



Identification, Isolation, and Structural Characterization of Novel Forced Degradation Products of Darunavir Using Advanced Analytical Techniques Like UPLC–MS, Prep-HPLC, HRMS, NMR, and FT-IR Spectroscopy

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Abstract

Since the stability of the pharmaceuticals plays a crucial role in efficacy and safety while using them in the treatment of disorders, the evaluation of purity and impurity profiling of pharmaceuticals is of utmost importance using efficient analytical techniques. The present study explains the identification, isolation, and characterization of stress degradation products of the anti-human immunovirus drug Darunavir. The degradation study was performed to evaluate the stability profile of Darunavir in different stress conditions like hydrolytic, oxidative, thermal, and photolytic conditions as per the ICH guidelines. Degradation products were identified using ultra-performance liquid chromatography coupled with mass spectrometry, isolated using semi-preparative high-performance liquid chromatography, and structural characterization by HRMS and ¹H, ¹³C NMR (1D, 2D). Darunavir is relatively stable in oxidative, thermal, and photolytic conditions; however, considerable degradation was observed in acid and base hydrolysis. A total of five degradation products were identified and isolated in acid and base degradation. DP-1, DP -2, & DP-3 were observed in acid conditions, whereas in base conditions, along with DP-2, two more DPs, i.e., DP-4 & DP-5, were identified. Among the five DPs, two degradation products, namely **DP-1**: N-(4-(N-(3-amino-2-hydroxy-4-phenylbutyl)-N-isobutylsulfamoyl) phenyl) acetimidamide. & **DP-3**: hexahydrofuro[2,3-b]furan-3-yl(4-((4-acetimidamido-N-isobutylphenyl)sulfonamido)-3-hydroxy-1-phenylbutan-2-yl)carbamate, are novel, remaining degradation products **DP-2**: 4-amino-N-(3-amino-2-hydroxy-4-phenylbutyl)-N-isobutylbenzenesulfonamide, **DP-4**: 4-amino-N-(((5S)-4-benzyl-2-oxooxazolidin-5-yl) methyl) -N-isobutyl benzenesulfonamide and **DP-5**: methyl ((3S)-4-((4-amino-N-isobutylphenyl) sulfonamido)-3-hydroxy-1-phenylbutan-2-yl) carbamate are already reported tentatively using a single analytical technique coupled with mass analysis without any evidence from NMR and IR data. Hence, the present study focused on using High-Resolution Mass, 1D, and 2D ¹H, ¹³C NMR data for concrete confirmation of structures for degradation products.

Keywords Darunavir · Force degradation studies · UPLC–MS · HRMS · NMR

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Introduction

The purity of pharmaceuticals is critical when using them to treat various ailments since the existence of unrelated products or impurities that may arise during the manufacturing, storage, or transportation of these pharmaceuticals and can cause major harmful or adverse effects. For instance, the acid hydrolysis degradation products of tetracycline produce reversible Fanconi syndrome (a disorder of kidney tubes), thermal degradation products of Isoniazid produce genotoxicity and carcinogenicity, and oxidative and photolytic degradation product of Ibuprofen is cytotoxic to fibroblast, etc. [1]. The monitoring of impurities for their acceptable limits is a challenging task since the availability of the methods may or may not be sensitive or selective for the separation and identification of possible impurities present along with the drug substance. The utilization of antiviral drugs is drastically increased against different viral infections that were emerging currently days including COVID-19. Darunavir (DAR) is an antiretroviral drug from the protease inhibitor class, which is used to treat infection of human immunodeficiency virus and also recently used in the treatment of COVID-19 along with cobicistat drug. It selectively inhibits the cleavage of HIV-encoded Gag-polyproteins in virus-infected cells, thereby preventing the formation of mature infectious virus particles [2].

DAR is marketed in ethanolate form under the brand name Prezista, in different dose strengths, such as 75, 150, 300, 400, and 600 mg. In addition, Prezista is available as an oral suspension with a 100 mg/mL dose strength [3]. Its molecular formula is $C_{27}H_{27}N_3O_7S$ (Mol. Wt.: 547.67 mol/gm) and the chemical name is (3R,3aS,6aR)-hexahydrofuro [2,3-b] furan-3-yl (4-((4-amino-N-isobutylphenyl)sulfonamido)-3-hydroxy-1-phenylbutan-2-yl) carbamate. DAR appears as a white, non-hygroscopic fine powder with a melting range of 111–115 °C.

The literature scrutiny revealed that few analytical methods were reported for the separation, identification, and estimation of related substances of DAR [4–7], DPs [2, 8], and for the estimation of the DAR in bulk and dosage forms along with other drugs [9–13]. LC–MS/MS studies on metabolites rat serum metabolism, and pharmacokinetics after administration of the drug were reported [14, 15].

The reported methods for separation, identification, and estimation of related substances and DPs of DAR were proposed structures of DPs of DAR only based on mass fragmentations, and none of them reported on the isolation, characterization, and complete structural conformation of DPs of DAR using any high-end analytical technique like nuclear magnetic resonance (NMR)

spectroscopy (1D, and 2D), high-resolution mass spectrometry, and IR spectroscopy. Hence, the present research endeavored to provide concrete evidence for the structures of the DPs of DAR, which includes the UPLC–MS method development for efficient separation and estimation of DAR and its five DPs, along with the characterization of the separated and isolated DPs by HRMS, IR, and NMR (1D, 2D) experiments.

Materials and Methods

Chemicals and Reagents

Darunavir is a gifted sample from one of the pharmaceutical organizations in Hyderabad, water purified by Millipore (Millipore, Netherlands) was used. Analytical grade reagents and solvents were used. Acetonitrile, methanol, and formic acid, which are HPLC grade purchased from Merck India Ltd. Dimethyl sulfoxide- d_6 was purchased from Cambridge isotope laboratories, hydrochloric acid, sodium hydroxide, hydrogen peroxide, which are AR grade purchased from Merck India Ltd.

Instrumentation and Software

The analytical instruments and supported software used in the present research work are LC–MS instruments manufactured by Waters, single quadrupole mass detector coupled with Acquity UHPLC front end, and Maslynx 4.2 software. Purification instrument was from Waters binary module 2545, auto-sampler 2707 with detector-2489 and Chromscope-2.1 software. HRMS instrument from Thermo Q-exactive Orbitrap MS with ESI ion source and Dionex ultimate 3000 LC front end was supported by Xcalibur software. NMR instrument was from Bruker Avance Neo 400 MHz and Topspin 4.11 software. Infrared spectroscopy instrument was from Shimadzu IR-Afinity-1S supported by lab solution software and analytical balance from Sartorius-SQP-F.

Ultra-Performance Liquid Chromatography–Mass Spectrometry (UPLC–MS)

Liquid chromatography separation was performed on Waters make Single Quadrupole Detector (SQD2) coupled with Acquity UPLC & Diode array detector (DAD) front end. Waters SQ Detector-2 single quadrupole mass spectrometer operating in dual polarity-positive and negative with electrospray ionization source (ESI) is used for mass spectral analysis. Full-scan mode 100–1500 Daltons (Da) was used for the MS optimization. The capillary voltage and source temperature were set to 3.5 kV and 140 °C, respectively. The

desolvation temperature and gas flow were set as 350 °C and 650 L/h respectively, while the cone gas flow was set at 50 L/h. The liquid chromatography–mass spectrometer instrument was controlled by the Mass Lynx V4.2 application manager. The injection volume was 0.5 µL with sample manager temperature maintained at 18 °C and the chromatographic runtime of 7.0 min.

UPLC–MS Method Development (Optimization of Chromatographic Conditions)

In pursuit of a suitable, reproducible, and reliable method, different UPLC columns using different buffers were screened to optimize the resolution. During copious trials, 0.05% formic acid buffer and 0.05% formic acid acetonitrile with Acquity CSH C18 showed positive and encouraging results. Though API and 4 DPs (DP-1, DP-2, DP-3, & DP-5) were resolved in Acquity CSH C₁₈ 50×2.1 mm, 1.7 µ, DP-4 was not separated. Further, extensive trials were made on Acquity; BEH C₁₈ column with various flow rate and gradient conditions. The optimum separation of all degradation impurities and API was achieved on Acquity BEH C₁₈ 100×2.1 mm, 1.7 µ, with 0.05% Formic acid in aqueous and 0.05% Formic acid acetonitrile, flow rate- 0.3 mL/min; column temperature-35 °C; with Binary gradient Time/ B conc. (%): 0/20, 0.5/20, 3/40, 4.5/40, 6.0/90, 7/90, 7.1/10. All 6 peaks were separated with good resolution and peak shape in this condition.

Optimized Chromatographic Conditions

The chromatographic condition used was Acquity UPLC BEH C₁₈ column (100×2.1 mm, 1.7 µm), which was procured from Waters India Ltd. The mobile phase components were mobile phase-A: 0.05% formic acid in aqueous, mobile Phase-B: acetonitrile, separation was accomplished in a gradient elution program {Time (min)/B conc (%): 0/20, 0.5/20, 3/40, 4.5/40, 6/90, 7/90 at a flow rate of 0.3 mL/ min, Column temperature 35 °C. The chromatographic eluents were monitored using a photodiode array (PDA) detector, and the injection volume of 0.5 µL was used for sample injections.

Water and acetonitrile in the ratio of 50:50 (v/v) were used as a diluent. The typical chromatogram is shown in in Fig. 1.

High-Resolution Mass Spectrometry (HRMS)

Samples were analyzed on Thermo Q Exactive orbitrap MS with ESI ion source. The front end is UHPLC Dionex Ultimate 3000 instrument comprising the binary pump, column manager, and PDA detector. Instrument parameters (source) were Spray Voltage: 3200 V; Capillary Temperature: 300 °C; Aux gas flow rate: 14; Aux gas heater Temperature: 440 °C. Sheath gas flow rate: 50; Sweep gas flow rate: 3. Reserpine (monoisotopic mass: 608.2734 Da) was used to check the mass accuracy. Mass data were acquired using Xcalibur software.

Preparative HPLC

Waters preparative HPLC equipped with ChromScope-2.1 software, 2545 pump module, 2489 dual UV detector, and 2707 sample manager with auto-fraction collector-III. In-house packed Luna C18 10µ, 150×25 mm (Phenomenex) was used to isolate the degradation products, with a flow rate of 18 mL/min and mobile phase 0.1% v/v formic acid in aqueous and acetonitrile. All isolated fractions were lyophilized using a Lyofreeze lyophilizer.

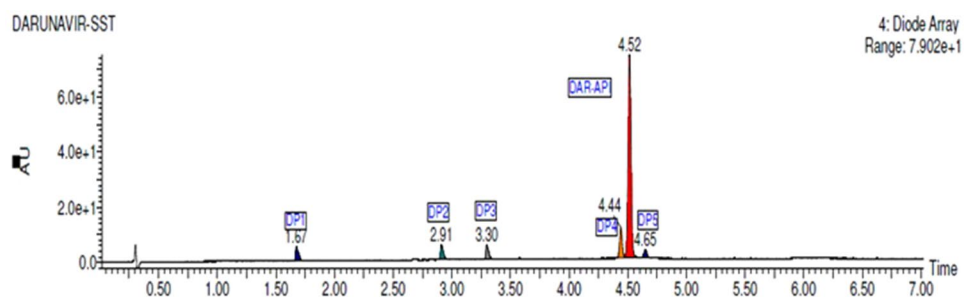
Nuclear Magnetic Resonance Spectroscopy (NMR)

¹H, ¹³C, and 2D NMR spectra of Darunavir and degradation products were recorded in DMSO-d₆ solvent on Bruker Avance Neo 400 MHz NMR instrument equipped with 5 mm i- probe (Broad Band Observance) with Z-gradient shim system, which has the sensitivities of 550:1 and 220:1. The ¹H NMR spectra referenced tetra-methyl-silane (TMS) singlet at zero (0) ppm and referenced DMSO-d₆ septet at 39.5 ppm in the ¹³C NMR spectrum.

FT-IR Spectroscopy

Shimadzu IR-Affinity-1S model with Lab solutions software was used to know the functional groups present in the

Fig. 1 Chromatogram of Darunavir and its Degradation Products



compounds. KBr was used as a medium to make the sample pellets.

Degradation Behavior of DAR

DAR was subjected to various forced degradation stress conditions, and the compound showed highly stable in oxidative, thermal, and photolytic conditions and did not form any degradation products, which confirms the stability of DAR toward the above conditions. The drug was found to be labile to acid and base hydrolysis in the presence of Acetonitrile and Methanol. As a result, ~15–20% of degradation was observed in both acid (1 N HCl with stirring at room temperature, up to 48 h) and base (1 N NaOH with stirring at room temperature, up to 36 h) conditions. Detailed degradation conditions [16–21] and results are displayed in Table 1, and degradation chromatograms of acid and base hydrolysis are shown in Fig. 2. In acid condition, 3 degradation products were formed (DP-1, DP-2, & DP-3), whereas in base conditions, along with DP2, 2 more additional degradation products were developed (DP-4 & DP-5). The confirmed structures of all the DPs are shown in Fig. 3. The rate of

degradation was influenced by temperature. Increasing the temperature increased the rate of degradation, and the percentage of DPs formed also increased.

Preparation of Degradation Samples for Purification

Degradation was observed in acidic and base conditions. The acid-degraded sample was neutralized with 5 N sodium bicarbonate solution, and the resultant solution was lyophilized to get a crude solid sample. The same was dissolved in 4–5 mL of Acetonitrile: Water (1:1 v/v). For base degradation, the degraded sample was neutralized with 5 N HCl solution, and the resultant solution was lyophilized to get a free solid. The same sample was dissolved in 4–5 mL of Methanol: water (1:1 v/v) for preparative HPLC purification.

Results and Discussion

Using liquid chromatography–mass spectrometry, individual samples were analyzed to determine the results of all the stress studies. All degradation products formed over a

Table 1 Darunavir forced degradation studies

Conditions	DP-1	DP-2	DP-3	DP-4	DP-5	API
Darunavir API (ACN + MeOH)	–	–	–	–	–	99
Acid (1 N HCl stirring at rt up to 48 h) (ACN + MeOH)	8	5	6	–	–	81
Base (1 N NaOH stirring at rt up to 36 h) (ACN + MeOH)	–	6	–	8	6	80
Oxidation (30% H ₂ O ₂ stirring at rt up to 48 h) (ACN + MeOH)	–	–	–	–	–	98
Thermal (explore to 100 °C up to 48 h) (ACN + MeOH)	–	–	–	–	–	98
Photolytic (explore at 254 nm for 48 h) (ACN + MeOH)	–	–	–	–	–	98

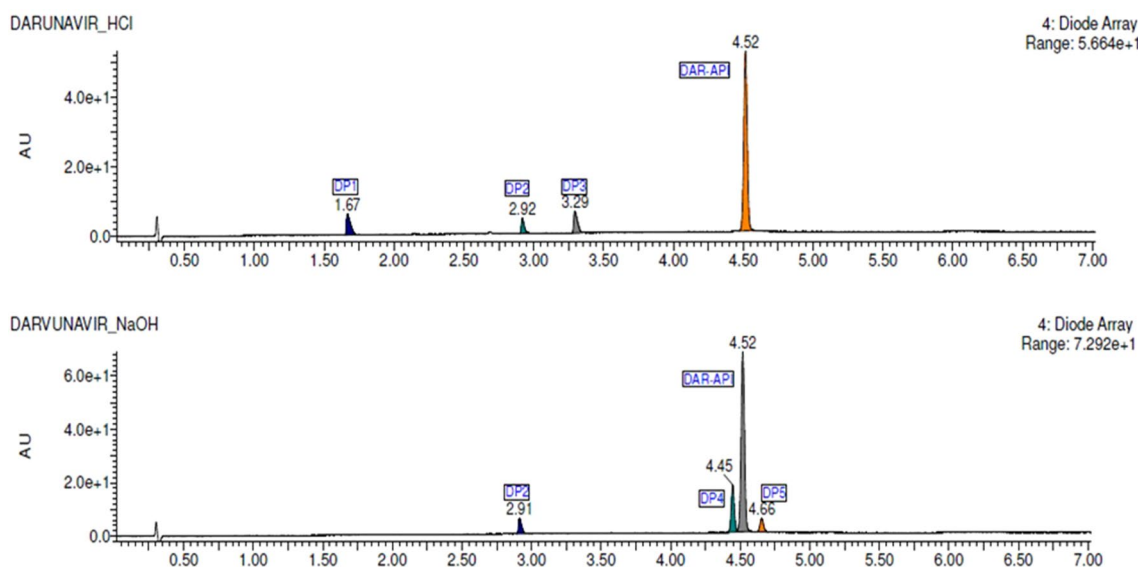


Fig. 2 Degradation behavior of Darunavir in Acid & Base

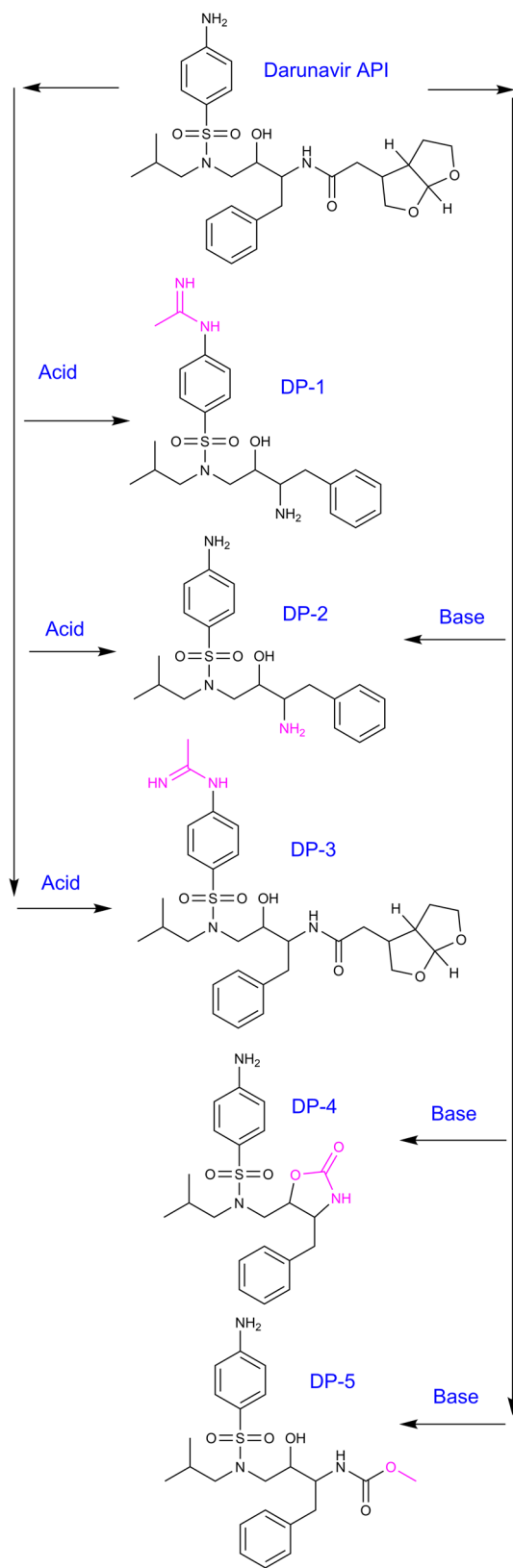


Fig. 3 Structure of Darunavir and its degradation products

period, and from each constraint, study conditions are outlined in the experimental section. Five significant degradation products developed in the study were isolated, identified, and characterized by UPLC–MS, HRMS, NMR (1D and 2D), and FT-IR techniques.

Isolation of Degradation Products

Degradation was observed under acid and base stress conditions with a considerable percentage of impurity formation > 5%. Purification was carried out using 0.1% formic acid in aqueous and acetonitrile as a mobile phase with In-house packed Luna C18 10 μ , 150 \times 25 mm (Phenomenex). Crude sample solutions were injected in consecutive injections, and the fractions were collected based on UV response and later mass confirmed by UPLC–MS. After completion of the degradation procedure, the degradation products were purified in Preparative HPLC to collect all the fractions of various degradation products separately and lyophilized to get a free solid.

Structural Confirmation of Degradation Products

To get the structural information of DAR API, all analytical data were recorded for reference purposes. Under positive ESI–MS conditions, $[M + H]^+$ at m/z 548.24, which confirms the molecular formula for DAR is $C_{27}H_{37}N_3O_7S$. The confirmed data of DAR are mentioned at HRMS (Fig. 4A), HRMS (Table 2), IR (Table 3), and NMR 1D & 2D (Table 4). The structural conformation of DAR by 2D-NMR is shown in Fig. 5. All the other degradation products are characterized based on the comparison of this structural elucidation data.

Degradation Product (DP-1) Characterization

The DAR API compound on treatment with acid, a degradation product-1 (DP-1), has been formed. This degradation product has been isolated and its mass was confirmed as $(M + H)^+$ 433.2249 with 1.21 ppm error in HRMS. For the calculated molecular formula, $C_{22}H_{32}O_3N_4S$ is shown in in Fig. 4B. The complete structure of the impurity was characterized by NMR experiments. The sample was prepared in a DMSO- D_6 -TFA-D mixture of solvents (0.5 ml of DMSO + 0.1 ml of TFA) and conducted NMR analysis. In proton NMR, a few proton signals are broadened, hence adding 0.1 ml of TFA to the DMSO solution to enhance the relaxation. In proton NMR, a total of 27 protons were observed, of which 9 are aromatic protons at 7.0 ppm to 8.0 ppm, and 18 protons are present in the aliphatic region at 0.6 ppm to 4.1 ppm. Two ortho-doublet signals at 7.52 ppm, 7.89 ppm with two protons integration, belong to a 1,4-disubstituted benzene ring in the structure, benzyl protons

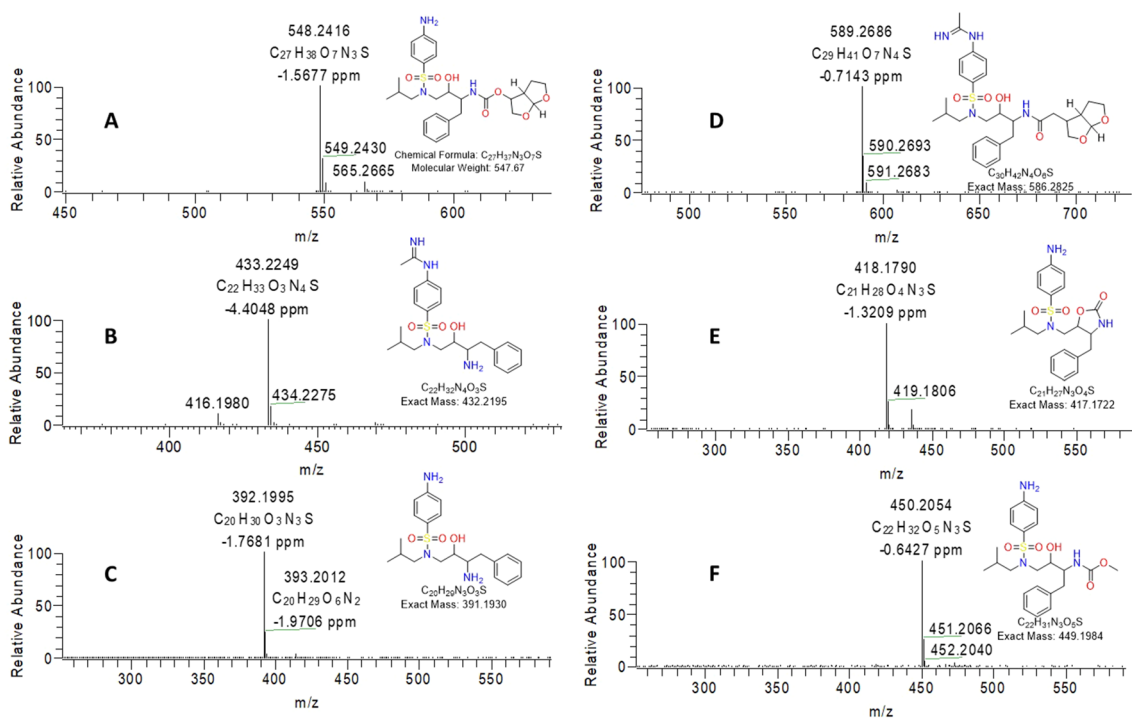


Fig. 4 HRMS data of Darunavir and its degradation products

Table 2 HRMS and melting range data for all degradation products

Products name	Formula	<i>m/z</i> Calculated	<i>m/z</i> Obtained	Mass error (ppm)	Melting range (°C)
Darunavir	C ₂₇ H ₃₈ O ₇ N ₃ S	548.2430	548.2416	1.56	111–115
DP-1	C ₂₂ H ₃₂ N ₄ O ₃ S	433.2273	433.2249	1.21	97–101
DP-2	C ₂₀ H ₂₉ N ₃ O ₃ S	392.2008	392.1995	1.77	95–99
DP-3	C ₂₉ H ₄₀ N ₄ O ₇ S	589.2696	589.2686	0.71	131–135
DP-4	C ₂₅ H ₂₇ N ₅ O ₅ S	418.1800	418.1790	1.32	105–109
DP-5	C ₂₂ H ₃₁ N ₃ O ₅ S	450.2062	450.2054	0.64	64–68

Table 3 Comparative FTIR data of Darunavir and its degradation products

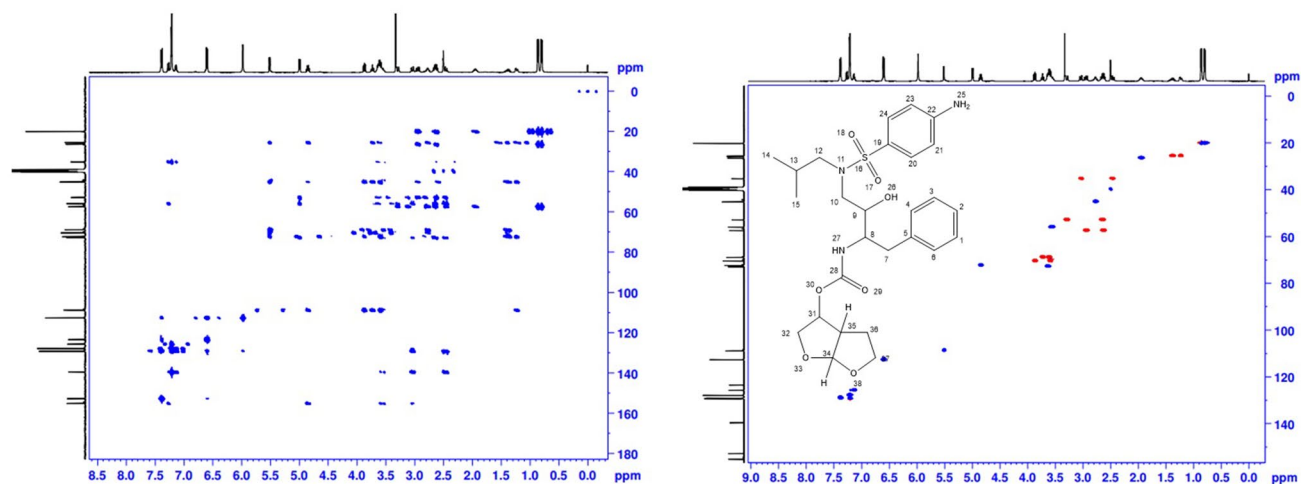
Assignment	Region (cm ⁻¹)	API	DP-1	DP-2	DP-3	DP-4	DP-5
O–H/N–H stretching	3700–3300	~3368	~3410	~3370	~3349	~3470,3368	~3367
SP ³ C–H stretching	2850–3000	~2965	~2957	~2963	~2962	~2960	~2959
C=O stretching	1760–1630	~1699	~1694	–	~1707	~1754	~1707
S=O asymmetric stretching	1300–1420	~1338	~1338	~1348	~1331	~1317	~1315
S=O symmetric stretching	1000–1200	~1148	~1154	~1145	~1153	~1148	~1146
C–O stretching in 2° alcohol	1087–1024	~1088	~1089	~1088	~1090	~1087	~1088

observed at 7.28 ppm and 7.35 ppm with one proton and four protons integration. A singlet signal with three proton integration at 2.39 ppm confirms the presence of a methyl group (acetamidine). Two doublets with 6 protons integration at 0.76, 0.82 ppm correspond to methyl groups forming the isobutyl group. In ¹³C NMR, a total of 17 carbon signals

were observed, out of which 9 signals were observed around 120 to 165 ppm, and 8 carbons were around 15 to 70 ppm, due to symmetry, the four aromatic signals' (Benzyl and 1,4-disubstituted benzene ring) intensity was high and considered as eight aromatic protonated carbons, most down-field carbon at 164.62 ppm corresponding to acetamidine

Table 4 Relative NMR assignments for Darunavir API

DAR-API (Recorded in DMSO-D6 solvent)					
Atom serial number	Name of the atom	Proton resonance frequency(ppm) and coupling constant J (Hz)	Carbon resonance frequency (ppm)	^1H - ^1H -gDQCOSY	^1H - ^{13}C -HMBC
1,3	CH	7.20(m,2H)	127.85	7.13	139.51
2	CH	7.13(m,1H)	125.66	7.2	129.2
4,6	CH	7.20(m,1H)	129.2	—	125.66, 35.15
5	C	—	139.51	—	—
7	CH ₂	2.43(dd,13.2 Hz,11.2 Hz,1H),3.01(dd,13.6 Hz,2.8 Hz,1H)	35.15	3.54	129.2, 139.51, 55.86, 72.8
8	CH	3.54(m,1H)	55.86	2.43,3.01,7.25	139.51
9	CH	3.64(m,1H)	72.8	4.98,2.65,3.27	—
10	CH ₂	2.65(m,1H),3.27(dd,14.8 Hz,3.2 Hz,1H)	52.76	3.64	57.33, 55.86
12	CH ₂	2.60(m,1H),2.91(dd,13.2 Hz,8.4 Hz,1H)	57.33	1.94	52.76, 26.34, 20.05
13	CH	1.94(m,1H)	26.34	2.60,2.91&0.78,0.85	57.33, 20.05
14.15	CH	0.78,0.85(d,6.4 Hz,6H)	20.05	1.94	20.05, 57.33

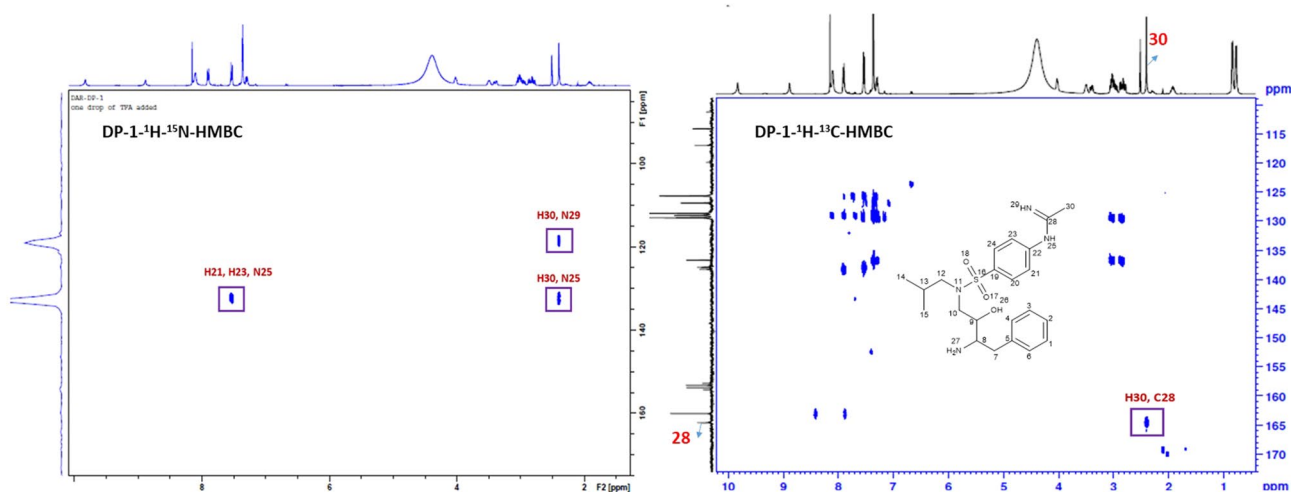
**Fig. 5** Darunavir- ^1H - ^{13}C -HMBC & HSQC spectrum

carbon. In edited g-HSQC experimental data, confirmed the presence of 9 aromatic protonated methine groups, 3 methyls, 3 methylene groups, and 3 methine groups in the aliphatic region. In gDQ-COSY experiment, H13 methine proton shows a correlation with H12 methylene and H14,15 methyl groups, and H8 methine proton shows a correlation with H7 methylene and H9 methine and H9 methine showing a correlation with H10 methylene protons, H8 proton. In g-HMBC experiment, a key 3 J correlation is observed from H30 protons to C28 carbon. In ^1H - ^{15}N -HMBC experiment, H30 methyl protons showed 3j correlation with N29(118.47 ppm) and N25(132.43 ppm) Nitrogen, as well, H21,23 protons showed connectivity to N25 Nitrogen. One excess methyl group and missing hexahydrofuro[2,3-b]furan ring protons in proton NMR, presence of extra

carbon at 164.62 ppm, key correlation in g-HMBC and ^1H - ^{15}N -HMBC experiments, all these NMR studies concluded that hexahydrofuro[2,3-b]furan cleaved at carbamate link and formed N-(4-(N-(3-amino-2-hydroxy-4-phenyl butyl)-N-isobutylsulfamoyl) phenyl) acetimidamide. The proton, carbon chemical shifts, and COSY, HMBC experiment connectivity were captured in Table 5, and the structural elucidation is represented in Figs. 6, 7, 8, 9, 10. In FTIR spectra, stretching frequency observed at 3410 cm^{-1} indicates the presence of NH group, the stretching frequency at 1089 cm^{-1} indicates the presence of secondary alcohol, and stretching frequency at 1338 cm^{-1} , 1154 cm^{-1} indicates the presence of the sulfoxide group, as shown in Table 3. The melting range of DP-1 is $97\text{--}101\text{ }^\circ\text{C}$. The possible

Table 5 Relative NMR assignments for DP-1

DAR-DP-1 (Recorded in DMSO-D6 + 1 drop of TFA-D solvent)					
Atom serial number	Name of the atom	Proton resonance frequency (ppm) and coupling constant J (Hz)	Carbon resonance frequency (ppm)	^1H - ^1H -gDQCOSY	^1H - ^{13}C -HMBC
1,3	CH	7.35 (m,2H)	128.68	7.28	136.69
2	CH	7.28 (m,2H)	126.91	7.35	129.44
4,6	CH	7.36 (m,2H)	129.44	–	126.91, 32.60
5	C	–	136.69	–	–
7	CH ₂	2.84 and 3.04 (m, 2H)	32.6	3.49	136.69, 129.44, 55.16 and 68.49
8	CH	3.49 (broad hump, 1H)	55.16	2.84, 3.04 and 4.02	–
9	CH	4.02 (broad triplet, 1H)	68.49	3.49, 2.92 and 3.37	–
10	CH ₂	2.92 (dd,14.4 Hz,7.6 Hz, 1H and 3.37 (dd,14.4 Hz,4.0 Hz,1H)	50.96	4.02	–
12	CH ₂	2.79 (dd,14.0 Hz,6.8 Hz,1H and 3.01(m, 1H)	56.95	1.92	50.96, 26.34 and 19.9
13	CH	1.92 (M,1H)	26.34	2.79, 3.01, 0.76 and 0.82	56.95, 19.9
14,15	CH ₃	0.76 and 0.82 (d,6.4 Hz,6H)	19.9	1.92	56.95, 26.34
19	C	–	137.87	–	–
20,24	CH	7.89 (d, 8.4 Hz, 2H)	129.03	7.52	138.22
21,23	CH	7.52 (d, 8.4 Hz, 2H)	125.69	7.89	137.87
22	C	–	138.22	–	–
28	C	–	164.62	–	–
30	CH ₃	2.39 (s, 3H)	19.15	–	164.62

**Fig. 6** ^1H - ^{15}N -HMBC & ^1H - ^{13}C -HMBC data of DP-1

mechanism of DP-1 formation from DAR in acidic conditions is explained in Fig. 11.

Degradation Product (DP-2) Characterization

Degradation product-2 (DP-2) has been formed on treating DAR with acid as well as base, and this DP-2 is isolated and its mass was confirmed as $(\text{M} + \text{H})^+ 392.1995$ with

1.77 ppm error in HRMS for the calculated molecular formula $\text{C}_{20}\text{H}_{30}\text{O}_3\text{N}_3\text{S}$ shown in Fig. 4C. The complete study of DP-2 has been done by 1D and 2D NMR studies. We have recorded needed NMR experiments in 400 MHz instrument, dissolved ~ 10 mg of DP-2 compound in DMSO-D6 and recorded ^1H , ^{13}C , g-COSY, g-HSQC and g-HMBC experiments. In proton NMR, a total of 26 protons were observed, in which, 9 protons from the aromatic region, 15

Table 6 Relative NMR assignments for DP-2

DAR-DP-2 (Recorded in DMSO-D6 solvent)					
Atom serial number	Name of the atom	Proton resonance frequency (ppm) and coupling constant J (Hz)	Carbon resonance frequency (ppm)	^1H - ^1H -gDQCOSY	^1H - ^{13}C -HMBC
1,3	CH	7.31 (m, 2H)	128.42	7.23	138.59
2	CH	7.23 (m, 1H)	126.33	7.31	129.43
4,6	CH	7.33 (m, 2H)	129.43	—	126.33, 35.43
5	C	—	138.59	—	—
7	CH ₂	2.55 (dd, 13.6 Hz, 8.8 Hz, 1H) and 2.91 (dd, 1.6 Hz, 4.0 Hz, 1H)	35.43	3.16	138.59, 129.43, 55.59 and 70.98
8	CH	3.16 (broad peak, 1H)	55.59	2.55, 2.91 & 3.78	—
9	CH	3.78 (broad peak, 1H)	70.98	3.16, 2.75 & 3.30	—
10	CH ₂	2.75 (dd, 14.4 Hz, 7.2 Hz, 1H) and 3.30 (14.8 Hz, 4.4 Hz, 1H)	52	3.78	55.59, 70.98 and 57.4
12	CH ₂	2.62 (dd, 13.6 Hz, 6.8 Hz, 1H) and 2.84 (dd, 13.2 Hz, 8.0 Hz, 1H)	57.4	1.9	52, 26.59 and 20.1
13	CH	1.90 (m, 1H)	26.59	2.62, 2.84, 0.76 and 0.81	57.4 and 20.1
14,15	CH ₃	0.76 and 0.81 (d, 6.4 Hz, 6H)	20.1	1.9	57.4, 26.59
19	C	—	123.5	—	—
20,24	CH	7.37 (d, 8.8 Hz, 2H)	129.09	6.6	152.84, 123.5
21,23	CH	6.60 (d, 8.8 Hz, 2H)	112.8	7.37	123.5
22	C	—	152.84	—	—
25	NH ₂	5.99 (broad singlet, 2H)	—	—	—

protons from the aliphatic region, and D₂O-Exchangeable protons primary amine showed at 5.99 ppm. There are two doublet signals at 6.60 ppm and 7.37 ppm with integration 2 protons, benzyl protons are present at 7.37 ppm with five proton integration, methyl protons from the isobutyl group show at 0.76 ppm with integration value 6, methine proton of the isobutyl is showing at 1.9 ppm, alcohol group attached proton is at 3.78 ppm, aliphatic amine attached methine is at 3.16 ppm, aryl group attached methylene group is at 2.55 and 2.91 ppm, N-attached methylene groups are at 2.75,

3.30, 2.62 and 2.84 ppm. In ^{13}C NMR, 8 aromatic carbons and 7 carbons from the aliphatic region were present, most downfield carbamate carbon is missing. g-HSQC experiment confirmed the presence of three methine groups, three methylenes, two methyls from the aliphatic side, and 9 methines from the aromatic region, ^1H - ^{15}N -HSQC experiment confirm the presence of primary amine, which is present on the aryl ring. The g-HMBC experiment has been conducted to check overall connectivity in structure, and data compiling with the given structure, all 1D and 2D NMR experiment results

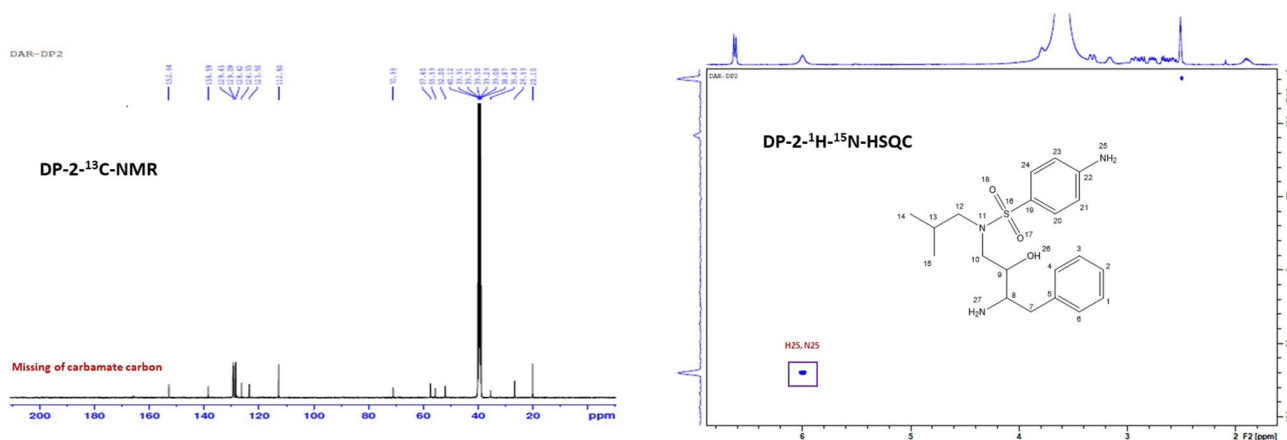
**Fig. 7** ^{13}C & ^1H - ^{15}N -HSQC data of DP-2

Table 7 Relative NMR assignments for DP-3DAR-DP-3 (Recorded in DMSO-D₆ + 1 drop of TFA-D solvent)

Atom serial number	Name of the atom	Proton resonance frequency (ppm) and coupling constant J (Hz)	Carbon resonance frequency (ppm)	¹ H- ¹ H-gDQCOSY	¹ H- ¹³ C-HMBC
1,3	CH	7.22 (m, 2H)	128.17	7.15	139.5
2	CH	7.15 (m, 1H)	126.04	7.22	129.42
4,6	CH	7.23 (m, 2H)	129.42	–	126.04, 35.41
5	C	–	139.5	–	–
7	CH ₂	2.49 (dd, 13.6 Hz, 11.2 Hz, 1H) and 3.02 (dd, 14.0 Hz, 2.8 Hz, 1H)	35.41	3.57	139.5, 129.42, 55.92, 72.7
8	CH	3.57 (m, 1H)	55.92	2.49, 3.02	–
9	CH	3.64 (m, 1H)	72.7	2.89	–
10	CH ₂	2.89 (dd, 14.8 Hz, 8.0 Hz, 1H) and 3.39 (dd, 14.8 Hz, 2.4 Hz, 1H)	52.25	3.64	55.92, 72.7, 56.68
12	CH ₂	2.83 (m, 1H) and 3.09 (dd, 13.6 Hz, 8.8 Hz, 1H)	56.68	1.97	52.25, 26.36, 20.02, 20.10
13	CH	1.97 (m, 1H)	26.36	2.83, 3.09, 0.81, 0.86	56.68, 20.2, 20.10
14,15	CH ₃	0.81 and 0.86 (d, 6.4 Hz, 6H)	20.02 and 20.10	1.97	26.36, 56.68
19	C	–	138.7	–	–
20,24	CH	7.91 (d, 8.4 Hz, 2H)	129.19	7.51	137.9
21,23	CH	7.51 (d, 8.4 Hz, 2H)	125.79	7.91	138.7
22	C	–	137.9	–	–
28	CO	–	155.45	–	–
31	CH	4.85 (m, 1H)	72.36	3.58, 3.83, 2.76	155.45, 109.04, 25.75
32	CH ₂	3.58 (m, 1H) and 3.83 (dd, 9.6 Hz, 6.0 Hz, 1H)	70.68	4.85	109.04, 45.28, 72.36
34	CH	5.51 (d, 5.2 Hz, 1H)	109.04	2.76	69.09, 45.28, 70.68, 72.36, 25.75
35	CH	2.76 (m, 1H)	45.28	4.85, 5.51, 1.24, 1.40	69.09, 70.68
36	CH ₂	1.24 and 1.40 (m, 2H)	25.75	2.76, 3.63, 3.73	109.04, 69.09, 72.36, 45.28
37	CH ₂	3.63 (m, 1H) and 3.73 (td, 8.0 Hz, 1.6 Hz, 1H)	69.09	1.24, 1.40	109.04, 45.28
39	C	–	164.73	–	–
41	CH ₃	2.36 (s, 3H)	19.3	–	164.73

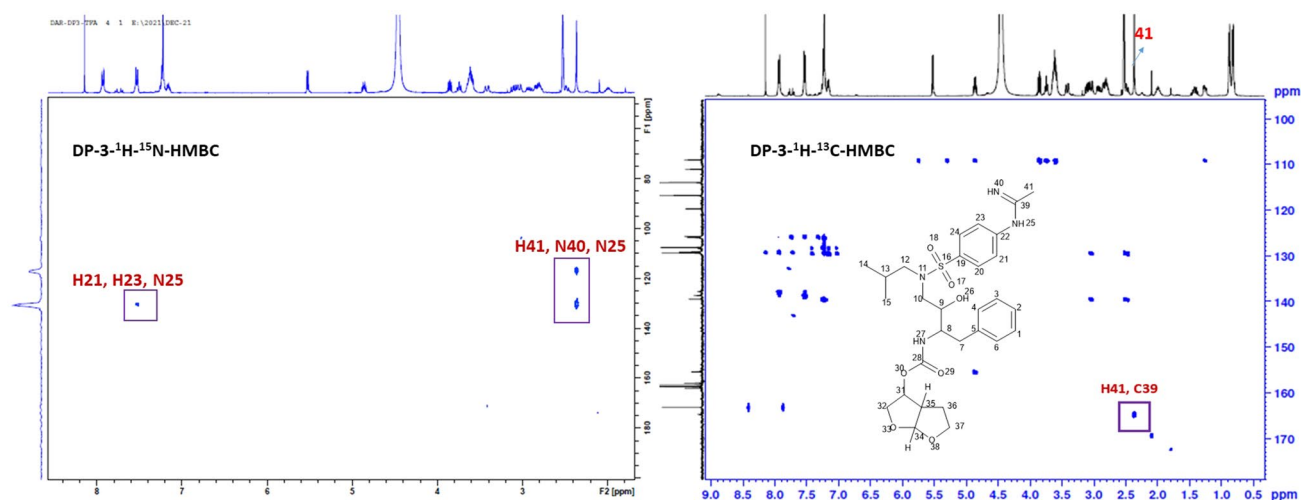
**Fig. 8** ¹H-¹⁵N-HMBC & ¹H-¹³C-HMBC data of DP-3

Table 8 Relative NMR assignments for DP-4

DAR-DP-4 (Recorded in DMSO-D ₆ solvent)					
Atom Serial number	Name of the atom	Proton resonance frequency (ppm) and coupling constant J (Hz)	Carbon resonance frequency (ppm)	¹ H- ¹ H-gDQCOSY	¹ H- ¹³ C-HMBC
1,3	CH	7.33 (t, 7.2 Hz, 2H)	128.51	7.27	137.4
2	CH	7.27 (t, 7.2 Hz, 2H)	126.4	7.33	128.81
4,6	CH	7.23 (d, 8.0 Hz, 2H)	128.81	7.33	126.4, 35.03
5	C	–	137.4	–	–
7	CH ₂	2.69 (14.4 Hz, 7.6 Hz, 1H) and 2.85 (14.4 Hz, 6.8 Hz, 1H)	35.03	4.26	137.4, 128.81, 54.25 and 77.58
8	CH	4.23 (q, 7.2 Hz, 1H)	54.25	2.69, 2.85 and 4.69	137.4, 35.03, 77.58 and 48.88, 157.64
9	CH	4.69 (td, 10 Hz, 2.4 Hz, 1H)	77.58	4.23, 3.14 and 3.23	157.64, 48.88, 54.25 and 35.03
10	CH ₂	3.14 (dd, 15.2 Hz, 9.6 Hz, 1H) and 3.23 (dd, 15.2 Hz, 2.4 Hz, 1H)	48.88	4.69	54.25, 77.58, 57.02
12	CH ₂	2.57 (dd, 13.6 Hz, 6.0 Hz, 1H) and 2.79 (dd, 13.6 Hz, 8.80 Hz, 1H)	57.02	1.84	48.88, 26.27 and 19.84, 19.93
13	CH	1.84 (m, 1H)	26.27	2.57, 2.79 and 0.78, 0.84	57.02, 19.84, 19.93
14,15	CH ₃	0.78 and 0.84 (d, 6.8 Hz, 6H)	19.84 and 19.93	1.84	57.03, 26.27
19	C	–	122.74	–	–
20,24	CH	7.19 (d, 8.8 Hz, 2H)	129.01	6.56	122.74, 152.9
21,23	CH	6.56 (d, 8.8 Hz, 2H)	112.76	7.19	122.74
22	C	–	152.9	–	–
25	NH ₂	6.00 (s, 2H)	–	–	112.76
27	NH	7.65 (s, 1H)	–	–	54.25, 77.58 and 157.64
28	CO	–	157.64	–	–

confirmed the accurate structure of DP-2 as 4-amino-N-(3-amino-2-hydroxy-4-phenyl butyl)-N-isobutyl benzenesulfonamide and represented in Fig. 7. Chemical shifts of ¹H and ¹³C are given in Table 6. In FTIR spectra, stretching frequency observed at 3370 cm⁻¹ indicates the presence of the NH group, the stretching frequency at 1088 cm⁻¹ indicates

the presence of 2° alcohol, and the stretching frequency at 1348 cm⁻¹, 1154 cm⁻¹ indicates the presence of the sulfoxide group, as shown in Table 3. The melting range of DP-2 is 95–99 °C. The possible degradation mechanism of DP-2 formation from DAR in basic conditions is explained in Fig. 12.

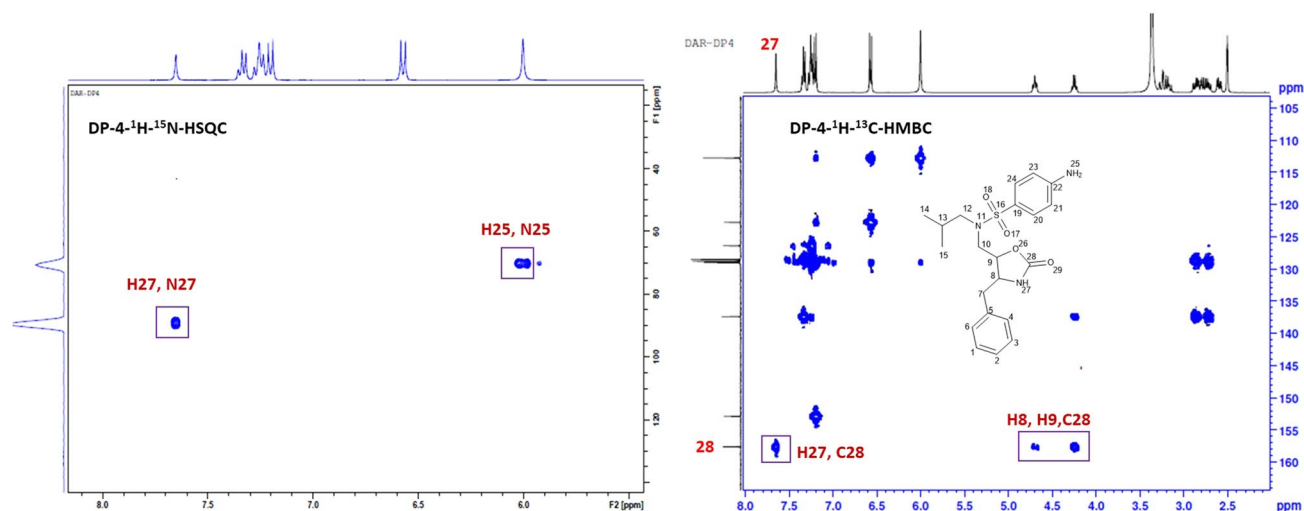
**Fig. 9** ¹H-¹⁵N-HSQC & ¹H-¹³C-HMBC data of DP-4

Table 9 Relative NMR assignments for DP-5

Atom serial number	Name of the atom	Proton resonance frequency(ppm) and coupling constant J (Hz)	Carbon resonance frequency (ppm)	$^1\text{H}-^1\text{H-gDQCOSY}$	$^1\text{H}-^{13}\text{C-HMBC}$
1,3	CH	7.22 (m, 2H)	127.89	7.14	139.63
2	CH	7.14 (m, 1H)	125.7	7.22	129.12
4,6	CH	7.23 (m, 2H)	129.12	–	125.7, 34.86
5	C	–	139.63	–	–
7	CH ₂	2.47 (m, 1H) and 2.97 (14.0 Hz, 2.8 Hz, 1H)	34.86	3.54	139.63, 129.12, 55.69
8	CH	3.54 (m, 1H)	55.69	2.47, 2.97, 7.02, 3.64	–
9	CH	3.64 (m, 1H)	72.44	2.66, 3.25 and 3.54, 4.95	–
10	CH ₂	2.66 and 3.25 (m, 2H)	52.67	3.64	72.44, 57.24
12	CH ₂	2.59 (dd, 13.2 Hz, 6.4 Hz, 1H) and 2.90 (13.2 Hz, 8.4 Hz, 1H)	57.24	1.93	52.67, 26.32 and 20.05
13	CH	1.93 (m, 1H)	26.32	2.59, 2.90 and 0.78, 0.84	57.24, 20.05
14,15	CH ₃	0.78 and 0.84 (d, 6.4 Hz, 6H)	20.05	1.93	57.24, 26.32
19	C	–	123.45	–	–
20,24	CH	7.37 (d, 8.8 Hz, 2H)	129.02	6.59	152.7
21,23	CH	6.59 (d, 8.8 Hz, 2H)	112.64	7.37	123.45
22	C	–	152.7	–	–
25	NH ₂	5.97 (s, 2H)	–	–	112.64
26	OH	4.95 (d, 6.4 Hz, 1H)	–	3.64	–
27	NH	7.02 (d, 9.2 Hz, 1H)	–	3.54	–
28	CO	–	156.29	–	–
31	OCH ₃	3.39 (s, 3H)	51.09	–	156.29

Degradation Product (DP-3) Characterization

DP-3 impurity has been formed from DAR on treatment with acid. Then the DP-3 is isolated and its mass was confirmed as $(M+H)^+$ 589.2686 with 0.71 ppm

error in HRMS for the calculated molecular formula of $\text{C}_{29}\text{H}_{41}\text{O}_7\text{N}_4\text{S}$ shown in Fig. 4D. It has excess 41 units than API, to find the accurate structure of DP-3, it was examined with the NMR experiments, in ^1H NMR, one of the key methyl groups shows broaden, hence added

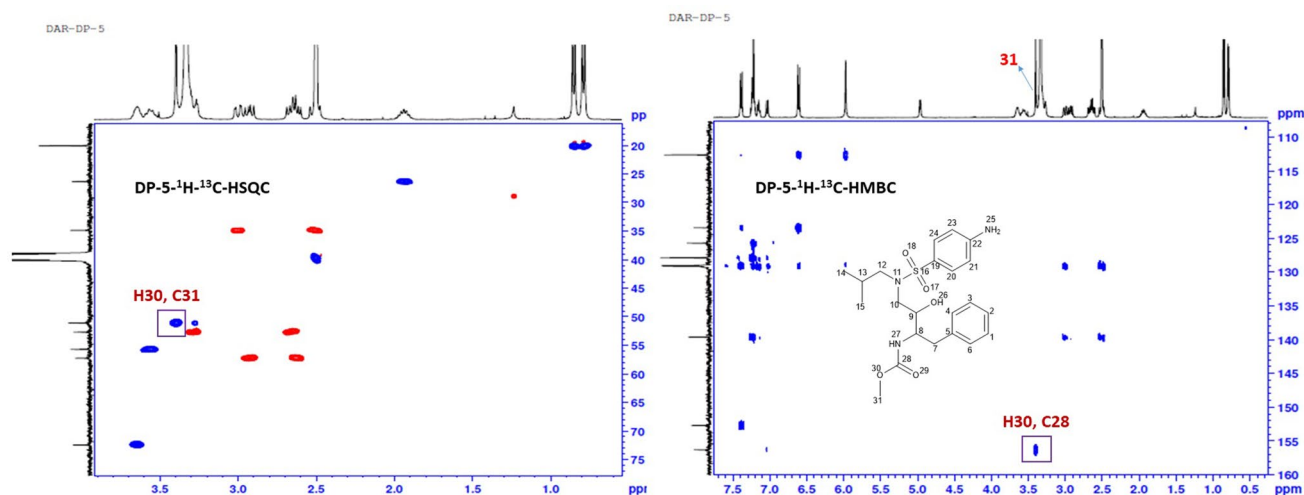


Fig. 10 $^1\text{H}-^{13}\text{C}$ HSQC & $^1\text{H}-^{13}\text{C}$ -HMBC of DP-5

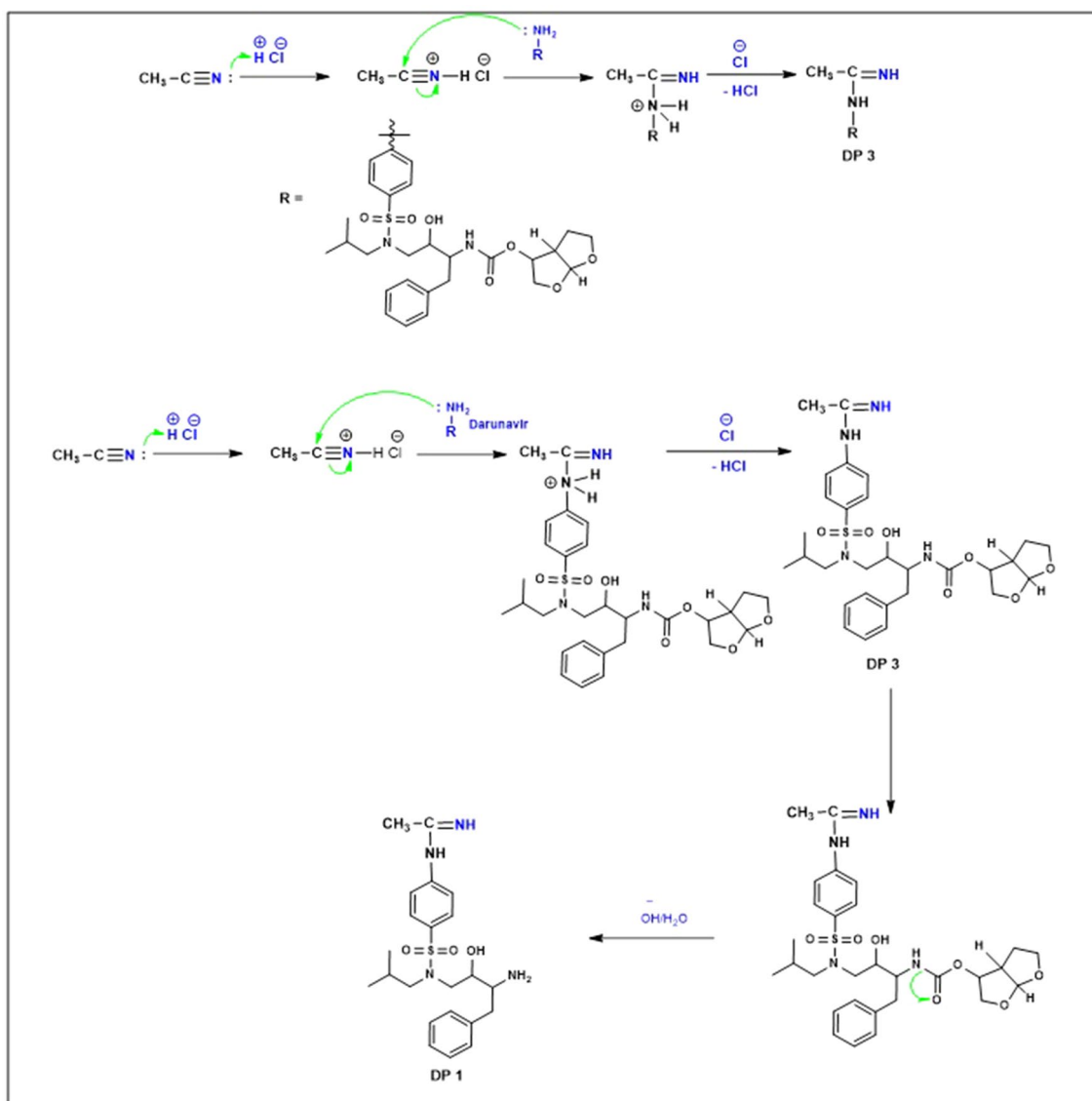


Fig. 11 Proposed mechanism for Acid degradation (DP-1 & DP-3)

one drop of TFA to the DMSO to improve the relaxation and recorded 1D and 2D experiment for the same sample, in ¹H NMR, 9 aromatic protons are shown in that 5 protons belong to benzyl group with integration 5 at 7.19 ppm and 1,4-disubstituted benzene ring protons observed at 7.51 ppm, 7.91 ppm with integration of each 2, one of the key important methyl group protons observed at 2.36 ppm, and isobutyl group methyls are at 0.81 ppm 0.86 ppm, remaining 18 methine and methylene protons were observed between 1.0 and 6.0 ppm, in ¹³C NMR, a total 25 carbon signals were observed, in that 10 carbons are from aromatic and 15 from aliphatic region, most downfield carbons are amidine carbon showing at 164.73 ppm, carbamate carbon is at 155.45 ppm,

remaining aryl carbons between 100 and 140 ppm, N-attached, O-attached methine and methylene carbons are at 45 pm to 75 ppm; methyls, isobutyl methine and methylenes carbons are between 15 and 45 ppm, most up-field carbon is acetamidine at 19.30 ppm; in g-HSQC experiment, 6 methines, 6 methylenes, and 3 methyls were confirmed in aliphatic region, and benzyl and 1,4-disubstituted benzene protonated carbons were confirmed in aromatic region. 2 J and 3 J correlations of ¹H-¹³C are identified by ¹H-¹³C-HMBC, and 2 J and 3 J correlations of Proton to Nitrogen were confirmed in ¹H-¹⁵N-HMBC experiment, the most important observation in g-HMBC experiment is H41 protons showing connectivity to C39 carbon, and ¹H-¹⁵N-HMBC experiment H41 protons showing

connectivity to N25 (130.44 ppm) and N40 (116.65 ppm) nitrogen; as well, H21 and H23 protons showed connectivity to N25 Nitrogen. This key connectivity in ^{15}N -HMBC strongly confirms the structure of DP-3, here primary amine group was converted as acetimidine and formed as hexahydrofuro[2,3-b]furan-3-yl(4-((4-acetimidamido-N-isobutylphenyl)sulfonamido)-3-hydroxy-1-phenylbutan-2-yl)carbamate, ^1H and ^{13}C assignment details are given in Table 7 and confirmed structure shown in Fig. 8. In FTIR spectra, stretching frequency observed at 3349 cm^{-1} indicates the presence of the NH group, the stretching frequency at 1090 cm^{-1} indicates the presence of 2° alcohol, stretching frequency at 1331 cm^{-1} , and 1153 cm^{-1} indicates the presence of the sulfoxide group, as shown in Table 3. The melting range of DP-3 is $131\text{--}135^\circ\text{C}$. The possible degradation mechanism of DP-3 formation from API in acidic conditions is explained in Fig. 11.

Degradation Product (DP-4) Characterization

API compound on treated with base DP-4 impurity has been formed. This DP-4 is isolated and its mass confirmed as $(\text{M} + \text{H})^+$ 418.1790 with 1.32 ppm error in HRMS for the calculated molecular formula of $\text{C}_{21}\text{H}_{28}\text{O}_4\text{N}_3\text{S}$ shown in

Fig. 4E. Mass is less than parent API compound 124 units and to find the precise structure of DP-4 1D and 2D NMR, experiments have been conducted, used DMSO- D_6 solvent to solubilize the compound and acquired in 400 MHz instrument, under the 1D experiments conducted ^1H and ^{13}C experiments; in proton NMR, a total 27 protons are observed, in that 9 protons are from aromatic region, 15 protons from aliphatic, and 3 labile protons, in aromatic region at benzyl protons are at 7.25 ppm and 7.33 ppm with respective 3 and 2 protons integration, 1,4 di-substituted ring protons present at 6.56 ppm and 7.19 ppm with the integration of two protons each and primary amine protons are at 6.00 ppm and oxazolidone ring proton is at 7.65 ppm as a singlet, isobutyl group methyls and methine protons are at 0.78, 0.84 ppm and 1.82 ppm, remaining methine and methylenes are between 2.5 ppm and 5.0 ppm. In ^{13}C NMR, a total 17 carbons are present in that 9 carbons are aromatic region between 110 and 160 ppm, most downfield carbon is 2-Oxazolidone carbon at 157.64 ppm, and primary amine attached carbon is at 152.90 ppm, symmetric carbons in phenyl and 1,4-disubstituted ring carbons are showing high-intensity signals, in aliphatic region, carbon signals are between 15 and 80 ppm and most unfilled carbons are isobutyl group methyl carbons and downfield

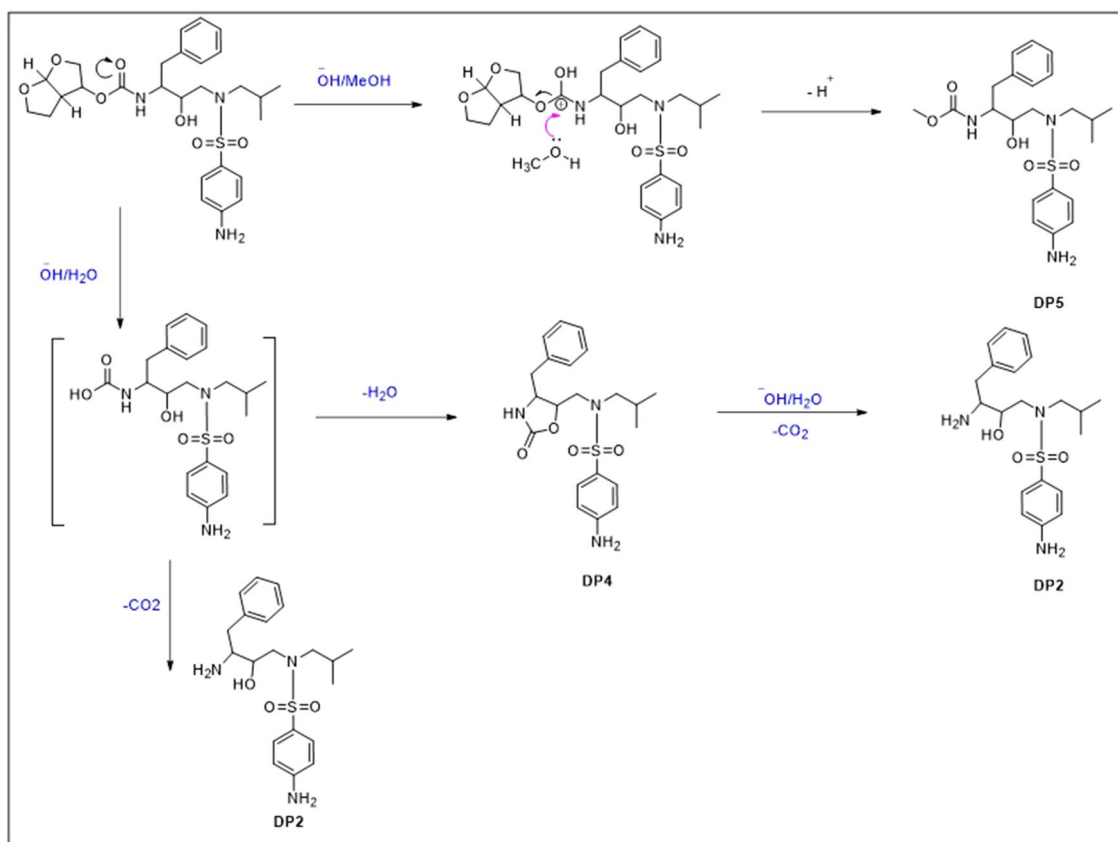


Fig. 12 Proposed mechanism for Base degradation (DP-2, DP-4 & DP-5)

carbon is O-attached methine showing at 77.58 ppm, Under the 2D experiments conducted g-HSQC, ^1H - ^{15}N -HSQC and g-HMBC experiments, to identify proton attached carbons performed g-HSQC experiment, here got the clear information about every methylene carbons and methine carbons, further conducted ^1H - ^{15}N -HSQC experiment, it gave clarity on the presence of amine and amide protons in the structure, and in g-HMBC experiment observed, one of the key correlations is H8,H9 and H27 protons showing connectivity to C28 carbon. Here H9 to C28 correlation should possible in cyclized structure only, all the experiments data confirmed that carbamate carbon got cyclized with secondary alcohol group and formed 2-Oxazolidone ring contained structure as 4-amino-N-(((5S)-4-benzyl-2-oxooxazolidin-5-yl)methyl)-N-isobutylbenzenesulfonamide and structure is given at Fig. 9. Its detailed proton and carbon values are captured in Table 8. In FTIR spectra, stretching frequency observed at 3470 cm^{-1} , 3368 cm^{-1} indicates presence of NH group, stretching frequency at 1087 cm^{-1} indicates the presence of 2° alcohol, stretching frequency at 1317 cm^{-1} , 1148 cm^{-1} indicates the presence of sulfoxide group, as shown in Table 3. The melting range of DP-4 is $105\text{--}109^\circ\text{C}$. The possible degradation mechanism of DP-4 formation from DAR in the basic condition is explained in Fig. 12.

Degradation Product (DP-5) Characterization

API compound on treatment with base DP-5 impurity has been formed, this degradation product is isolated and its mass confirmed as $(\text{M} + \text{H})^+ 450.2054$ with 0.64 ppm error in HRMS for the calculated molecular formula of $\text{C}_{22}\text{H}_{32}\text{O}_5\text{N}_3\text{S}$ shown in Fig. 4F. Compound mass shows 98 units, it is lower than API compound, to confirm the precise structure of DP-5 performed NMR experiments. In ^1H NMR, the most significant observation is no protons observed from hexahydrofuro[2,3-b]furan ring, and singlet signal is shown at 3.39 ppm with integration value 3. It clearly indicates the presence of O-methoxy group in the structure, two doublet methyls observed at 0.78 ppm & 0.84 ppm corresponding to isobutyl group, 3 methylene groups and 3 methines groups are present between 1.5 and 4.0 ppm, secondary alcohol proton is showing at 4.95 ppm and amidic -NH proton is at 7.02 ppm, benzyl ring protons are showing at 7.22 ppm with integration 5 protons, 1,4-di-substituted benzene ring protons are shown at 6.59 ppm and 7.37 ppm with integration each signal two protons. The total number of protons in the structure is 31, in ^{13}C NMR, most downfield carbon corresponding to methyl carbamate is shown at 156.29 ppm, and benzyl and 1,4-di-substituted benzene ring carbons are shown between 110 and 152 ppm, and a total number of aromatic carbons are 9, in aliphatic region, 8 carbons are present, O-methoxy carbon is at 51.09 ppm and alcohol group attached carbon is at 72.44 ppm, and isobutyl group methyl

carbons are shown at 20.05 ppm, N-attached methylene carbons are at 52.67 ppm & 57.24 ppm, benzyl attached methylene group carbon is at 34.86 ppm, methine carbons are at 55.69 & 26.32 ppm, conducted HSQC experiment for confirmation of methine, methylene and methyl carbons. Here H31 protons attached carbon is shown at 51.09 ppm. This chemical shift confirms the presence of O-methoxy group in the compound, in g-HMBC experiment, O-methoxy protons are shown 3 J connectivity to carbamate carbon, and this key correlation confirms the position of O-methoxy position. Based on above key correlations, structure of DP5 is methyl ((3S)-4-((4-amino-N-isobutylphenyl)sulfonamido)-3-hydroxy-1-phenylbutan-2-yl)carbamate. Detailed assignment of proton and carbon values is captured in Table 9, and structure is shown at Fig. 10. In FTIR spectra, stretching frequency observed at 3367 cm^{-1} , indicates the presence of the NH group, the stretching frequency at 1088 cm^{-1} indicates the presence of 2° alcohol, and the stretching frequency at 1315 cm^{-1} , and 1146 cm^{-1} indicates the presence of sulfoxide group, as shown in Table 3. The melting range of DP-5 is $64\text{--}68^\circ\text{C}$. The possible degradation mechanism of DP-5 formation from DAR in the basic condition is explained in Fig. 12.

Conclusion

The stress degradation study of Darunavir was examined as per ICH prescribed guidelines. The drug was subjected to acidic, alkaline, oxidative, thermolytic, and photolytic degradation conditions. The drug was stable in oxidative, thermal, and photolytic conditions as it did not show any impurity formation. However, five degradation products were formed in acid and base stress hydrolysis conditions. These degradation products were isolated and fully characterized by various analytical techniques like NMR (1D & 2D experiments) and HRMS experiments. FT-IR data gave an additional add-on to confirm the structures. Among five DPs, DP-1, DP-3 are novel products, and DP-2, DP-4, and DP-5 are reported degradation products with limited data as the structures were characterized only by mass spectral data, and no structural elucidation was done by 2D-NMR and HRMS. The current study provides the complete structural interpretation of Darunavir and all 5 degradation impurities using HRMS, FTIR, and 2D-NMR studies. It also reports well-developed UPLC-MS method to separate all the impurities with good resolution. The method used is simple, sensitive, and stability indicating with very short runtime i.e., 7.0 min. Using this we can perform qualitative and quantitative analyses of degradation products. The complete structural characterization of degradation products by HRMS, NMR, and FTIR will help the complete studies and the nature of the degradation products, which will help in the process, shelf-life

time, and safety of the API products. These stress studies will provide knowledge about possible degradation products and the pathways of API and help to elucidate the structure of the degradation products. This stress degradation can be a useful tool to predict the stability of a drug product with effects on purity, potency, and safety. It is important to know the impurity profile and behavior of a drug product under various stress conditions. The isolation method of the degradation products will help to know the individual characterization of degradation products. The complete spectral analysis of $^1\text{H NMR}$, $^{13}\text{C NMR}$, 2D-NMR, FT-IR, and HR-MS was reported for the first time and confirmed the proposed chemical structures of degradation impurities.

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Author contributions Arun Kumar Modinia carried out experimentation work and prepared the manuscript. Sathish Kumar Konidala planned and guided the work and prepared the manuscript. Mahesh Ranga, Umamaheshwar Puppala, Muralidharan Kaliyapermal, and Mahesh Kumar Reddy Geerreddy did data collection, analysis. Ramu Samineni and parul Grover did the manuscript review and suggestions.

Data availability statement The data can be available as the supplementary data file attached to this article.

Declarations

Conflict of Interest The authors declare that they have no conflict of interests.

Consent for Publication We authorize to publish the article without any conflict.

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