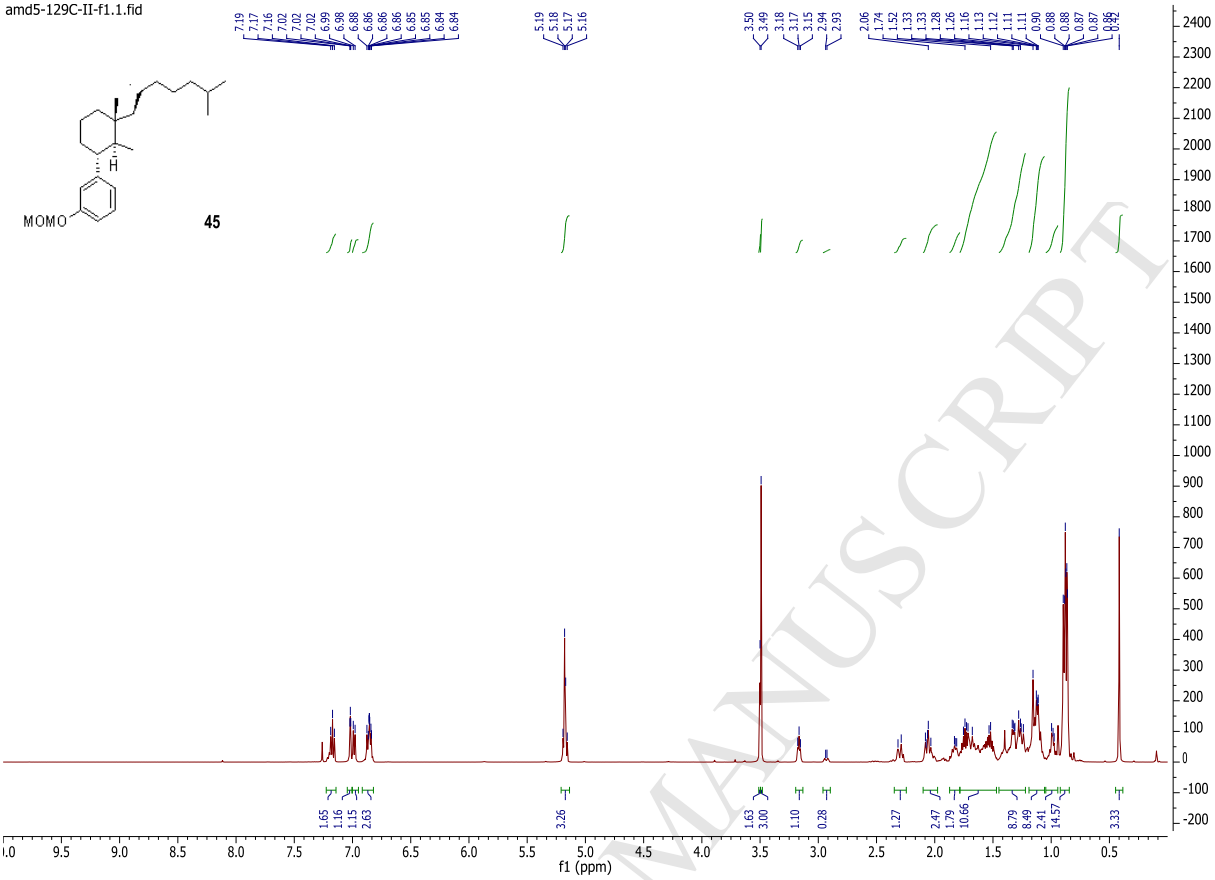


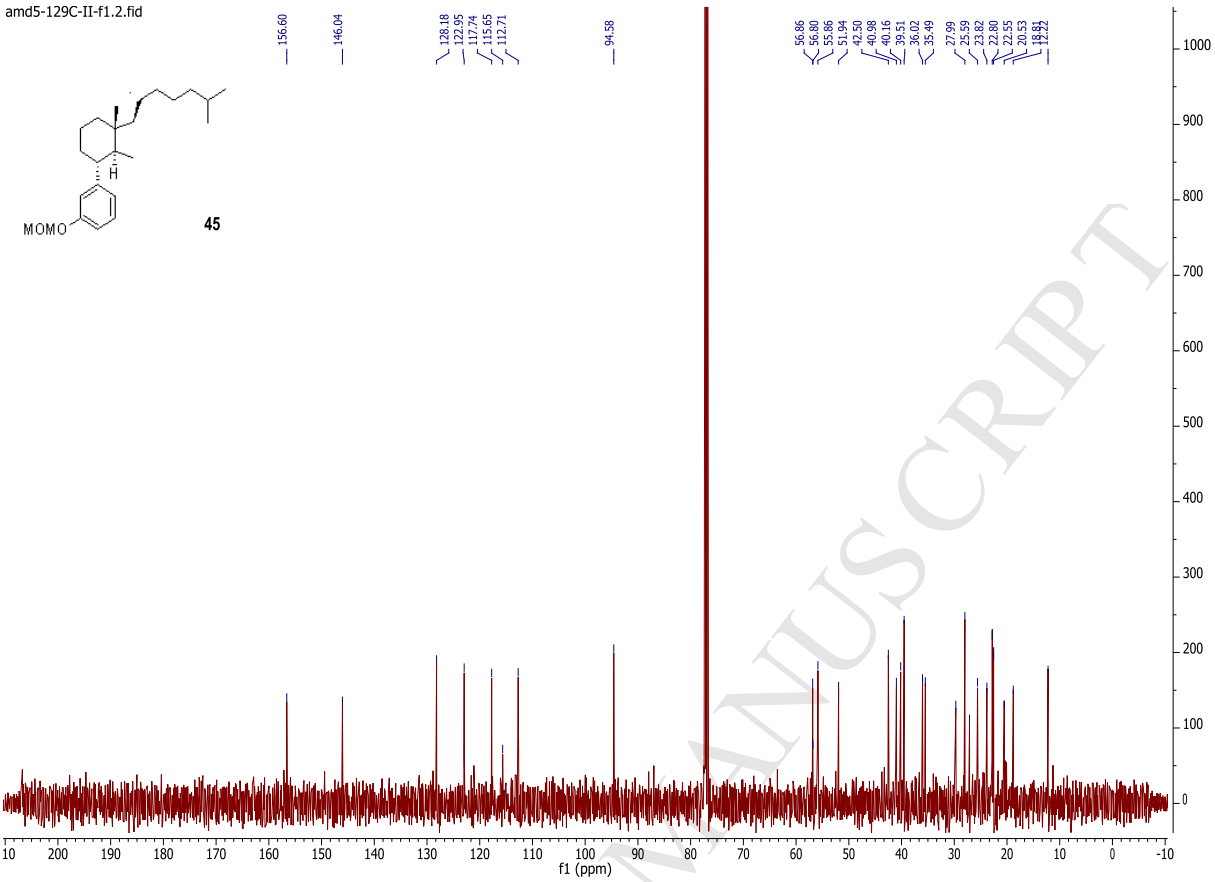


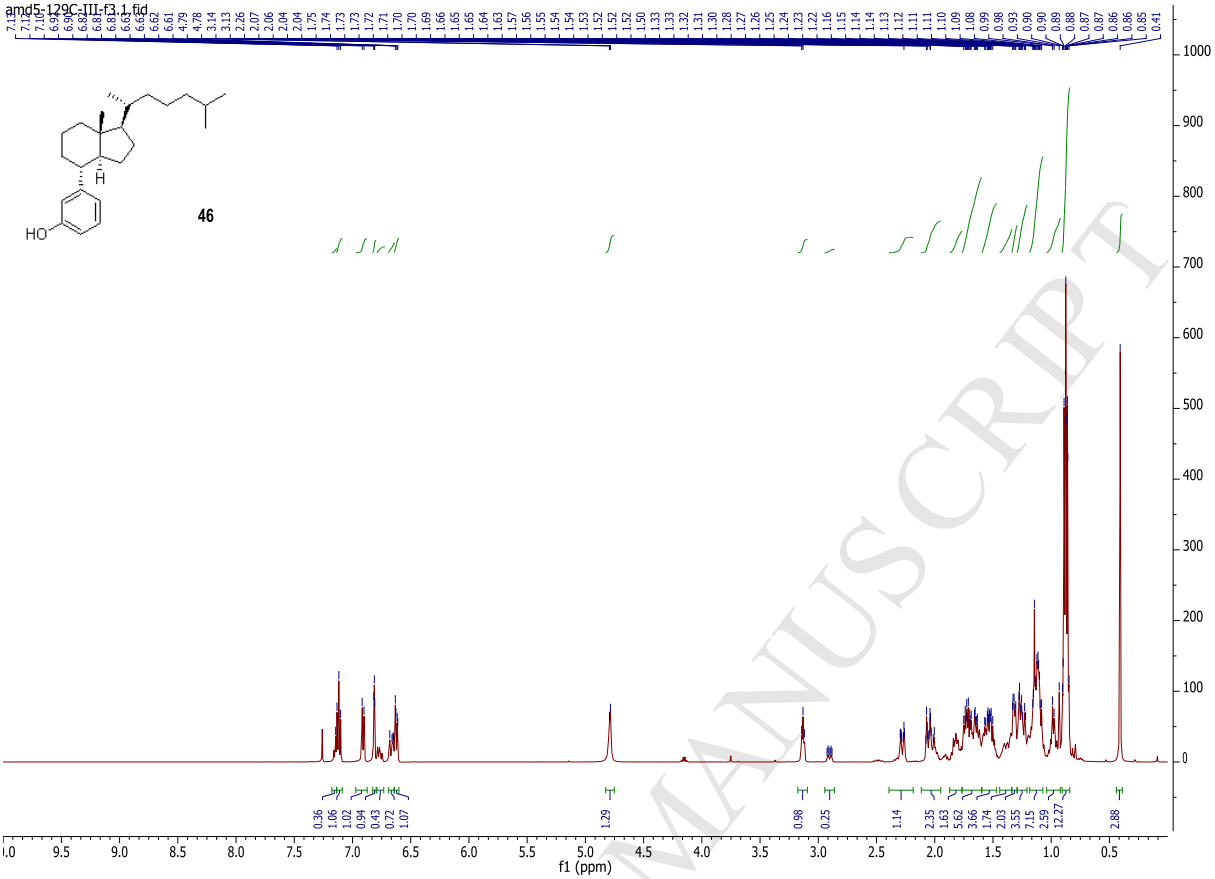


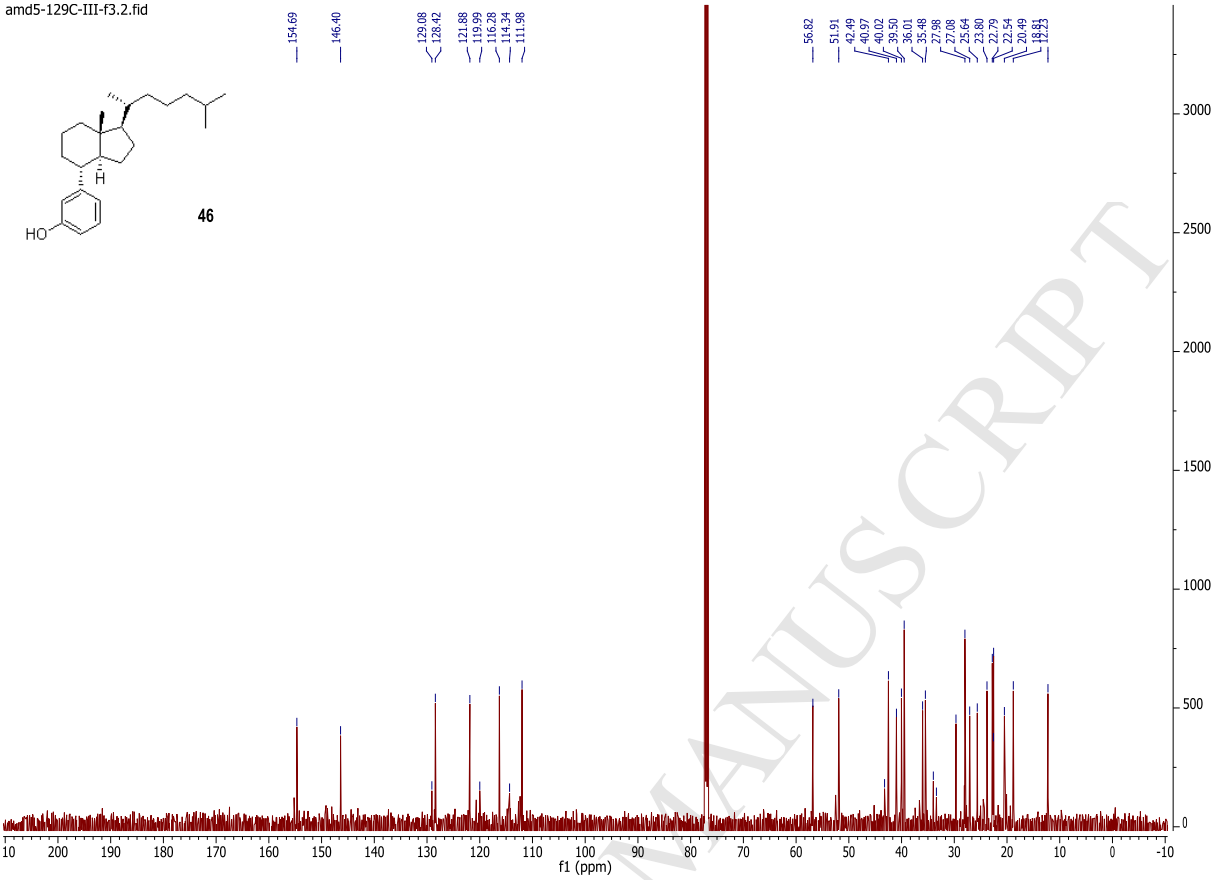


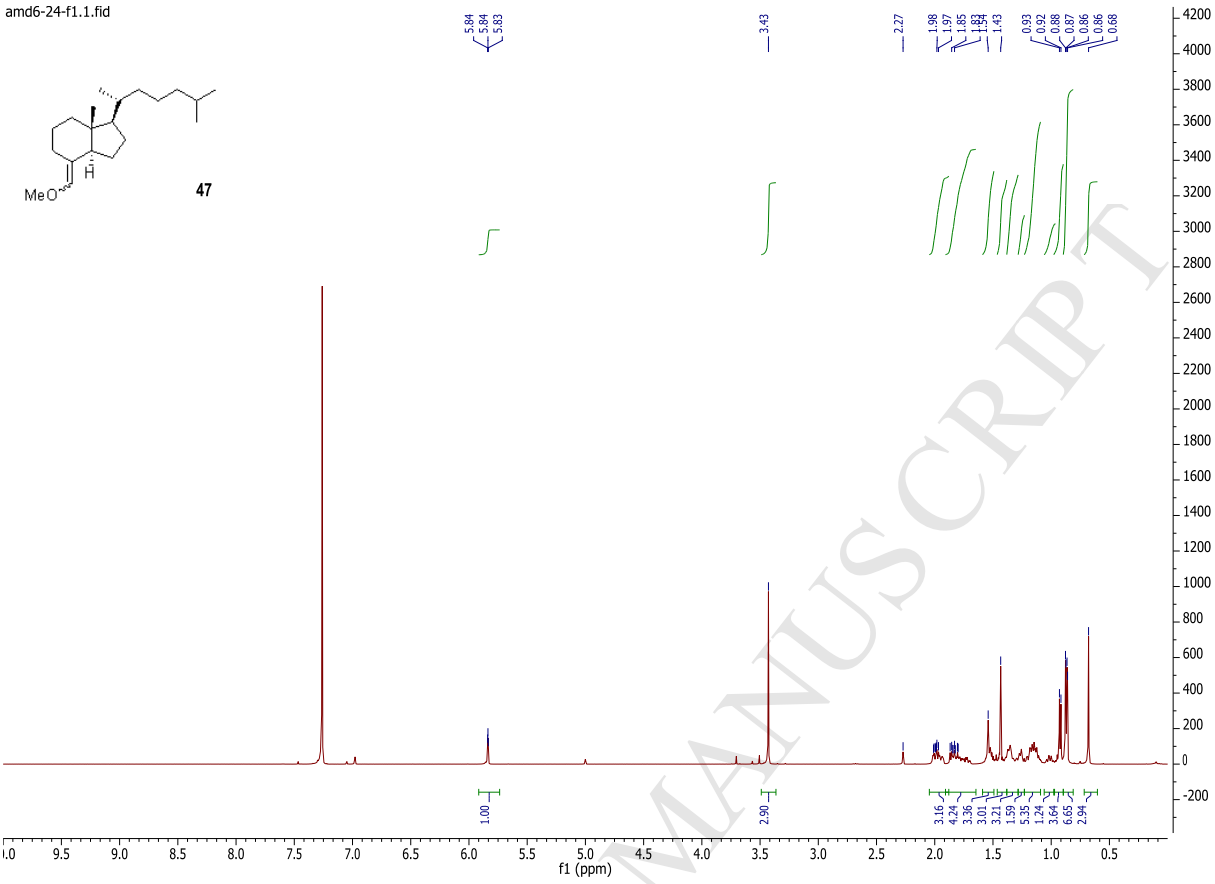


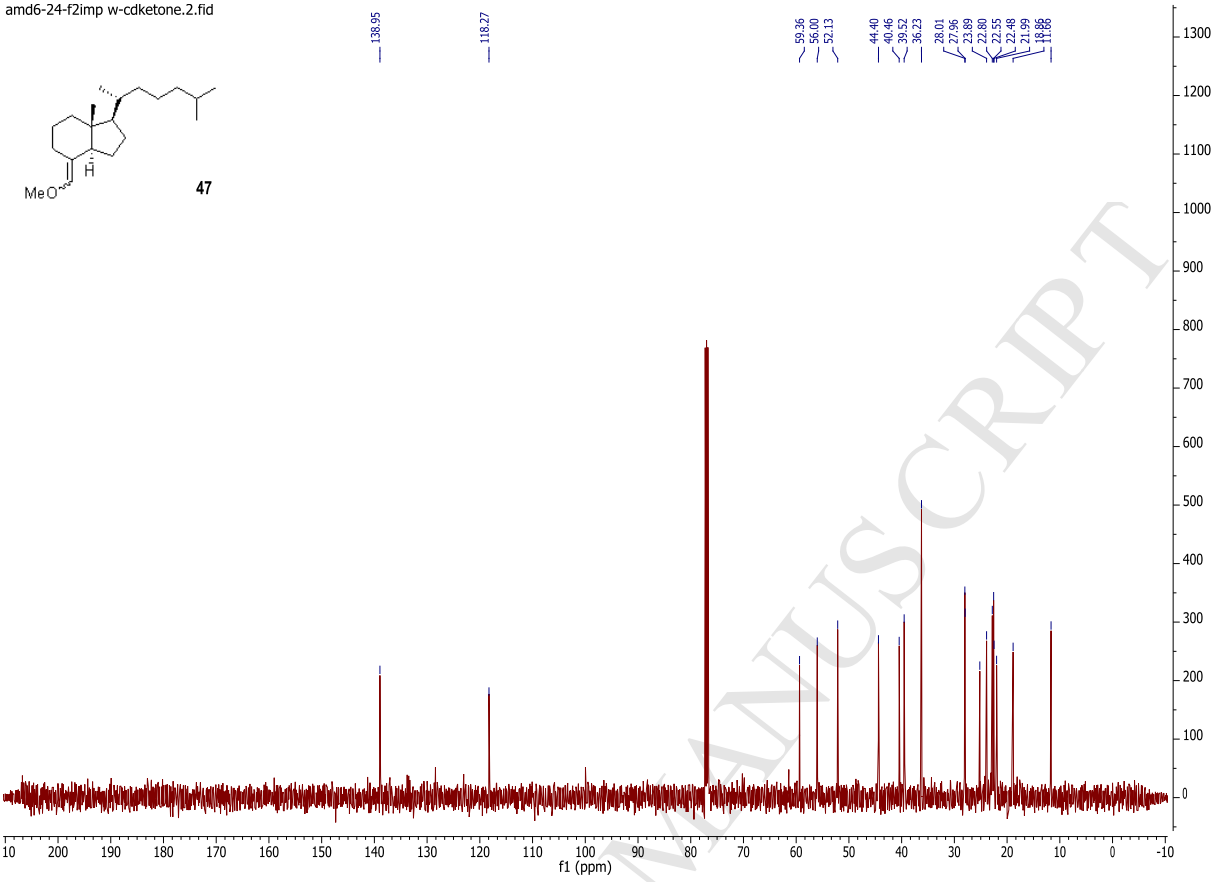


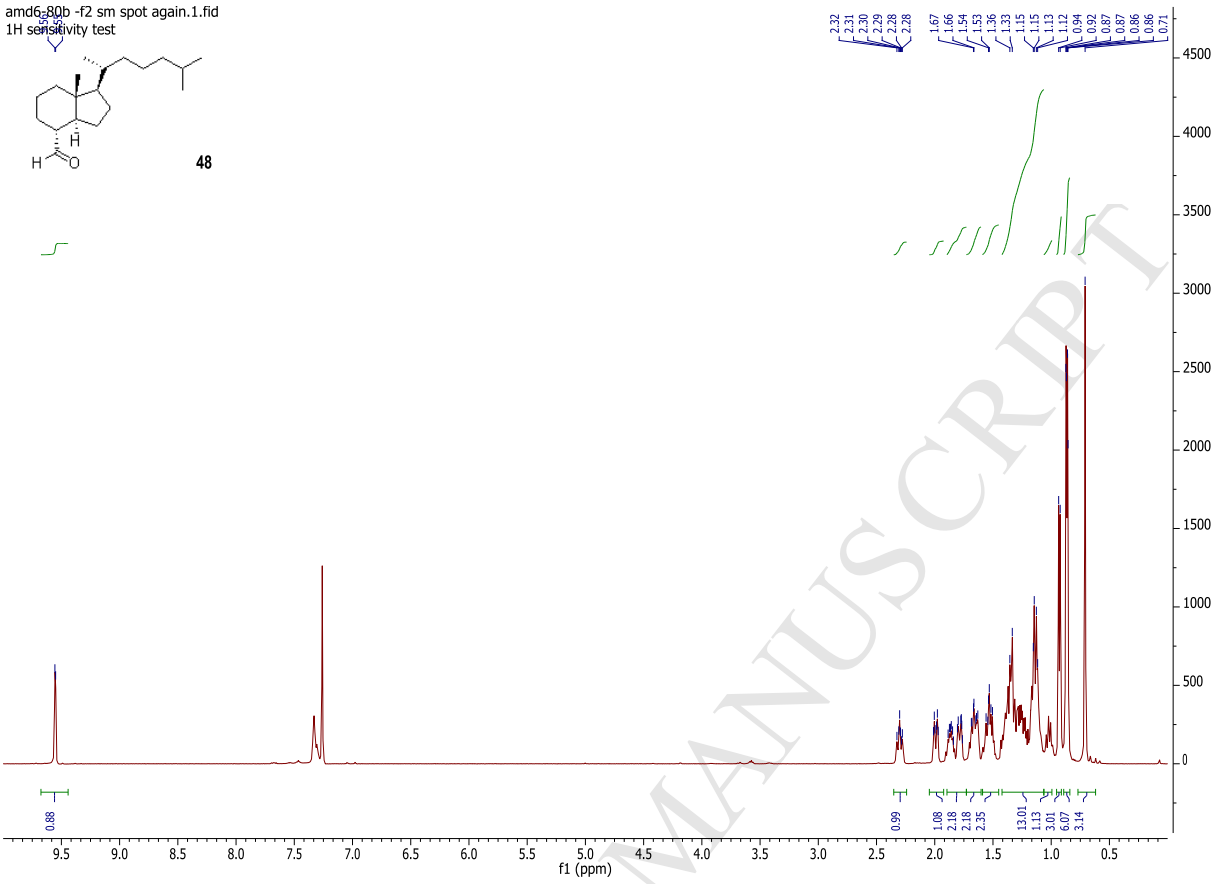


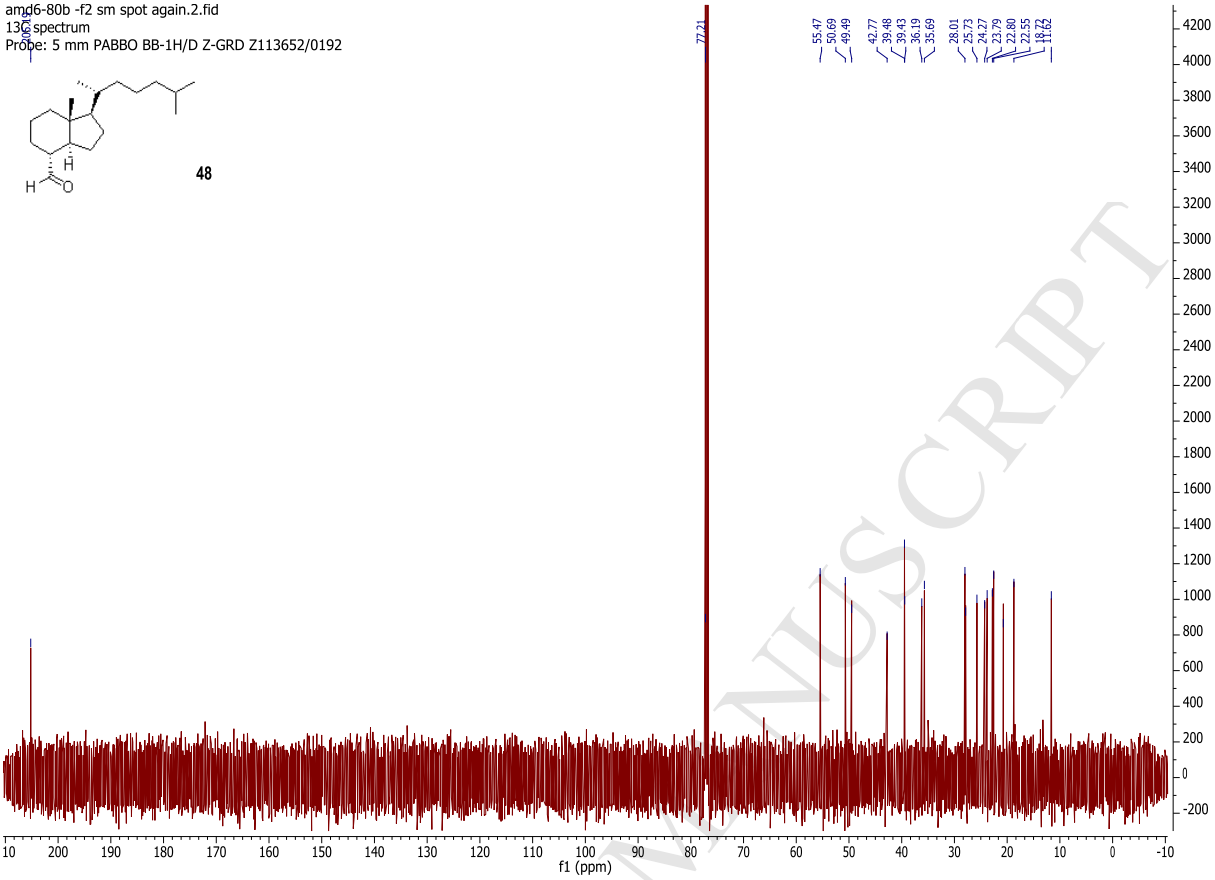


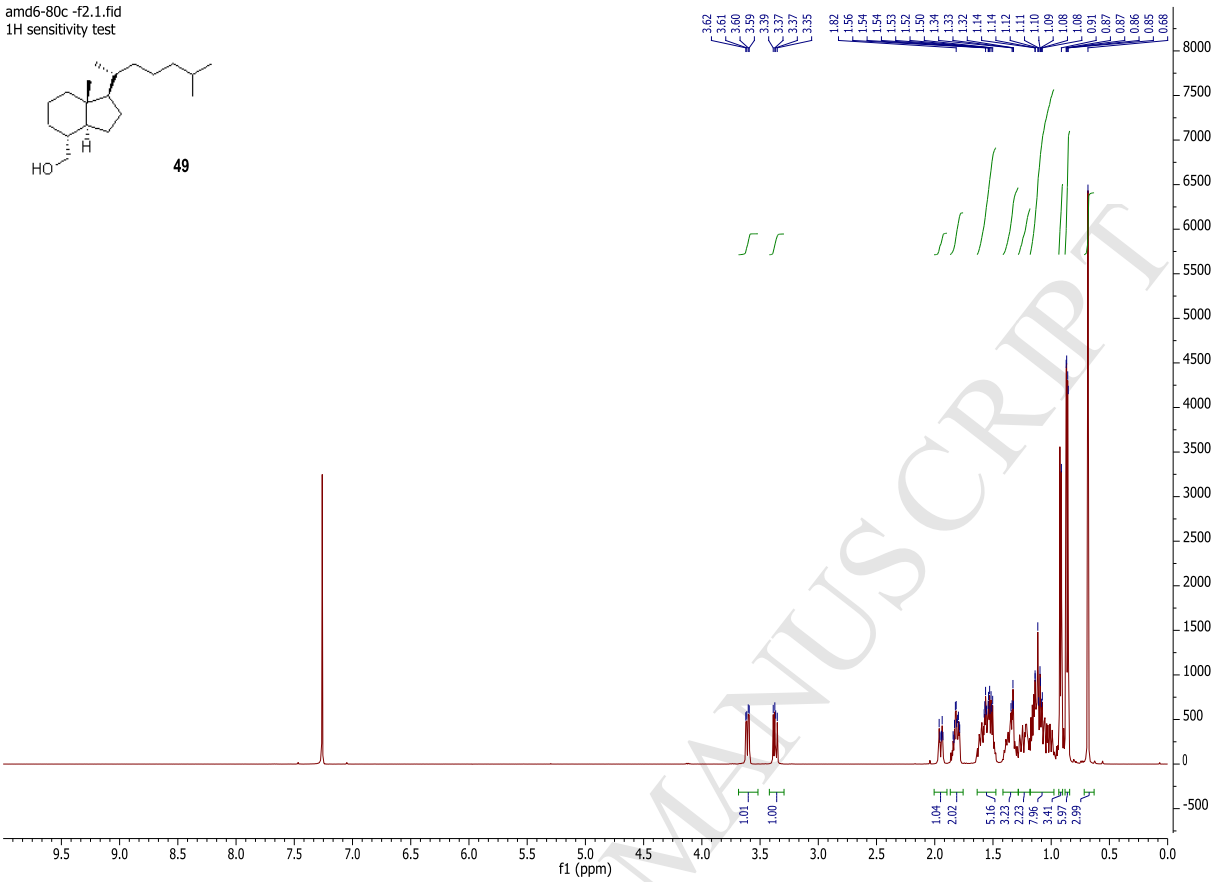


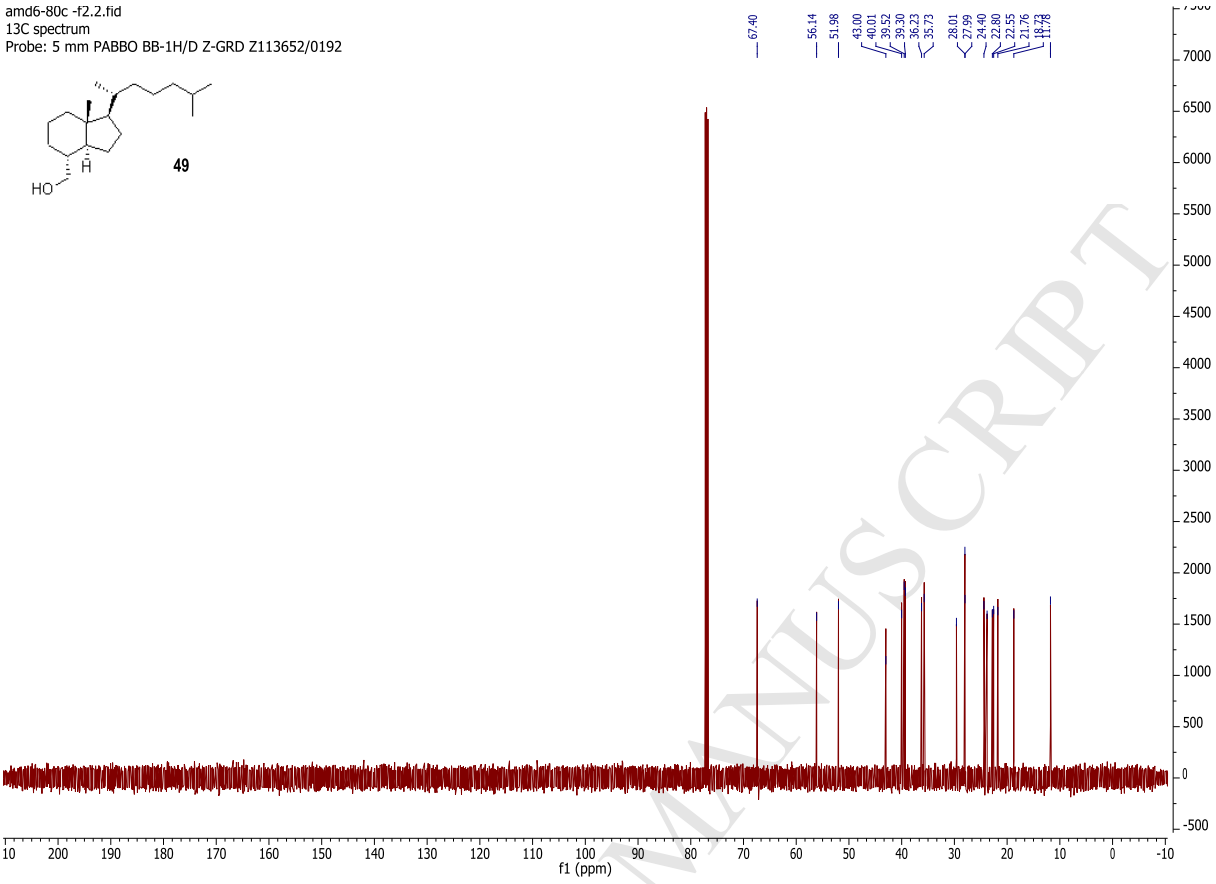


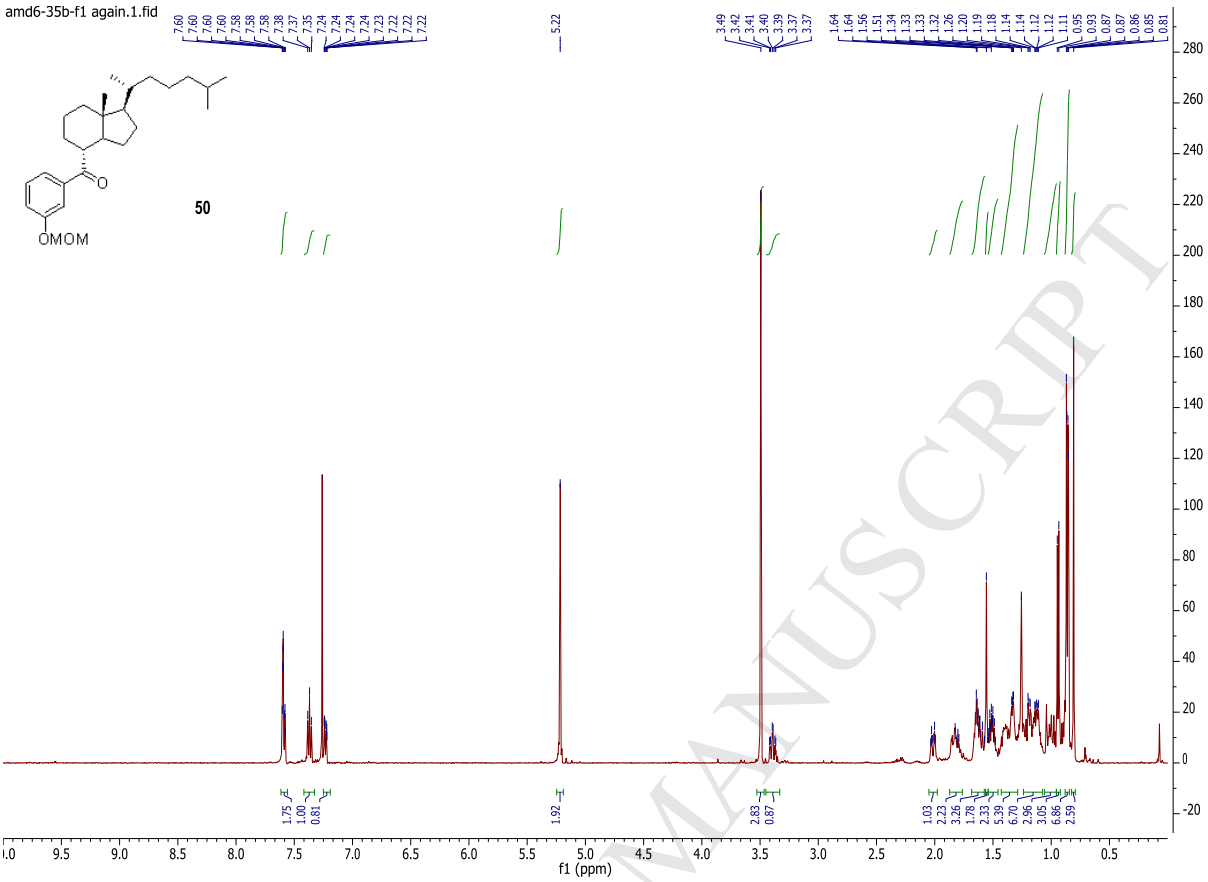


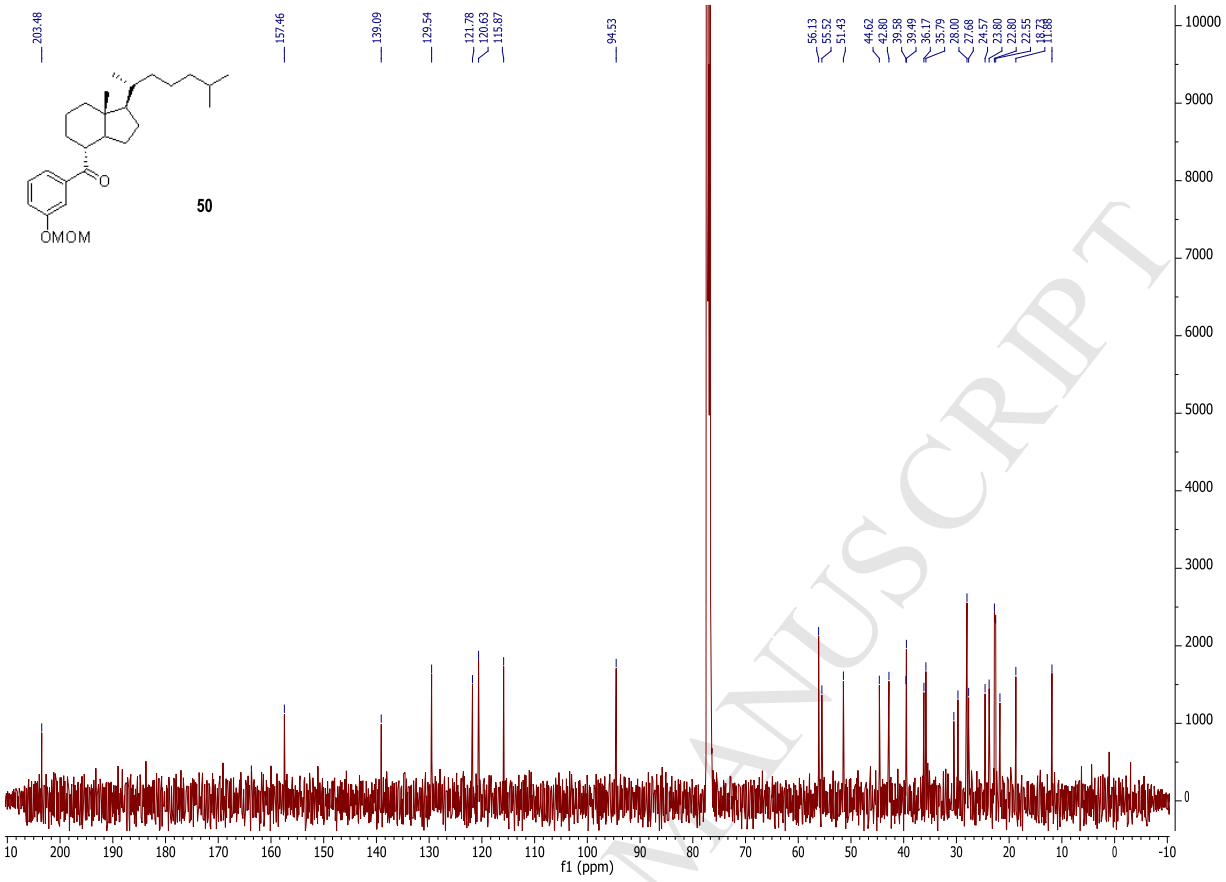


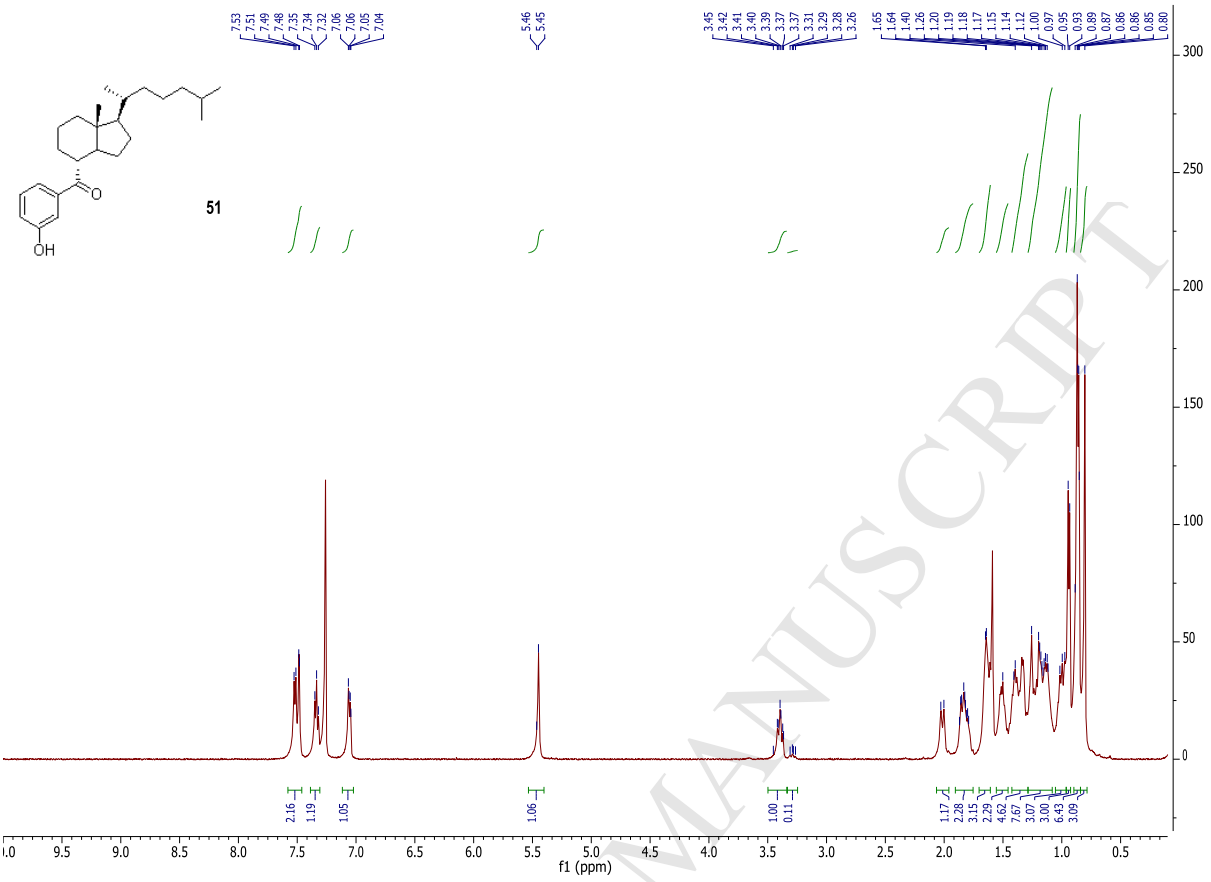


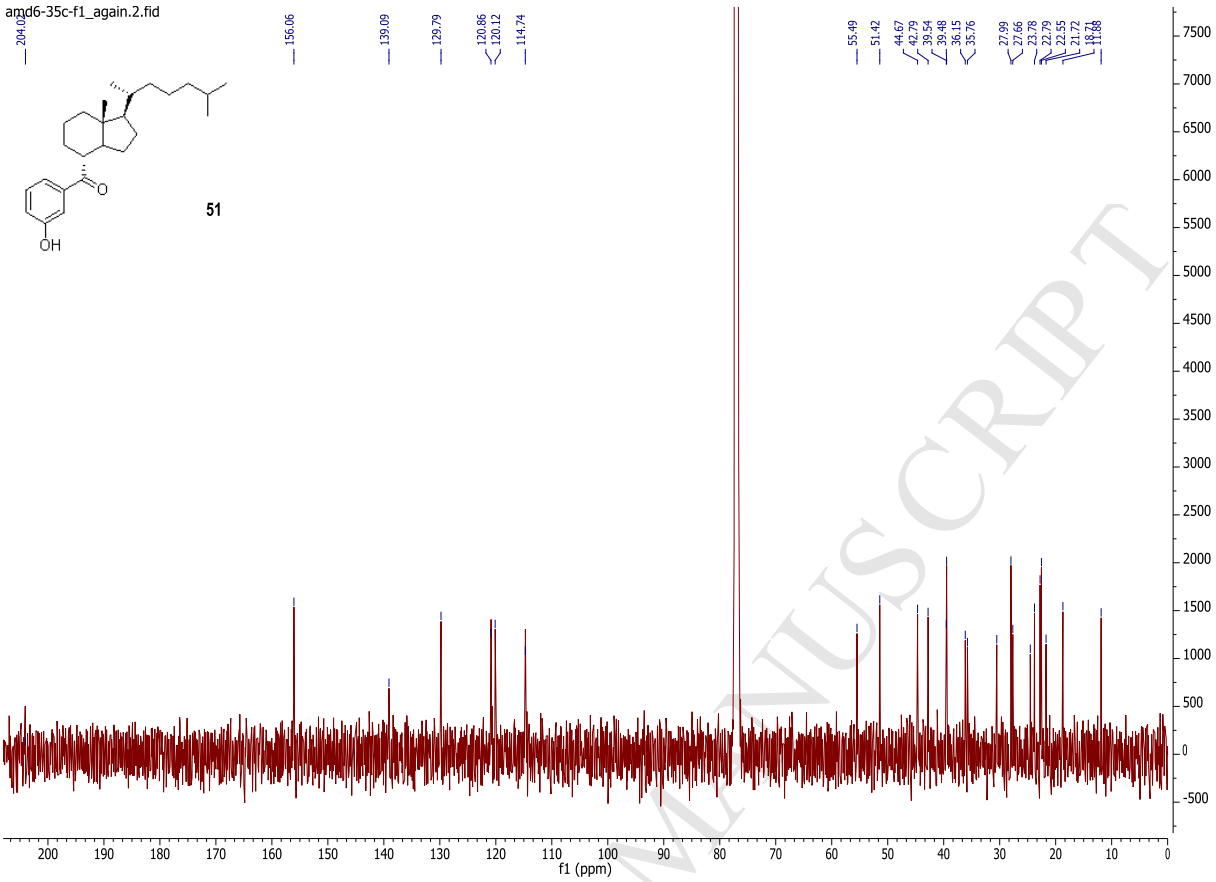


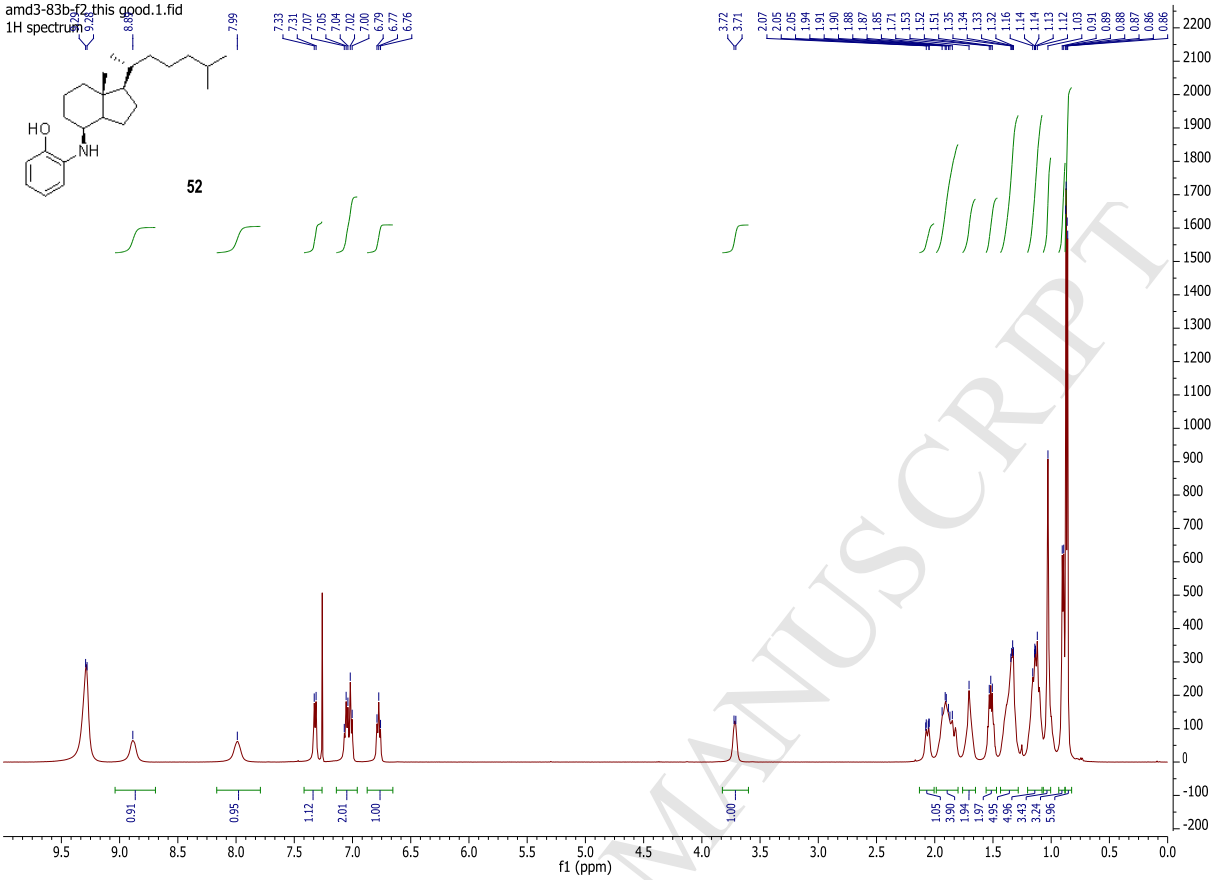


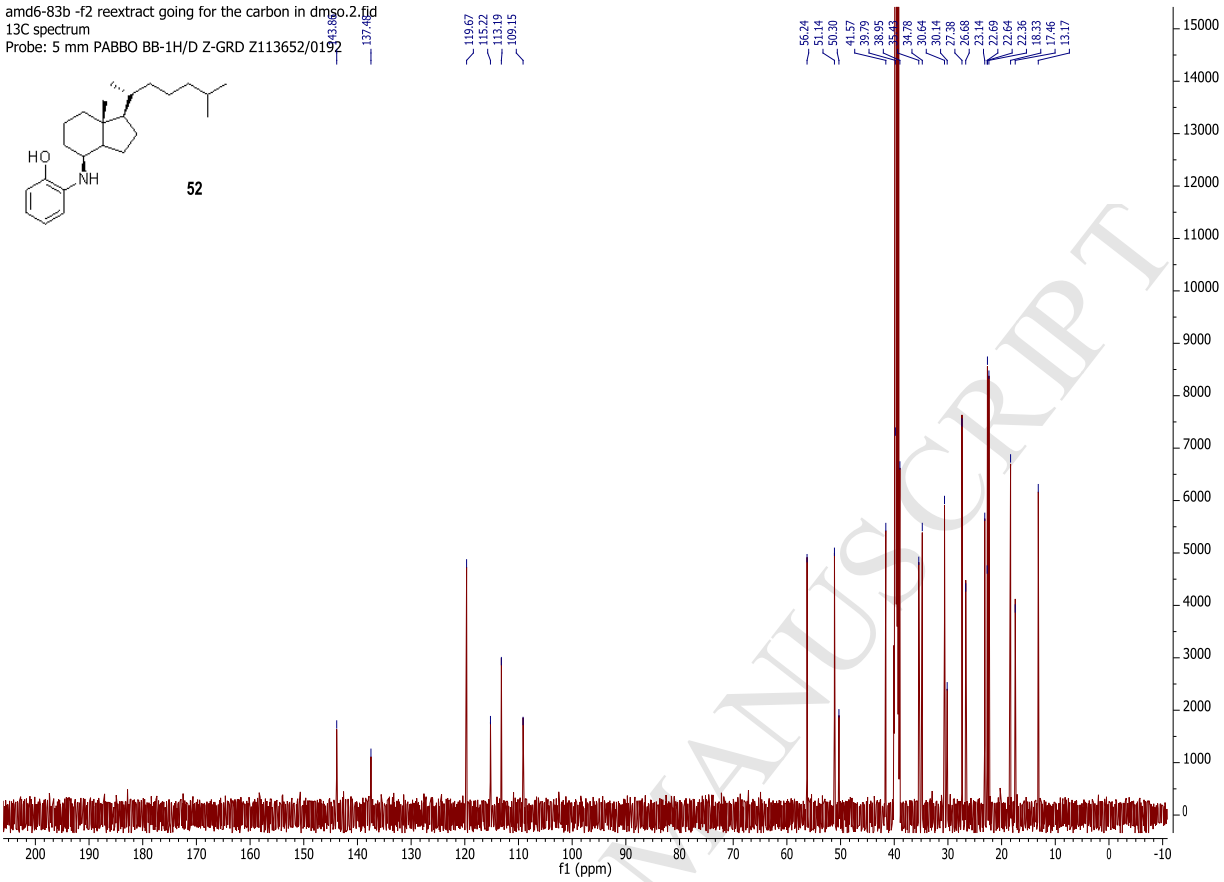


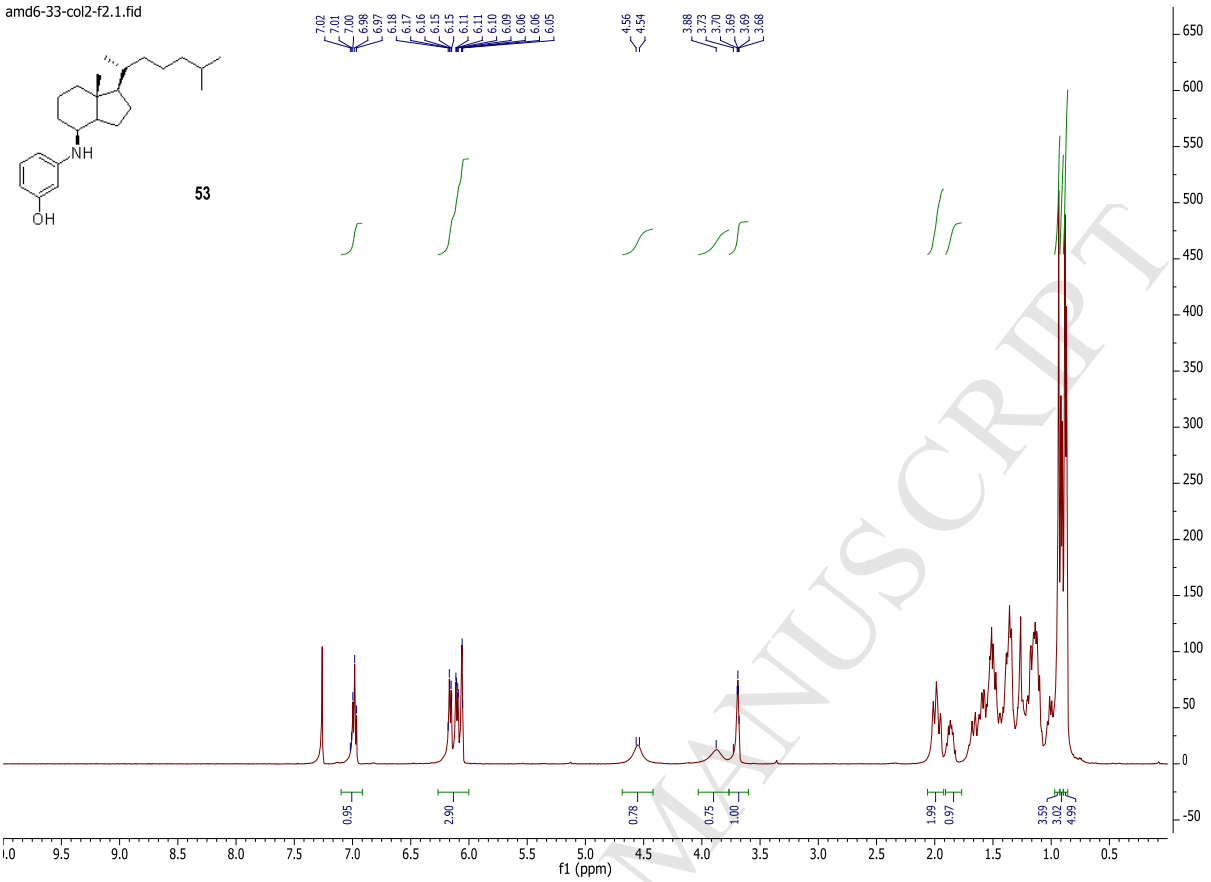


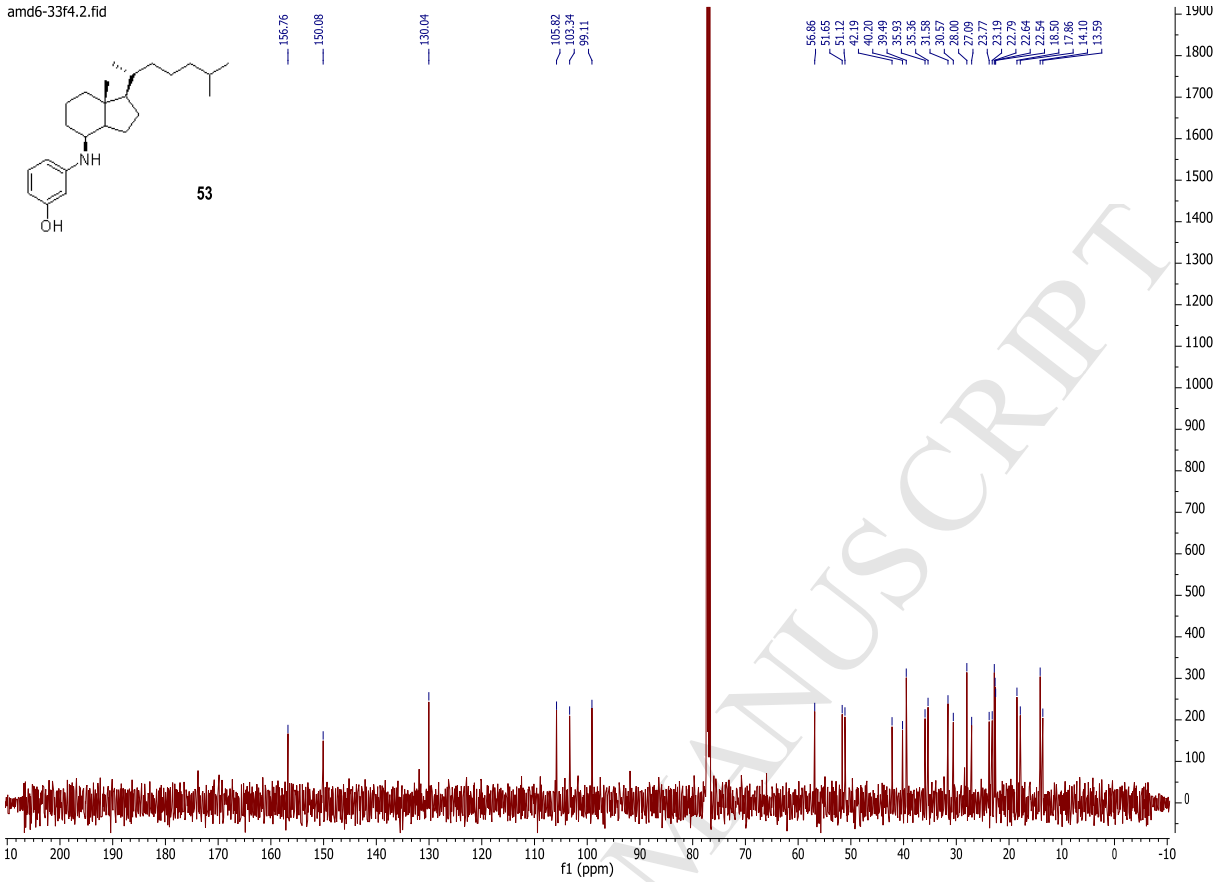




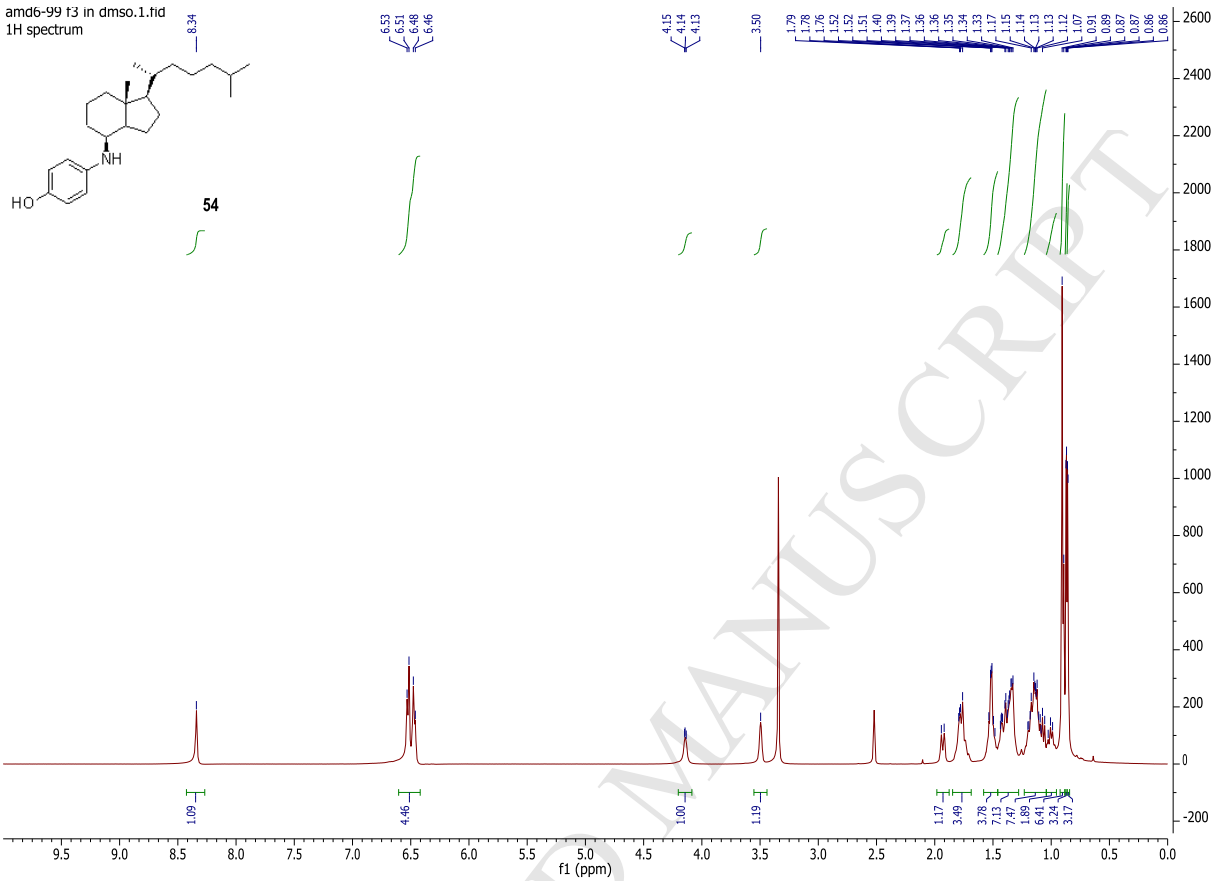




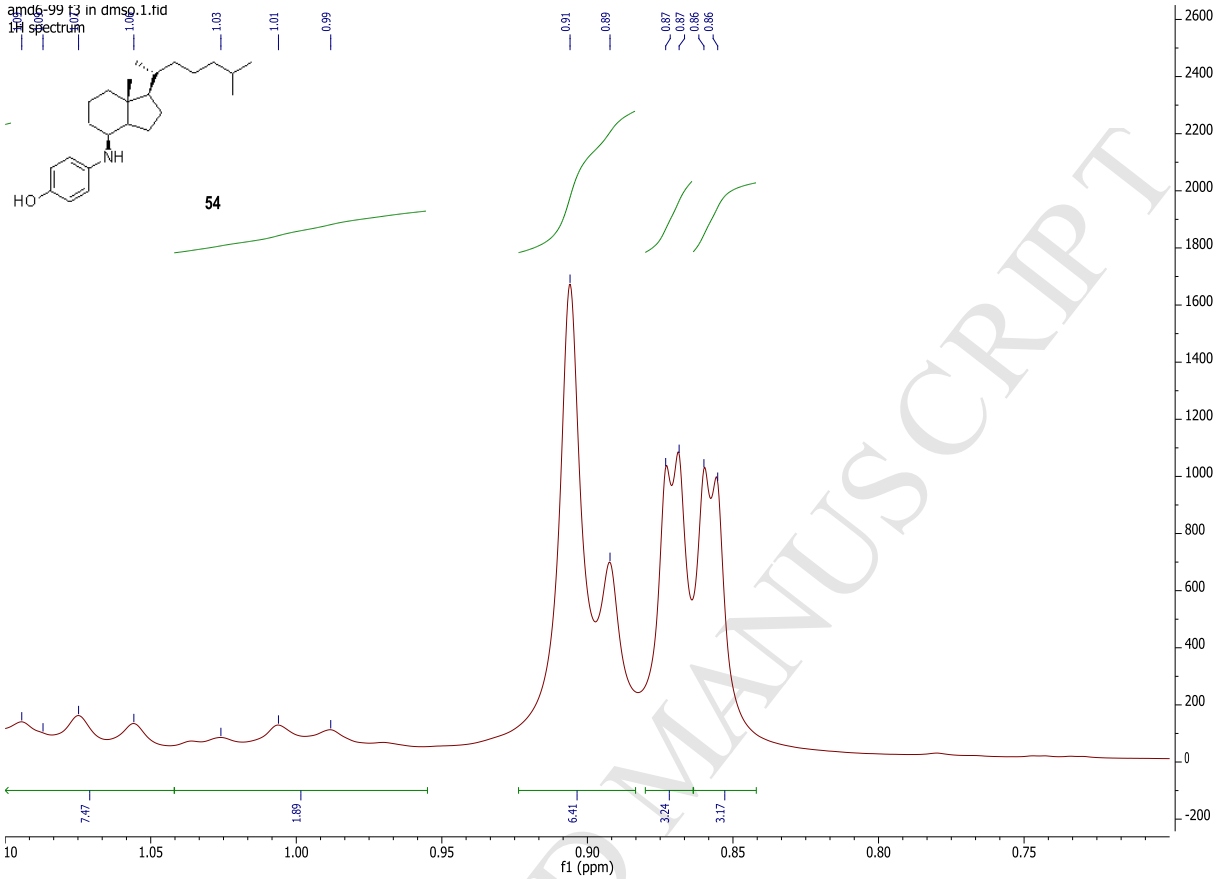


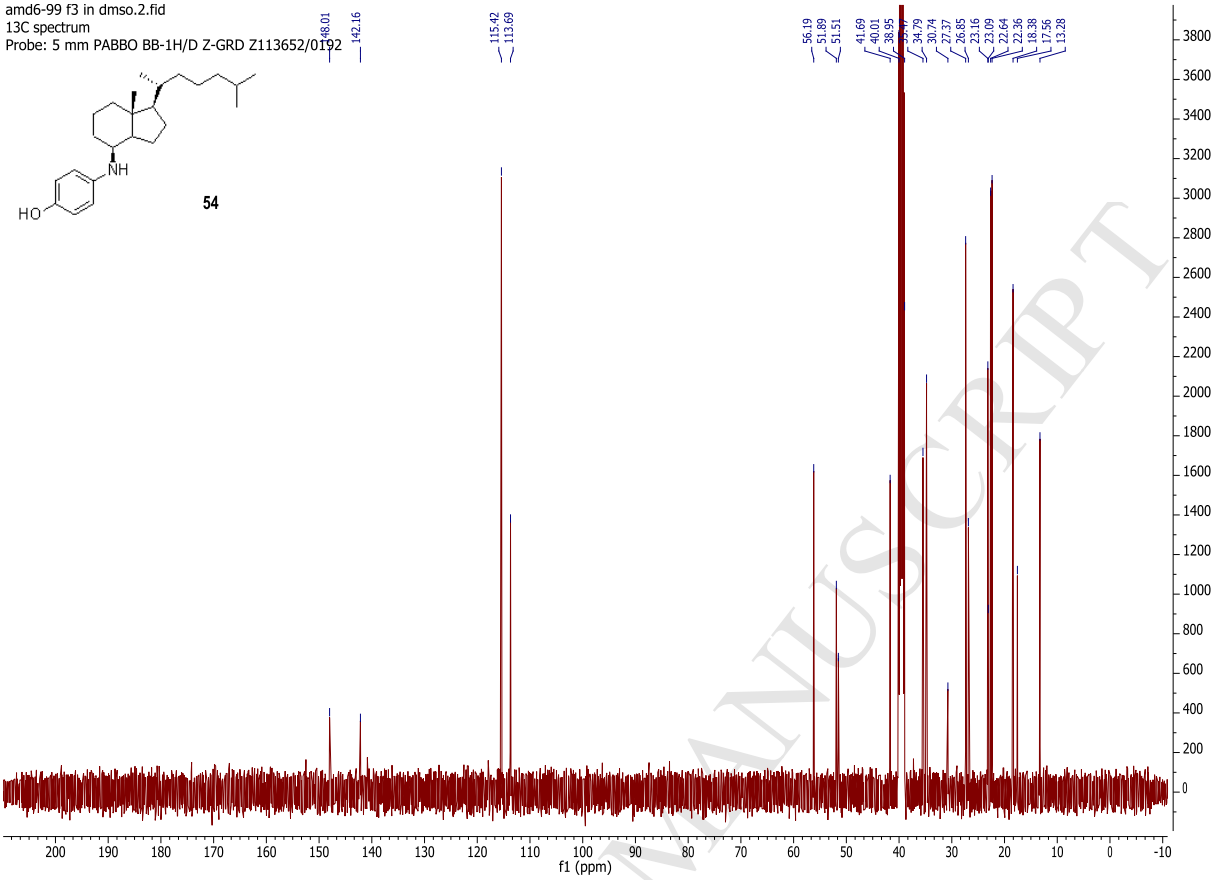


54: (full ^{1}H NMR spectrum in d-DMSO)

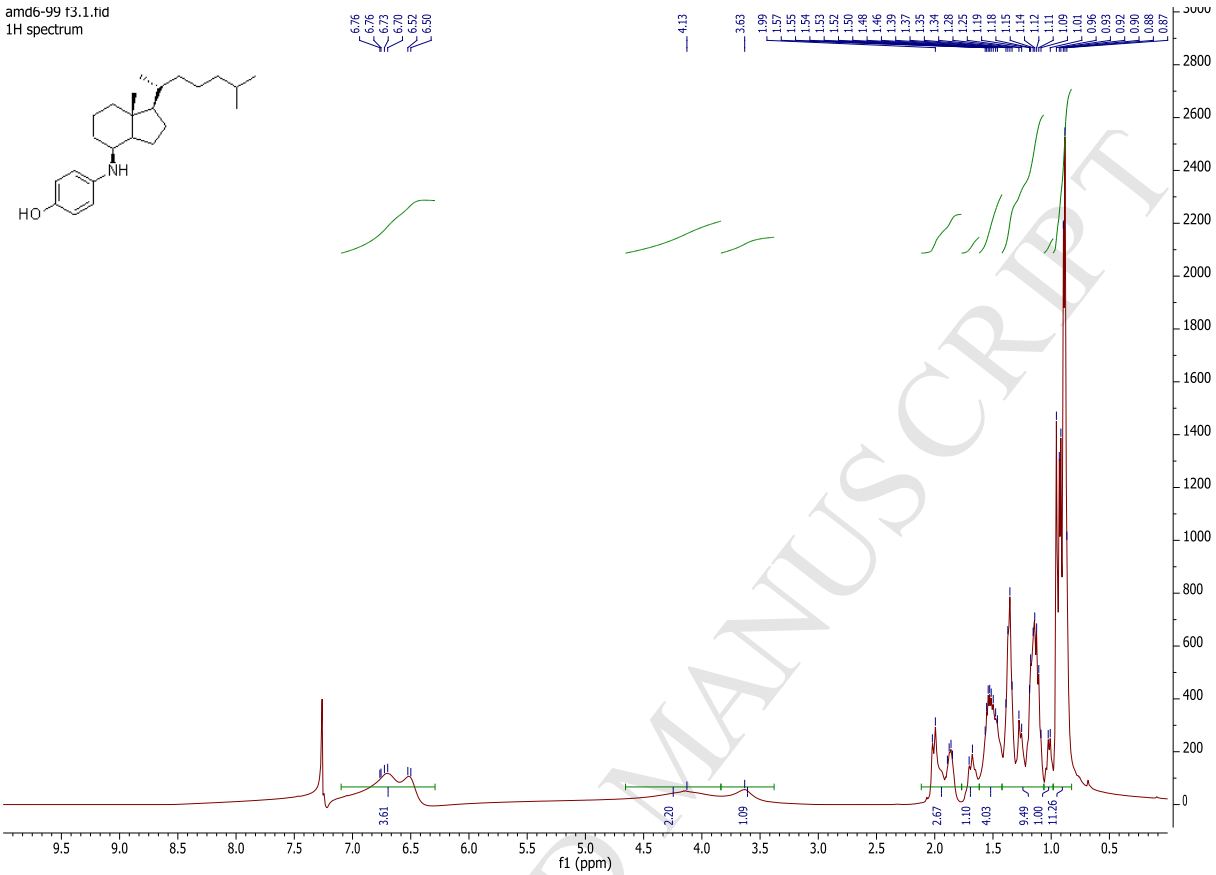


54: (expanded ^{1}H NMR spectrum in DMSO)

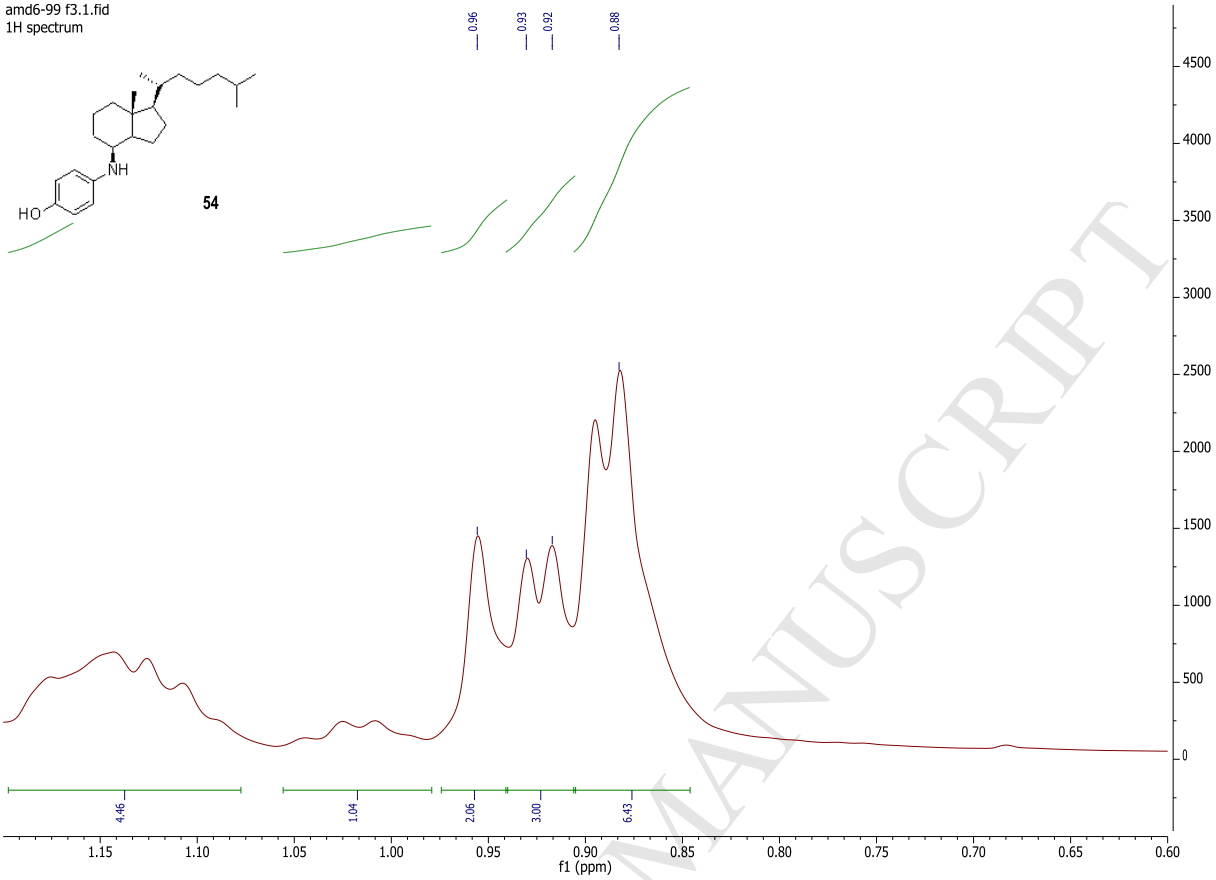




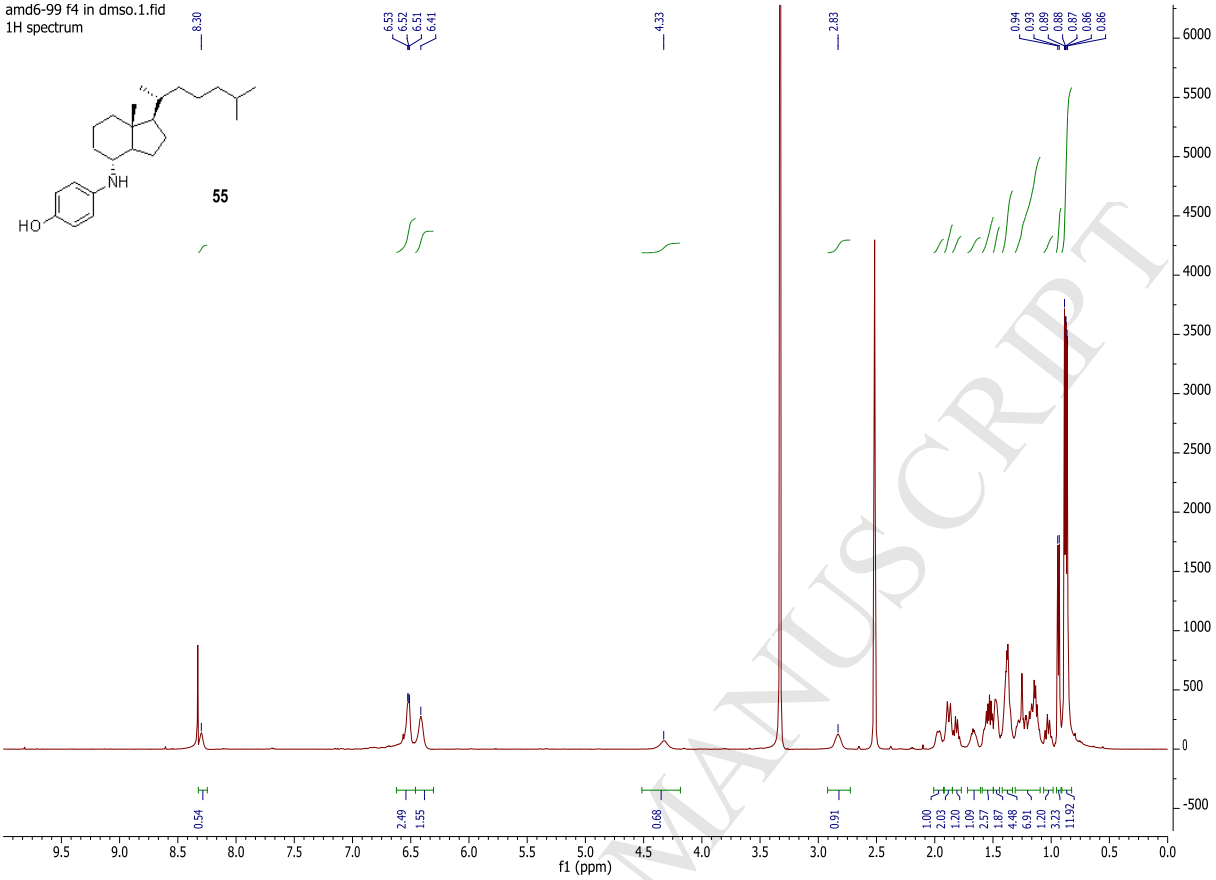
54: (full ^{1}H NMR spectrum in $CDCl_3$)



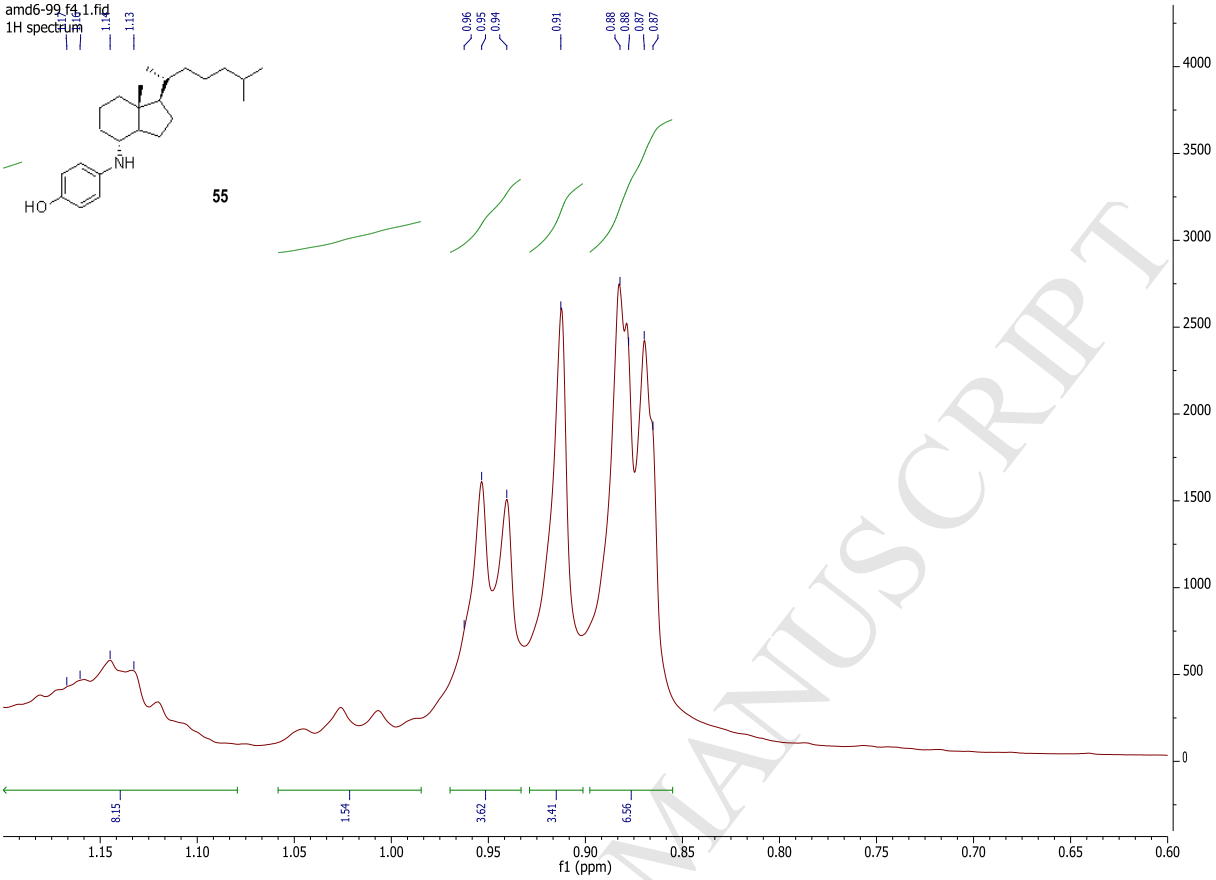
54: (expanded ^{1}H NMR spectrum in $CDCl_3$)

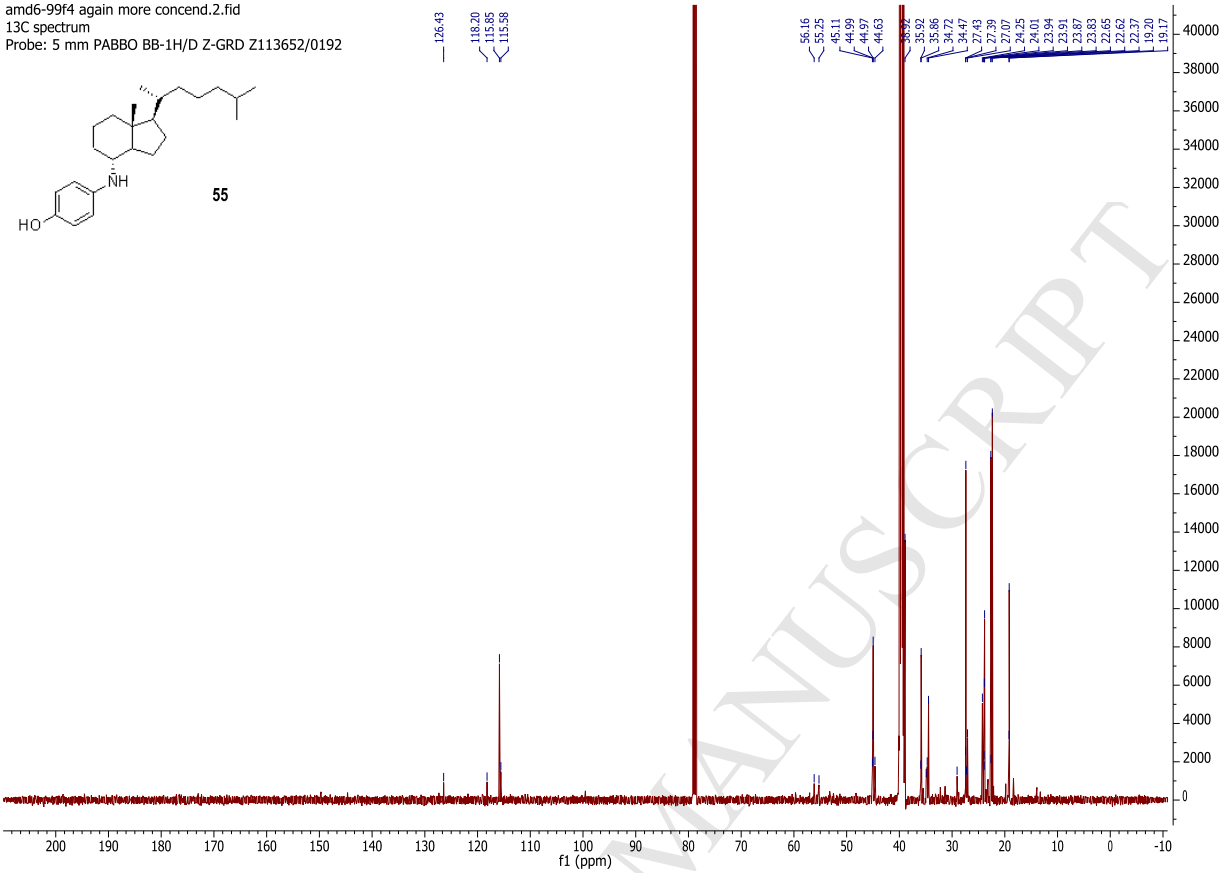


55: (full ^{1}H NMR spectrum in DMSO)

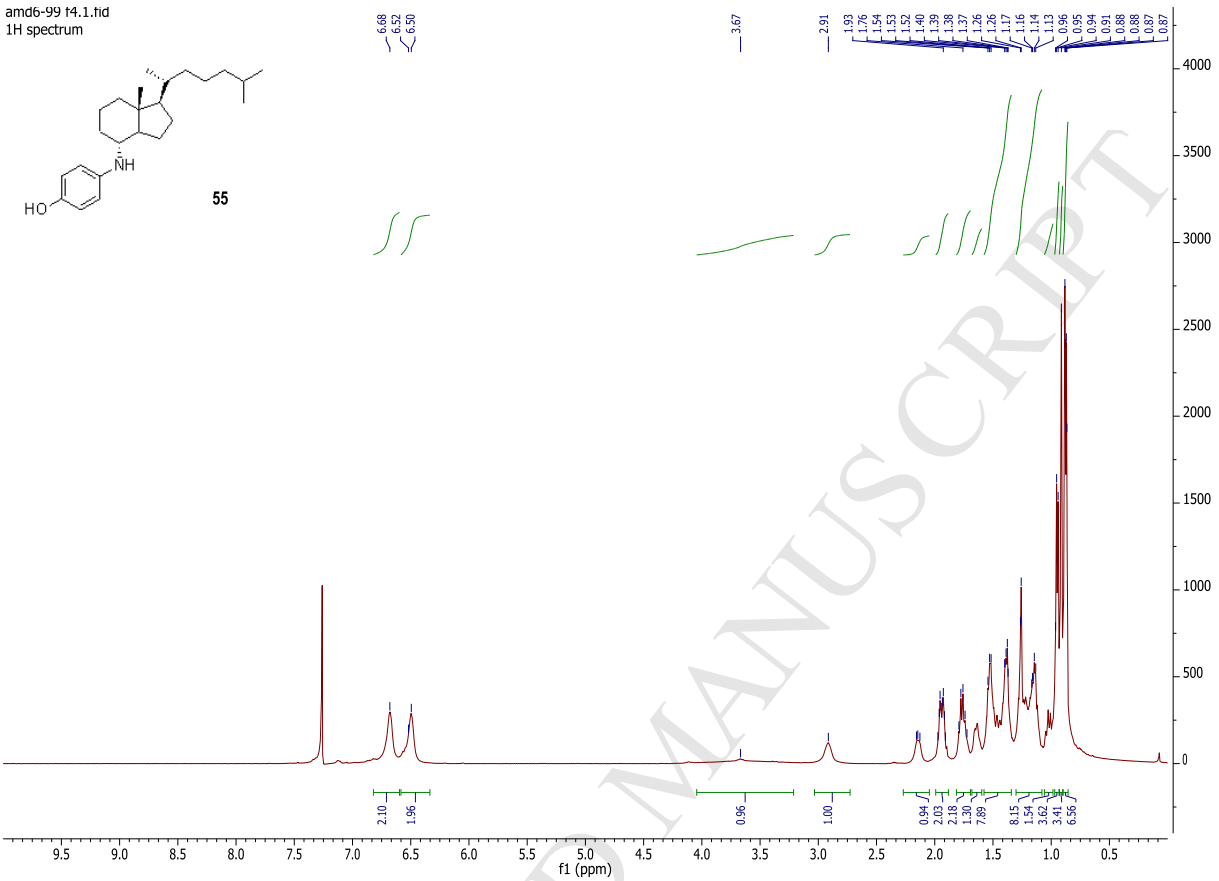


55: (expanded ^{1}H NMR spectrum in DMSO)





55: (full ^{1}H NMR spectrum in $CDCl_3$)



55: (expanded ^{1}H NMR spectrum in $CDCl_3$)