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Research Article

Preformulation Study of NSC-726796

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Abstract. A stability-indicating high-performance liquid chromatography method to quantify 2-(2,4-difluorophenyl)-4,5,6,7-tetrafluoroisoindoline-1,3-dione (NSC-726796) and its three main degradation products was developed. This method was used to investigate its degradation kinetics and mechanism. The reaction follows first-order kinetics and appears to be base catalyzed with the maximum stability at pH 1. The products were identified as 2-(2,4-difluorophenylcarbamoyl)-3,4,5,6-tetrafluorobenzoic acid (NSC-749820), 2,4-difluoroaniline, and tetrafluorophthalic acid. The parent drug, NSC-726796, was also found to react with methanol and ethanol. NSC-726796 demonstrates antiangiogenic activity, however, when its degradant NSC749820 does not show antiangiogenic activity.

KEY WORDS: CPS49; NSC-726796; NSC-749820; preformulation; stability.

INTRODUCTION

The thalidomide analog 2-(2,4-difluorophenyl)-4,5,6,7-tetrafluoroisoindoline-1,3-dione (NSC-726796), also known as CPS49, is currently being investigated by the National Cancer institute for its anticancer activity. It demonstrates antiangiogenic activity, as well as direct cytotoxic effects against lymphocytic leukemia, multiple myeloma, lung cancer, prostate cancer, and endothelial cells (1–6). Drug instability can cause lost of potency or toxicity. Therefore, it is important to determine how much drug is lost with time and what are its degradants. The purpose of this study is to develop a stability-indicating high-performance liquid chromatography method for the quantitation of NSC-726796 and to determine the effect of solution pH on its stability. The chemical structure of NSC-726796 is shown in Table I. It is a substituted phthalimide with a melting point of 144 °C, a molecular weight of 331, and a calculated octanol/water partition coefficient (ClogP) of 3.26. It is not ionizable in the 1–14 pH range.

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METHODS

Chemicals and Reagents

NSC-726796 was provided by the Pharmaceutical Resources Branch, National Cancer Institute (Bethesda, MD, USA). Hydrochloric acid, trifluoroacetic acid (TFA), sodium hydroxide, citric acid monohydrate, sodium citrate, monosodium phosphate, sodium phosphate dibasic, sodium carbonate, sodium bicarbonate, sodium citrate, tetrafluorophthalic acid, 2,4-difluoroaniline, tetraflurophthalic anhydride, and dichloromethane were purchased from Sigma-Aldrich. Sodium chloride and acetonitrile were obtained from Fisher Chemicals. TRIS (base) and 4-dimethylaminopyridine were purchased from J.T. Baker and Fluka, respectively. All agents were used without further purification. Water was purified using a Milli-Q system (18.2 MΩ cm, Millipore, Billerica, MA, USA).

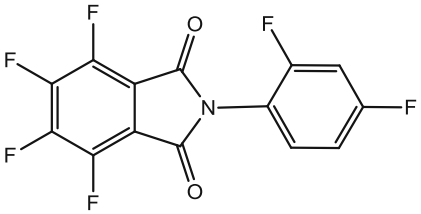
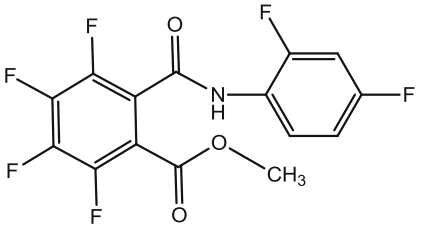
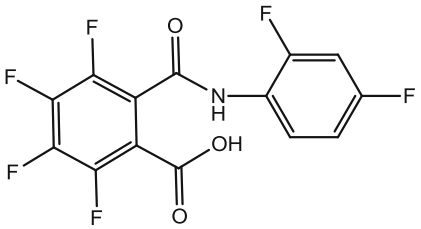
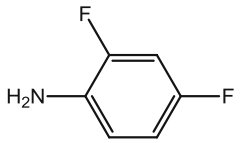
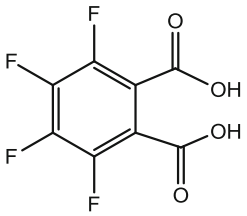
Analytical Procedures

High-Performance Liquid Chromatography Conditions

Chromatographic separation was achieved using reversed-phase chromatography with isocratic elution. A Discover® HS F5 column (150×4.6 mm, 3 μm; Supelco) was used as the stationary phase.

The mobile phase, consisting of acetonitrile and DI water (0.1 % TFA; 60/40 *v*/*v*) was filtered and degassed through a 0.45 μm filter and pumped at a flow rate of 0.5 ml/min. The column temperature was 20 °C and the injection volume was 5 μl. The high-performance liquid chromatography (HPLC) system used was Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary pump, an automatic injector, and a diode array detector. The system was

Table I. List of Compound, Structure, HPLC Retention Time, ClogP, and Molecular Weight

No.	Compound	Structure	Retention Time (min)	ClogP	Molecular Weight
	NSC-726796		12.2	3.26	331.17
I	Methanol adduct of NSC-726796		10.2	2.46	363.21
II	NSC-749820		6.6	1.57	349.18
III	2,4-difluoroaniline		6.1	1.50	129.11
IV	tetrafluorophthalic acid		4.5	0.65	238.09

controlled by ChemStation® software. Each peak area was computed by the integrator. The UV detection wavelength was set at 222 nm with 360 nm as the reference.

Mass Spectrometry

The degradation products of NSC-726796 were analyzed by Thermo Finnigan TSQ Quantum Ultra (San Jose, CA, USA) operated in MS mode using electrospray ionization in positive/negative ionization mode scanning from 50 to 800 amu. Samples were manually injected using the injection valve on the instrument and infused into the electrospray source at 0.3 mL/min with a loop size of 5 μl. The instrument settings were as follows: spray voltage at 4,500 V, sheath gas (nitrogen) of 35 arbitrary units, auxiliary gas (nitrogen) of five arbitrary units, ion transfer capillary temperature at 390 °C, and source collision energy at 11 V.

Thermal Analysis

The differential scanning calorimetry (DSC) thermograms of the solid samples were obtained using a Q1000 differential scanning calorimeter (TA Instruments, New

Table II. List of Buffer Solution Prepared for Stability Studies

pH	Buffer	Buffer strength (mM)	Ionic strength (mM)
1	HCl	100	300
2.5	Citrate	100	300
4	Citrate	100	300
5.5	Citrate	100	300
7	Phosphate	100	300
8.5	Tris	100	300
10	Carbonate	100	300

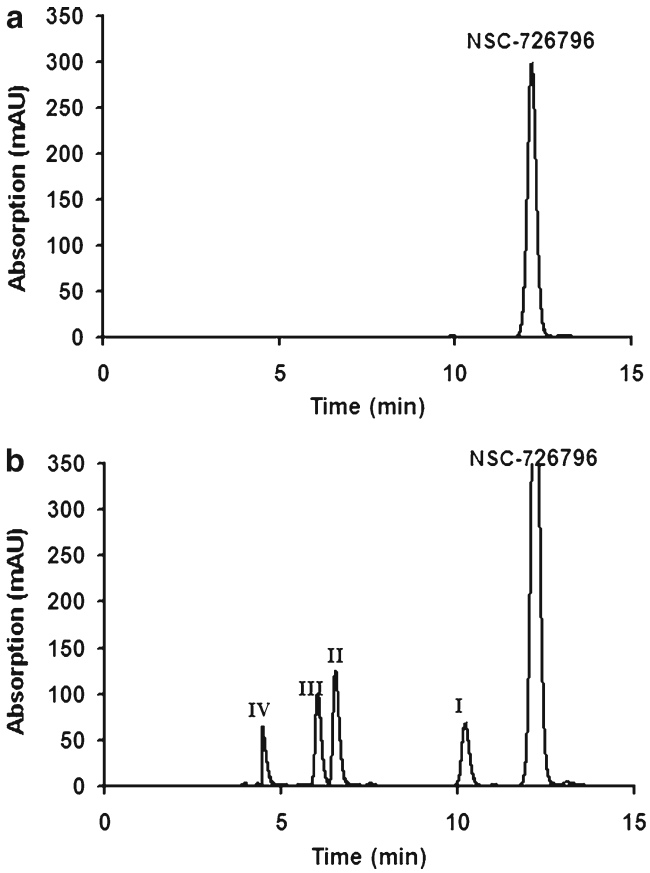


Fig. 1. Chromatograms of NSC-726796 standard solution **a** and NSC-726796 solution spiked with related compounds **b** as listed in Table I

Table III. Linearity Results of Standard Curve

Concentration (μg/mL)	Mean peak area	%RSD
10.0	514.24	2.32
20.0	1,084.41	1.28
50.1	2,693.37	0.87
80.2	4,386.26	0.96
100.2	5,314.83	1.58

Castle, DE, USA) equipped with Universal Analysis 2000 software. Approximately, 5 mg of the drug was sealed in a hermetically crimped aluminum pan. The sample was heated with a linear ramp of 5 °C/min and purged with nitrogen at a flow rate of 40 mL/min. The melting temperature was noted as the point on the temperature scale corresponding to the maximum deviation from the baseline.

Experimental Procedures

Stock Solutions

Stock solutions of NSC-726796 were prepared by dissolving the appropriate amount of the compounds in acetonitrile. Stock solutions were stored at 4 °C until required.

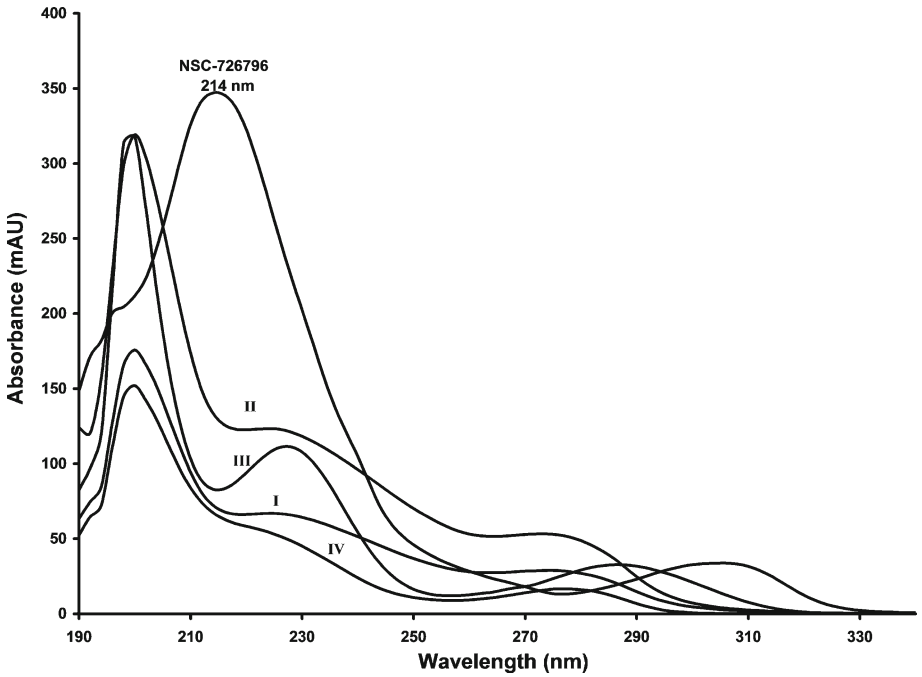


Fig. 2. UV spectra of all compounds

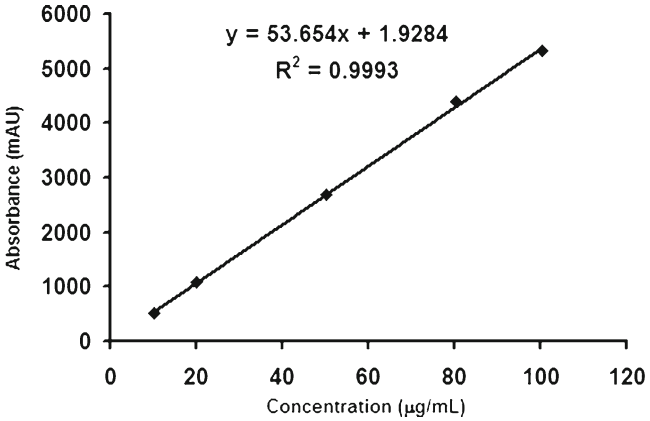


Fig. 3. Standard curve of NSC-726796

Preparation of Buffer Solutions

The buffer systems used for the pH stability profile studies are listed in Table II. The buffers used were: hydrochloric acid, citric acid–sodium citrate, and monosodium phosphate–disodium phosphate, sodium carbonate–sodium bicarbonate, and TRIS (base). A constant ionic strength of 300 mM was maintained for each buffer by adding an appropriate amount of NaCl. The buffer solutions were freshly prepared and the pH values were measured by an Accumet® pH meter and an H1 1083 glass electrodes. The pH meter was standardized with pH 2.0, 4.0, 7.0, and 10.0 solutions before each measurement.

Validation Procedure

The HPLC method was validated with respect to the following parameters: specificity, linearity, accuracy, precision, limit of detection, and limit of quantitation. A set of five standards of NSC-726796 at the following concentrations were prepared: 10.0, 20.0, 50.1, 80.2, and 100.2 µg/mL. Linearity was evaluated using calibration curves obtained during 5 days. Each of the five standards was analyzed in six replicates each day.

Accuracy was confirmed by spiking a control sample of NSC-726796 at 20, 50, and 60 µg/mL. Triplicates were prepared for each concentration. The recovery percentage was calculated using the following equation:

$$Recovery(\%) = \frac{Measured\ concentration}{Expected\ concentration} \times 100$$

Precision throughout the calibration range was evaluated in terms of intraday repeatability and interday reproducibility with the same set of solutions used for linearity. The intraday repeatability study was carried out by injecting the same sample three times on the same day. The interday reproducibility was investigated on five consecutive days. The peak areas obtained were used to calculate mean, standard deviation, and relative standard deviation (%RSD). Evaluation of the limit of detection (LOD) and limit of quantification (LOQ) determination was performed according to guidelines of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (Validation of Analytical Procedures: Text and Methodology (R1), 27 October, 1994).

The standard deviation of the *y*-intercept (σ) and the slope (S) of the calibration curve was used to calibrate LOD and LOQ with the following equations:

$$LOD = \frac{3\sigma}{S} \qquad LOQ = \frac{10\sigma}{S}$$

Reaction Kinetic Studies

The formation of hydrolysis products of NSC-726796 was carried out in aqueous solution at ambient temperature. The effect of pH on the observed degradation constant, k_{obs} was determined using the buffer systems listed in Table II with pH value ranging from 1 to 10. Duplicate sample at each pH value were prepared by quenching 10 µL of the stock solution in the buffer solution. The resulting solutions were immediately filtered through 0.45 µm filters. Stressed sample were injected for HPLC analysis at intervals such that at least four datapoints could be depicted on the kinetic plots.

Synthetic Studies

One of the hydrolysis products of NSC-726796, is the ring-open acid, 2-(2,4-difluorophenylcarbamoyl)-3,4,5,6-tetrafluorobenzoic acid (NSC-749820). Since it was not commercially available, it was synthesized by coupling tetraflurophthalic anhydride (1 g, 4.54 mmol) and 2,4-difluoroaniline (0.59 g, 4.54 mmol) using 4-dimethylaminopyridine (56 mg, 0.454 mmol) as the catalyst and dry dichloromethane (10 ml) as the solvent. The mixture was stirred at ambient temperature for 72 h. After the reaction was complete, the reaction mixture was partitioned between ethyl acetate and 2 N HCl. The aqueous phase

Table IV. Peak Area Values for NSC-726796 from Intraday Precision Studies

Concentration (µg/mL)	Area					
	A_1	A_2	A_3	Mean	SD	%RSD
10.0	515.8	514.6	511.5	514.0	2.2	0.4
20.0	1,070.2	1,069.1	1,068.9	1,069.4	0.7	0.1
50.1	2,659.5	2,642.2	2,647.1	2,649.6	8.9	0.3
80.2	4,352.8	4,345.5	4,343.1	4,347.2	5.1	0.1
100.2	5,252.8	5,243.0	5,239.6	5,245.1	6.9	0.1

Table V. Mean Area Values (n=5) for NSC-726796 from Interday Precision Studies

Concentration (µg/mL)	Mean area							
	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	%RSD
10.0	513.5	524.6	495.4	513.1	524.5	514.2	11.9	2.3
20.0	1,070.0	1,095.6	1,099.4	1,086.9	1,070.1	1,084.4	13.9	1.3
50.1	2,651.5	2,703.8	2,703.8	2,703.8	2,703.8	2,693.4	23.4	0.9
80.2	4,347.3	4,433.3	4,430.1	4,364.3	4,356.3	4,386.3	41.9	1.0
100.2	5,237.3	5,399.1	5,218.3	5,328.9	5,390.6	5,314.8	84.2	1.6

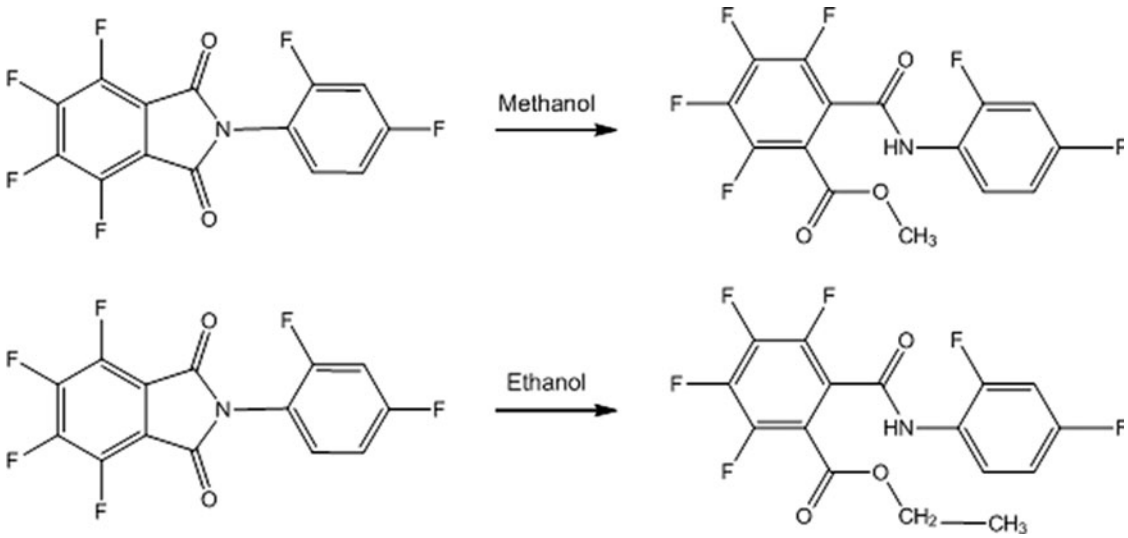


Fig. 4. Degradation pathways of NSC-726796 in methanol and ethanol

was then extracted twice more with ethyl acetate. The combined extracts were evaporated to a solid *in vacuo*. The solid was treated with diethyl ether at room temperature and the undissolved solids were discarded. The remaining solution was evaporated and then triturated with cold diethyl ether to dissolve any remaining anhydride and filtered. The resulting white powder was characterized by MS and X-ray crystallography.

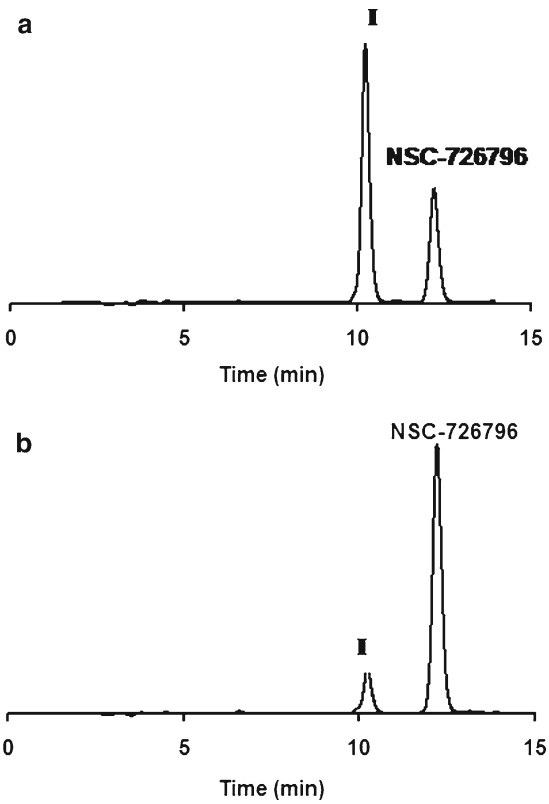


Fig. 5. Chromatograms of methanol adduct in acetonitrile; **a** 0 min and **b** 270 min

Reverse Reaction Kinetics at Low pH

The reaction kinetics for conversion from ring-open mono acid degradant to NSC-726796 was investigated in aqueous solutions at pH 1 and 2.5. Aliquots (10 μL) of the mono acid stock solution in acetonitrile were quenched in pH 1 and 2.5 buffers. Duplicates for each pH were prepared and immediately filtered. The resulting solutions were analyzed by HPLC at various time intervals.

Rat Aortic Ring Angiogenesis Assay

The rat aortic ring assay was performed, as previously reported, to assess antiangiogenic activity (7). NSC-726796 was tested at a concentration of 100 μM, applied to the embedded ring either immediately after dilution into aqueous cell growth media or 30 min after dissolution, to allow for hydrolysis to occur. NSC-749820 was also tested at a concentration of 100 μM. After 4 days of treatment, rings were photographed and microvessel growth quantified using Adobe Photoshop. The pixel count of each ring was normalized to the control ring for each plate, to determine the percentage of growth inhibition. Groups were compared by *t* test with a *P* value of <0.05 considered to be significant.

RESULTS AND DISSCUSION

HPLC Validation

Method specificity was demonstrated by the separation of the NSC-726796, related adduct, and degradants listed in Table I. The structure, molecular weight, Clog P, and chromatography retention time of these compounds are included.

Representative chromatograms of NSC-726796 solution and an NSC-726796 solution spiked with related compounds I, II, III, and IV are shown in Fig. 1. The chromatograms illustrate that the NSC-726796 peak is free from any interference of its related compounds and these four compounds are also well separated from each other. The order of retention times for the compounds follows their polarity. Thus, the HPLC method presented in this study is selective for NSC-726796 and other compounds, which might coexist as degradation products. The UV spectra of the compounds (Fig. 2) were obtained with the online diode array detector. NSC-726796 shows maximum absorption at about 214 nm. However, at this wavelength, other degradation products have low absorption. Hence, the wavelength of 222 nm at which all compounds are detectable was chosen. The peak purity was verified by scanning along each peak.

Linearity was constructed with from the mean value of 5 days. The peak areas of NSC-726796 at different concentrations are shown in Table III. The calibration curve shown in Fig. 3 was established by plotting the peak area against the concentration using linear regression analysis. The linearity exists over the concentration range studied with a slope of 53.65, a *y*-intercept of 1.93 and a correlation coefficient of 0.9993.

The accuracy of an analytical method is the closeness of the test results to the true value. The average recovery at three concentration levels was 99.7 with %RSD <2 %.

Data obtained from five different concentrations for three replicate injections during intra-analyst precision studies are given in Table IV. The %RSD of multiple injections ranged from 0.1 to 0.4 %. The corresponding mean data for interday precision studies are given in Table V. As the intra- and interday precision are within the acceptance criteria of 5 %, the method is precise. The values for LOD and LOQ were estimated at 1.5 and 4.6 μg/mL, respectively.

Degradation in Alcohols

The proposed reaction scheme of NSC-726796 with methanol is shown in Fig. 4. A similar reaction occurs when the drug

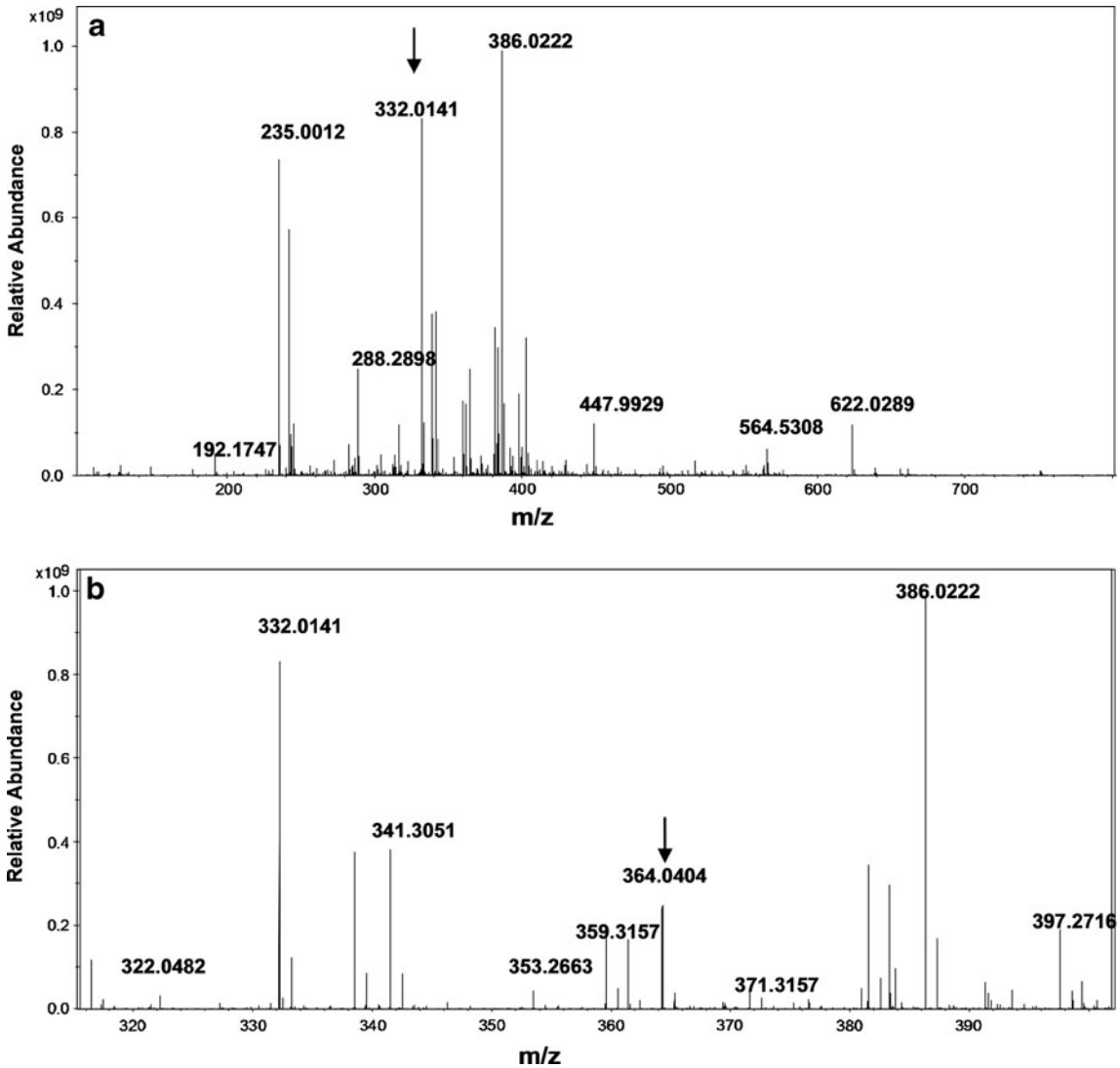


Fig. 6. Positive electrospray ionization-MS spectra of **a** NSC-726796 and **b** methanol adduct of NSC-726796

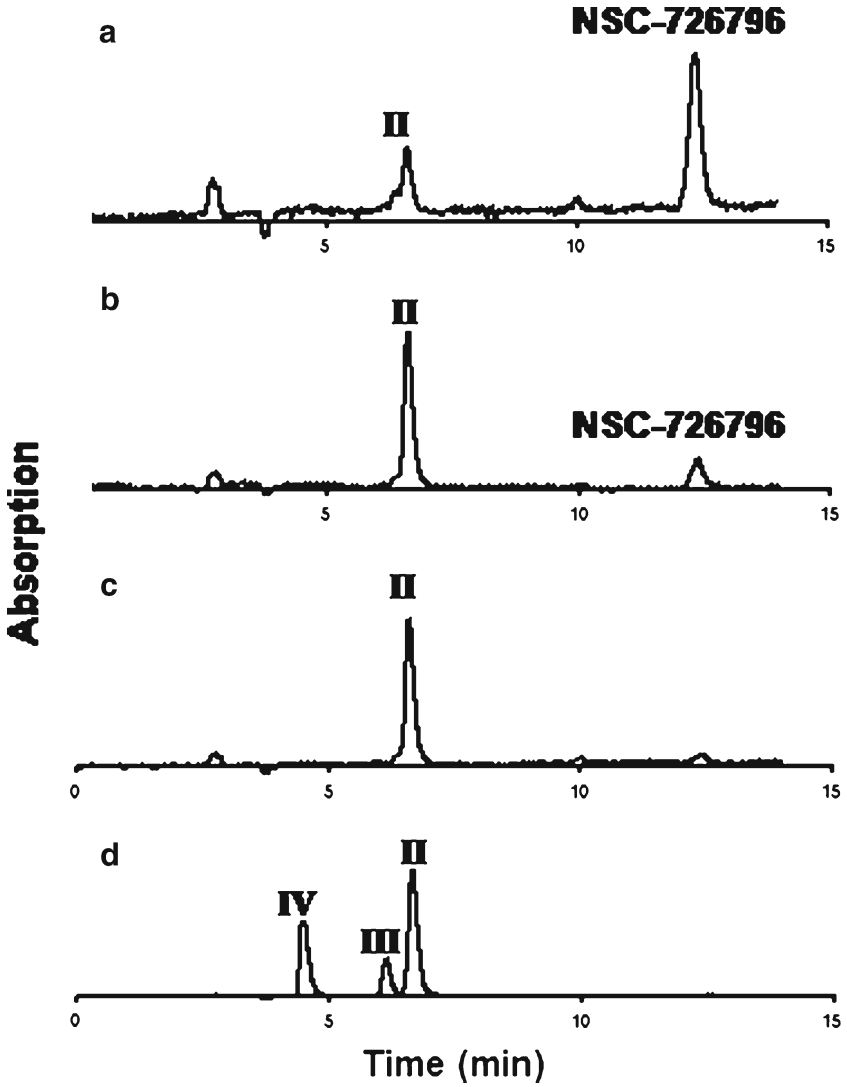


Fig. 7. Chromatograms of the NSC-726796 in DI water at different time **a** 0 min, **b** 15 min, **c** 45 min, and **d** 21 days

is in contact with ethanol. Since these reactions occur rapidly, primary alcohols are not suitable for use as a mobile phase or a solubilization agent for the drug.

The methanol adduct (I in Table I) was isolated by dissolving NSC-726796 in methanol followed by slow evaporation of the solvent. The molecular weight determined by mass spectrometry (Fig. 5) is consistent with the structure provided proposed. When the methanol adduct is dissolved in pure acetonitrile, there is some immediate conversion to the parent compound. The reaction is nearly complete after 4.5 h as shown in Fig. 6.

Degradation in Aqueous Solutions

NSC-726796 degrades rapidly upon contact with water. This is illustrated in Fig. 7 by the representative HPLC chromatograms of the NSC-726796 in DI water at 0, 15, and 45 min as well as 21 days. The parent compound degrades to compound II, which elutes at 6.6 min and has a molecular weight of 349 (Fig. 8a). In 15 min, most of the parent drug is lost. After 45 min, the loss is almost complete. Compound II further degrades to III and IV with retention time of 6.1 and 4.5 min, respectively. Degradant II was later confirmed to be 2-(2,4-difluorophenylcarbamoyl)-3,4,5,6-tetrafluorobenzoic acid by comparison to the synthesized standard. Degradants III and IV were identified as 2,4-difluoroaniline and 3,4,5,6-tetrafluorophthalic acid by mass spectrometry (Fig. 8b, c) and by comparing their retention times and UV spectra with the reference compounds purchased from Sigma-Aldrich.

Degradation Kinetics in Aqueous Solutions

The stability of NSC-726796 at pH=1.0, 2.5, 4.0, 5.5, 7.0, 8.5, and 10.0 was investigated for up to 6 h. The percentage remaining in the solution is plotted in Fig. 9 for each pH.

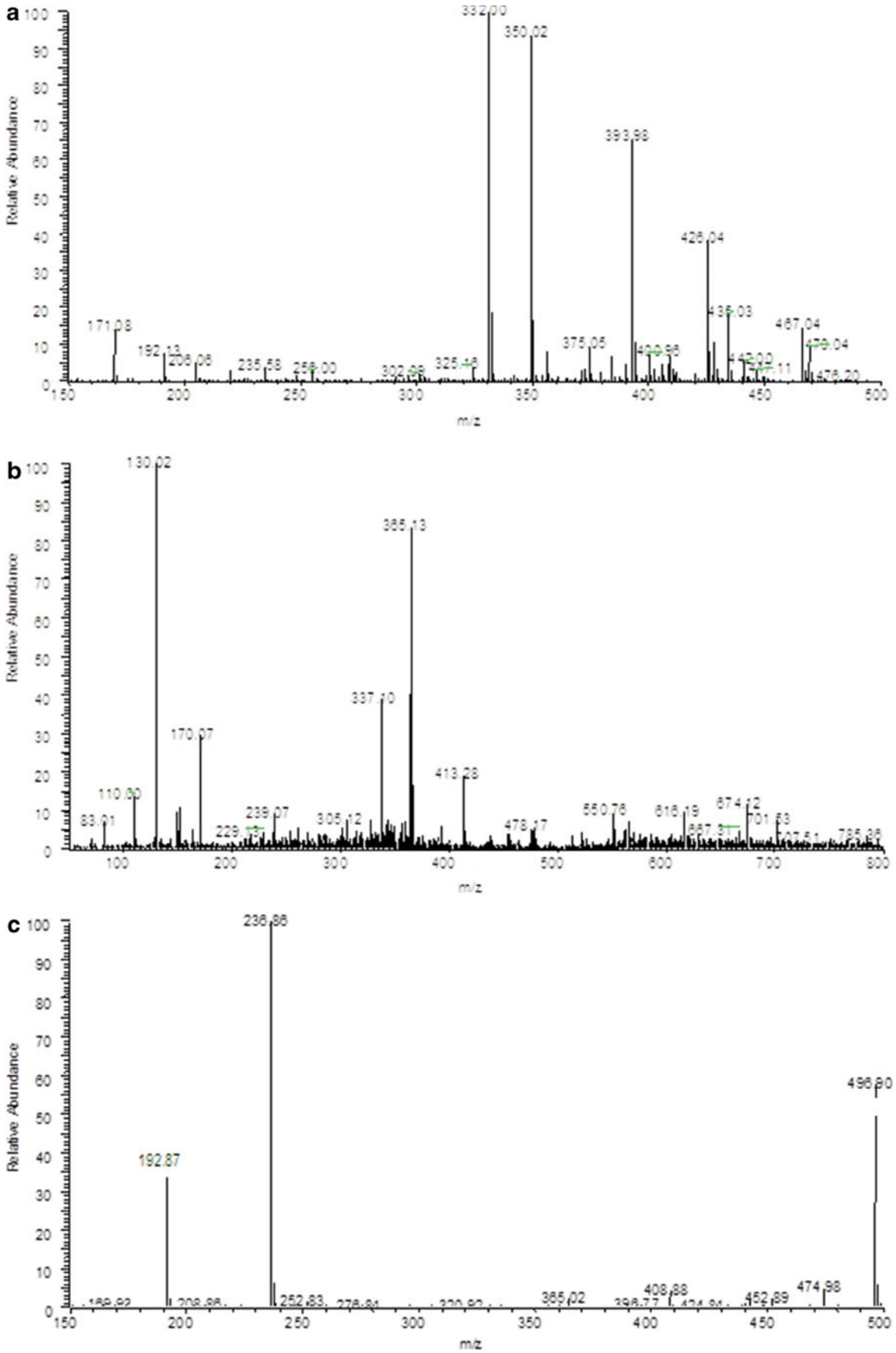


Fig. 8. Electrospray ionization-MS spectra of **a** 2-(2,4-difluorophenylcarbamoyl)-3,4,5,6-tetrafluorobenzoic acid (position mode), **b** 2,4-difluoroaniline (positive mode), and **c** tetrafluorophthalic acid (negative mode)

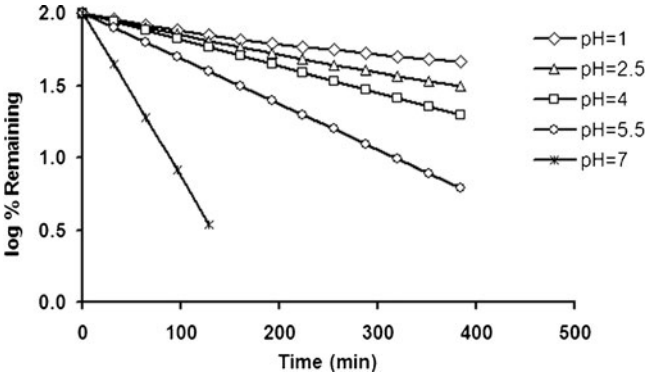


Fig. 9. Percentage of NSC-726796 remaining (log scale) *vs.* time over 360 min

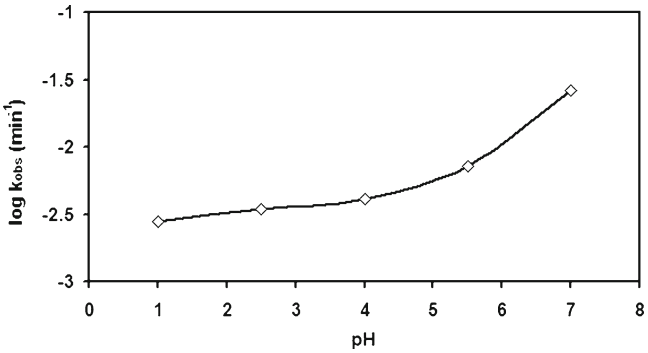


Fig. 10. Degradation rate constant (k_{obs} min^{-1}) *vs.* pH of NSC-726796

It is clear from Fig. 9 that above pH 4, the degradation follows a first-order mechanism, where the slope corresponds to the $\left(\frac{k_{obs}}{2.303}\right)$ term in the following equation

$$\log[D] = \log[D_0] - \left(\frac{k_{obs}}{2.303}\right)t$$

$[D_0]$ and $[D]$ are the initial and time (t) concentrations of drug, respectively, and k_{obs} is the observed degradation rate constant.

However, at pH 1 and 2.5, a slope change occurs after 200 min. Only the initial slope was used to calculate k_{obs}. Table VI lists the degradation rate constant data at the investigated pH values. The reason for the slope change will be discussed later.

The k_{obs} values are plotted against pH in Fig. 10. The degradation of the drug appears to be base-catalyzed hydrolysis. It shows that the drug is more stable at pH 1 with about 50 % drug loss after 6 h, while more than 90 % drug was lost at pH 5.5. The degradation rate above pH 8.5 is too fast to measure using the procedures described herein.

Synthesis of NSC-749820

A white powder was obtained using the synthetic scheme described in Fig. 11. Mass spectrometry indicates the powder has a molecular weight of 349.18. The powder was assayed by HPLC and found to be greater than 97 % pure. The major peak has the same retention time, spectrum, and molecular weight as degradant II from NSC-726796 and the single crystal crystallography (reference) are consistent with the proposed structure. The mono acid was assigned an internal identification number by National Cancer Institute as NSC-749820.

Table VI. Observed NSC-726796 Degradation Rate Constants and Half Life in Aqueous Buffers

pH	k_{obs} (min^{-1})	$T_{1/2}$ (min)
1	0.00276	334
2.5	0.00345	231
4	0.00415	167
5.5	0.00714	97
7	0.0263	26
8.5	Degrades too fast to quantitate	<5
10	Degrades too fast to quantitate	<1

Degradation Mechanism

The degradation pathway of NSC-726796 is proposed in Fig. 12. The phthalimide moiety is readily hydrolyzed to the mono acid NSC-749820. The mono acid is further hydrolyzed to produce 2,4-difluoroaniline and tetrafluorophthalic acid.

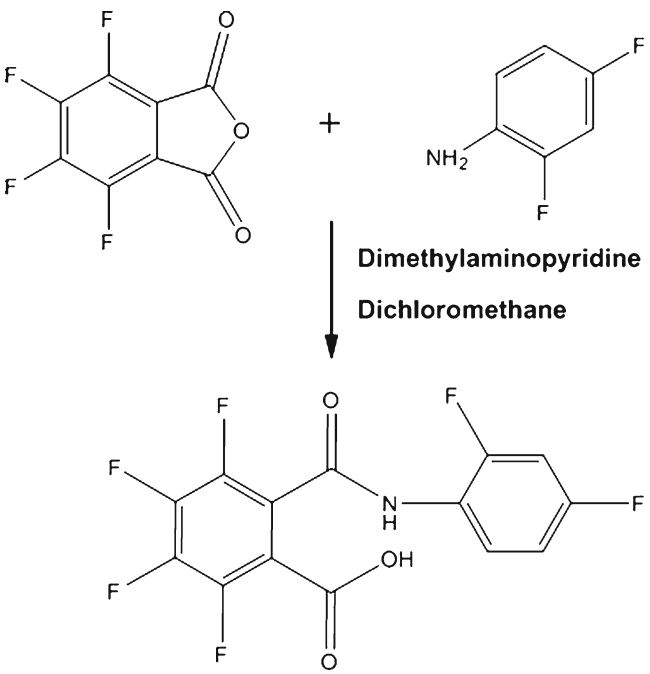


Fig. 11. Synthetic scheme of 2-(2,4-difluorophenylcarbamoyl)-3,4,5,6-tetrafluorobenzoic acid (NSC-749820)

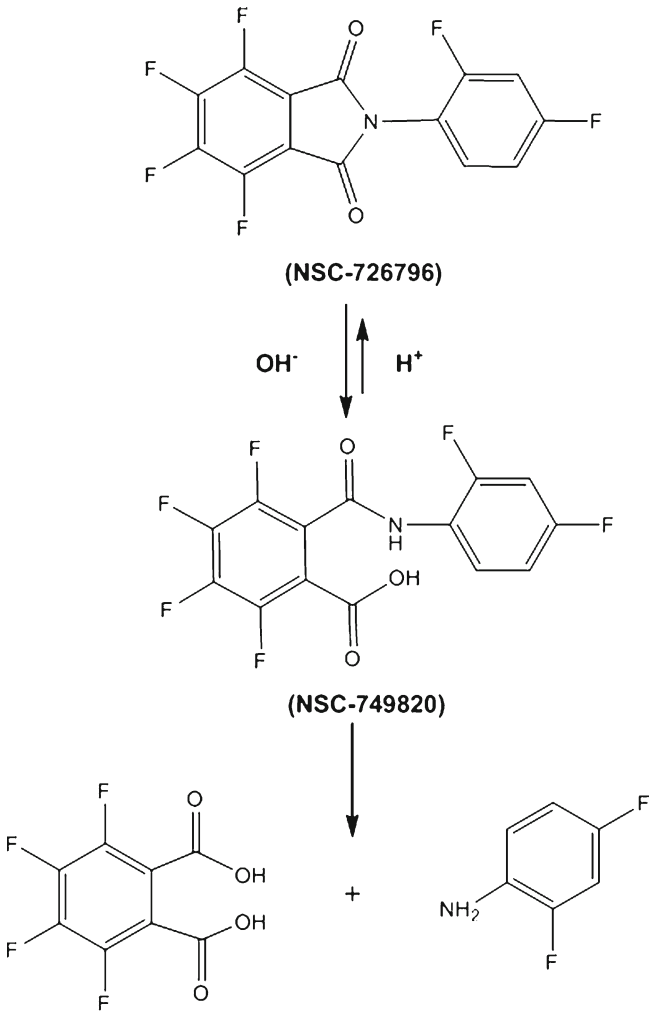


Fig. 12. Degradation mechanism of NSC-72796

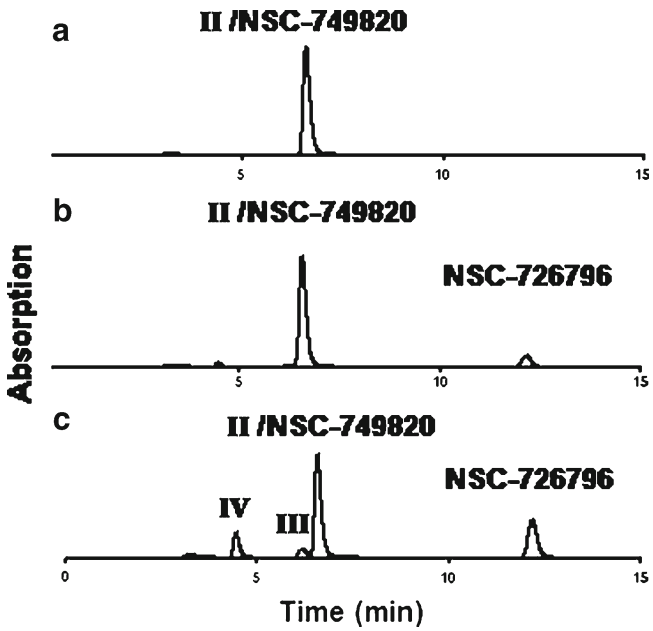


Fig. 13. Chromatograms of the NSC-749820 in pH 1; **a** 0 min, **b** 60 min, and **c** 240 min

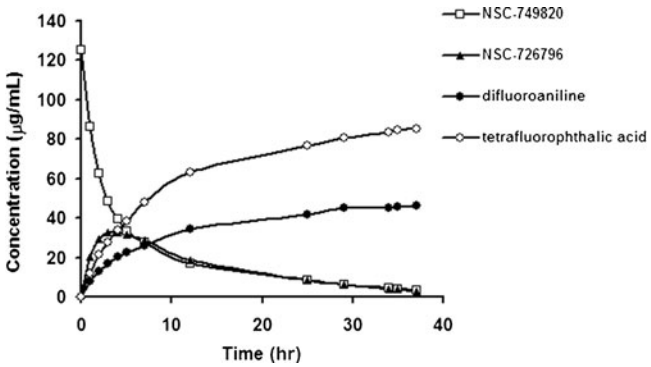


Fig. 14. Conversion of NSC-749820 to NSC-726796, 3,4,5,6-tetrafluorophthalic acid and 2,4-difluoroaniline at pH=1

Reverse Reaction Kinetics at Low pH

The slope change in the degradation of NSC-726796 at pH 1.0 and 2.5 shown in Fig. 10 is very likely due to the ring-open degradant NSC-749820 converting back to the parent drug. To confirm this, the synthesized mono acid was dissolved in acetonitrile and then quenched with pH 1 buffer. These samples were filtered and analyzed by HPLC. The HPLC chromatogram in Fig. 13a for a fresh solution at pH 1 shows only one peak for the ring-open compound. Figure 13b shows that the ring-closed form is present after 1 h. Additional degradants, tetrafluorophthalic acid and 2,4-difluoroaniline, appear after 4 h as illustrated in Fig. 13c. The amount of each compound was monitored at selected time intervals and plotted in Fig. 14. The interconversion between the ring-open and closed form was also observed at pH 2.5, but not at neutral and basic solutions. The partial conversion of the mono acid compound to the parent drug at low pH can be ascribed to the acid-catalyzed esterification as the reversed reaction of hydrolysis illustrated in Fig. 15. Mass balance of the reaction at pH 1.0 was confirmed.

DSC Results

DSC studies of both compounds shown in Fig. 16 indicate that NSC-726796 and the degradant have a melting point of 144 and 169 °C, respectively. The higher melting point of the ring-open compound is due to its ability to bond hydrogen, even though it has less symmetry than the parent phthalimide.

Rationale

The ring-open degradant is more polar (ClogP=1.60) than that of the parent compound (ClogP=3.26). Using the general solubility equation (8) and the melting points shown above, the intrinsic solubility of ring-open degradant is about 100 times higher than the parent drug. Furthermore, while NSC-726796 is not ionizable between pH 1 and 14, the mono acid degradant has an acidic pK_a of 1.79 and it will be largely ionized and more soluble at blood pH. For these reasons, the ring-open degradant was synthesized in the hope of its having anticancer activity.

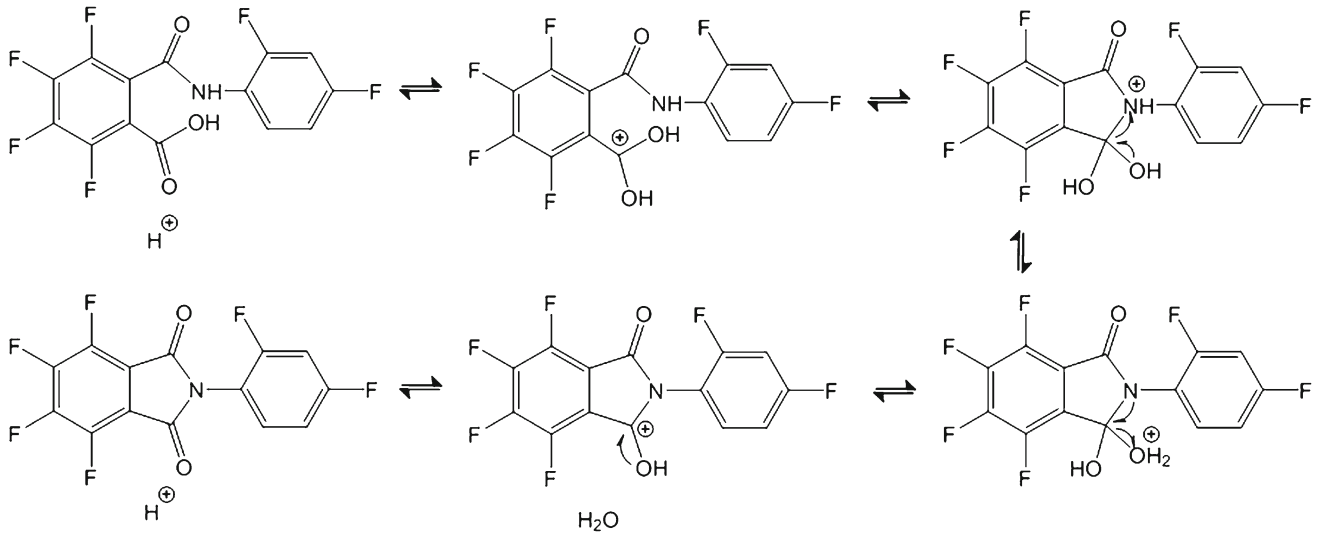


Fig. 15. Hydrolysis of phthalimide and esterification of the mono acid

Antiangiogenic Activity

The rat aortic ring detects both antiangiogenic and direct cytotoxic activity. As shown in Fig. 17 and Table VII, NSC-726796 significantly inhibited microvessel outgrowth. Conversely, when NSC-726796 was diluted in aqueous buffer for 30 min prior to ring application, no activity was observed. Similarly, no significant activity was observed when rings were treated with NSC-749820, the mono acid hydrolysis product. These results suggest that it is indeed the parent drug that is the active moiety. Unfortunately, its rapid degradation in aqueous media precludes its use as an anticancer agent.

CONCLUSION

The hydrolytic degradation kinetics and mechanism of NSC-726796 was investigated at various pH values at

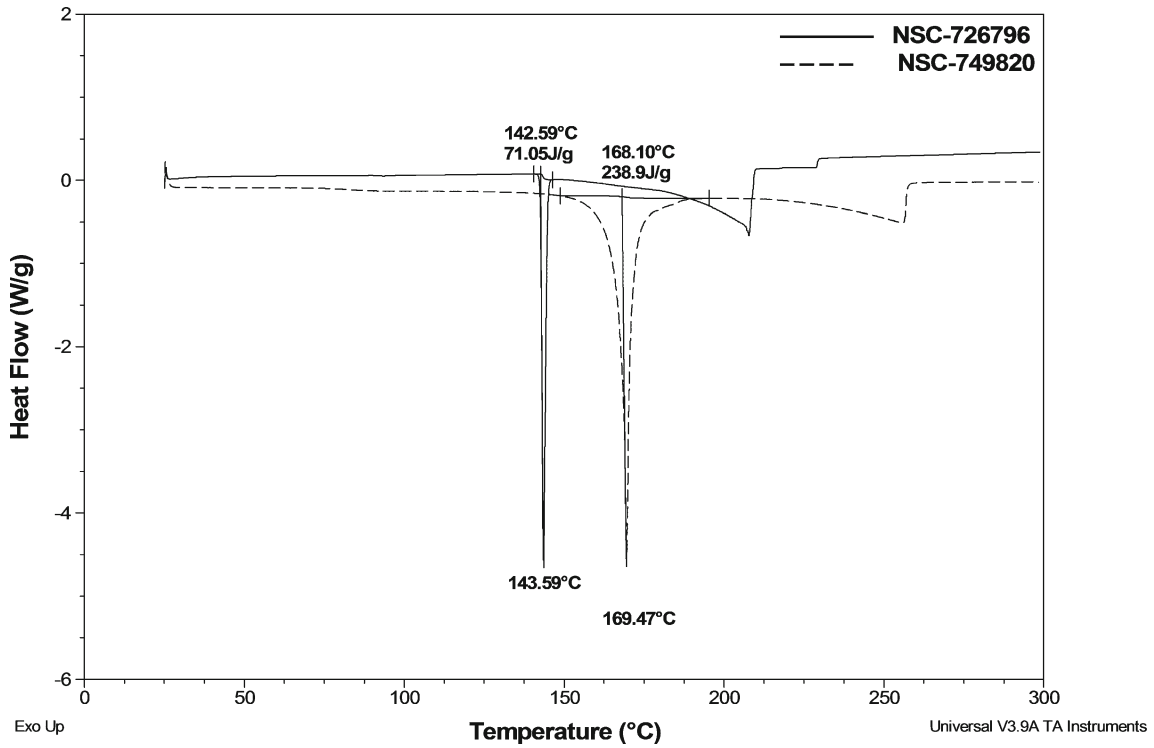


Fig. 16. DSC thermograms of NSC-726796 and NSC-749820

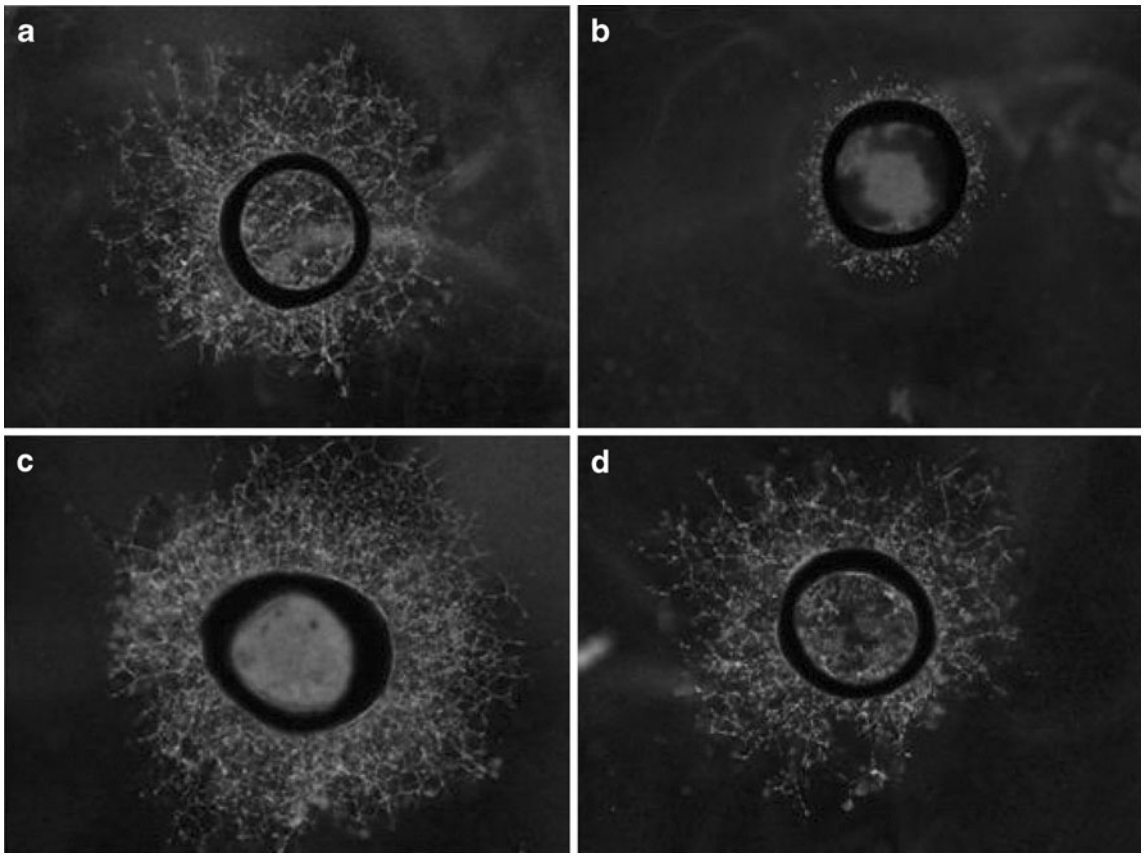


Fig. 17. Rat aortic cross-sections, after 4 days of treatment with **a** vehicle control, **b** 100 μM NSC-726796, **c** 100 μM NSC-726796, after 30 min in aqueous buffer and **d** 100 μM NSC-749820

ambient temperature. From the log k_{obs}–pH profile, it is concluded that the hydrolysis is mainly base catalyzed. The ring-open degradant was synthesized and identified as 2-(2,4-difluorophenylcarbamoyl)-3,4,5,6-tetrafluorobenzoic acid which is also known as NSC-749820. This mono acid can be converted back to NSC-726796 in acidic conditions, but eventually degrades to tetrafluorophthalic acid and 2,4-difluoroaniline. It appears that NSC-726796 has anticancer activity. However, it is rapidly hydrolyzed and the hydrolysis products, including NSC-749820, are inactive.

Table VII. Rat Aortic Ring Microvessel Outgrowth Inhibition with Various Treatments

Treatment	Microvessel outgrowth (%)	*P* value[a]
Vehicle control	100	–
CAI (positive control)	10.3±3.9	0.011
NSC 726796	22.1±14.9	0.002
NSC 726796 30 min post	102.4±16.8	0.87
NSC 749820	83.9±25.3	0.30

[a] As compared to vehicle control

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