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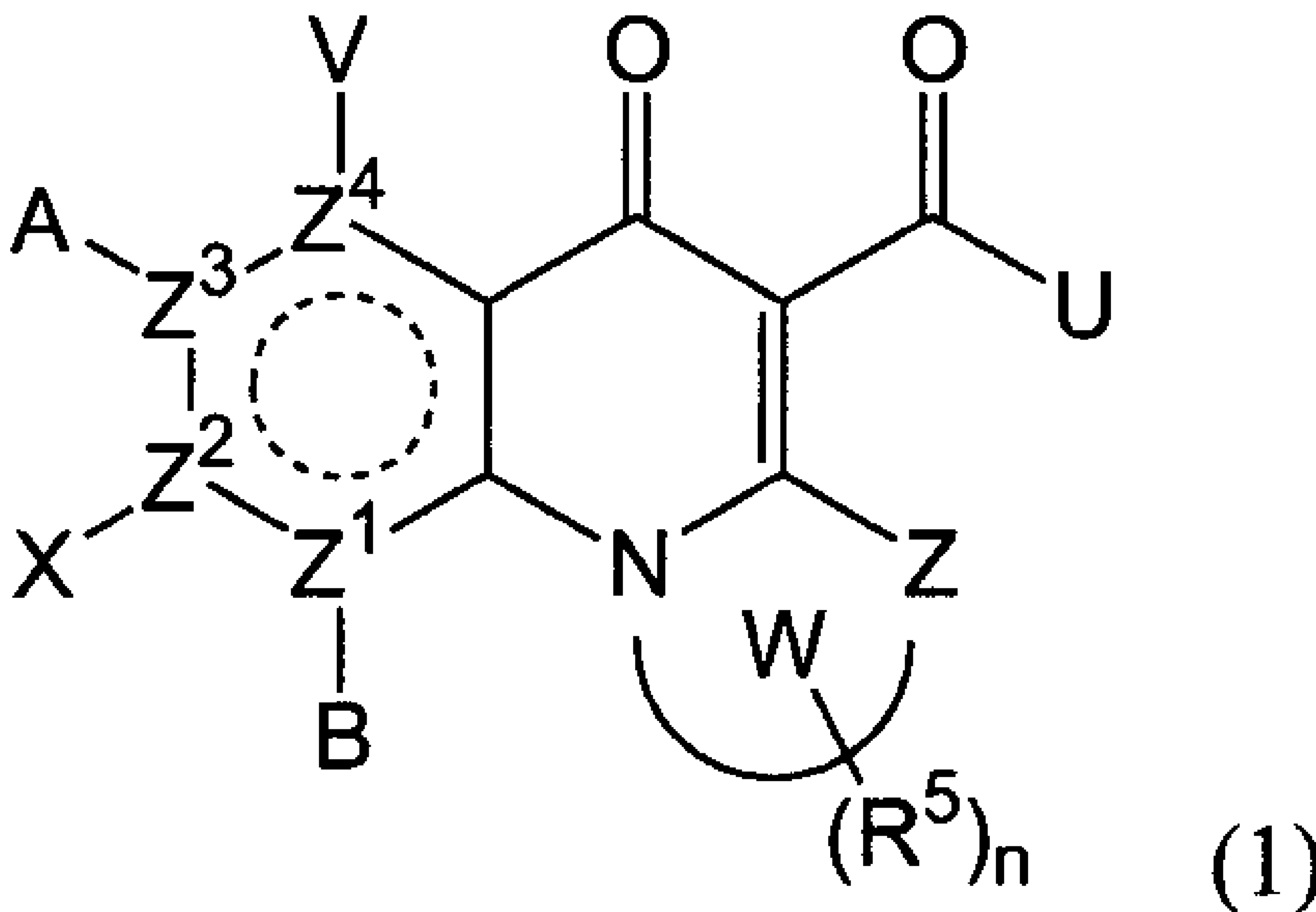
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(54) Titre : ANALOGUES DE LA QUINOLONE

(54) Title: QUINOLONE ANALOGS



(57) Abrégé/Abstract:

The present invention provides quinolone analogs of formula (I) which may inhibit cell proliferation and/or induce cell apoptosis. The present invention also provides methods of preparing quinolone analogs, and methods of using the same.



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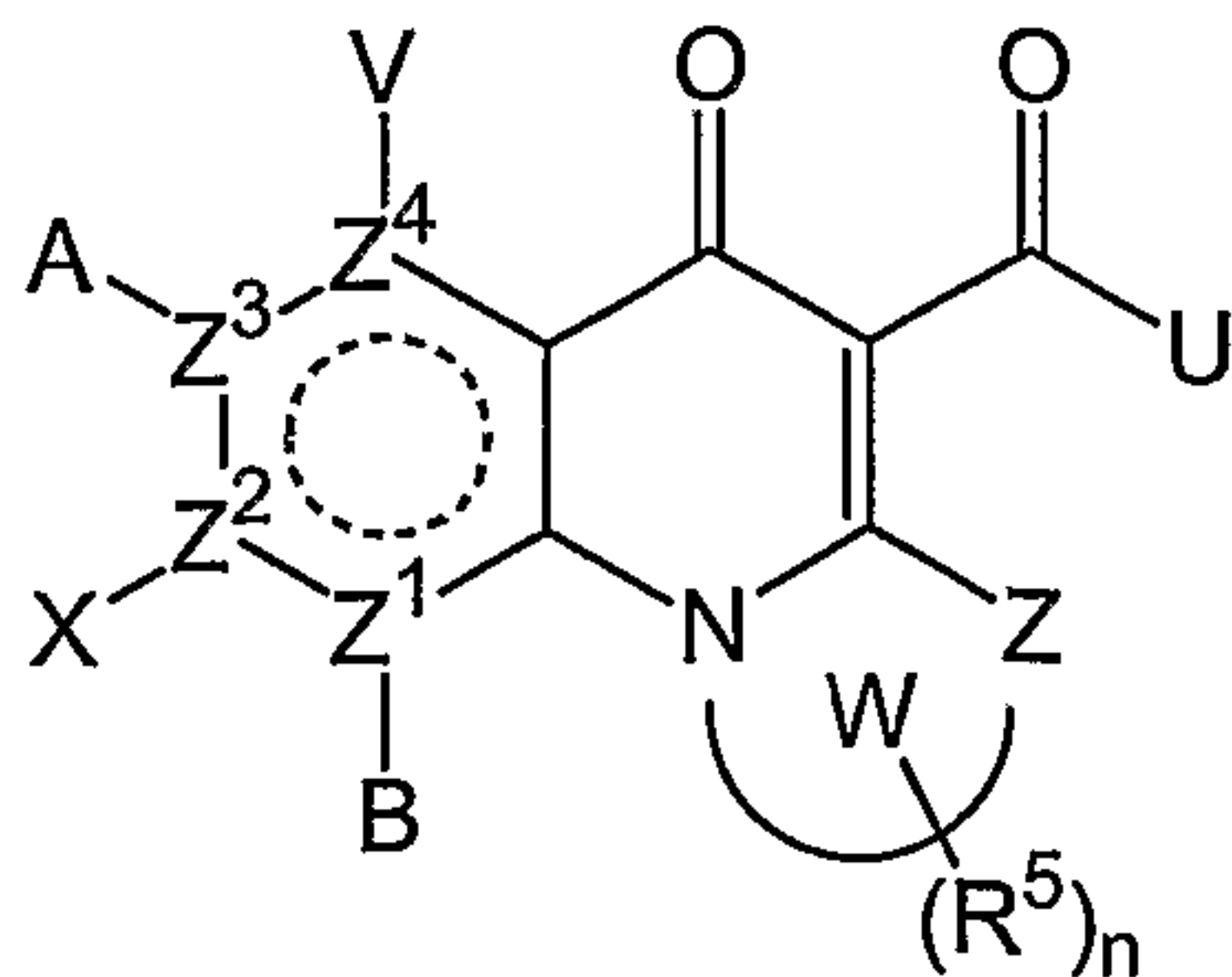
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## (54) Title: QUINOLONE ANALOGS AS CELL PROLIFERATION INHIBITORS



(1)

(57) Abstract: The present invention provides quinolone analogs of formula (I) which may inhibit cell proliferation and/or induce cell apoptosis. The present invention also provides methods of preparing quinolone analogs, and methods of using the same.

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## QUINOLONE ANALOGS

### Field of the Invention

[0002] The invention relates to quinolone analogs and uses thereof. The invention also relates to methods of preparing quinolone analogs.

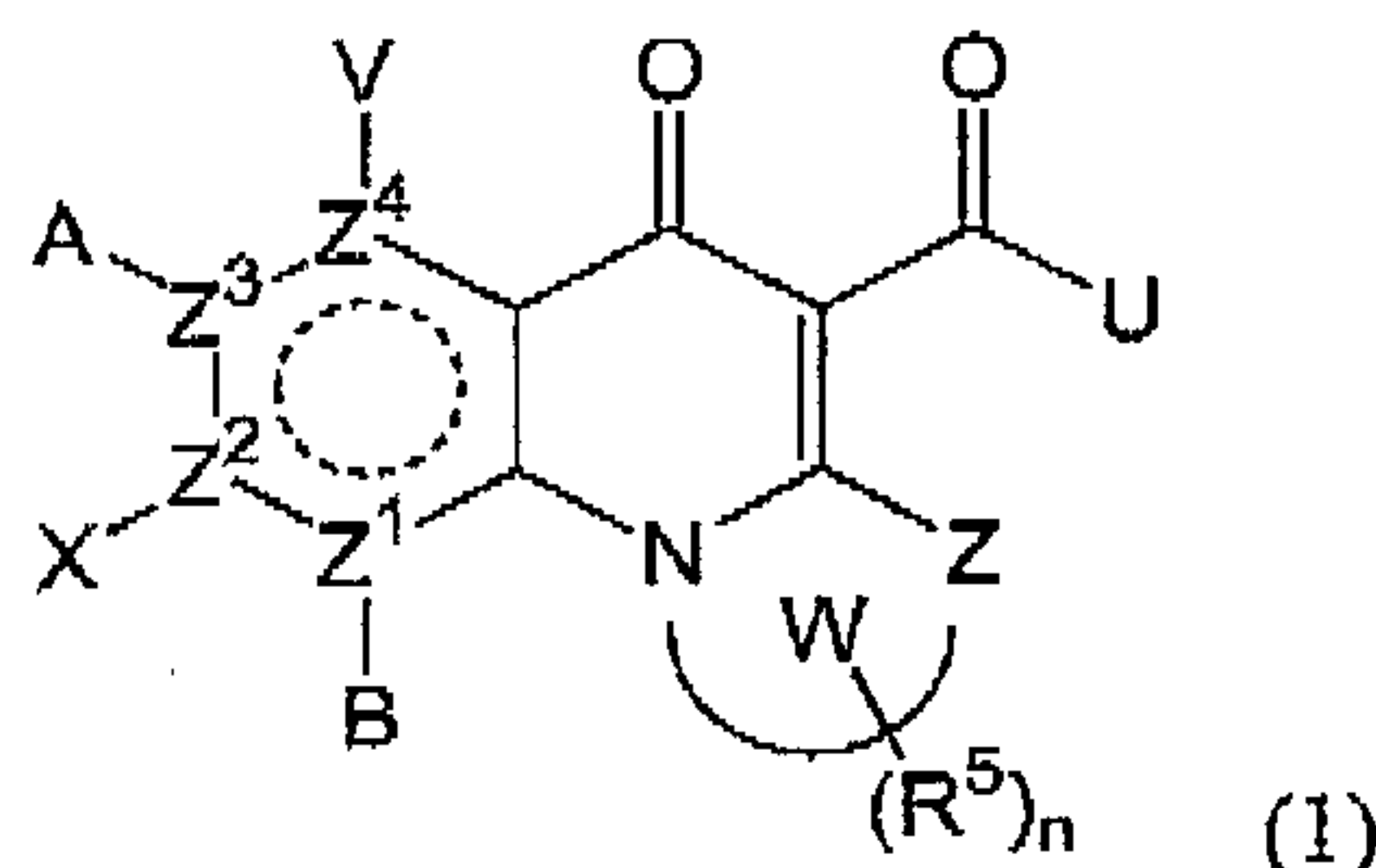
### Background

[0003] Evidence suggests quadruplex structures can exist in vivo in specific regions of the genome, including the telomeric ends of chromosomes and oncogene regulatory regions (Han, *et al.*, *Trends Pharm. Sci.* (2000) 21:136-142). Quadruplex structures can form in purine-rich strands of nucleic acids. In duplex nucleic acids, certain purine rich strands are capable of engaging in a slow equilibrium between a typical duplex helix structure and in unwound and non-B-form regions. These unwound and non-B forms can be referred to as “paranemic structures.” Some forms are associated with sensitivity to S1 nuclease digestion, which can be referred to as “nuclease hypersensitivity elements” or “NHEs.” A quadruplex is one type of paranemic structure and certain NHEs can adopt a quadruplex structure.

### Summary of the Invention

[0004] The present invention provides quinolone analogs which may inhibit cell proliferation and/or induce cell apoptosis. The present invention also provides methods of preparing quinolone analogs, and methods of using the same.

[0004A] Various embodiments of this invention provide a compound having formula (1):



and pharmaceutically acceptable salts and esters thereof;

wherein B, X, A, or V is absent if  $Z^1$ ,  $Z^2$ ,  $Z^3$ , and  $Z^4$  are N, respectively, and independently H, halo, azido,  $R^2$ ,  $\text{CH}_2\text{R}^2$ ,  $\text{SR}^2$ ,  $\text{OR}^2$  or  $\text{NR}^1\text{R}^2$  when  $Z^1$ ,  $Z^2$ ,  $Z^3$ , and  $Z^4$  are C; or

A and V, A and X, or X and B may form a carbocyclic ring, heterocyclic ring, aryl or heteroaryl, each of which may be optionally substituted and/or fused with a cyclic ring;

Z is O, S,  $\text{NR}^1$ ,  $\text{CH}_2$ , or  $\text{C}=\text{O}$ ;

$Z^1$ ,  $Z^2$ ,  $Z^3$  and  $Z^4$  are C or N, provided any two N are non-adjacent;

W together with N and Z forms an optionally substituted 5- or 6-membered ring that is fused to an optionally substituted saturated or unsaturated ring that optionally contains a heteroatom and is monocyclic or fused with a single or multiple carbocyclic or heterocyclic rings;

U is  $\text{NR}^1\text{R}^2$ ,  $\text{NR}^1 - (\text{CR}^1_2)_n - \text{NR}^3\text{R}^4$ , or  $\text{N}=\text{CR}^1\text{R}^2$ , wherein in  $\text{N}=\text{CR}^1\text{R}^2$ ,  $\text{R}^1$  and  $\text{R}^2$  together with C may form a ring,

provided when U is  $\text{NH}_2$ , then at least one of  $Z^1$ - $Z^4$  is N;

in each  $\text{NR}^1\text{R}^2$ ,  $\text{R}^1$  and  $\text{R}^2$  together with N may form an optionally substituted ring;

in  $\text{NR}^3\text{R}^4$ ,  $\text{R}^3$  and  $\text{R}^4$  together with N may form an optionally substituted ring;

$\text{R}^1$  and  $\text{R}^3$  are independently H or  $\text{C}_{1-6}$  alkyl;

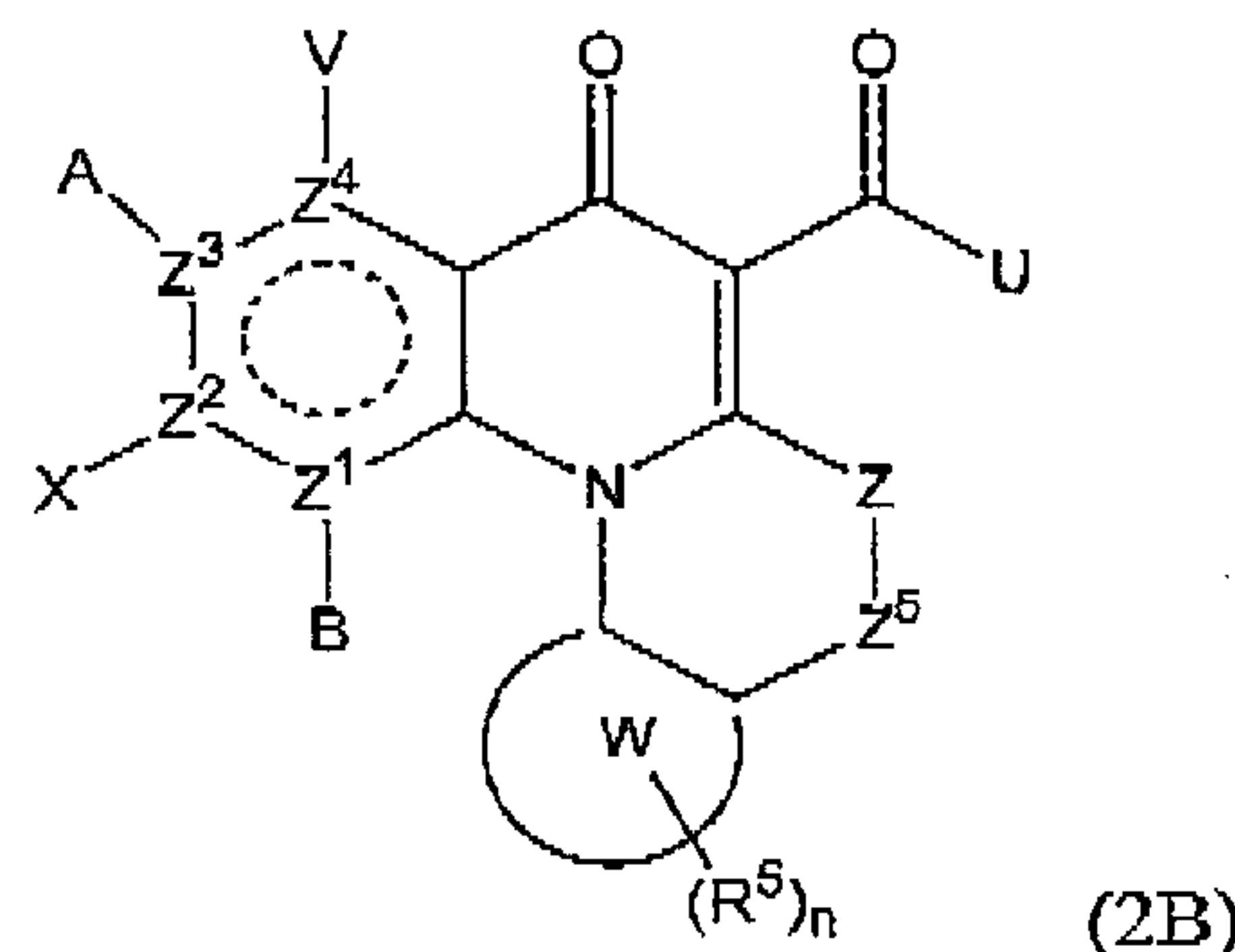
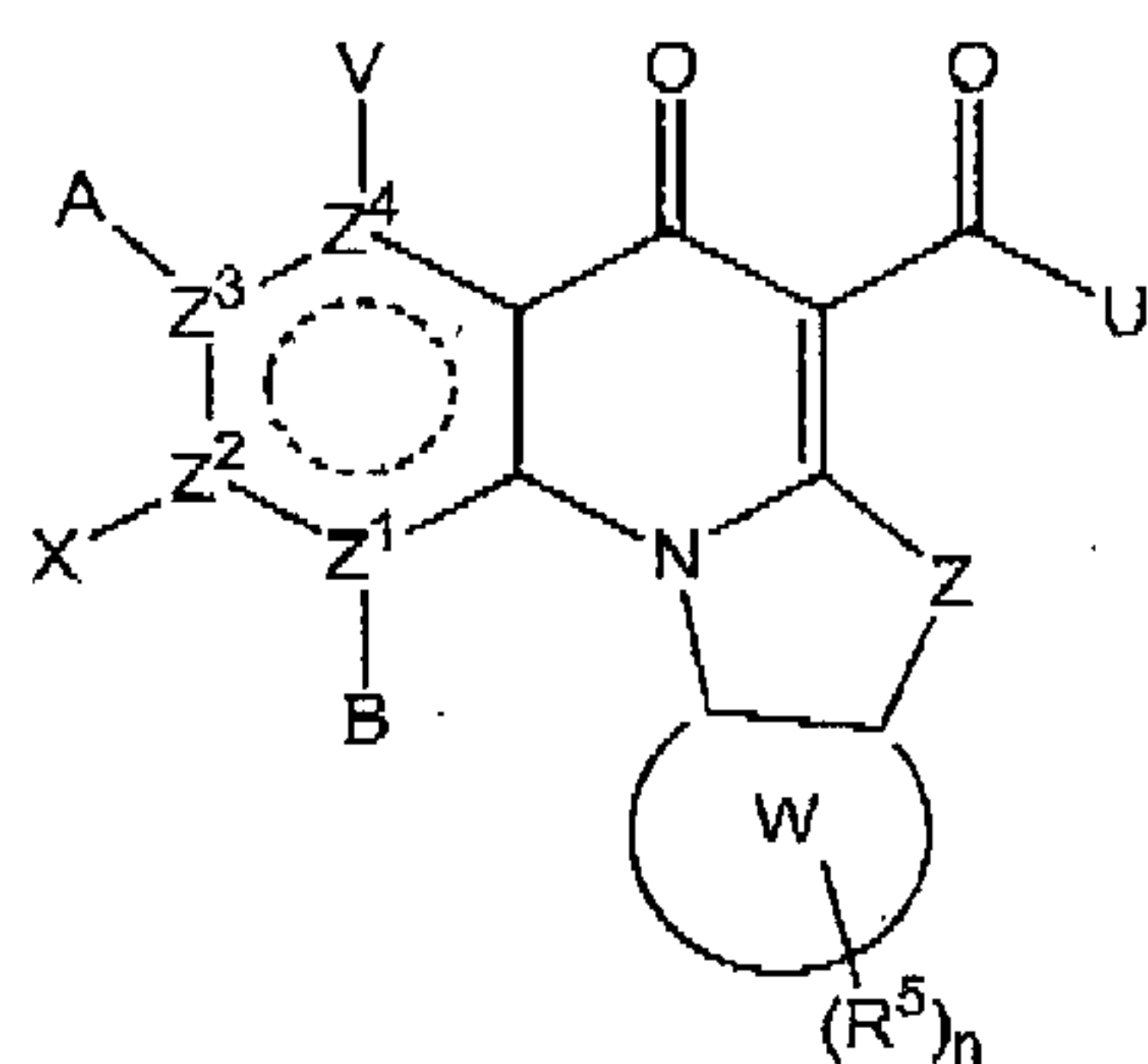
each  $\text{R}^2$  is H, or a  $\text{C}_{1-10}$  alkyl or  $\text{C}_{2-10}$  alkenyl each optionally substituted with a halogen, one or more non-adjacent heteroatoms, a carbocyclic ring, a heterocyclic ring, an aryl or heteroaryl, wherein each ring is optionally substituted; or  $\text{R}^2$  is an optionally substituted carbocyclic ring, heterocyclic ring, aryl or heteroaryl;

$R^4$  is H, a  $C_{1-10}$  alkyl or  $C_{2-10}$  alkenyl optionally containing one or more non-adjacent heteroatoms selected from N, O and S, and optionally substituted with a carbocyclic or heterocyclic ring; or  $R^3$  and  $R^4$  together with N may form an optionally substituted ring;

each  $R^5$  is a substituent at any position on ring W; and is H,  $OR^2$ , amino, alkoxy, amido, halogen, cyano or an inorganic substituent; or  $R^5$  is  $C_{1-6}$  alkyl,  $C_{2-6}$  alkenyl,  $C_{2-6}$  alkynyl,  $-CONHR^1$ , each optionally substituted by halo, carbonyl or one or more non-adjacent heteroatoms; or two adjacent  $R^5$  are linked to obtain a 5-6 membered optionally substituted carbocyclic or heterocyclic ring that may be fused to an additional optionally substituted carbocyclic or heterocyclic ring; and

n is 1-6.

**[0004B]** Various embodiments of this invention provide a compound having formula 2A or 2B:



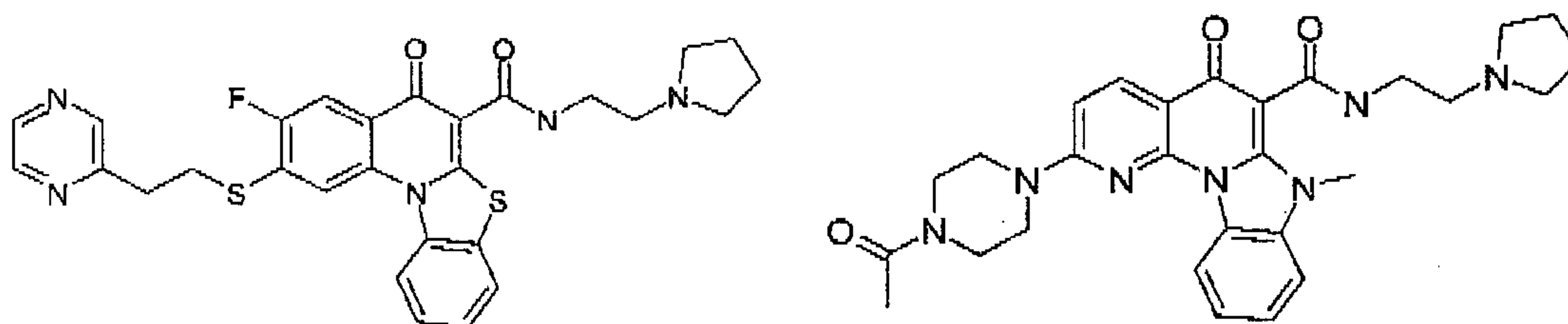
wherein A, B, V, X, U, Z,  $Z^1$ ,  $Z^2$ ,  $Z^3$ ,  $Z^4$  and n are as described above;

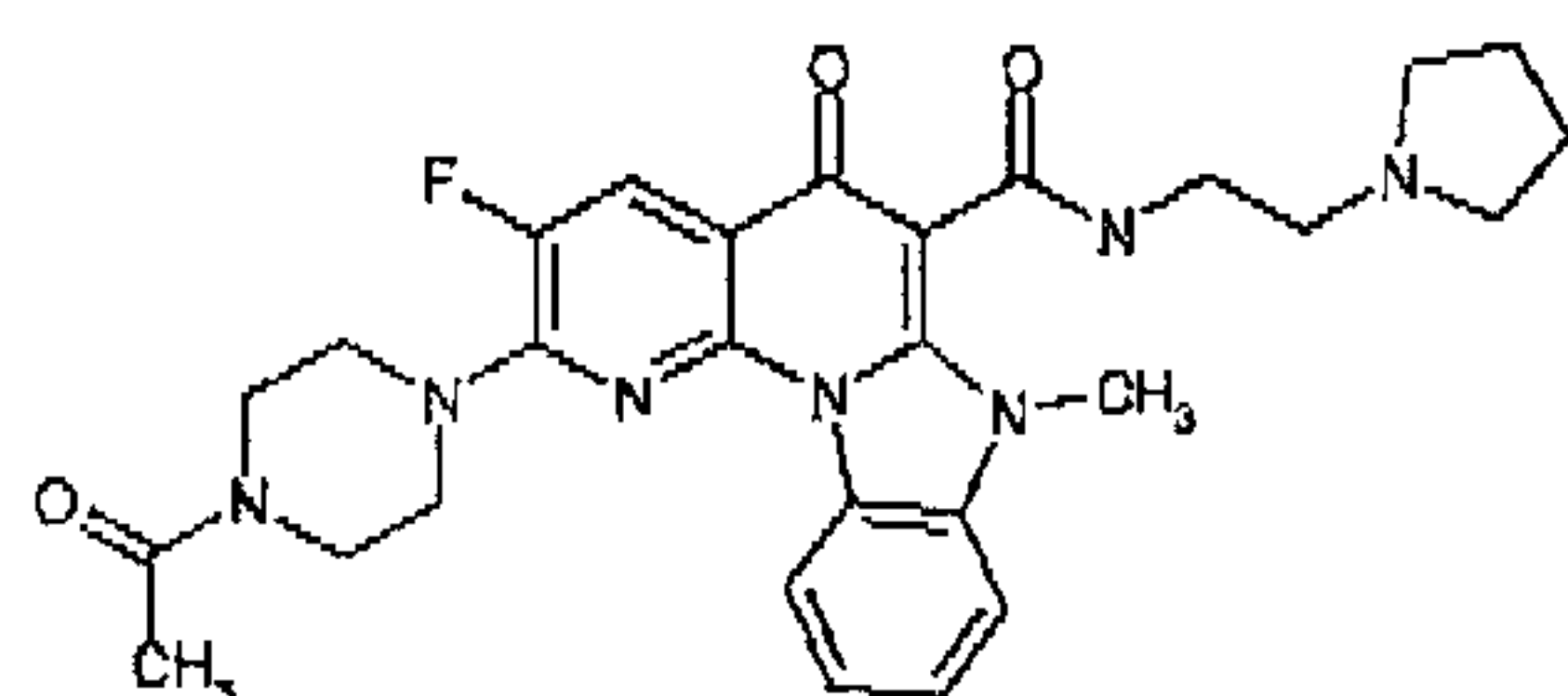
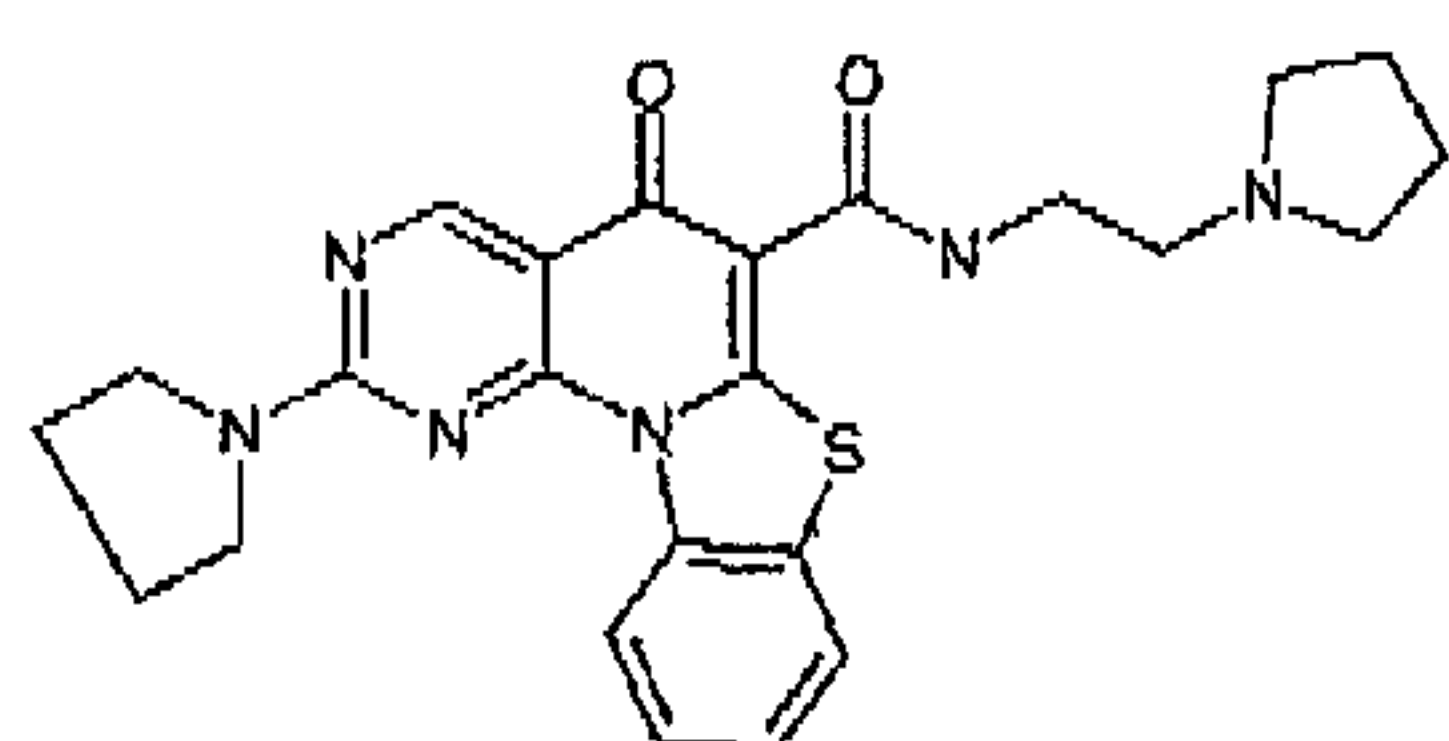
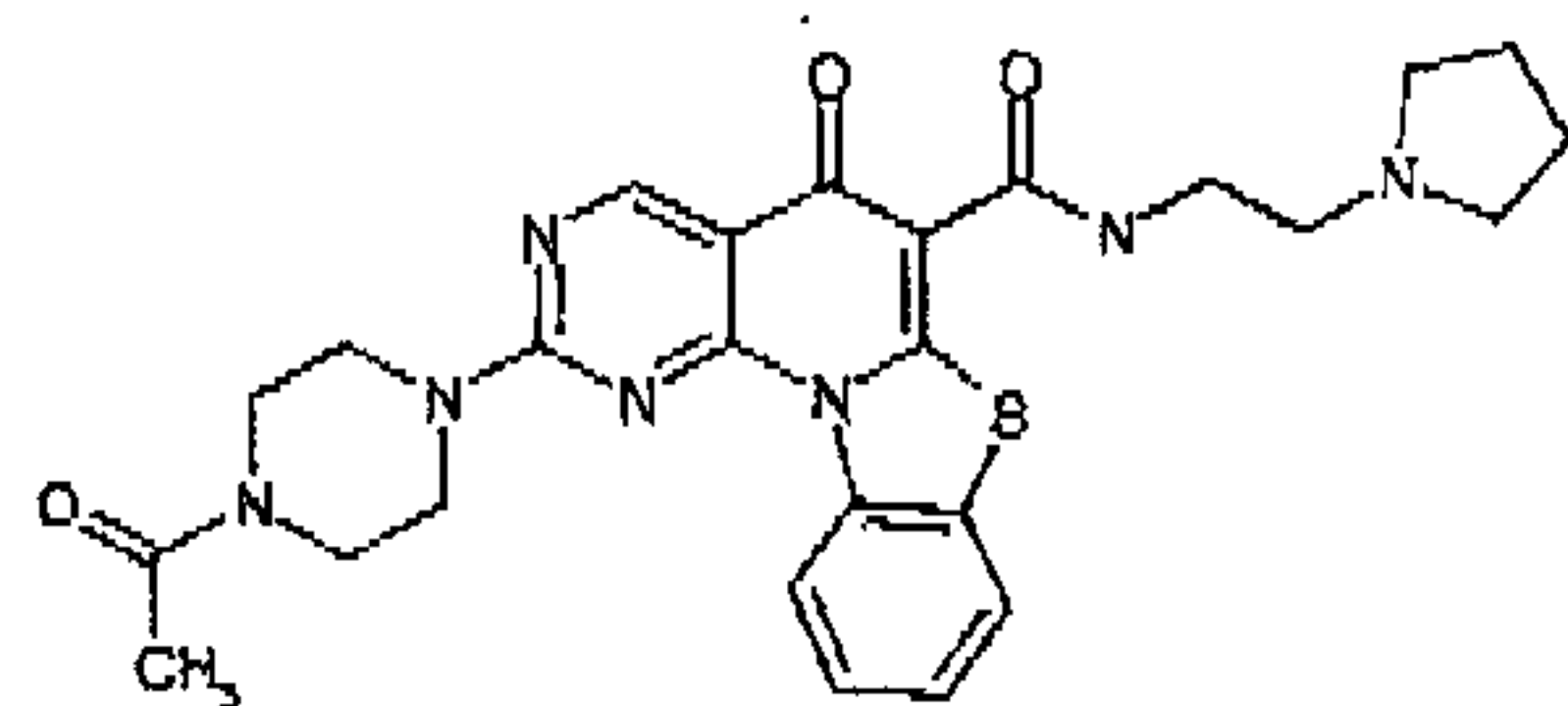
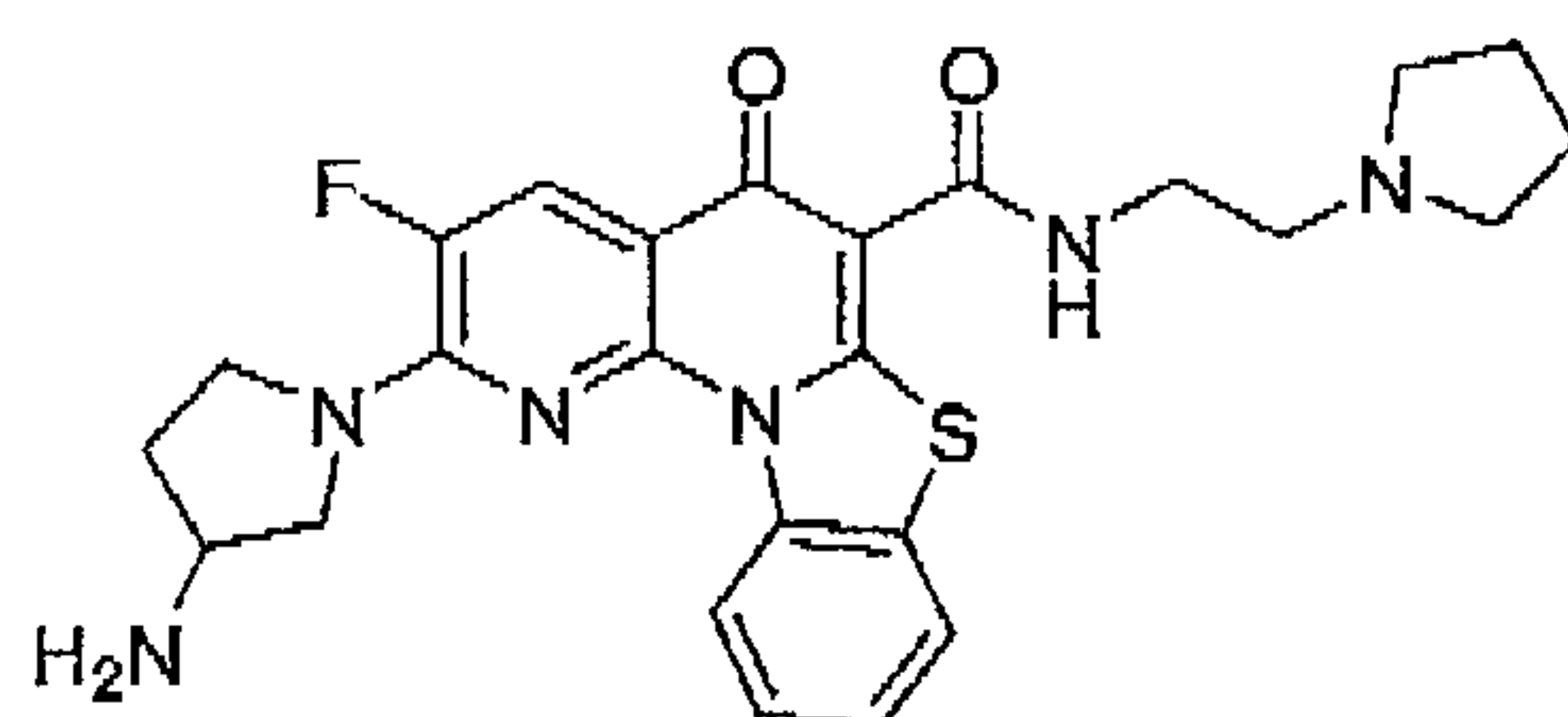
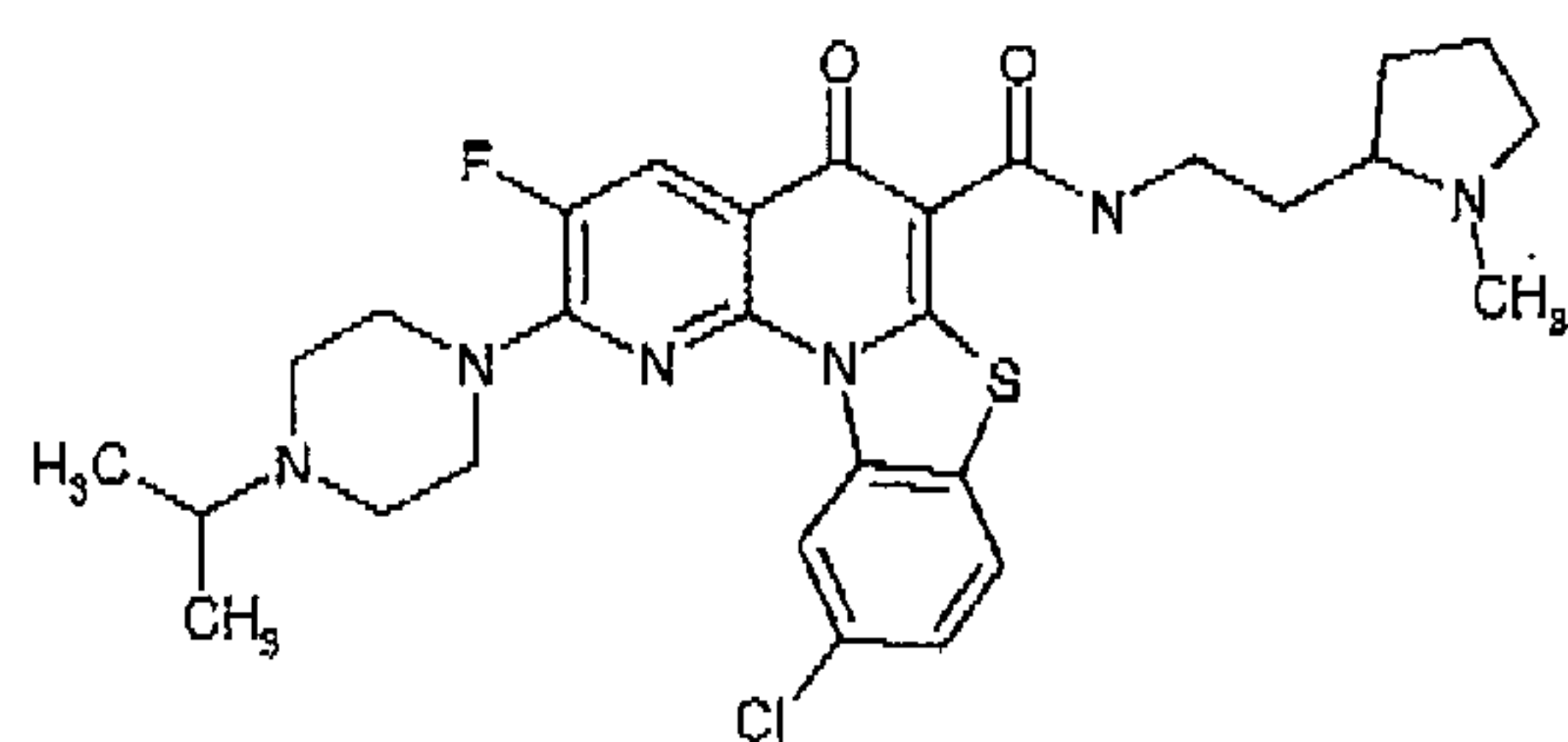
$Z^5$  is O,  $NR^1$ ,  $CR^6$ , or  $C=O$ ;

$R^6$  is H,  $C_{1-6}$  alkyl, hydroxyl, alkoxy, halo, amino or amido; and

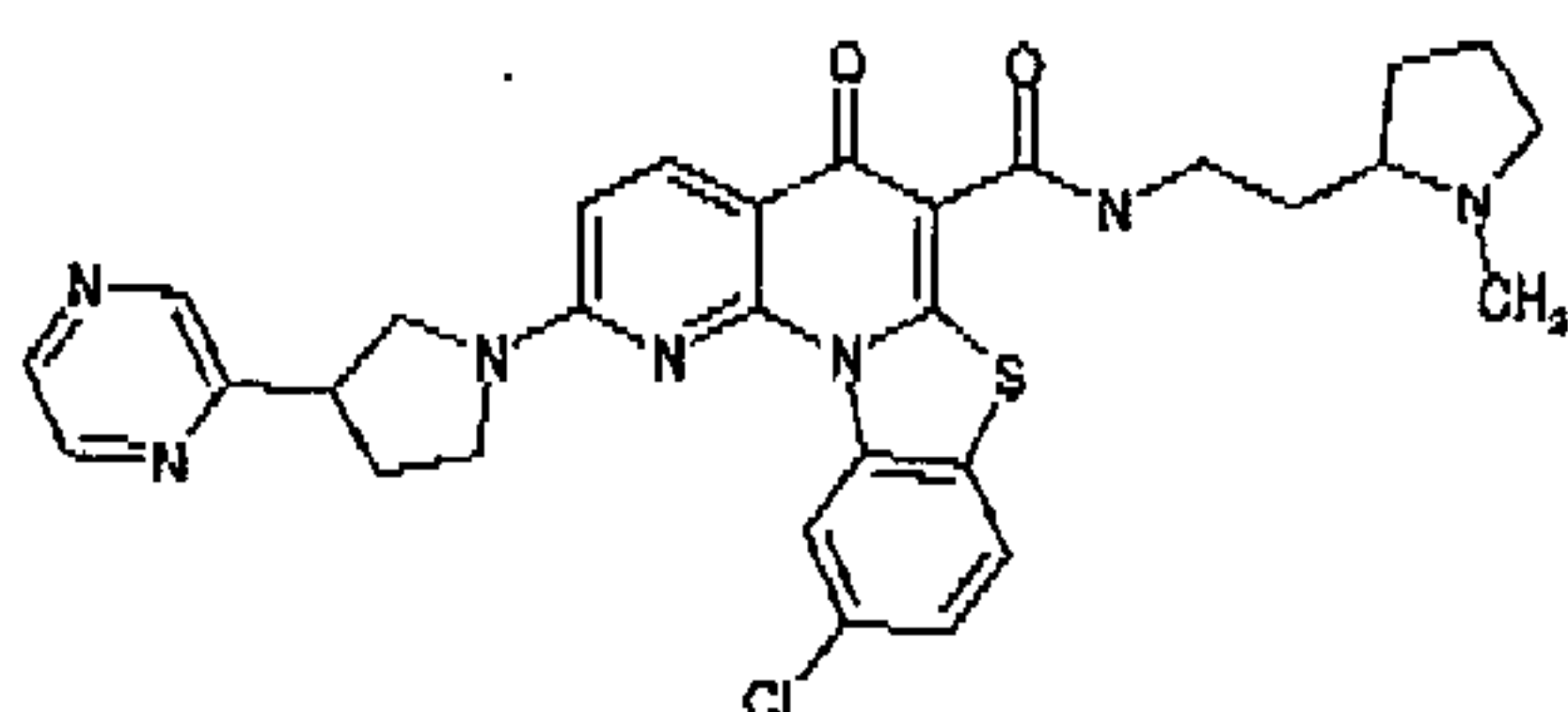
Z and  $Z^5$  may optionally form a double bond.

**[0004C]** Various embodiments of this invention provide a compound having the formula:





, or



[0004D] Various embodiments of this invention provide pharmaceutical compositions comprising a compound of this invention and a pharmaceutically acceptable carrier.

[0004E] Various embodiments of this invention provide use of a compound or composition of this invention optionally with a procedure and/or chemotherapeutic agent, to reduce cell proliferation and/or ameliorate a cell proliferative disorder or for a preparation of a medicament for such use.

[0004F] Various embodiments of this invention provide use of a compound or composition of this invention optionally with an antimicrobial agent to reduce microbial titers and/or ameliorate a microbial infection or to prepare a medicament for such reduction or amelioration.

[0004G] Various embodiments of this invention provide use of a compound or composition of this invention optionally with a procedure and/or chemotherapeutic agent to induce cell death and/or induce apoptosis. The use may be for preparation of a medicament for such induction.

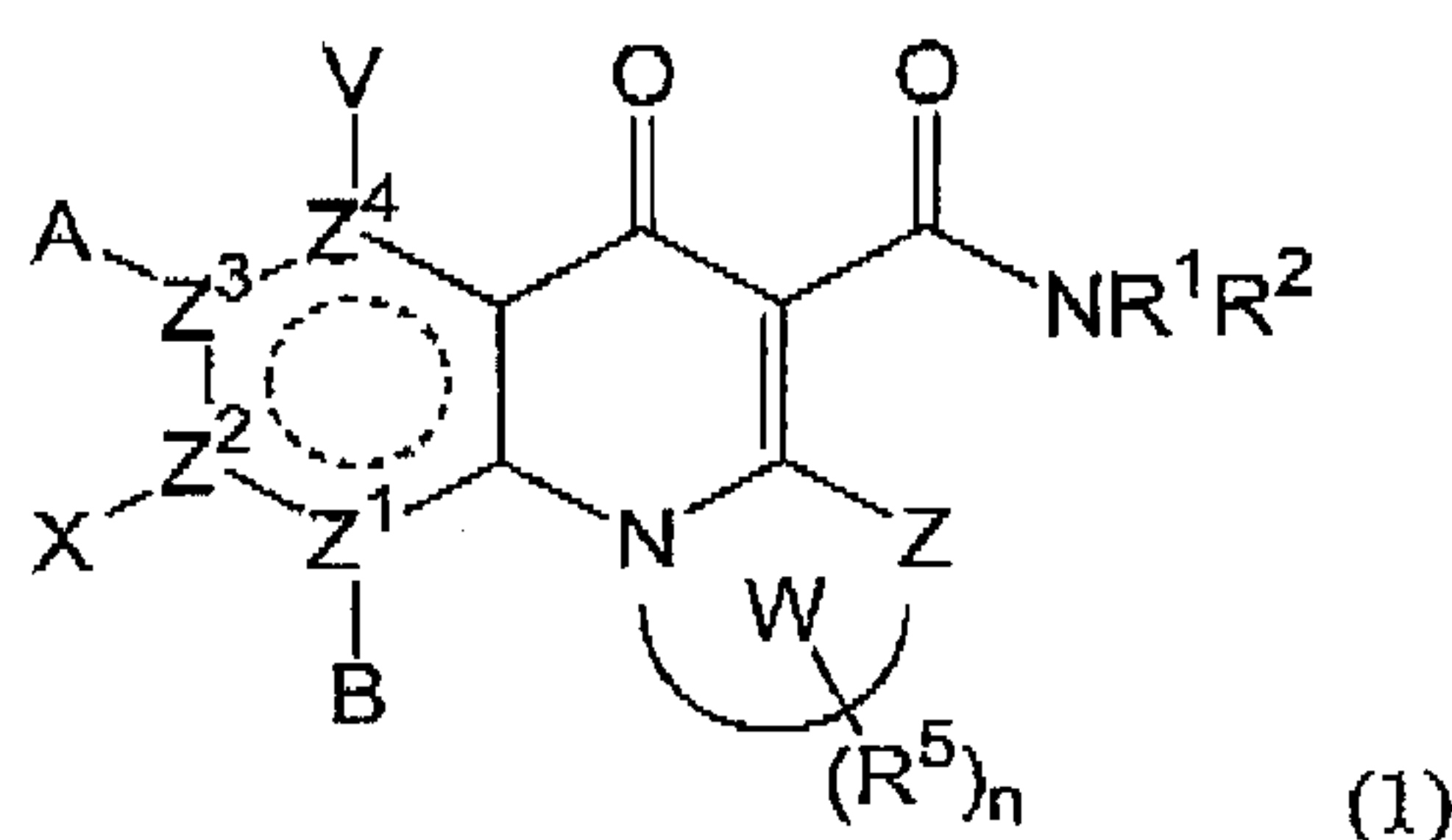
[0004H] Various embodiments of this invention provide an *in vitro* method for reducing cell proliferation comprising administering to a system an effective amount of a compound or composition of this invention and optionally with a procedure and/or a chemotherapeutic agent, thereby reducing cell proliferation in the system.

[0004I] Various embodiments of this invention provide an *in vitro* method for reducing microbial titers and/or ameliorating a microbial infection, comprising contacting a system with an effective amount of a compound or composition of this invention and optionally with an antimicrobial agent, thereby reducing microbial titers and/or ameliorating said microbial infection.

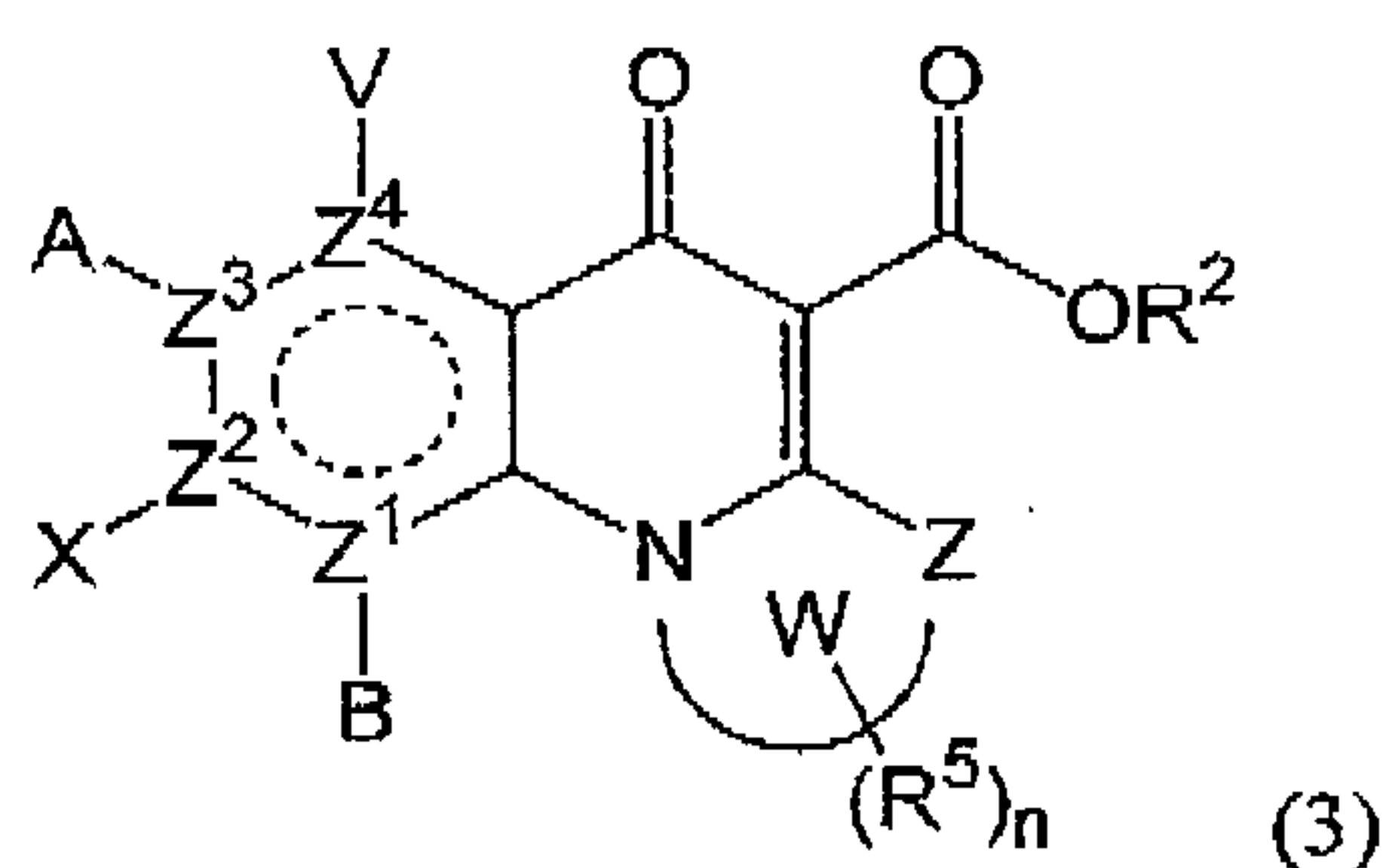
[0004J] Various embodiments of this invention provide an *in vitro* method for inducing cell death and/or inducing apoptosis, comprising administering to a system an effective amount of a compound or composition of this invention and optionally with a

procedure and/or a chemotherapeutic agent, thereby inducing cell death and/or inducing apoptosis.

[0004K] Various embodiments of this invention provide a method for preparing compounds having formula (1),



comprising contacting an ester having formula 3, an amine with formula  $\text{NHR}^1\text{R}^2$ , and a Lewis acid having formula  $\text{ML}_n$ , wherein L is a halogen atom or an organic radical, n' is 3-5, and M is a group III elemental atom, a group IV elemental atom, As, Sb, V or Fe,



wherein A, V, and X are independently H, halo, azido,  $\text{R}^2$ ,  $\text{CH}_2\text{R}^2$ ,  $\text{SR}^2$ ,  $\text{OR}^2$  or  $\text{NR}^1\text{R}^2$ ; or

wherein A and X, or A and V may form a carbocyclic ring, heterocyclic ring, aryl or heteroaryl, each of which may be optionally substituted and/or fused with a cyclic ring;

wherein in  $\text{NR}^1\text{R}^2$ ,  $\text{R}^1$  and  $\text{R}^2$  may form an optionally substituted ring;

each Z is  $\text{CH}_2$ , O, S, or  $\text{NR}^1$ ;

$\text{Z}^1$ ,  $\text{Z}^2$ ,  $\text{Z}^3$ , and  $\text{Z}^4$  are independently C or N, provided any two N are non-adjacent;

each  $\text{R}^1$  is H or a  $\text{C}_{1-6}$  alkyl;

each  $\text{R}^2$  is H or a  $\text{C}_{1-10}$  alkyl or  $\text{C}_{2-10}$  alkenyl optionally containing one or more non-adjacent heteroatoms selected from N, O, and S, and optionally substituted with a

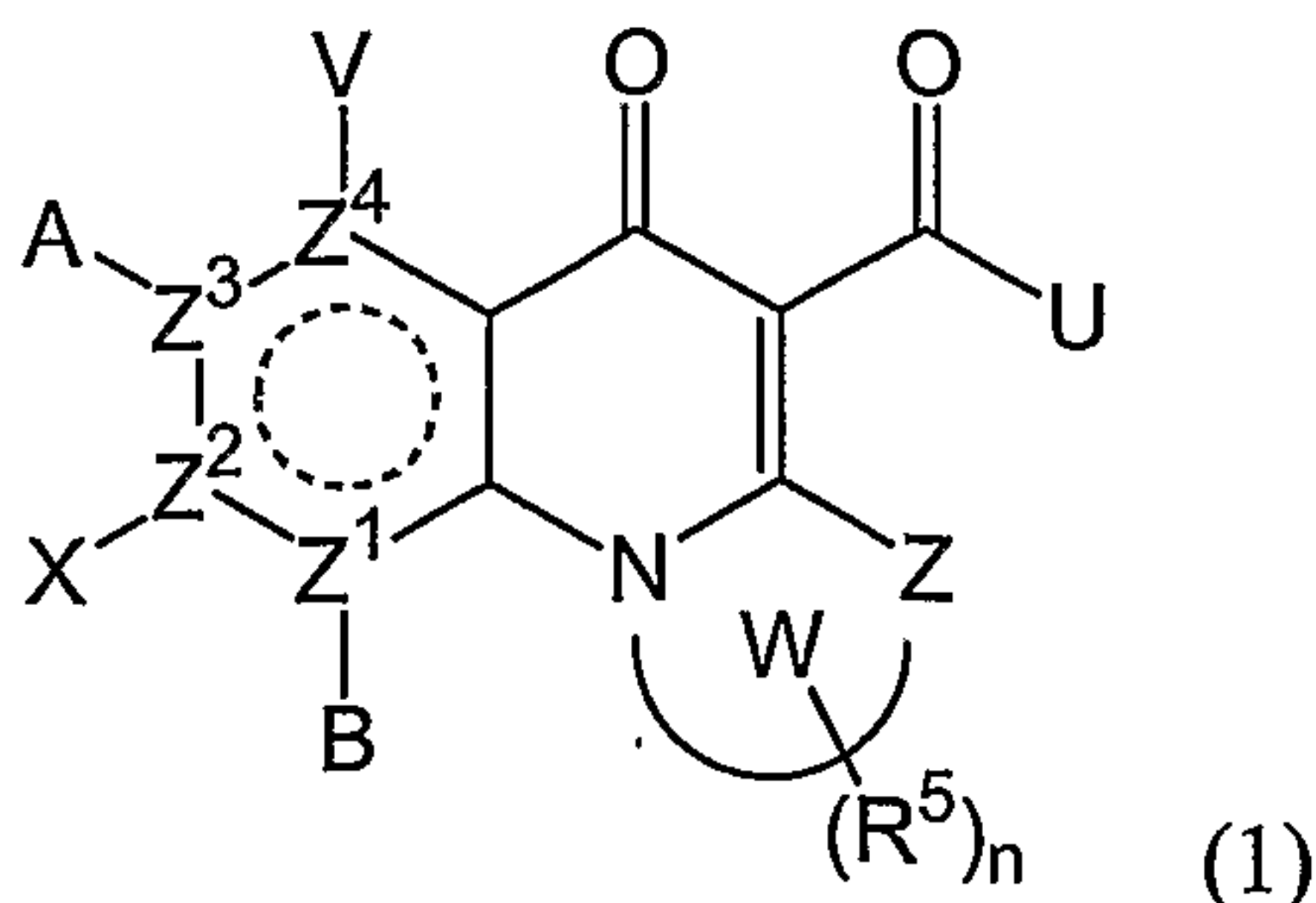
carbocyclic or heterocyclic ring; or  $R^2$  is an optionally substituted carbocyclic ring, heterocyclic ring, aryl or heteroaryl;

each B is H or halo;

W together with N and Z forms an optionally substituted 5- or 6-membered ring that is fused to an optionally substituted aryl or heteroaryl, wherein said aryl or heteroaryl is monocyclic or fused with a single or multiple ring which optionally contains a heteroatom;

and each  $R^5$  is a substituent at any position on W; and is H,  $OR^2$ ,  $NR^1R^2$ ,  $C_{1-6}$  alkyl,  $C_{2-6}$  alkenyl, each optionally substituted by halo, C=O or one or more heteroatoms; or  $R^5$  is an inorganic substituent; or two adjacent  $R^5$  is linked to obtain a 5-6 membered substituted or unsubstituted carbocyclic or heterocyclic ring, optionally fused to an additional substituted or unsubstituted carbocyclic or heterocyclic ring; and n is 1-6.

[0005] In one aspect, the present invention provides compounds having the general formula:



and pharmaceutically acceptable salts, esters and prodrugs thereof;

wherein B, X, A, or V is absent if  $Z^2$ ,  $Z^3$ , or  $Z^4$ , respectively, is N, and independently H, halo, azido,  $R^2$ ,  $CH_2R^2$ ,  $SR^2$ ,  $OR^2$  or  $NR^1R^2$  if  $Z^2$ ,  $Z^3$ , or  $Z^4$ , respectively, is C; or

A and V, A and X, or X and B may form a carbocyclic ring, heterocyclic ring, aryl or heteroaryl, each of which may be optionally substituted and/or fused with a cyclic ring;

Z is O, S,  $NR^1$ ,  $CH_2$ , or  $C=O$ ;

$Z^1$ ,  $Z^2$ ,  $Z^3$  and  $Z^4$  are C or N, provided any two N are non-adjacent;

W together with N and Z forms an optionally substituted 5- or 6-membered ring that is fused to an optionally substituted saturated or unsaturated ring; said saturated or unsaturated ring may contain a heteroatom and is monocyclic or fused with a single or multiple carbocyclic or heterocyclic rings;

U is  $R^2$ ,  $OR^2$ ,  $NR^1R^2$ ,  $NR^1 - (CR^1_2)_n - NR^3R^4$ , or  $N=CR^1R^2$ , wherein in  $N=CR^1R^2$   $R^1$  and  $R^2$  together with C may form a ring,

provided U is not H, and when U is OH,  $OR^2$  or  $NH_2$ , then at least one of  $Z^1$ - $Z^4$  is N;

in each  $NR^1R^2$ ,  $R^1$  and  $R^2$  together with N may form an optionally substituted ring;

in  $NR^3R^4$ ,  $R^3$  and  $R^4$  together with N may form an optionally substituted ring;

$R^1$  and  $R^3$  are independently H or  $C_{1-6}$  alkyl;

each  $R^2$  is H, or a  $C_{1-10}$  alkyl or  $C_{2-10}$  alkenyl each optionally substituted with a halogen, one or more non-adjacent heteroatoms, a carbocyclic ring; a heterocyclic ring, an aryl or heteroaryl, wherein each ring is optionally substituted; or  $R^2$  is an optionally substituted carbocyclic ring, heterocyclic ring, aryl or heteroaryl;

$R^4$  is H, a  $C_{1-10}$  alkyl or  $C_{2-10}$  alkenyl optionally containing one or more non-adjacent heteroatoms selected from N, O and S, and optionally substituted with a carbocyclic or heterocyclic ring; or  $R^3$  and  $R^4$  together with N may form an optionally substituted ring;

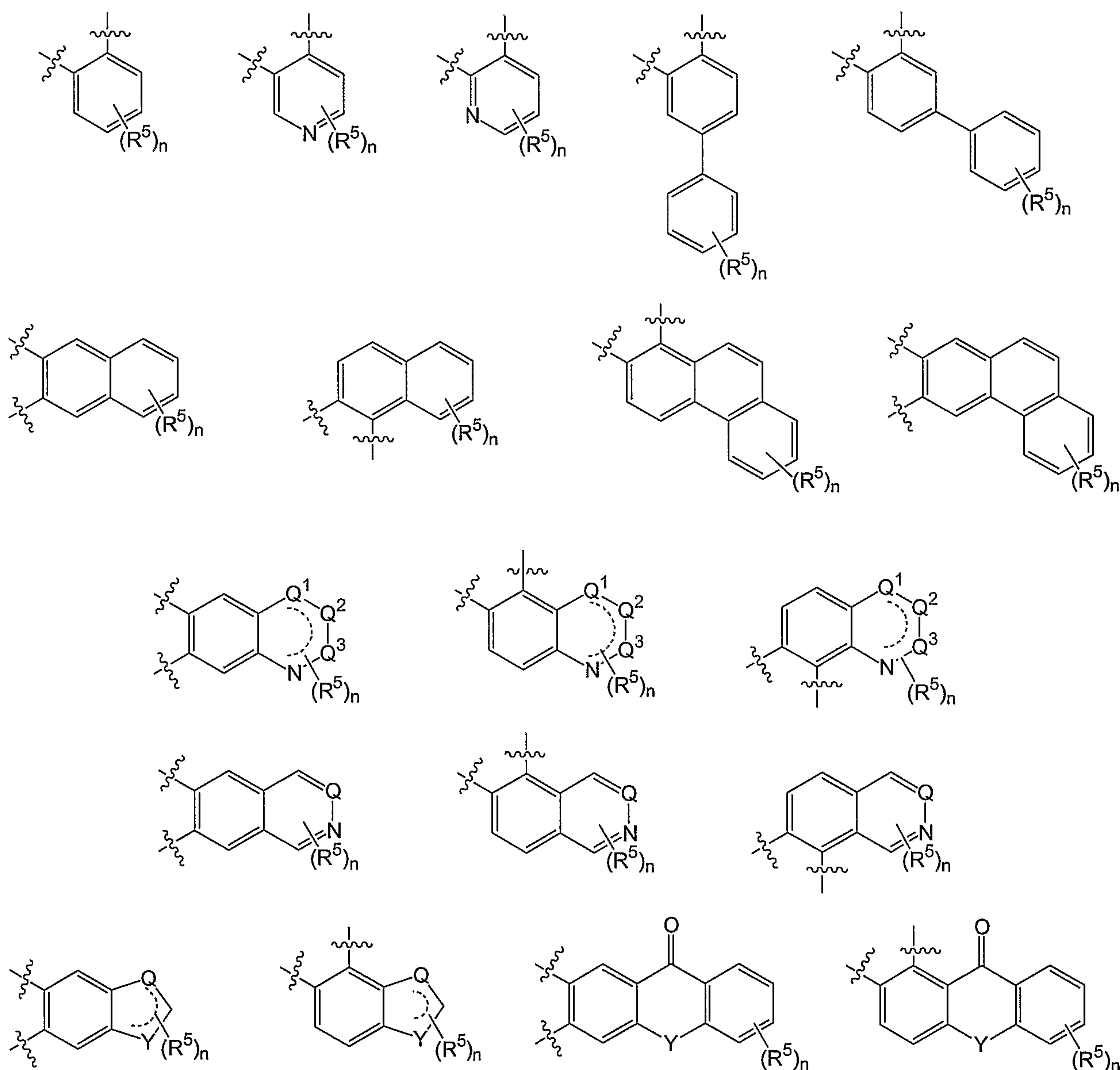
each  $R^5$  is a substituent at any position on ring W; and is H,  $OR^2$ , amino, alkoxy, amido, halogen, cyano or an inorganic substituent; or  $R^5$  is  $C_{1-6}$  alkyl,  $C_{2-6}$  alkenyl,  $C_{2-6}$  alkynyl,  $-CONHR^1$ , each optionally substituted by halo, carbonyl or one or more non-adjacent

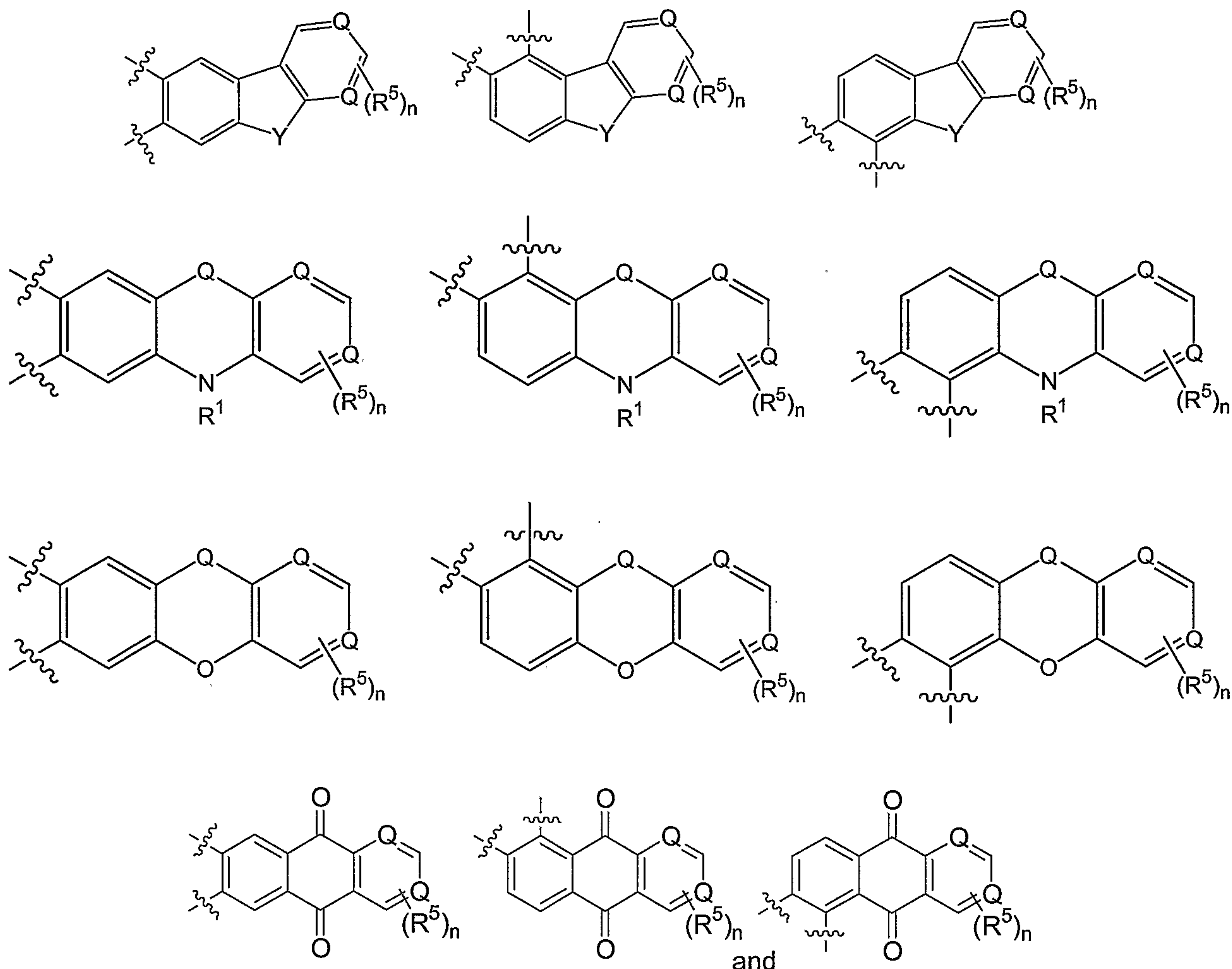
heteroatoms; or two adjacent  $R^5$  are linked to obtain a 5-6 membered optionally substituted carbocyclic or heterocyclic ring that may be fused to an additional optionally substituted carbocyclic or heterocyclic ring; and

$n$  is 1-6.

[0006] In the above formula (1), B may be absent when  $Z^1$  is N, or is H or a halogen when  $Z^1$  is C.

[0007] In the above formula (1), W together with N and Z forms an optionally substituted 5- or 6-membered ring that is fused to an optionally substituted aryl or heteroaryl selected from the group consisting of:



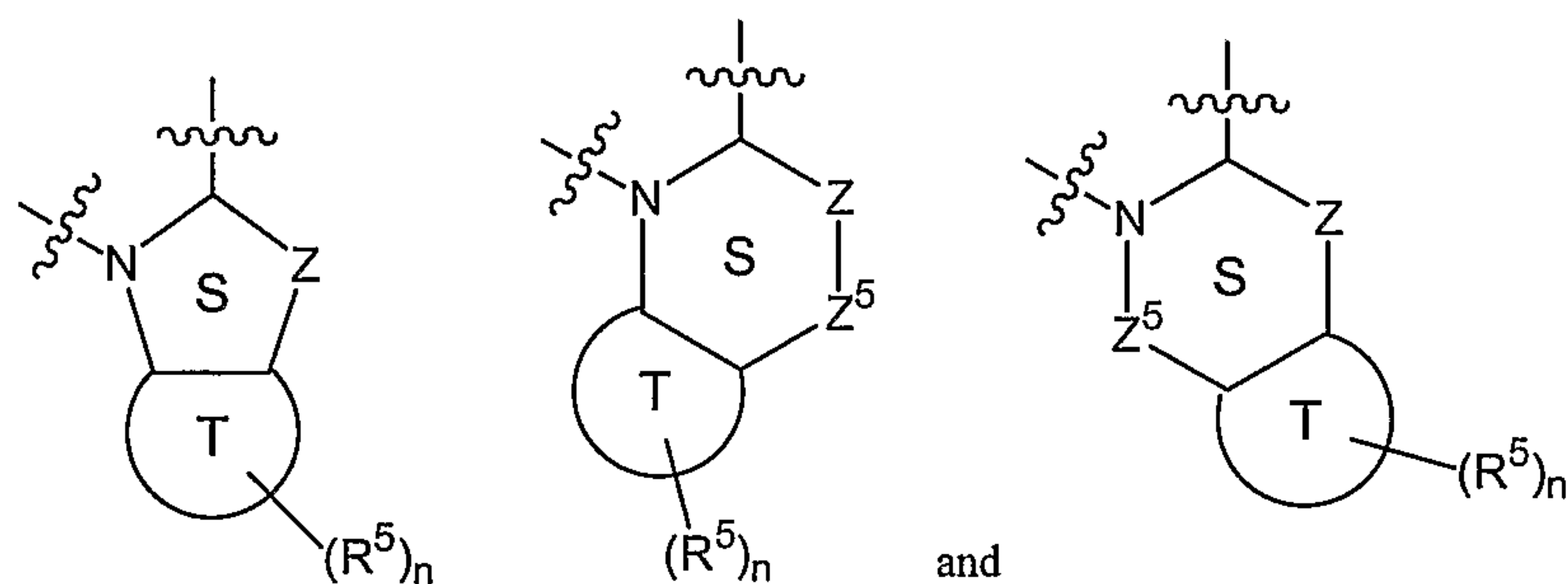


wherein each Q, Q<sup>1</sup>, Q<sup>2</sup>, and Q<sup>3</sup> is independently CH or N;

Y is independently O, CH, C=O or NR<sup>1</sup>;

n and R<sup>5</sup> is as defined above.

**[0008]** In other embodiments, W together with N and Z form a group having the formula selected from the group consisting of



wherein Z is O, S, CR<sup>1</sup>, NR<sup>1</sup>, or C=O;

each Z<sup>5</sup> is CR<sup>6</sup>, NR<sup>1</sup>, or C=O, provided Z and Z<sup>5</sup> if adjacent are not both NR<sup>1</sup>;

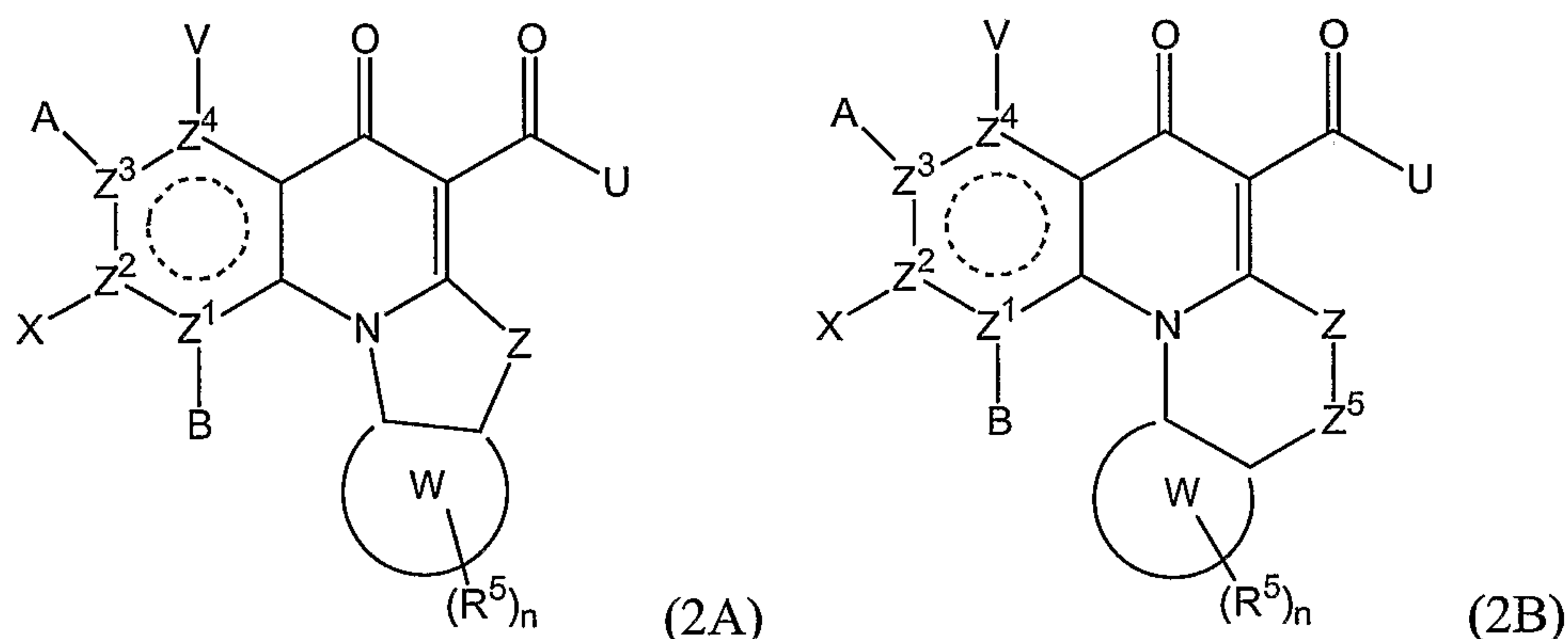
each R<sup>1</sup> is H, C<sub>1-6</sub> alkyl, COR<sup>2</sup> or S(O)<sub>p</sub>R<sup>2</sup> wherein p is 1-2;

$R^6$  is H, or a substituent known in the art, including but not limited to hydroxyl, alkyl, alkoxy, halo, amino, or amido; and

ring S and ring T may be saturated or unsaturated.

[0009] In some embodiments, W together with N and Z forms a 5- or 6-membered ring that is fused to a phenyl. In other embodiments, W together with N and Z forms a 5- or 6-membered ring that is optionally fused to another ring, when U is  $NR^1R^2$ , provided U is not  $NH_2$ . In certain embodiments, W together with N and Z forms a 5- or 6-membered ring that is not fused to another ring, when U is  $NR^1R^2$  (e.g.,  $NH_2$ ).

[0010] In yet another embodiment, the compounds of the present invention have the general formula (2A) or (2B):



wherein A, B, V, X, U, Z,  $Z^1$ ,  $Z^2$ ,  $Z^3$ ,  $Z^4$  and n are as described above;

$Z^5$  is O,  $NR^1$ ,  $CR^6$ , or  $C=O$ ;

$R^6$  is H,  $C_{1-6}$  alkyl, hydroxyl, alkoxy, halo, amino or amido; and

Z and  $Z^5$  may optionally form a double bond.

[0011] In the above formula (1), (2A) and (2B), U may be  $NR^1R^2$ , wherein  $R^1$  is H, and  $R^2$  is a  $C_{1-10}$  alkyl optionally substituted with a heteroatom, a  $C_{3-6}$  cycloalkyl, aryl or a 5-14 membered heterocyclic ring containing one or more N, O or S. For example,  $R^2$  may be a  $C_{1-10}$  alkyl substituted with an optionally substituted morpholine, thiomorpholine, imidazole, aminodithiadazole, pyrrolidine, piperazine, pyridine or piperidine. In other examples,  $R^1$  and  $R^2$  together with N form an optionally substituted piperidine, pyrrolidine, piperazine, morpholine, thiomorpholine, imidazole, or aminodithiazole.

[0012] In other embodiments, U is  $NR^1 - (CR^1_2)_n - NR^3R^4$ ; n is 1-4; and  $R^3$  and  $R^4$  in  $NR^3R^4$  together form an optionally substituted piperidine, pyrrolidine, piperazine, morpholine, thiomorpholine, imidazole, or aminodithiazole. In some examples, U is  $NH-(CH_2)_n-NR^3R^4$

wherein  $R^3$  and  $R^4$  together with N form an optionally substituted pyrrolidine, which may be linked to  $(CH_2)_n$  at any position in the pyrrolidine ring. In one embodiment,  $R^3$  and  $R^4$  together with N form an N-methyl substituted pyrrolidine. In some embodiments, U is 2-(1-methylpyrrolidin-2-yl)ethylamino or (2-pyrrolidin-1-yl)ethanamino.

**[0013]** In the above formula (1), (2A) and (2B), Z may be S or  $NR^1$ . In some embodiments, at least one of B, X, or A is halo and  $Z^1$ ,  $Z^2$ , and  $Z^3$  are C. In other embodiments, X and A are not each H when  $Z^2$  and  $Z^3$  are C. In the above formula (1), (2A) and (2B), V may be H. In particular embodiments, U is not OH.

**[0014]** In one embodiment, each of  $Z^1$ ,  $Z^2$ ,  $Z^3$  and  $Z^4$  are C. In another embodiment, three of  $Z^1$ ,  $Z^2$ ,  $Z^3$  and  $Z^4$  is C, and the other is N. For example,  $Z^1$ ,  $Z^2$  and  $Z^3$  are C, and  $Z^4$  is N. Alternatively,  $Z^1$ ,  $Z^2$  and  $Z^4$  are C, and  $Z^3$  is N. In other examples,  $Z^1$ ,  $Z^3$  and  $Z^4$  are C and  $Z^2$  is N. In yet other examples,  $Z^2$ ,  $Z^3$  and  $Z^4$  are C, and  $Z^1$  is N.

**[0015]** In another embodiment, two of  $Z^1$ ,  $Z^2$ ,  $Z^3$  and  $Z^4$  are C, and the other two are non-adjacent nitrogens. For example,  $Z^1$  and  $Z^3$  may be C, and  $Z^2$  and  $Z^4$  are N. Alternatively,  $Z^1$  and  $Z^3$  may be N, and  $Z^2$  and  $Z^4$  may be C. In other examples,  $Z^1$  and  $Z^4$  are N, and  $Z^2$  and  $Z^3$  are C. In particular examples, W together with N and Z forms a 5- or 6-membered ring that is fused to a phenyl.

**[0016]** In some embodiments, each of B, X, A, and V is H and  $Z^1$ - $Z^4$  are C. In many embodiments, at least one of B, X, A, and V is H and the corresponding adjacent  $Z^1$ - $Z^4$  atom is C. For example, any two of B, X, A, and V may be H. In one example, V and B may both be H. In other examples, any three of B, X, A, and V are H and the corresponding adjacent  $Z^1$ - $Z^4$  atom is C.

**[0017]** In certain embodiments, one of B, X, A, and V is a halogen (e.g., fluorine) and the corresponding adjacent  $Z^1$ - $Z^4$  is C. In other embodiments, two of X, A, and V are halogen or  $SR^2$ , wherein  $R^2$  is a  $C_{0-10}$  alkyl or  $C_{2-10}$  alkenyl optionally substituted with a heteroatom, a carbocyclic ring, a heterocyclic ring, an aryl or a heteroaryl; and the corresponding adjacent  $Z^2$ - $Z^4$  is C. For example, each X and A may be a halogen. In other examples, each X and A if present may be  $SR^2$ , wherein  $R^2$  is a  $C_{0-10}$  alkyl substituted with phenyl or pyrazine. In yet other examples, V, A and X may be alkynyls, fluorinated alkyls such as  $CF_3$ ,  $CH_2CF_3$ , perfluorinated alkyls, etc.; cyano, nitro, amides, sulfonyl amides, or carbonyl compounds such as  $COR^2$ .

**[0018]** In each of the above formulas, U, and X, V, and A if present may independently be  $NR^1R^2$ , wherein  $R^1$  is H, and  $R^2$  is a  $C_{1-10}$  alkyl optionally substituted with a heteroatom, a  $C_{3-6}$

cycloalkyl, aryl or a 5-14 membered heterocyclic ring containing one or more N, O or S. If more than one  $\text{NR}^1\text{R}^2$  moiety is present in a compound within the invention, as when both A and U are  $\text{NR}^1\text{R}^2$  in a compound according to any one of the above formula, each  $\text{R}^1$  and each  $\text{R}^2$  is independently selected. In one example,  $\text{R}^2$  is a  $\text{C}_{1-10}$  alkyl substituted with an optionally substituted 5-14 membered heterocyclic ring. For example,  $\text{R}^2$  may be a  $\text{C}_{1-10}$  alkyl substituted with morpholine, thiomorpholine, imidazole, aminodithiadazole, pyrrolidine, piperazine, pyridine or piperidine. Alternatively,  $\text{R}^1$  and  $\text{R}^2$  together with N may form an optionally substituted heterocyclic ring containing one or more N, O or S. For example,  $\text{R}^1$  and  $\text{R}^2$  together with N may form piperidine, pyrrolidine, piperazine, morpholine, thiomorpholine, imidazole, or aminodithiazole.

[0019] Illustrative examples of optionally substituted heterocyclic rings include but are not limited to tetrahydrofuran, 1,3-dioxolane, 2,3-dihydrofuran, tetrahydropyran, benzofuran, isobenzofuran, 1,3-dihydro-isobenzofuran, isoxazole, 4,5-dihydroisoxazole, piperidine, pyrrolidine, pyrrolidin-2-one, pyrrole, pyridine, pyrimidine, octahydro-pyrrolo[3,4-*b*]pyridine, piperazine, pyrazine, morpholine, thiomorpholine, imidazole, aminodithiadazole, imidazolidine-2,4-dione, benzimidazole, 1,3-dihydrobenzimidazol-2-one, indole, thiazole, benzothiazole, thiadiazole, thiophene, tetrahydro-thiophene 1,1-dioxide, diazepine, triazole, diazabicyclo[2.2.1]heptane, 2,5-diazabicyclo[2.2.1]heptane, and 2,3,4,4a,9,9a-hexahydro-1H- $\beta$ -carboline.

[0020] In one embodiment, the present invention provides compounds having formula (1), (2A) or (2B), wherein:

each of A, V and B if present is independently H or halogen (e.g., chloro or fluoro);

X is  $-(\text{R}^5)\text{R}^1\text{R}^2$ , wherein  $\text{R}^5$  is C or N and wherein in each  $-(\text{R}^5)\text{R}^1\text{R}^2$ ,  $\text{R}^1$  and  $\text{R}^2$  together may form an optionally substituted aryl or heteroaryl ring;

Z is NH or N-alkyl (e.g., N- $\text{CH}_3$ );

W together with N and Z forms an optionally substituted 5- or 6-membered ring that is fused with an optionally substituted aryl or heteroaryl ring; and

U is  $-\text{R}^5\text{R}^6-(\text{CH}_2)_n-\text{CHR}^2-\text{NR}^3\text{R}^4$ , wherein  $\text{R}^6$  is H or  $\text{C}_{1-10}$  alkyl and wherein in the  $-\text{CHR}^2-\text{NR}^3\text{R}^4$  moiety each  $\text{R}^3$  or  $\text{R}^4$  together with the C may form an optionally substituted heterocyclic or heteroaryl ring, or wherein in the  $-\text{CHR}^2-\text{NR}^3\text{R}^4$  moiety each  $\text{R}^3$  or  $\text{R}^4$  together with the N may form an optionally substituted carbocyclic, heterocyclic, aryl or heteroaryl ring.

**[0021]** In another embodiment, the present invention provides compounds having formula (1), (2A) or (2B), wherein:

A if present is H or halogen (*e.g.*, chloro or fluoro);

X if present is  $-(R^5)R^1R^2$ , wherein  $R^5$  is C or N and wherein in each  $-(R^5)R^1R^2$ ,  $R^1$  and  $R^2$  together may form an optionally substituted aryl or heteroaryl ring;

Z is NH or N-alkyl (*e.g.*, N-CH<sub>3</sub>);

W together with N and Z forms an optionally substituted 5- or 6-membered ring that is fused with an optionally substituted aryl or heteroaryl ring; and

U is  $-R^5R^6-(CH_2)_n-CHR^2-NR^3R^4$ , wherein  $R^6$  is H or alkyl and wherein in the  $-CHR^2-NR^3R^4$  moiety each  $R^3$  or  $R^4$  together with the C may form an optionally substituted heterocyclic or heteroaryl ring, or wherein in the  $-CHR^2-NR^3R^4$  moiety each  $R^3$  or  $R^4$  together with the N may form an optionally substituted carbocyclic, heterocyclic, aryl or heteroaryl ring.

**[0022]** In each of the above formula, each optionally substituted moiety may be substituted with one or more halo,  $OR^2$ ,  $NR^1R^2$ , carbamate, C<sub>1-10</sub> alkyl, C<sub>2-10</sub> alkenyl, each optionally substituted by halo, C=O, aryl or one or more heteroatoms; inorganic substituents, aryl, carbocyclic or a heterocyclic ring. Other substituents include but are not limited to alkynyl, cycloalkyl, fluorinated alkyls such as CF<sub>3</sub>, CH<sub>2</sub>CF<sub>3</sub>, perfluorinated alkyls, etc.; oxygenated fluorinated alkyls such as OCF<sub>3</sub> or CH<sub>2</sub>CF<sub>3</sub>, etc.; cyano, nitro,  $COR^2$ ,  $NR^2COR^2$ , sulfonyl amides;  $NR^2SOOR^2$ ;  $SR^2$ ,  $SOR^2$ ,  $COOR^2$ ,  $CONR^2_2$ ,  $OCOR^2$ ,  $OCOOR^2$ ,  $CONR^2_2$ ,  $NR^2COOR^2$ ,  $NR^2CONR^2_2$ ,  $NRC(NR)(NR^2_2)$ ,  $NR(CO)NR^2_2$ , and  $SOONR^2_2$ , wherein each  $R^2$  is as defined in formula 1.

**[0023]** The present invention also provides pharmaceutical compositions comprising a compound having any one of the above formula, and a pharmaceutically acceptable excipient. In one example, the composition comprises a compound having any one of the above formula, polyethylene glycol, and propylene glycol in a buffer solution.

**[0024]** Furthermore, the present invention relates to methods for reducing cell proliferation and/or inducing cell death, comprising contacting a system with an effective amount of a compound having any one of the above formula, or a pharmaceutical composition thereof and optionally in combination with a chemotherapeutic agent, thereby reducing cell proliferation and/or inducing cell death, such as apoptosis or apoptotic cell death, in said system. The system may be a cell or a tissue. In one embodiment, the system includes a pancreatic cell, such as a cell from a subject or a cultured cell (*e.g.*, *in vitro* or *ex vivo*). In particular

embodiments, the system includes a pancreatic cancer cell. In one embodiment, the system is a cell line such as PC3, HCT116, HT29, MIA Paca-2, HPAC, Hs700T, Panc10.05, Panc 02.13, PL45, SW 190, Hs 766T, CFPAC-1 and PANC-1.

**[0025]** The present invention also provides methods for ameliorating a cell proliferative disorder, comprising administering to a subject in need thereof an effective amount of a compound having any one of the above formula, or a pharmaceutical composition thereof and optionally in combination with a chemotherapeutic agent, thereby ameliorating said cell-proliferative disorder. For example, cell proliferation may be reduced, and/or cell death, such as apoptosis or apoptotic cell death, may be induced. The cell proliferative disorder may be a tumor or a cancer in a human or animal subject. In a particular embodiment, the cancer is pancreatic cancer, including non-endocrine and endocrine tumors. Illustrative examples of non-endocrine tumors include but are not limited to adenocarcinomas, acinar cell carcinomas, adenosquamous carcinomas, giant cell tumors, intraductal papillary mucinous neoplasms, mucinous cystadenocarcinomas, pancreatoblastomas, serous cystadenomas, solid and pseudopapillary tumors. An endocrine tumor may be an islet cell tumor.

**[0026]** The above methods for reducing cell proliferation and/or inducing cell death may also be practiced in combination with a procedure and/or a chemotherapeutic agent. Examples of procedures that may be used in combination with the methods of the present invention include but are not limited to radiotherapy or surgery. In certain embodiments, the compounds of the present invention are administered in combination with gemcitabine, and used to reduce cell proliferation, induce cell death, and/or ameliorate a cell proliferative disorder.

**[0027]** Furthermore, the present invention provides methods for reducing microbial titers, comprising contacting a system with an effective amount of a compound having any one of the above formula, or a pharmaceutical composition thereof and optionally with an antimicrobial agent, thereby reducing microbial titers. The system may be a cell or a tissue. The present invention also provides methods for ameliorating a microbial infection, comprising administering to a subject in need thereof an effective amount of a compound having any one of the above formula, or a pharmaceutical composition thereof and optionally with an antimicrobial agent, thereby ameliorating said microbial infection. The subject may be human or an animal. The microbial titers may be viral, bacterial or fungal titers.

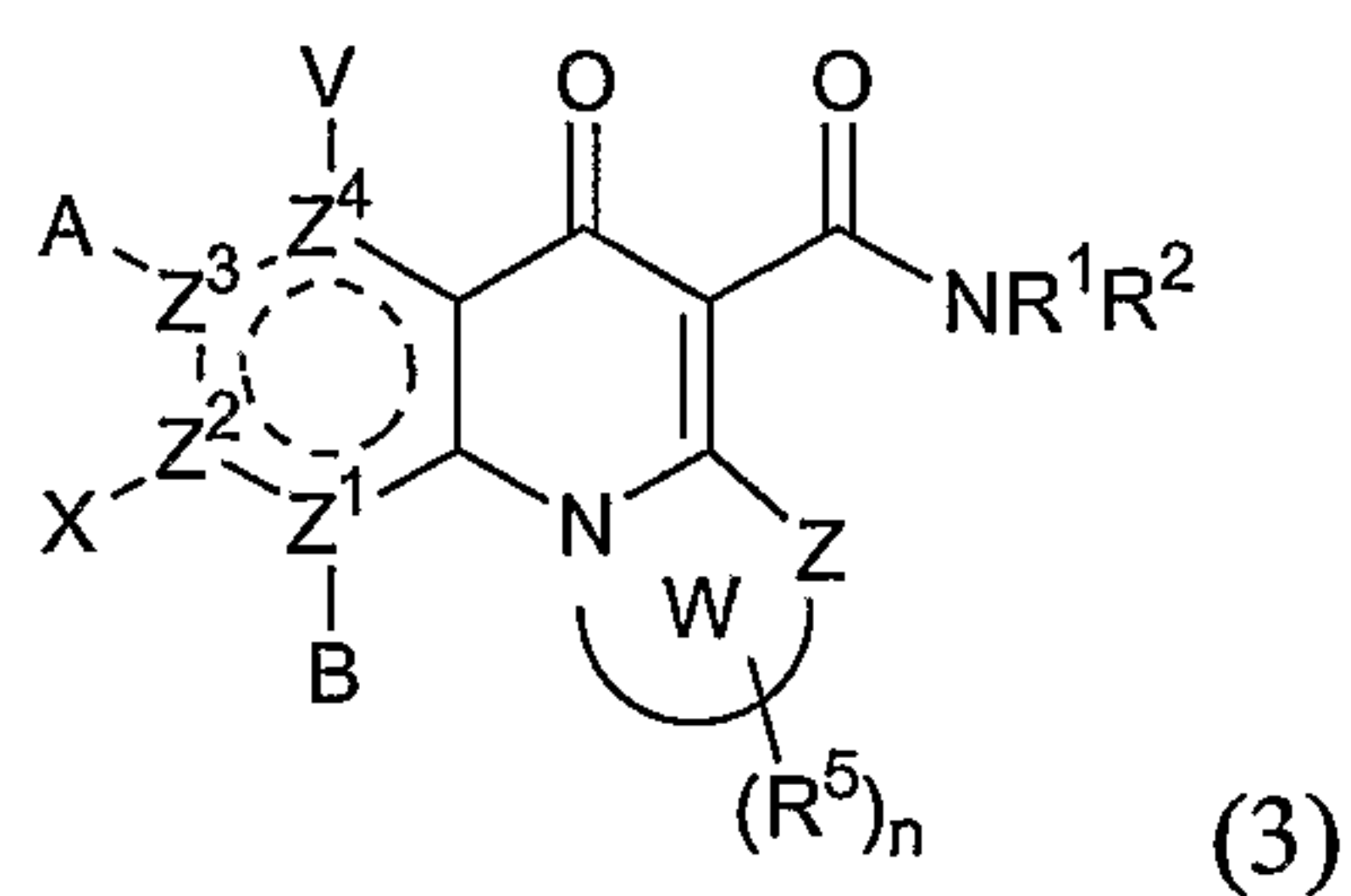
**[0028]** The present invention also relates to methods for determining interaction selectivity between a compound having any one of the above formula, and nucleic acids capable of forming a quadruplex structure, comprising: a) contacting a compound in the absence of a

competitor molecule with three or more nucleic acids capable of forming a quadruplex structure, wherein each nucleic acid is not a telomere nucleic acid; b) measuring a direct interaction between the compound and said three or more nucleic acids; and c) determining interaction selectivity from a comparison of the interaction measurements. In one example, three or more nucleic acids comprise a nucleotide sequence located 5' of an oncogene nucleotide sequence. The oncogene may be MYC, HIF, VEGF, ABL, TGF, PDGF $\alpha$ , MYB, SPARC, HER, VAV, RET, H-RAS, EGF, SRC, BCL-1, BCL-2, DHFR, or HMGA. In determining interaction selectivity, the compound may be separately contacted with each of said three or more nucleic acids in a different vessel. Furthermore, the interaction selectivity may be determined from a comparison of IC<sub>50</sub> values.

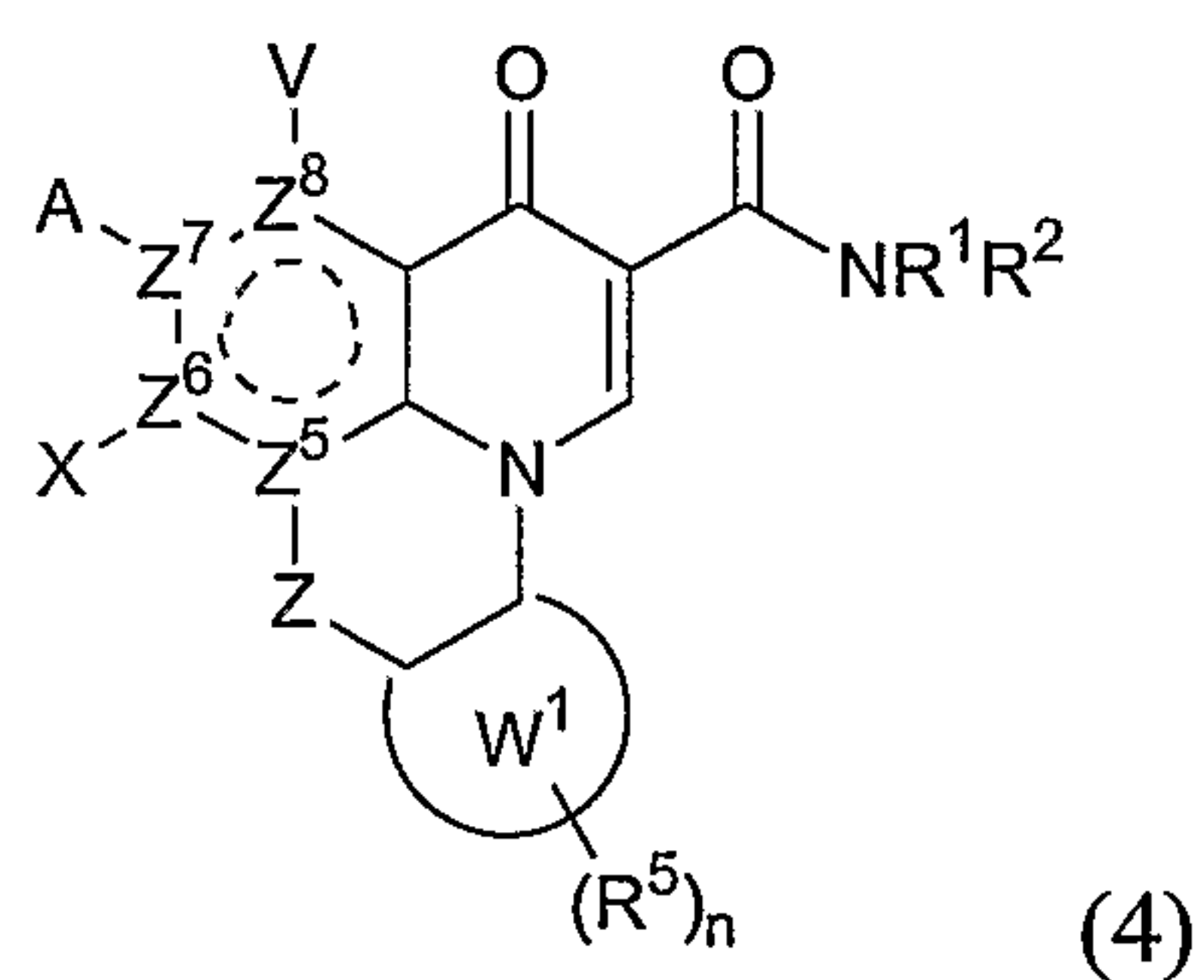
[0029] The compounds of the present invention may or may not interact with regions of DNA that can form quadruplexes. In certain embodiments, the compounds of the present invention may bind and/or stabilize a propeller quadruplex. Examples of propeller quadruplexes include but are not limited to H-RAS, RET, BCL-1, DHFR, TGF- $\beta$ , HIF-1 $\alpha$ , VEGF, c-Myc, or PDGF $\alpha$ . In another embodiment, the compound may bind and/or stabilize a chair-ell or a basket quadruplex. For example, the compound may bind and/or stabilize BCL-2.

[0030] The present invention also provides methods for inducing cell death, such as apoptotic cell death (apoptosis), comprising administering to a system or a subject in need thereof an effective amount of a compound having any one of the above formula, or a pharmaceutical composition thereof and optionally with a chemotherapeutic agent. The present invention also provides methods for treating or ameliorating a disorder mediated by oncogene overexpression, such as c-Myc overexpression, comprising administering to a system or a subject in need thereof an effective amount of a compound having any of the formula, or a pharmaceutical composition thereof and optionally with a chemotherapeutic agent. The subject may be human or an animal, and system may be a cell or a tissue.

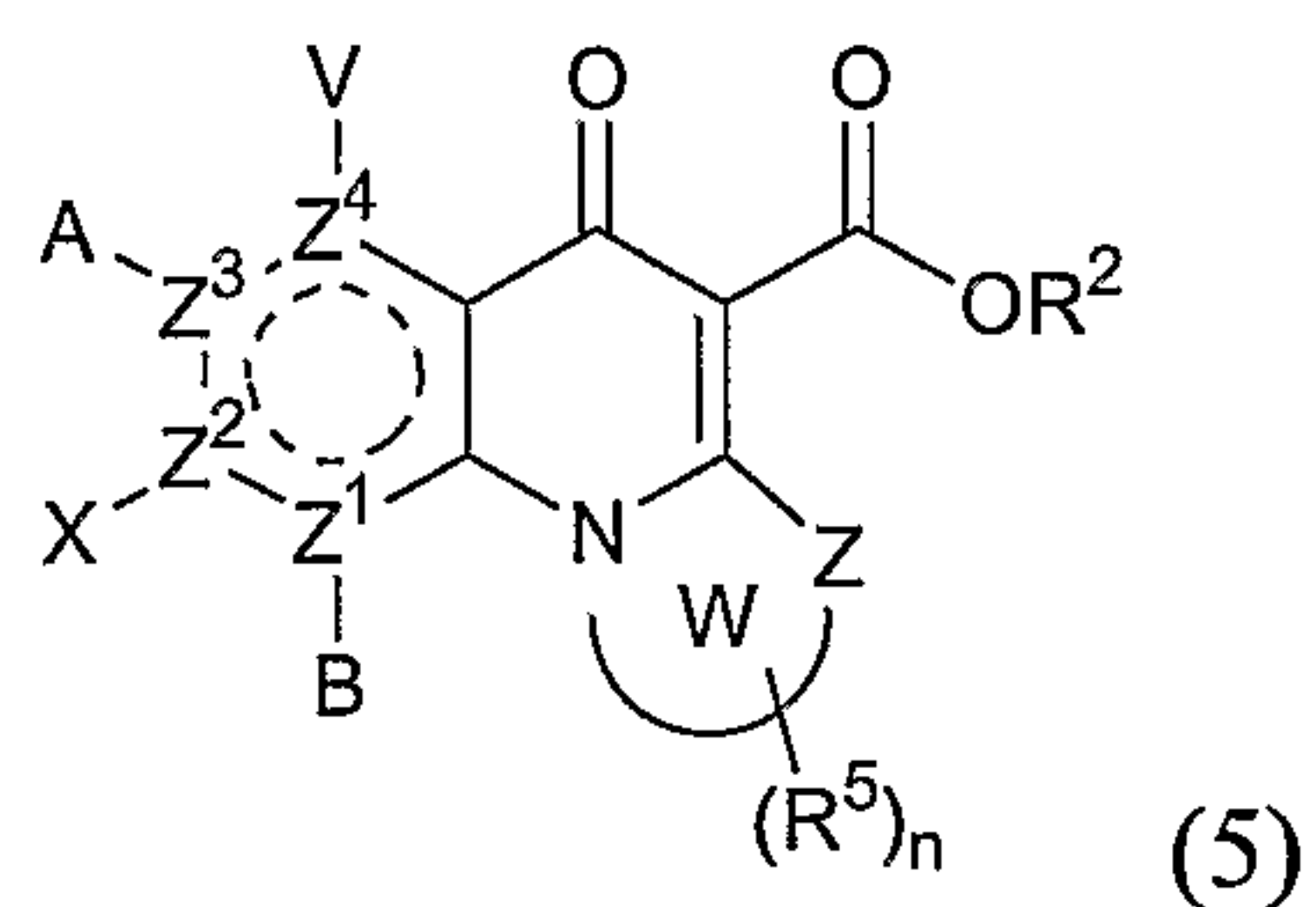
[0031] In another aspect, the present invention provides methods for preparing compounds having formula (3)



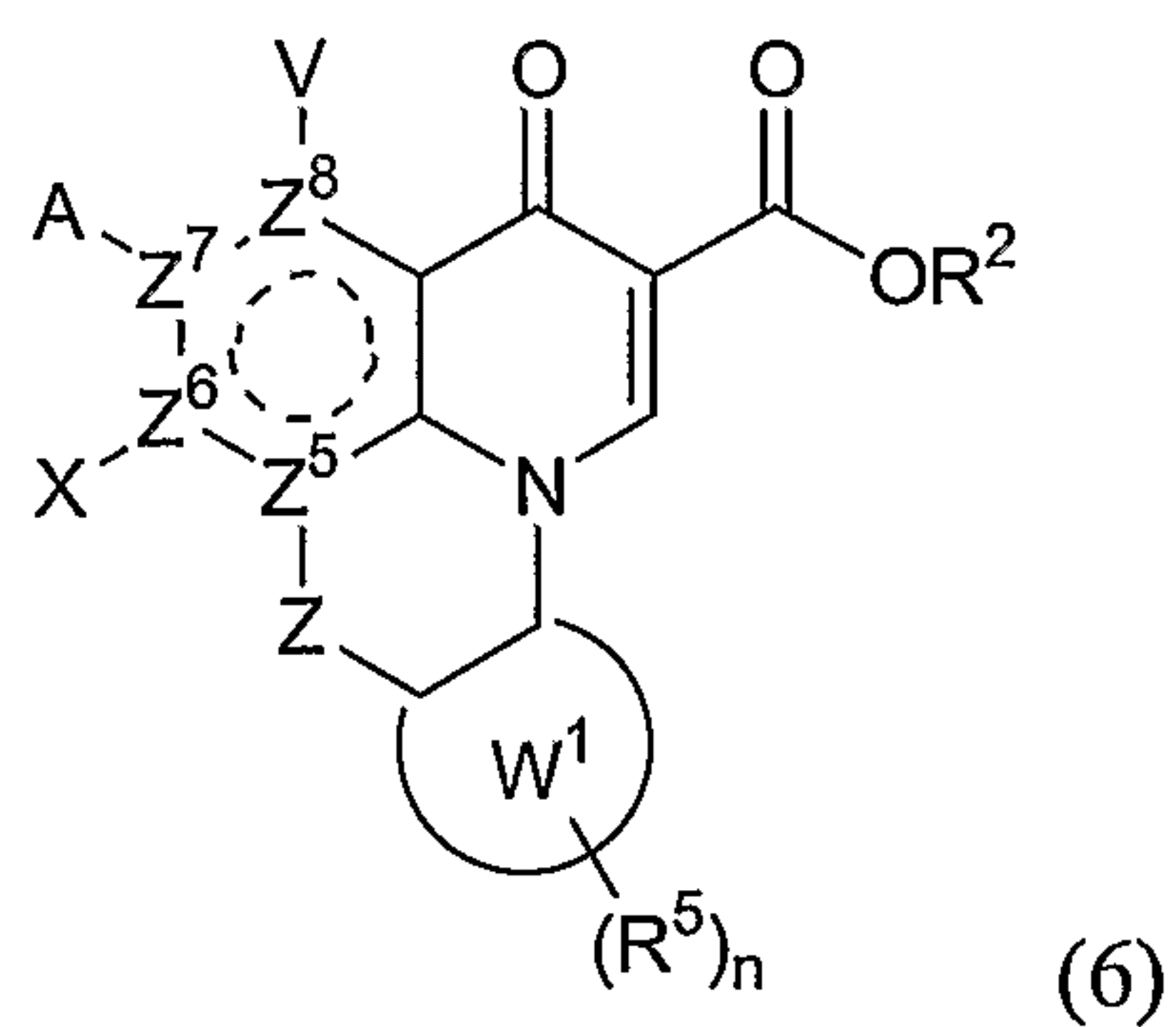
or formula (4)



comprising contacting an ester, NHR<sup>1</sup>R<sup>2</sup>, and a Lewis acid, wherein said ester has formula (5)



or formula (6)



wherein A, B, V, X, R<sup>1</sup>, R<sup>2</sup>, R<sup>5</sup>, Z, Z<sup>1</sup>, Z<sup>2</sup>, Z<sup>3</sup>, Z<sup>4</sup> and n are as described above in formula (1);

W together with N and Z forms an optionally substituted 5- or 6-membered ring that is fused to an optionally substituted aryl or heteroaryl, wherein said aryl or heteroaryl may be monocyclic or fused with a single or multiple ring, and wherein said ring optionally contains a heteroatom;

W<sup>1</sup> is an optionally substituted aryl or heteroaryl, which may be monocyclic, or fused with a single or multiple ring and optionally containing a heteroatom;

Z<sup>5</sup> is C or N, provided Z<sup>5</sup> is C if Z is O, S or NR<sup>1</sup>, and further provided that Z and Z<sup>6</sup> are not N if Z<sup>5</sup> is N; and

Z<sup>6</sup>, Z<sup>7</sup>, and Z<sup>8</sup> are independently C or N, provided any two N are non-adjacent.

[0032] The present methods for preparing compounds having formula (3) involve amide coupling of an ester with an amine in the presence of a Lewis acid such as aluminum chloride. Suitable Lewis acids may be selected by conducting a test reaction, and observing the amount of reaction product produced, as described hereafter. The present methods do not require hydrolysis of the ester to a carboxylic acid prior to amide coupling. Thus, the present methods are simpler. As shown in Example 29, the present methods also provide higher yields and purity than previous methods involving requiring hydrolysis of the ester to the acid (Example 30).

[0033] In one embodiment, the Lewis acid has formula ML<sub>n</sub>, wherein L is a halogen atom or an organic radical, n is 3-5, and M is a group III elemental atom, a group IV elemental atom, As, Sb, V or Fe.

[0034] In the above methods, the contacting step may be performed at room temperature. Alternatively, the ester, amine and Lewis acid may be contacted at cooler or elevated temperatures than room temperature, which may be determined by one skilled in the art.

[0035] In one example, the contacting step comprises contacting the ester and amine in an organic solvent to form a solution, and contacting the solution with a Lewis acid. In one example, the organic solvent is methylene chloride. The reaction may also be conducted using other suitable solvents known in the art.

[0036] In one embodiment, an excess of amine in relation to the ester may be used. For example, the ratio of the ester to the amine may be 1:2; 1:1.5; or 1:1.25.

[0037] In another embodiment, an equimolar amount of Lewis acid to the amine may be used. Alternatively, the amount of Lewis acid used may be more or less than the amine.

[0038] The above methods may further comprise isolating a compound having any one of the above formula. The isolated compounds may further be purified using any methods known

in the art. For example, the isolated compounds may be purified through column chromatography, recrystallization, or both.

[0039] In the above methods, the purity of the isolated compounds may be between 90 and 99%. For example, the isolated compounds may have a purity between 90 and 95 %.

[0040] In the above methods, the ester may be contacted with  $\text{NHR}^1\text{R}^2$ ,

wherein  $\text{R}^1$  is a  $(\text{CR}^3_2)_n$  group;

$\text{R}^2$  is  $\text{NR}^3\text{R}^4$ ;

$\text{R}^3$  is H or  $\text{C}_{1-6}$  alkyl;

$n$  is 1-6; and

$\text{R}^4$  is H or a  $\text{C}_{1-10}$  alkyl or  $\text{C}_{2-10}$  alkenyl optionally containing one or more non-adjacent heteroatoms selected from N, O and S, and optionally substituted with a carbocyclic or heterocyclic ring; and

wherein in  $\text{NR}^3\text{R}^4$ ,  $\text{R}^3$  and  $\text{R}^4$  may form an optionally substituted ring such as those previously described above.

#### Brief Description of the Drawings

[0041] Figures 1-10 show the activity of exemplary compounds of the present invention in an HCT-116 colorectal cancer xenograft model.

#### Definitions

[0042] As used herein, the term “alkyl” refers to a carbon-containing compound, and encompasses compounds containing one or more heteroatoms. The term “alkyl” also encompasses alkyls substituted with one or more substituents including but not limited to  $\text{OR}^1$ , amino, amido, halo,  $=\text{O}$ , aryl, heterocyclic groups, or inorganic substituents.

[0043] As used herein, the term “carbocycle” refers to a cyclic compound containing only carbon atoms in the ring, whereas a “heterocycle” refers to a cyclic compound comprising a heteroatom. The carbocyclic and heterocyclic structures encompass compounds having monocyclic, bicyclic or multiple ring systems.

[0044] As used herein, the term “aryl” refers to a polyunsaturated, typically aromatic hydrocarbon substituent, whereas a “heteroaryl” or “heteroaromatic” refer to an aromatic ring containing a heteroatom. The aryl and heteroaryl structures encompass compounds having monocyclic, bicyclic or multiple ring systems.

[0045] As used herein, the term “heteroatom” refers to any atom that is not carbon or hydrogen, such as nitrogen, oxygen or sulfur.

[0046] Illustrative examples of heterocycles include but are not limited to tetrahydrofuran, 1,3-dioxolane, 2,3-dihydrofuran, pyran, tetrahydropyran, benzofuran, isobenzofuran, 1,3-dihydro-isobenzofuran, isoxazole, 4,5-dihydroisoxazole, piperidine, pyrrolidine, pyrrolidin-2-one, pyrrole, pyridine, pyrimidine, octahydro-pyrrolo[3,4-*b*]pyridine, piperazine, pyrazine, morpholine, thiomorpholine, imidazole, imidazolidine-2,4-dione, 1,3-dihydrobenzimidazol-2-one, indole, thiazole, benzothiazole, thiadiazole, thiophene, tetrahydro-thiophene 1,1-dioxide, diazepine, triazole, guanidine, diazabicyclo[2.2.1]heptane, 2,5-diazabicyclo[2.2.1]heptane, 2,3,4,4a,9,9a-hexahydro-1H- $\beta$ -carboline, oxirane, oxetane, tetrahydropyran, dioxane, lactones, aziridine, azetidine, piperidine, lactams, and may also encompass heteroaryls. Other illustrative examples of heteroaryls include but are not limited to furan, pyrrole, pyridine, pyrimidine, imidazole, benzimidazole and triazole.

[0047] As used herein, the term “inorganic substituent” refers to substituents that do not contain carbon or contain carbon bound to elements other than hydrogen (*e.g.*, elemental carbon, carbon monoxide, carbon dioxide, and carbonate). Examples of inorganic substituents include but are not limited to nitro, halogen, sulfonyls, sulfinyls, phosphates, etc.

[0048] The terms “treat,” “treatment” and “therapeutic effect” as used herein refer to reducing or stopping a cell proliferation rate (*e.g.*, slowing or halting tumor growth) or reducing the number of proliferating cancer cells (*e.g.*, removing part or all of a tumor). These terms also are applicable to reducing a titre of a microorganism in a system (*i.e.*, cell, tissue, or subject) infected with a microorganism, reducing the rate of microbial propagation, reducing the number of symptoms or an effect of a symptom associated with the microbial infection, and/or removing detectable amounts of the microbe from the system. Examples of microorganism include but are not limited to virus, bacterium and fungus.

[0049] As used herein, the term “chemotherapeutic agent” refers to a therapeutic agent that may be used for treating or ameliorating a cell proliferative disorder such as tumors or cancer. Examples of chemotherapeutic agents include but are not limited to an antineoplastic agent, an alkylating agent, a plant alkaloid, an antimicrobial agent, a sulfonamide, an antiviral agent, a platinum agent, and other anticancer agents known in the art. Particular examples of chemotherapeutic agents include but are not limited to cisplatin, carboplatin, busulphan, methotrexate, daunorubicin, doxorubicin, cyclophosphamide, mephalan, vincristine, vinblastine, chlorambucil, paclitaxel, gemcitabine, and others known in the art. (See *e.g.*,

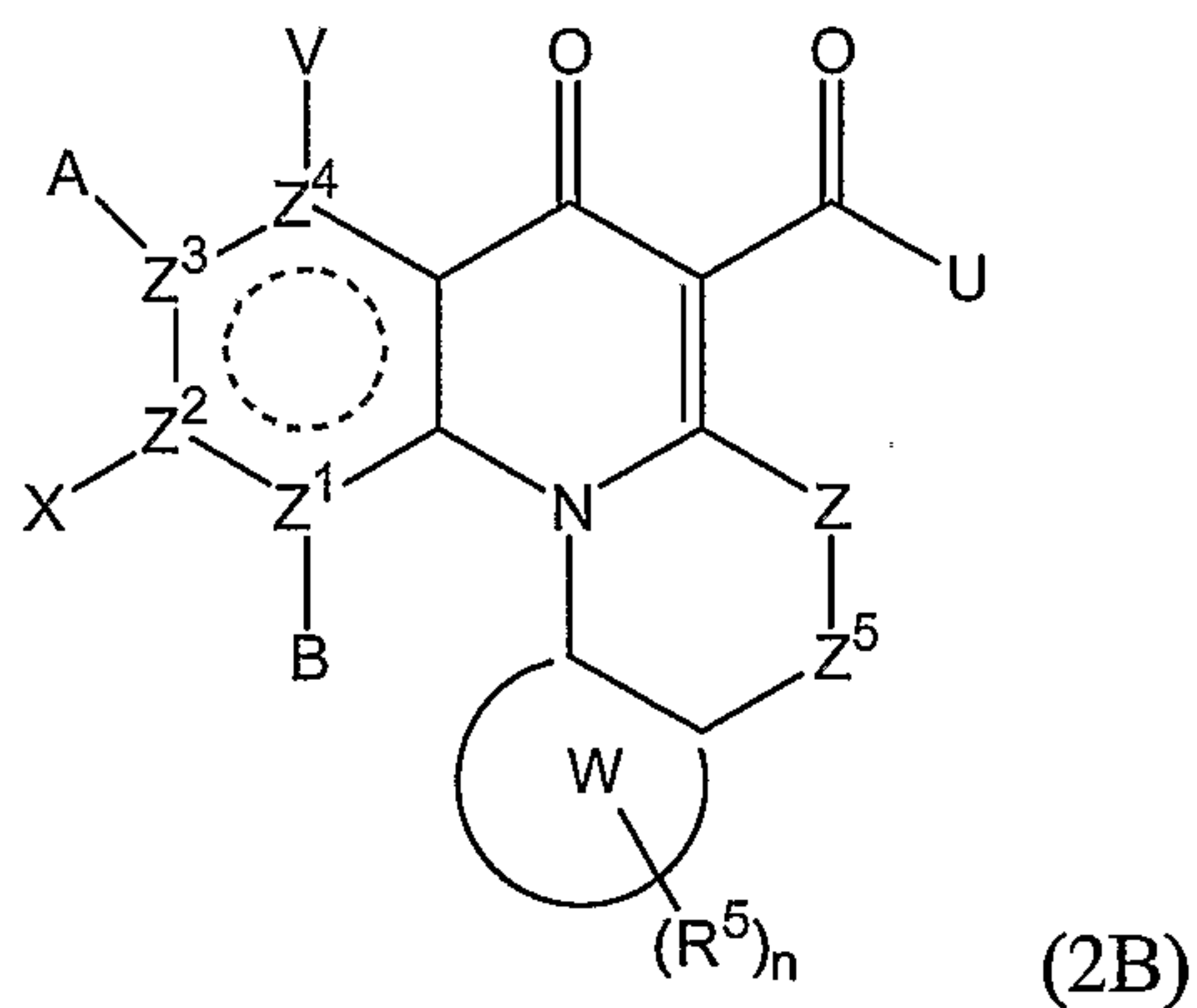
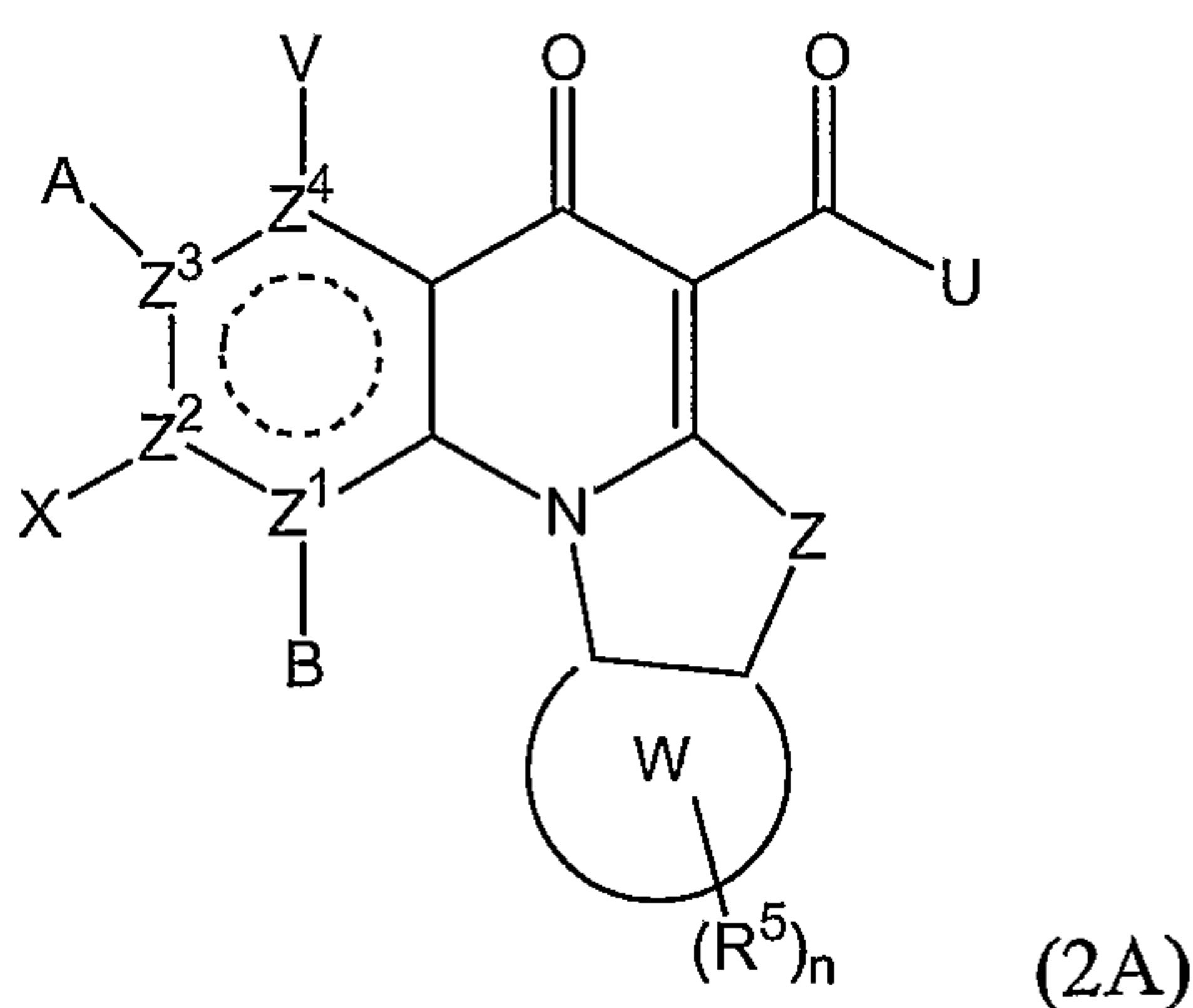
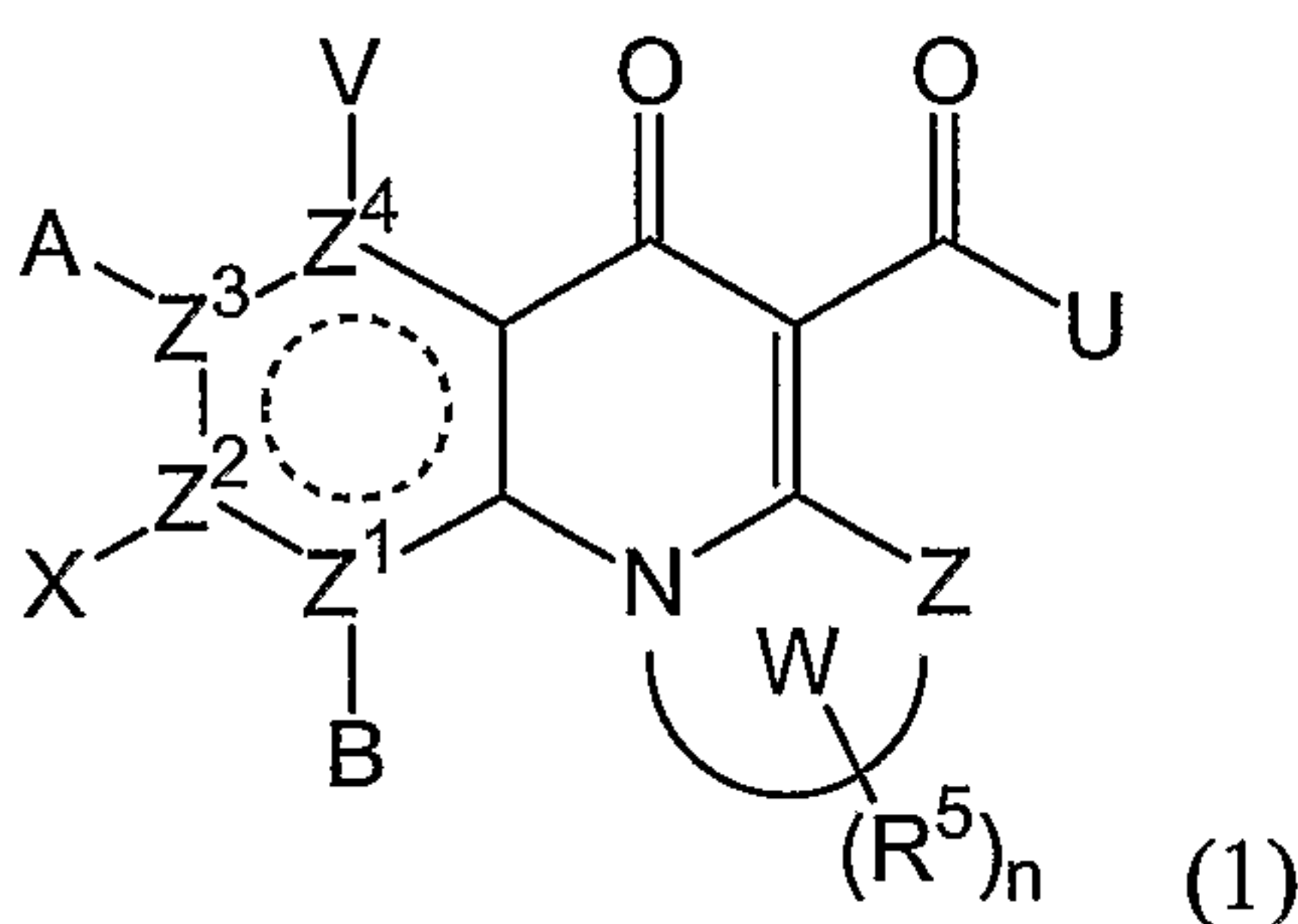
Goodman & Gilman's, The Pharmacological Basis of Therapeutics (9th Ed) (Goodman, *et al.*, eds.) (McGraw-Hill) (1996); and 1999 Physician's Desk Reference (1998)).

**[0050]** As used herein, the term “apoptosis” refers to an intrinsic cell self-destruction or suicide program. In response to a triggering stimulus, cells undergo a cascade of events including cell shrinkage, blebbing of cell membranes and chromatic condensation and fragmentation. These events culminate in cell conversion to clusters of membrane-bound particles (apoptotic bodies), which are thereafter engulfed by macrophages.

### Description of the Invention

**[0051]** The present invention relates to quinolone compounds having formula (1), (2A), and (2B), and pharmaceutically acceptable salts, esters, and prodrugs thereof. The present invention also relates to methods for using the compounds described herein, such as in screening and in treatment. The compounds of the present invention may or may not interact with regions of DNA that can form quadruplexes.

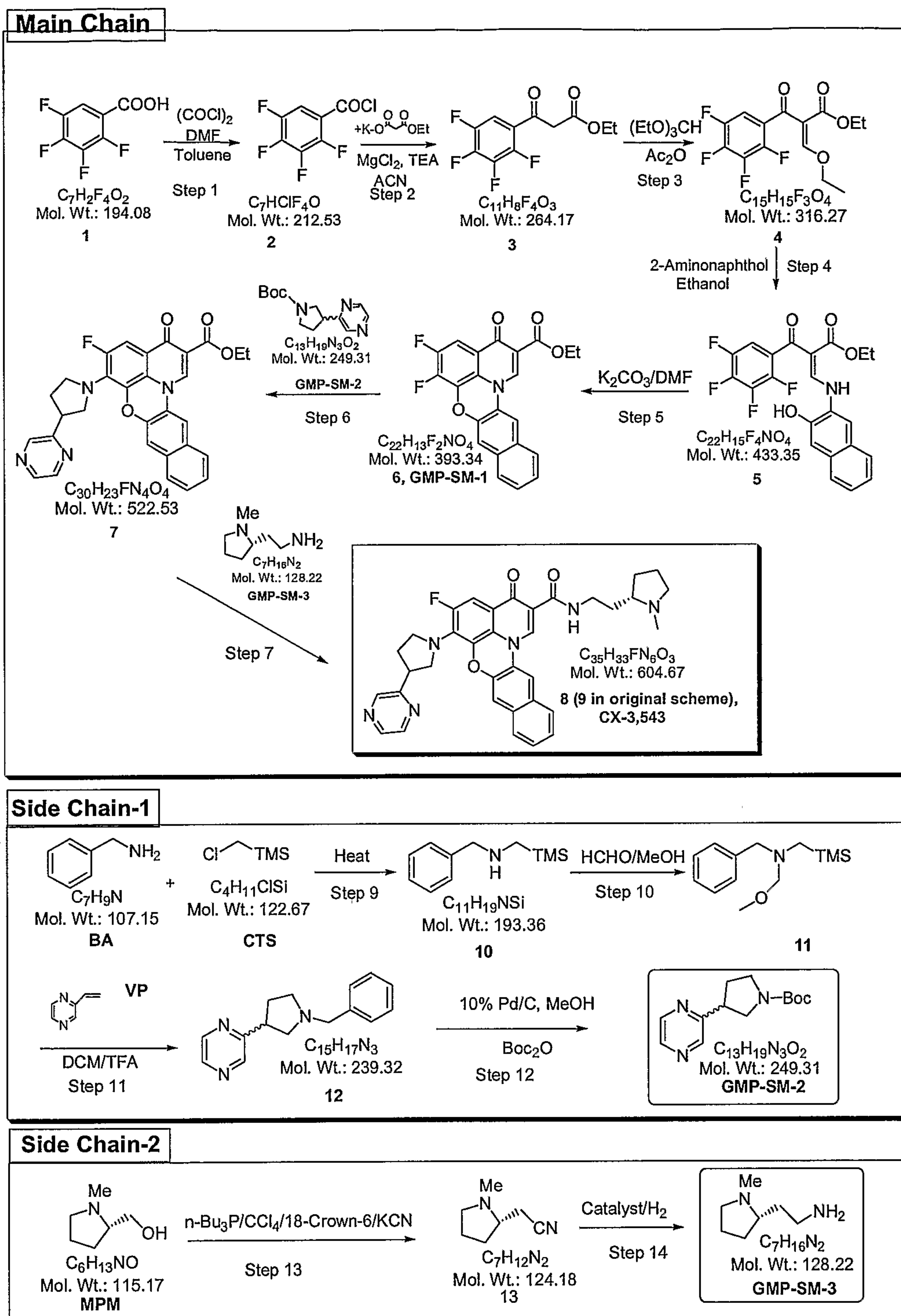
**[0052]** The compounds of present invention having formula (1), (2A), and (2B) are reproduced below:



wherein A, B, V, X, U, Z, Z<sup>1</sup>, Z<sup>2</sup>, Z<sup>3</sup>, Z<sup>4</sup>, X<sup>2</sup> and n are as described above.

**[0053]** Synthetic procedures for preparing the compounds of the present invention are illustrated in Scheme 1, and in the Examples. Other variations in the synthetic procedures known to those with ordinary skill in the art may also be used to prepare the compounds of the present invention. For example, various protecting groups may be used in the preparation of the intermediate illustrated in Side-Chain 1. (See, *e.g.*, Example 31.)

## Scheme 1



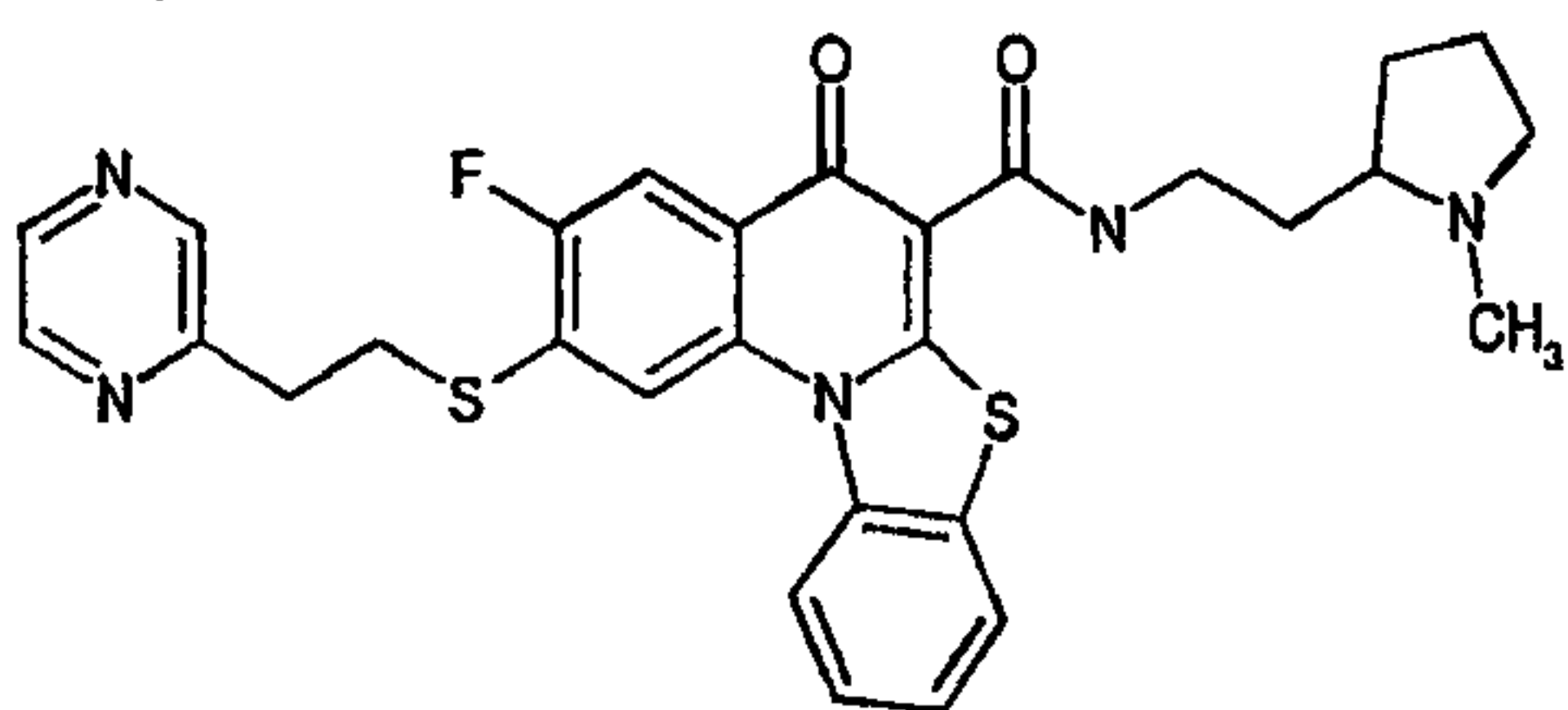
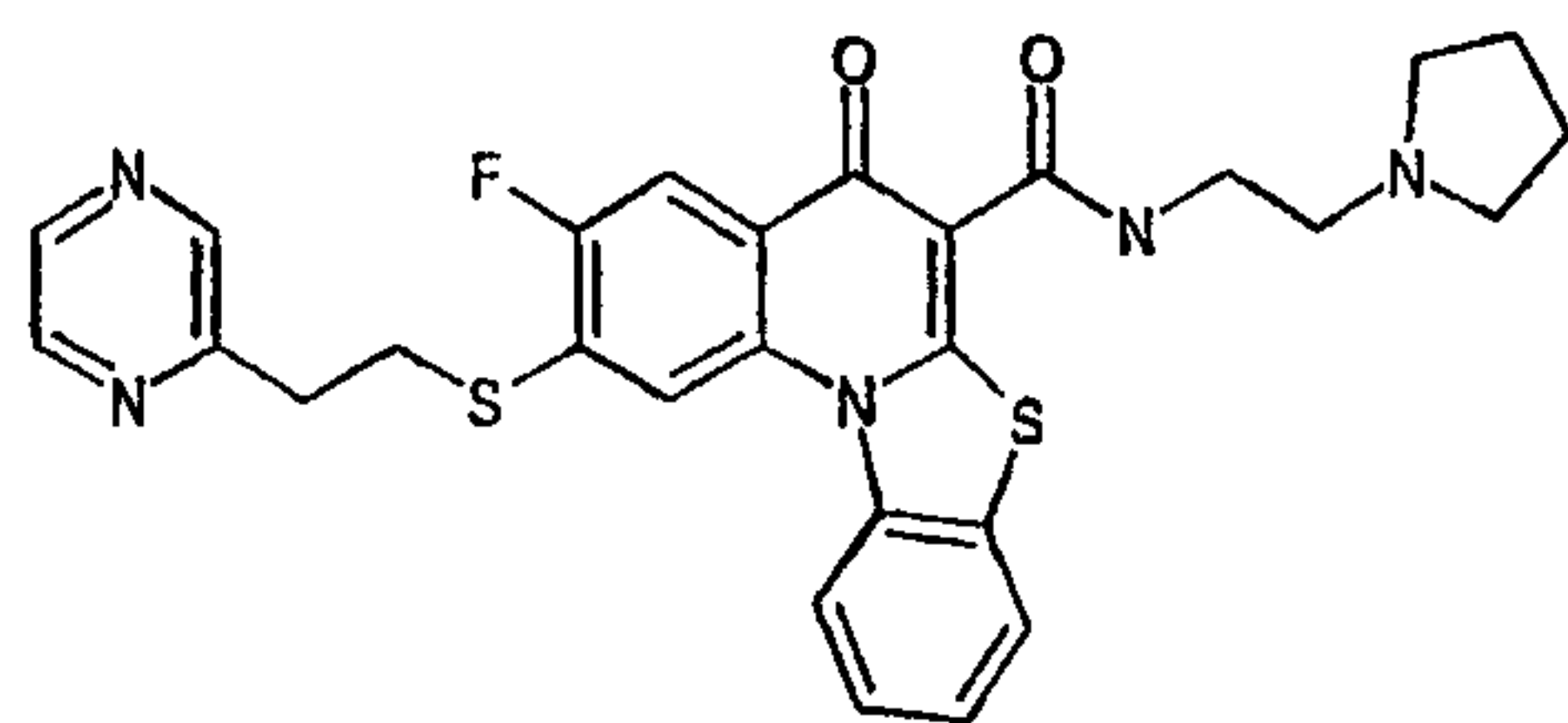
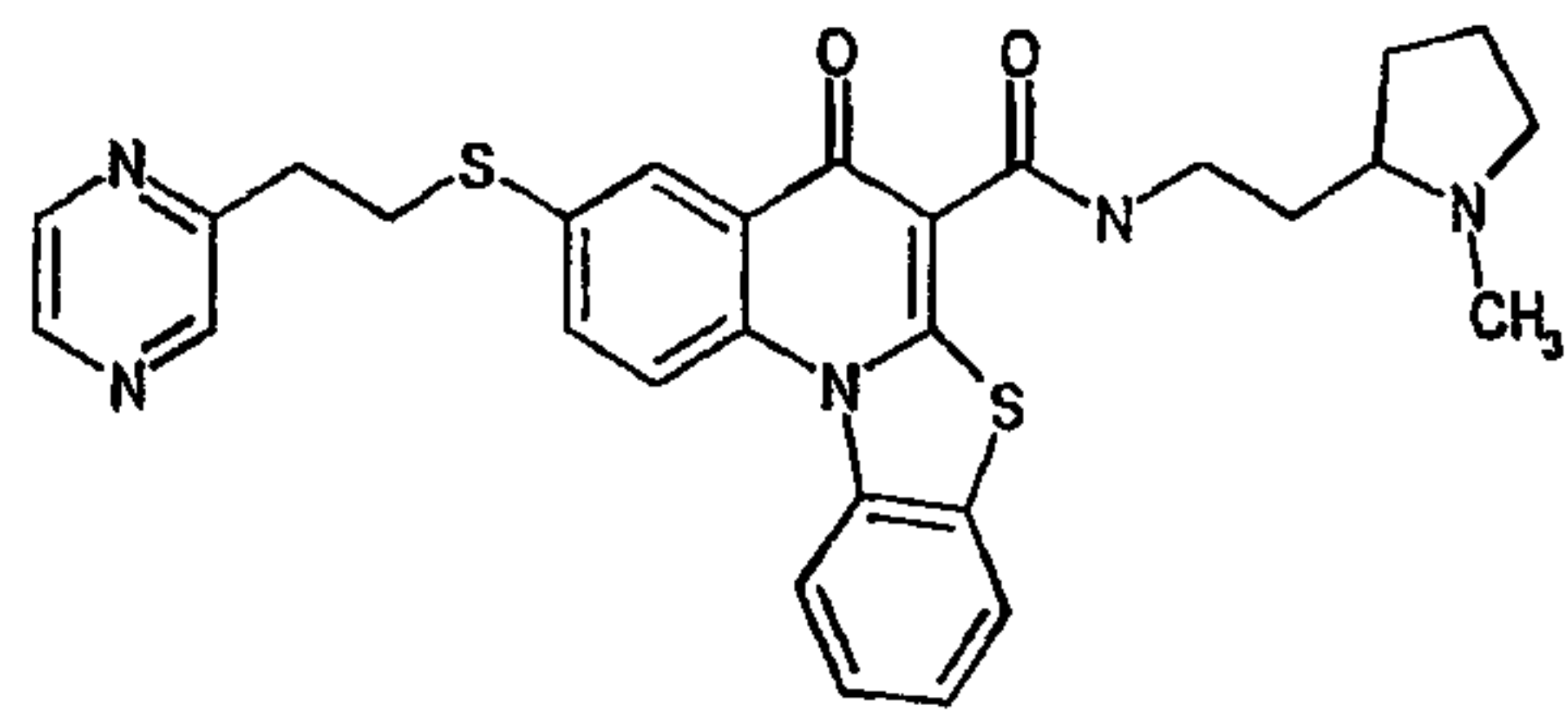
[0054] The compounds of the present invention may be chiral. As used herein, a chiral compound is a compound that is different from its mirror image, and has an enantiomer. Furthermore, the compounds may be racemic, or an isolated enantiomer or stereoisomer. Methods of synthesizing chiral compounds and resolving a racemic mixture of enantiomers are

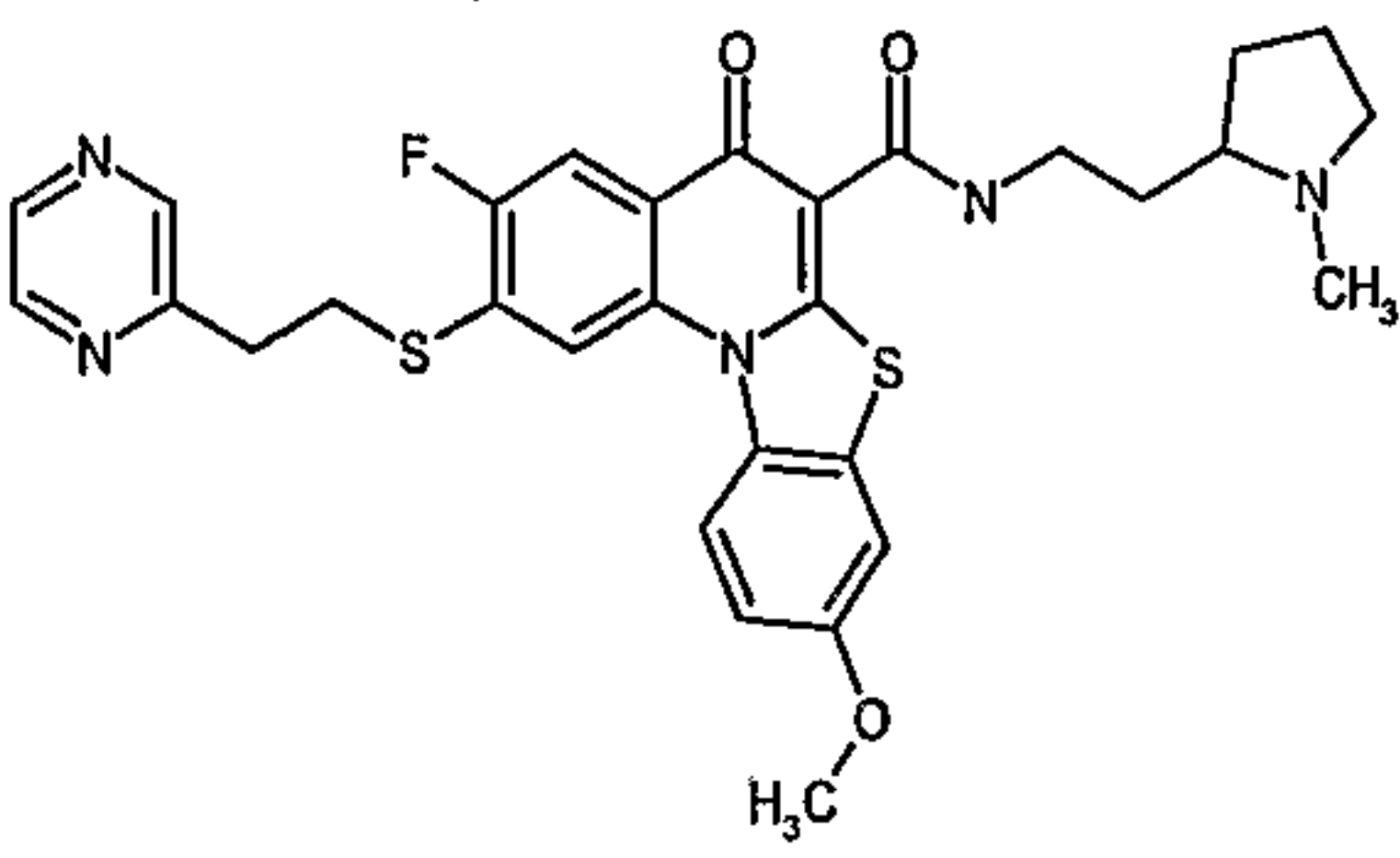
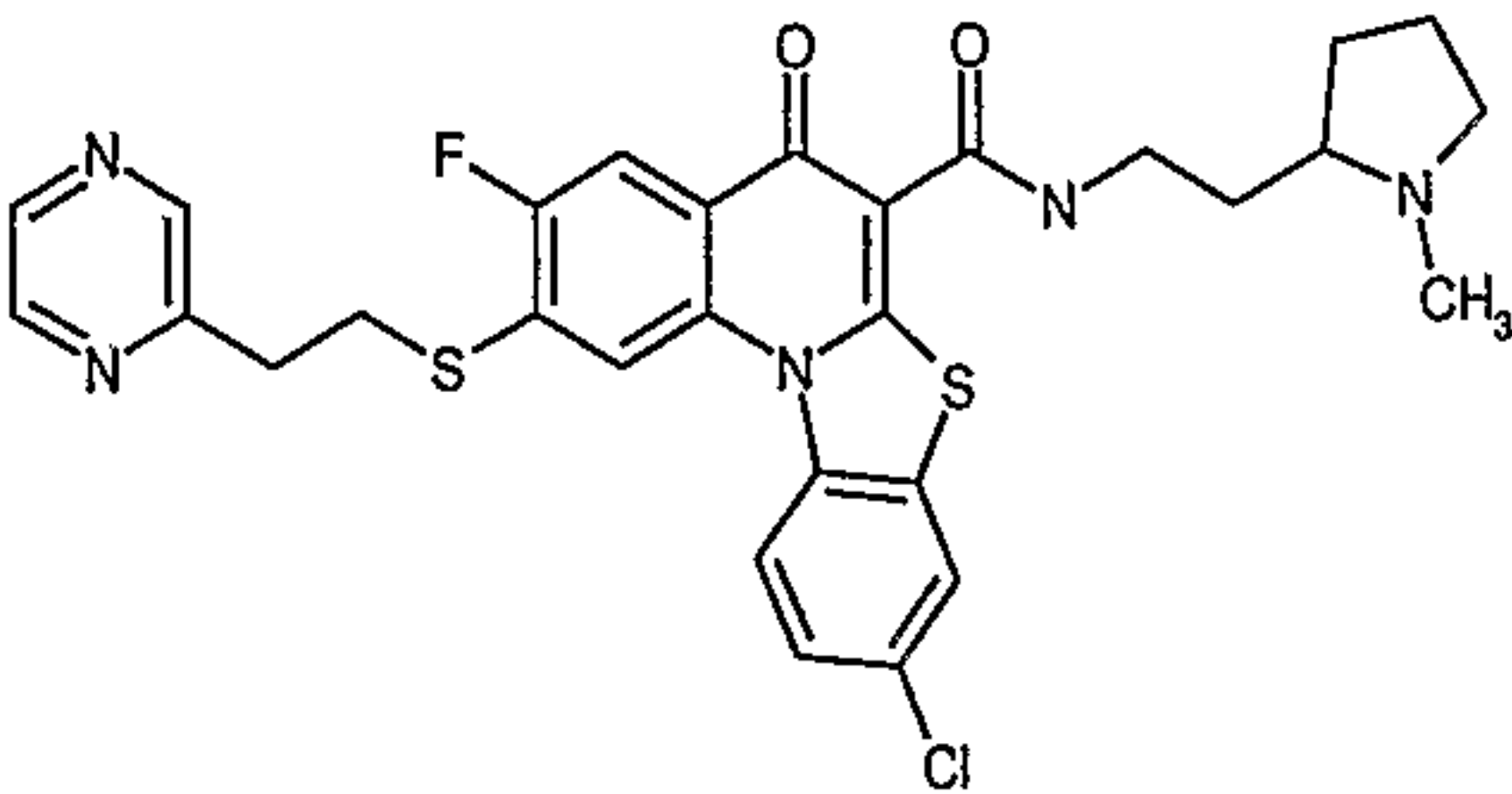
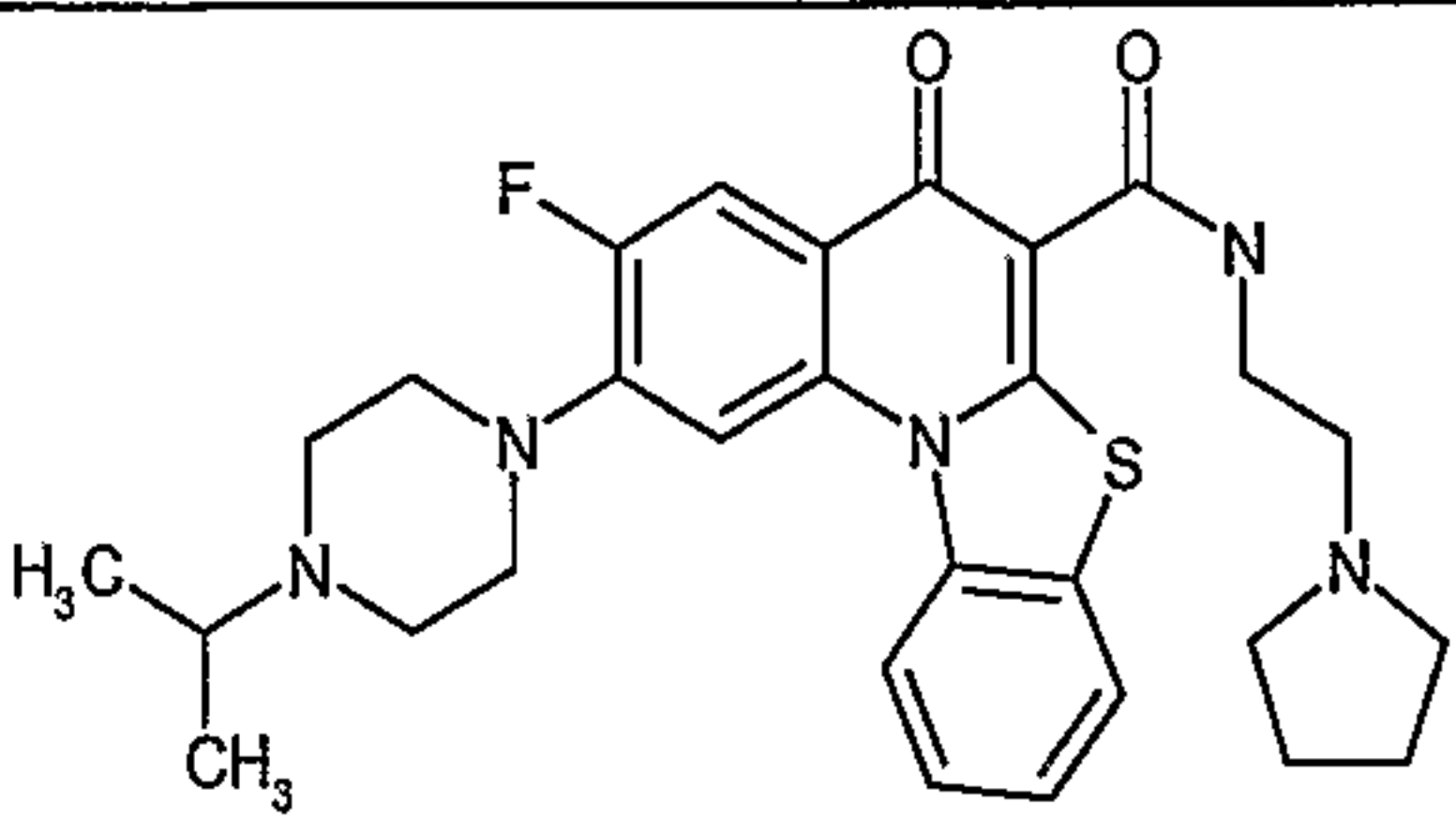
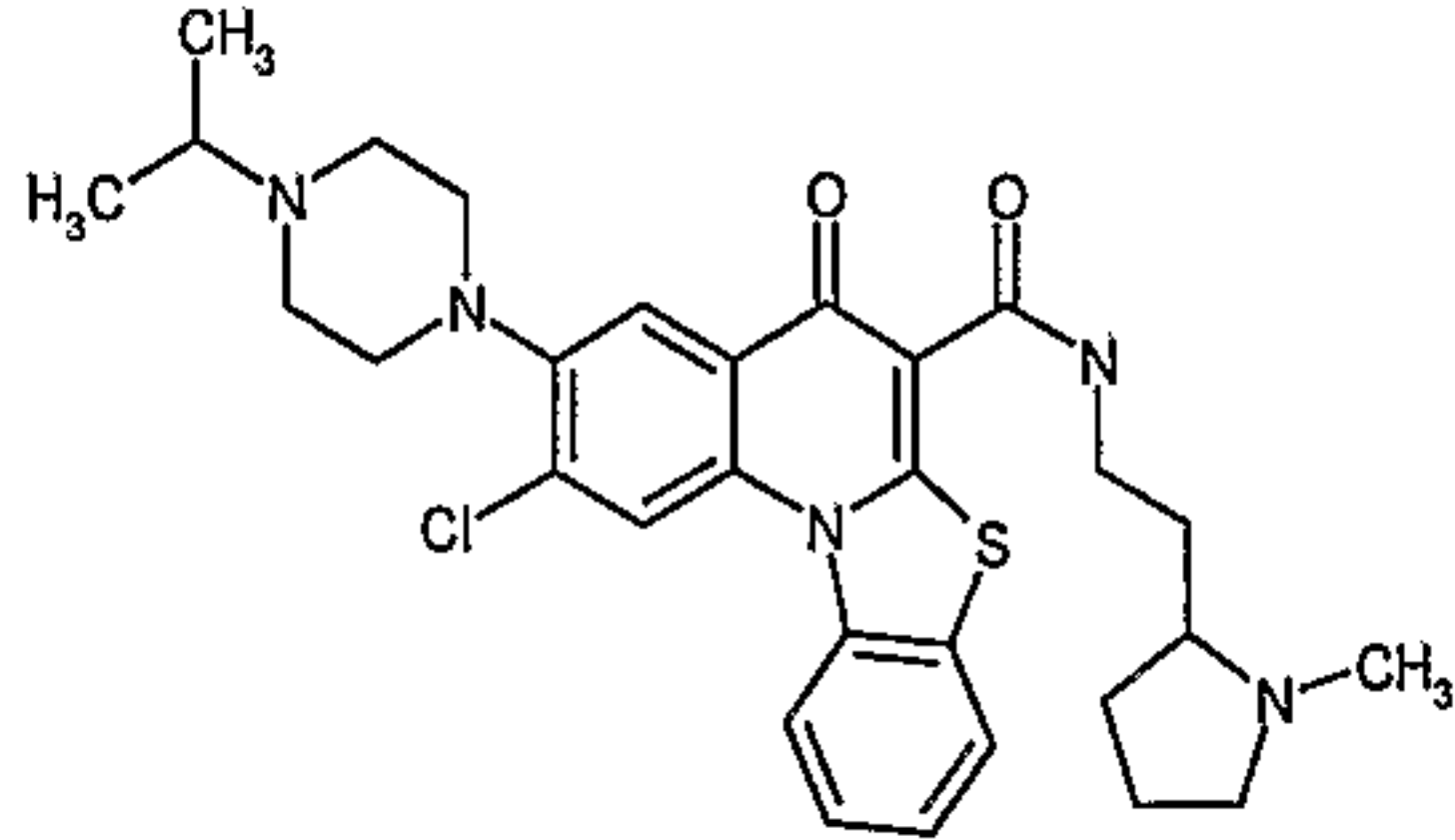
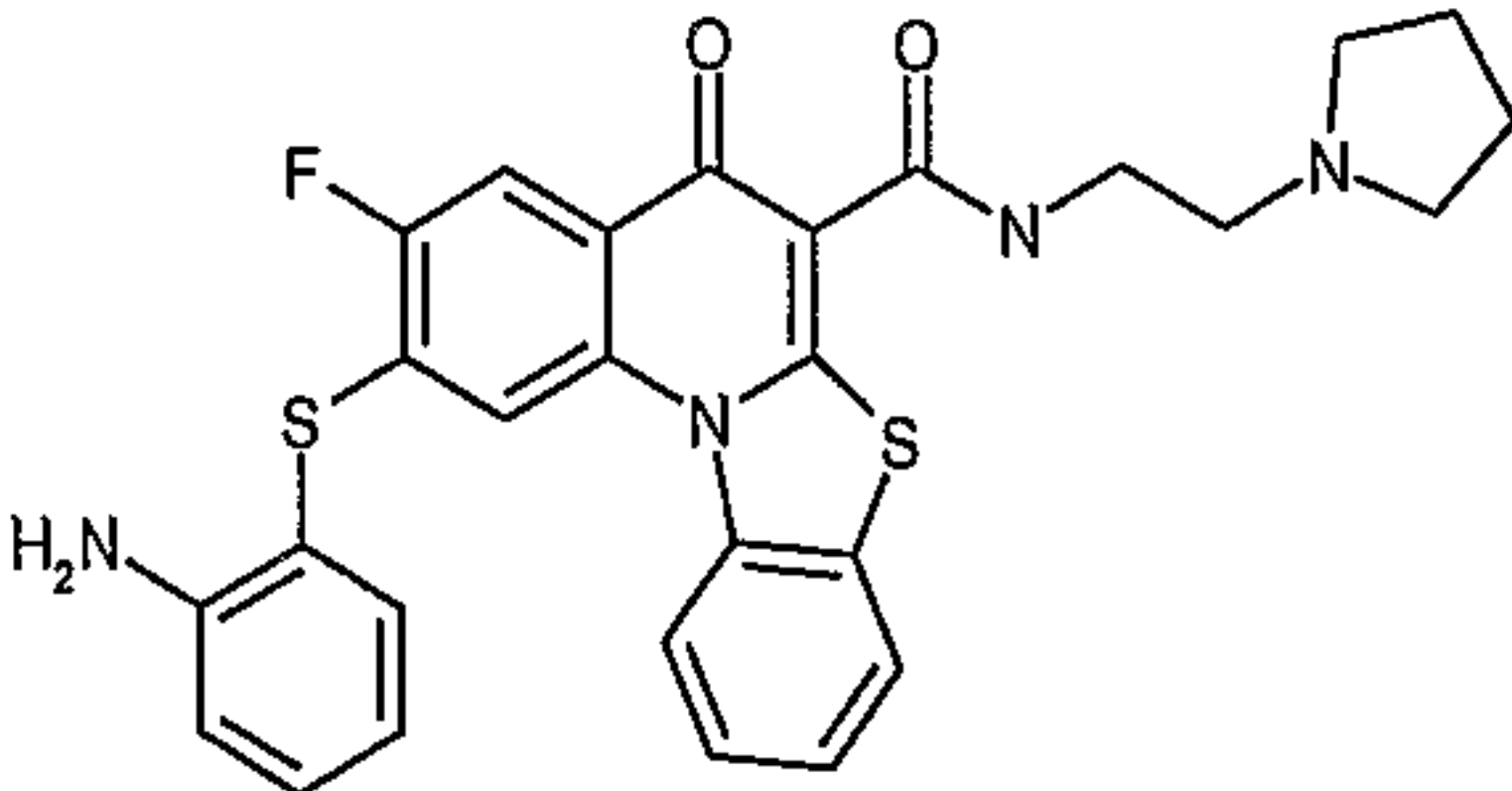
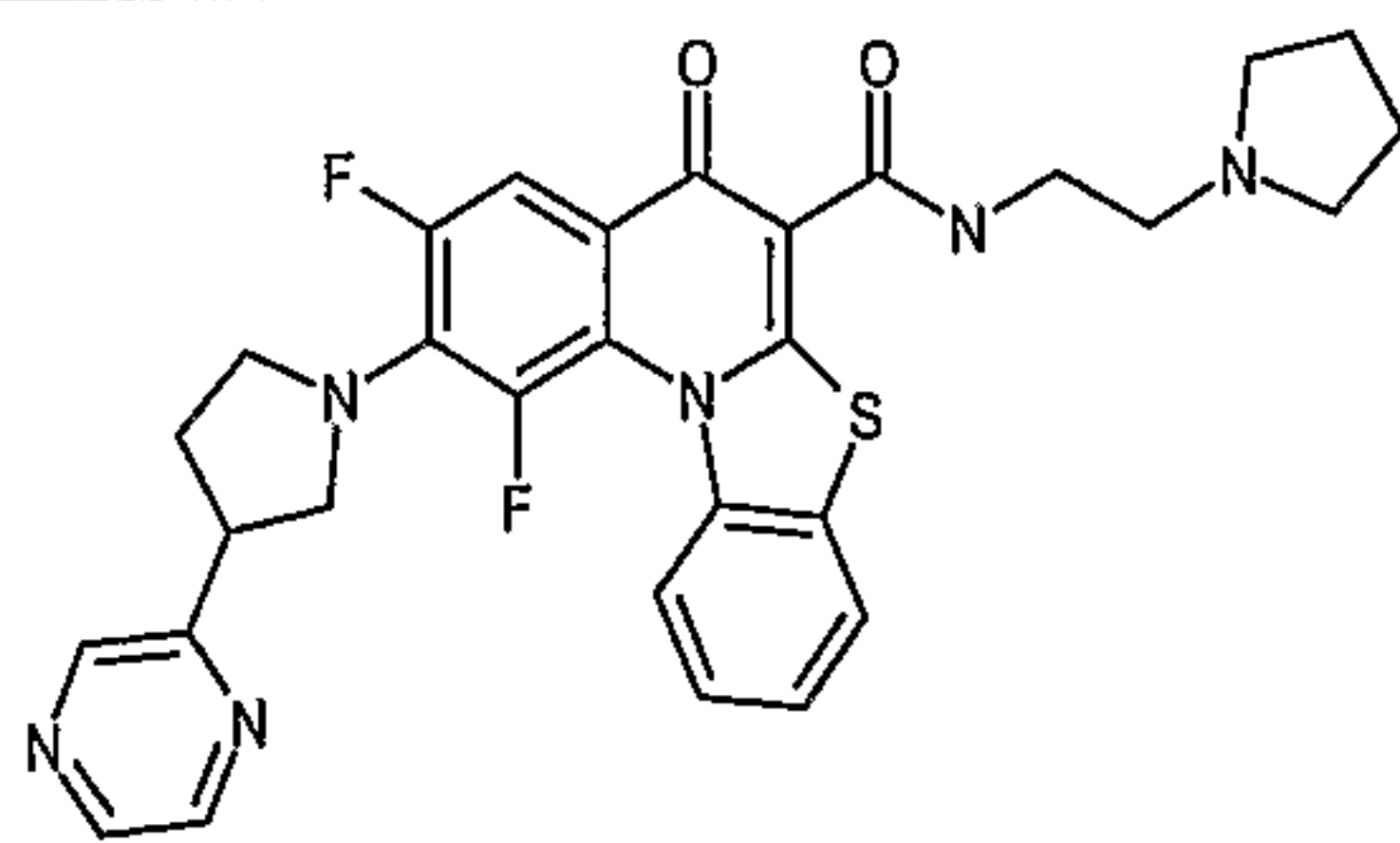
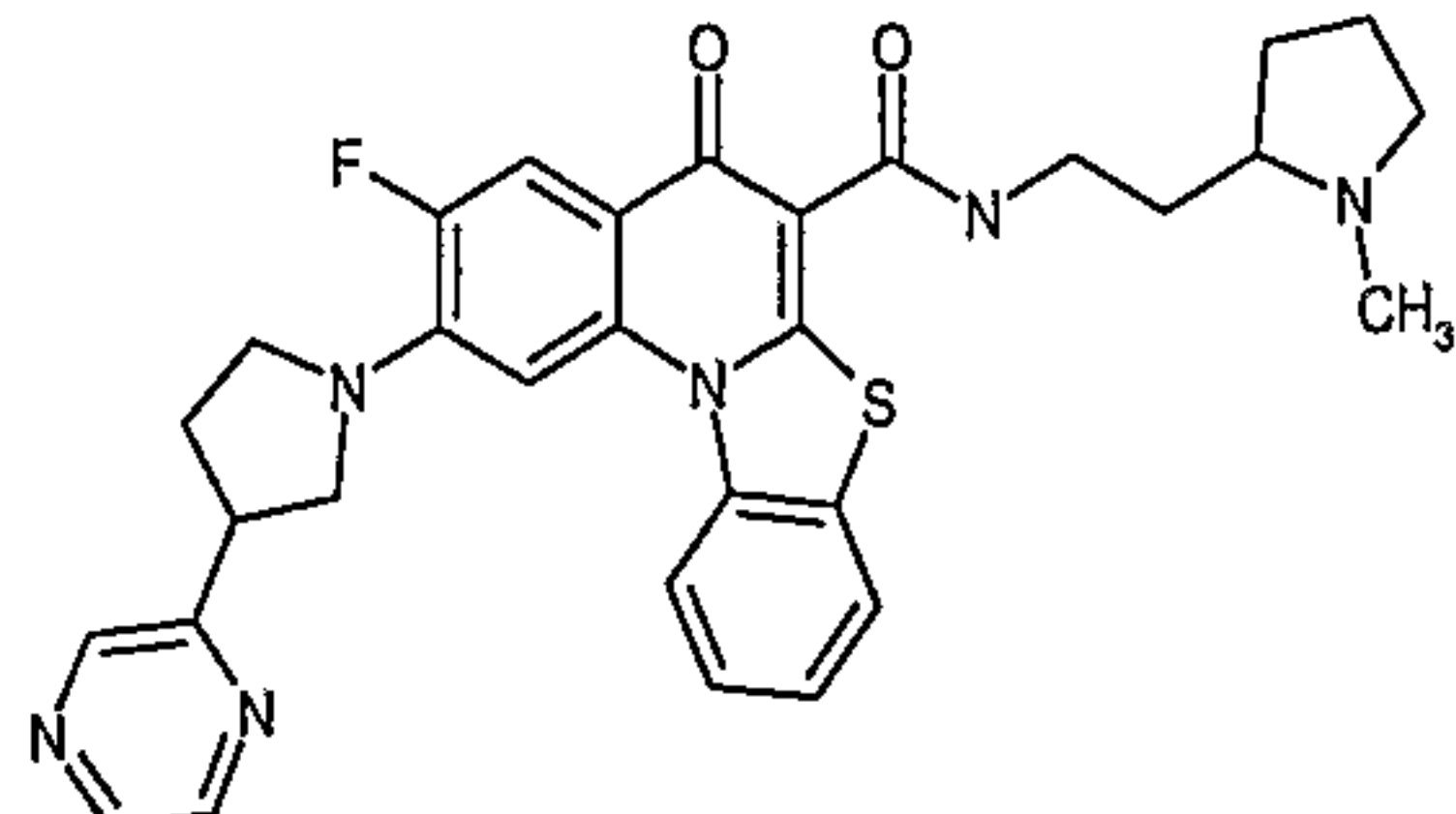
well known to those skilled in the art. See, *e.g.*, March, "Advanced Organic Chemistry," John Wiley and Sons, Inc., New York, (1985).

[0055] The compounds of the present invention were tested using screening assays such as those described herein. Figures 1-10 show the activity of an exemplary compound of the present invention in an HCT-116 colorectal cancer xenograft model. Some compounds did not show activity at a given dosage.

[0056] Illustrative examples of compounds having the above formula are shown in Table 1 (A-C), and in the Examples. The present invention also encompasses other compounds having any one formula (1), (2A) and (2B), comprising substituents U, A, X, V, and B independently selected from the substituents exemplified in Table 1 (A-C), and in the Examples. For example, the isopropyl substituent in the last two compounds shown in Table 1A may be replaced with an acetyl substituent, or the N-CH<sub>3</sub> in the fused ring may be replaced with an NH group. Furthermore, the fluoro group may be replaced with H. Thus, the present invention is not limited to the specific combination of substituents described in various embodiments below.

**Table 1A**

| MOLSTRUCTURE                                                                         | STOP $\mu$ M | HCT-116 MTS $\mu$ M |
|--------------------------------------------------------------------------------------|--------------|---------------------|
|  | 7.2          | 1.1                 |
|   | 5.8          | 0.57                |
|  | 10           | 6.1                 |

| MOLSTRUCTURE                                                                        | STOP $\mu$ M | HCT-116 MTS $\mu$ M |
|-------------------------------------------------------------------------------------|--------------|---------------------|
|    | 9.3          | 5.7                 |
|   | 1.7          | 3.57                |
|   | 9            | 0.3                 |
|  | > 15         |                     |
|  | 15           | 2.4                 |
|  | 11.6         | 3.5                 |
|  | 1.3          | 3.4                 |

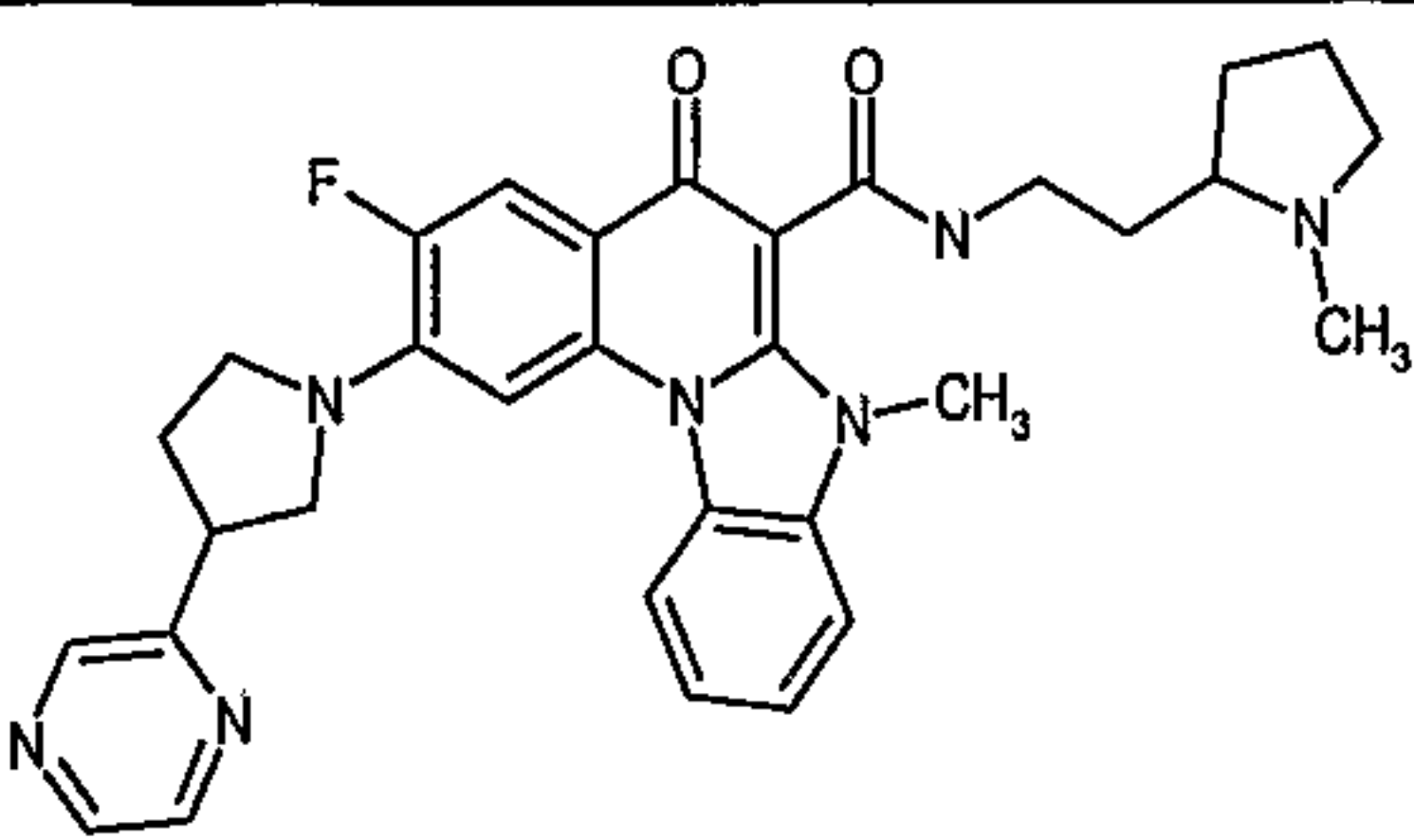
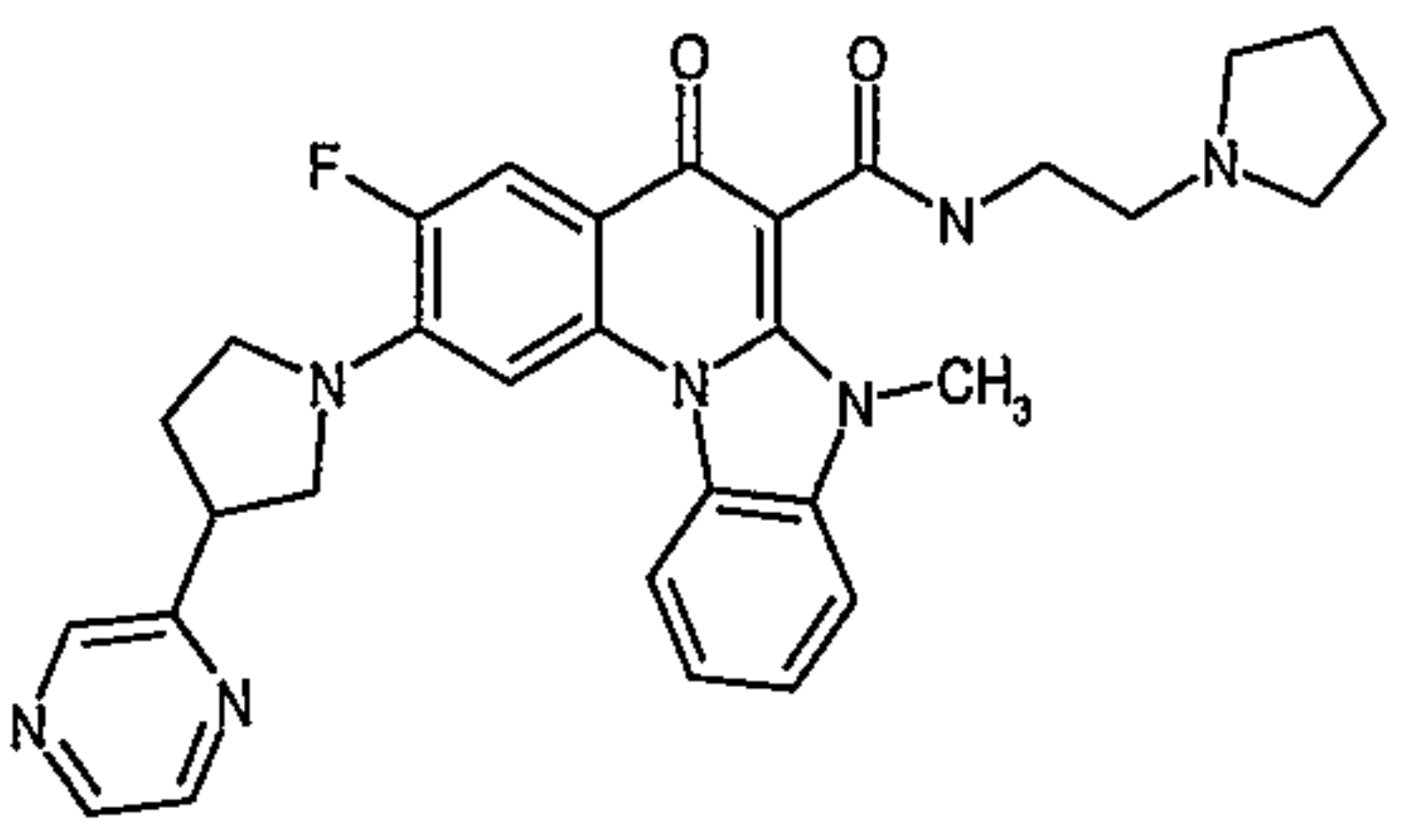
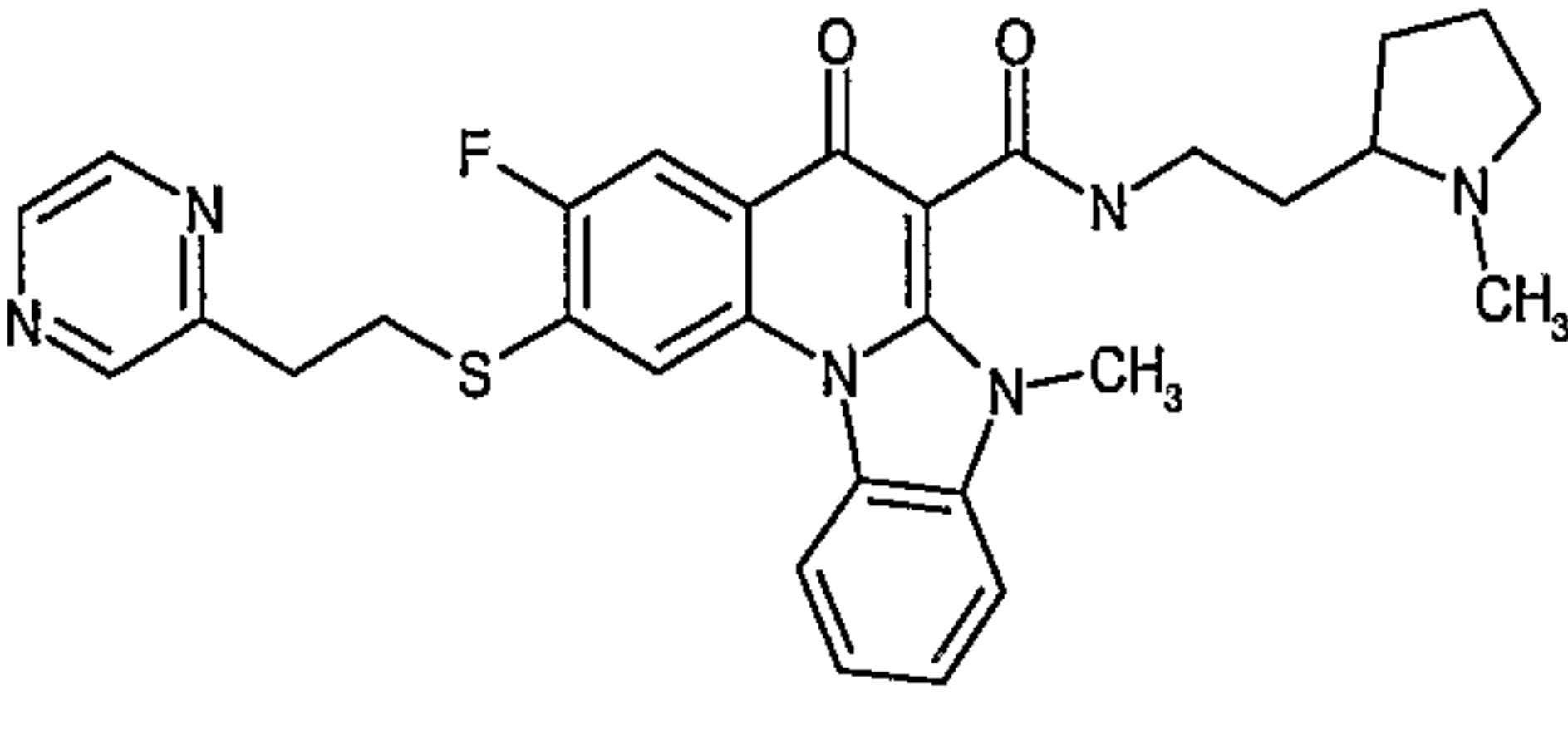
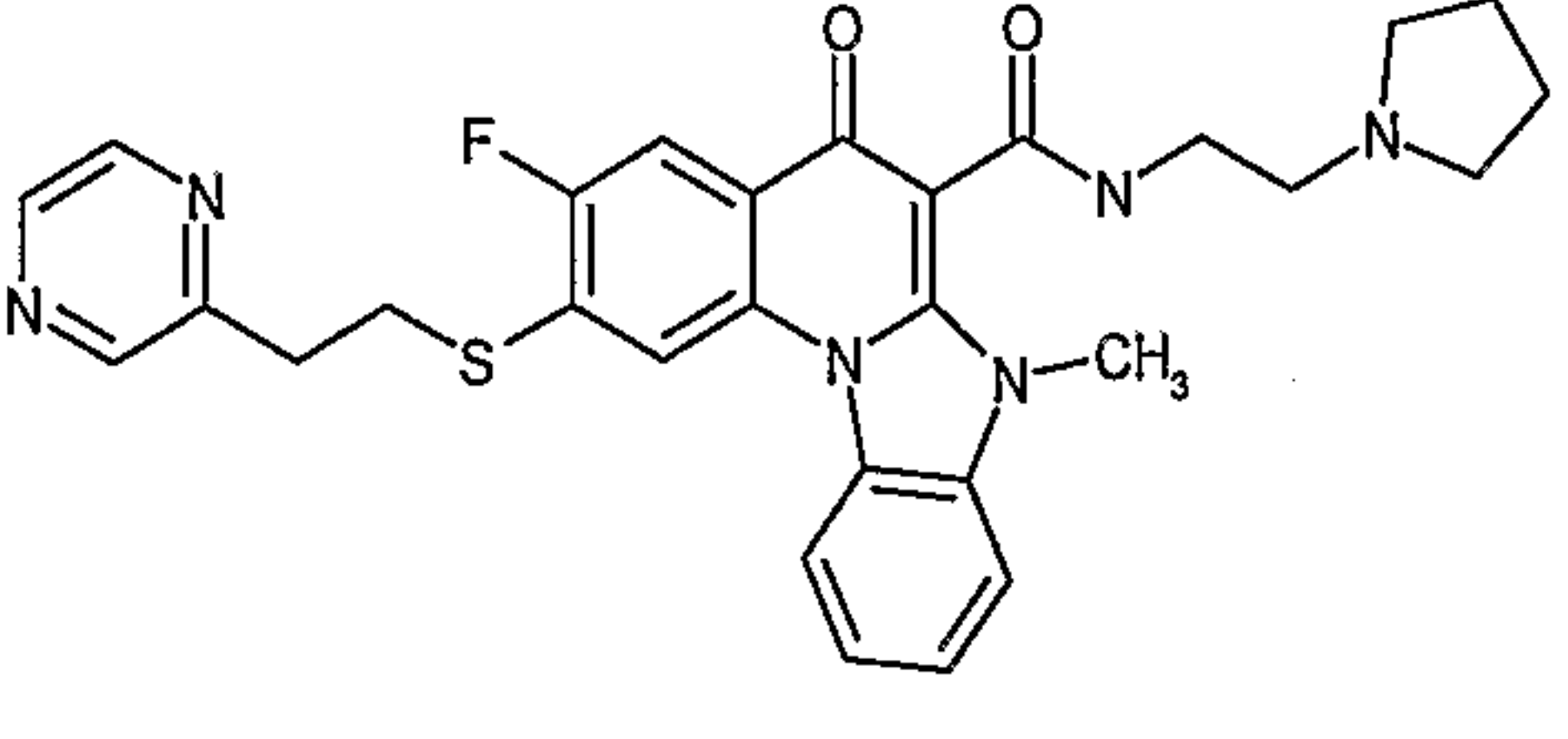
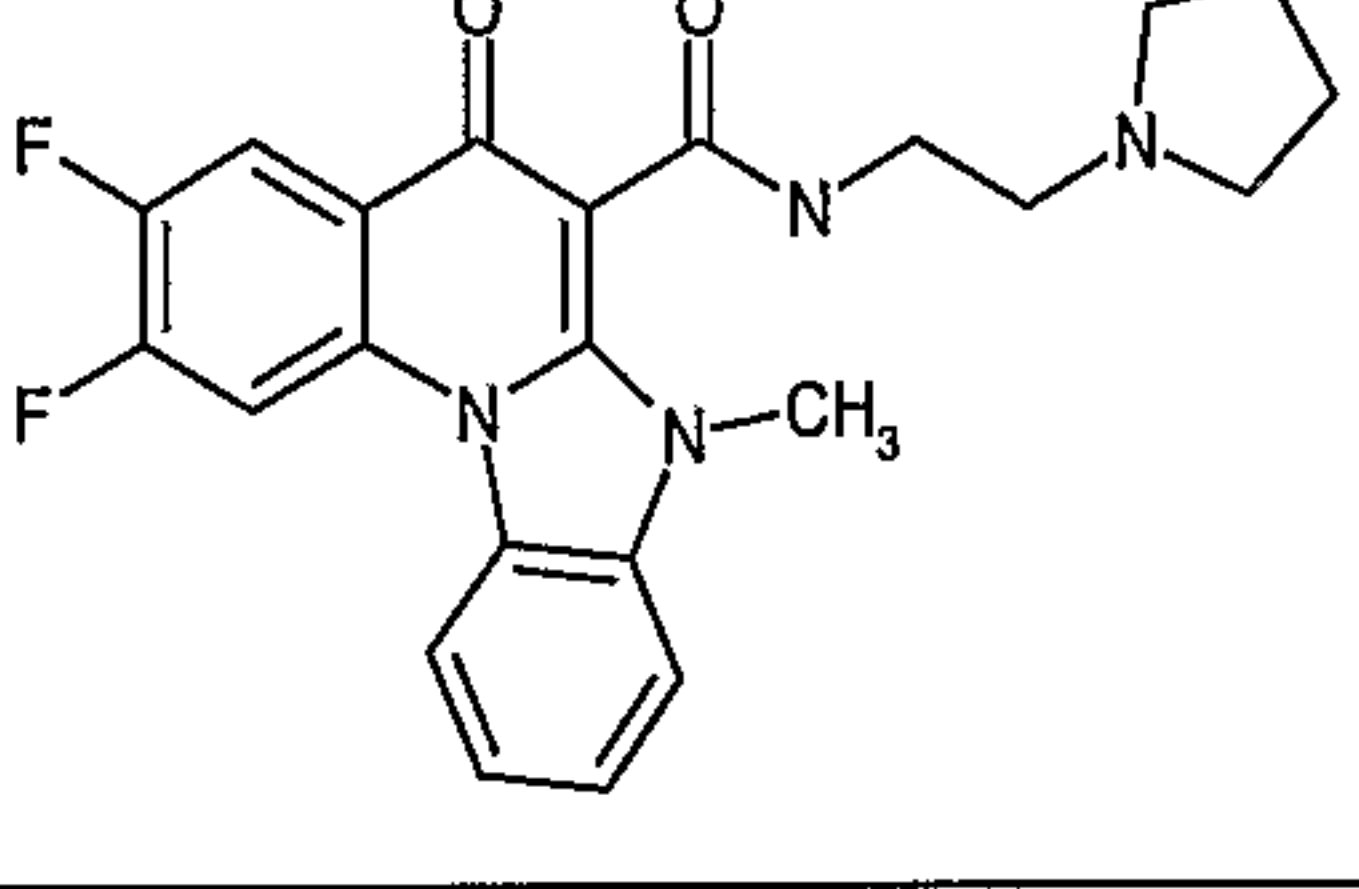
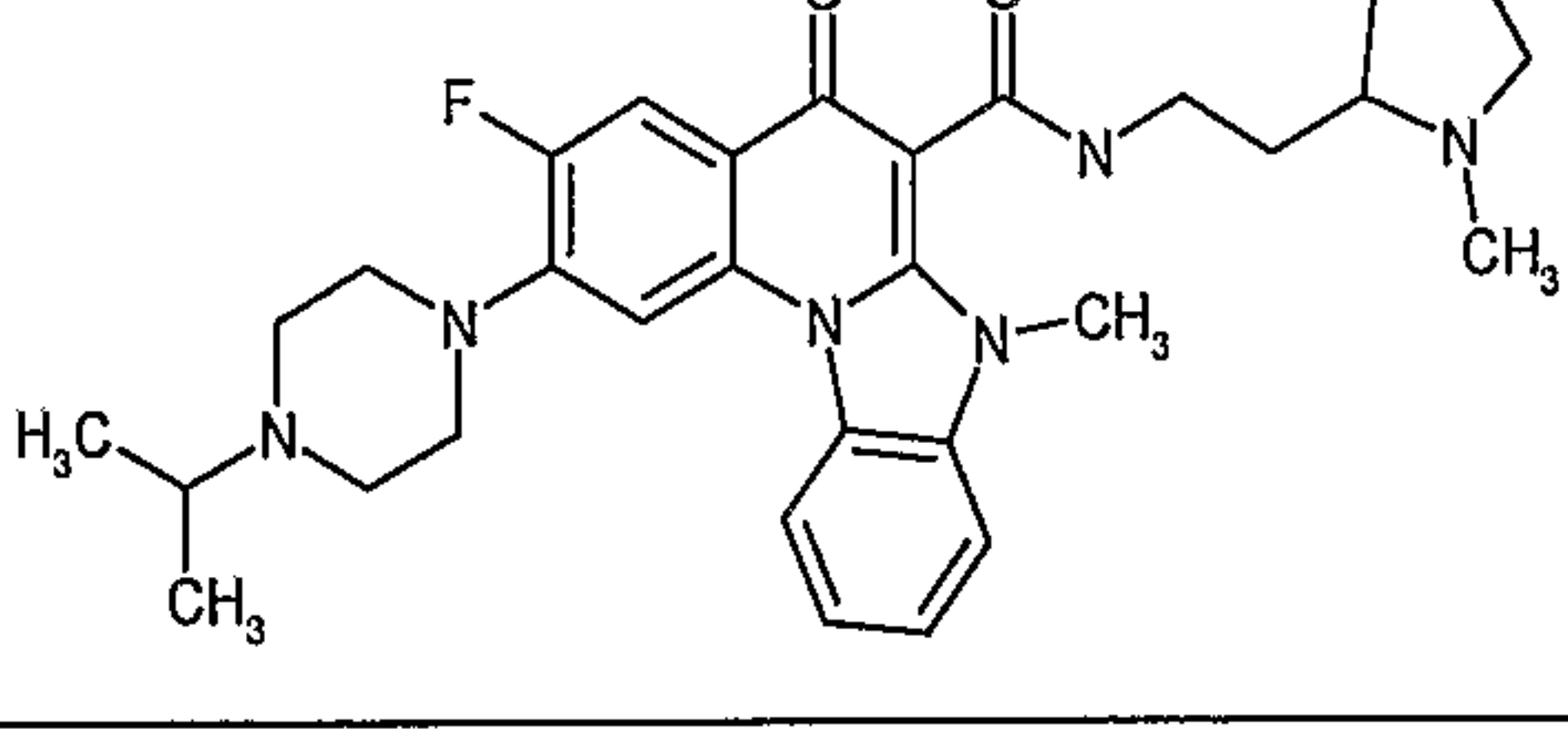
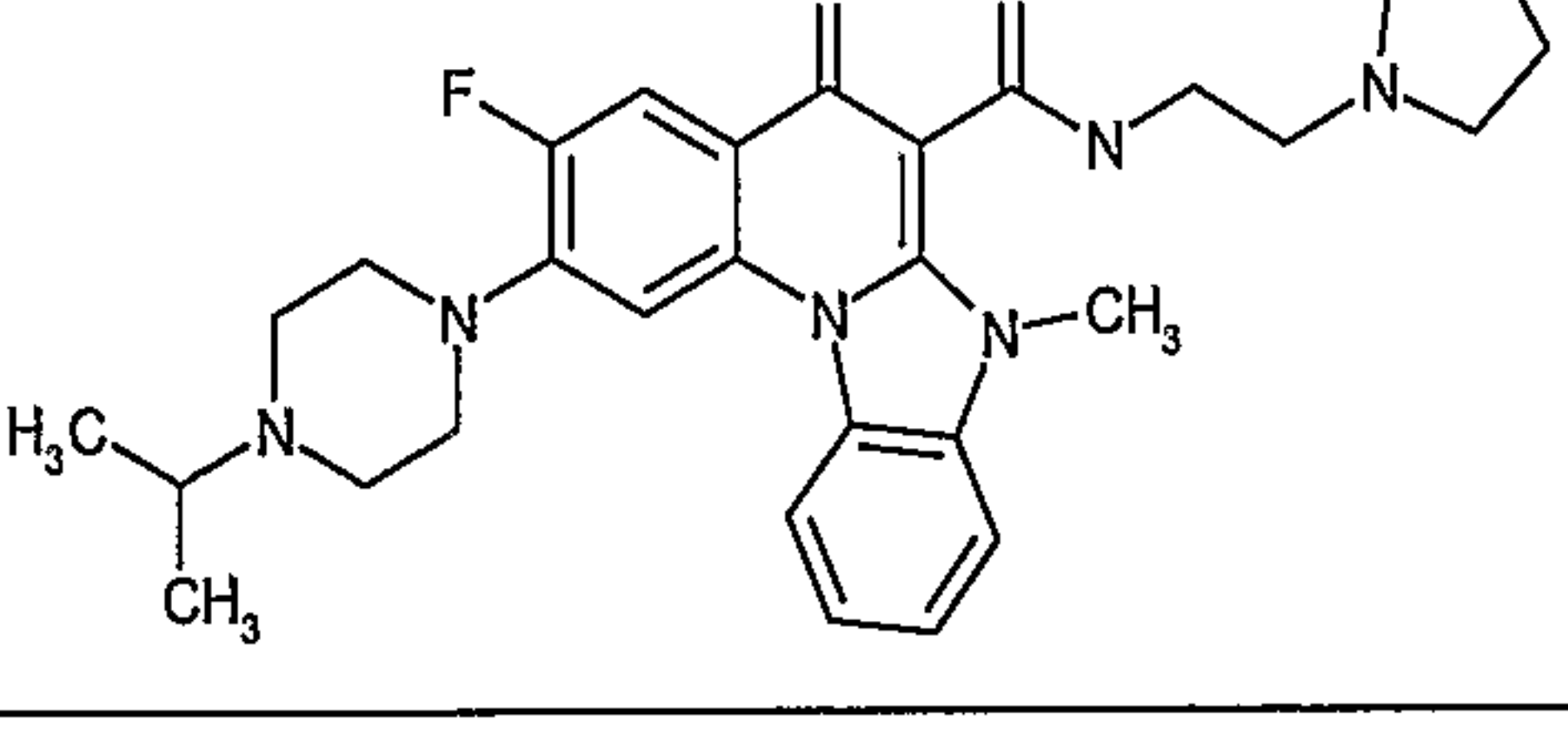
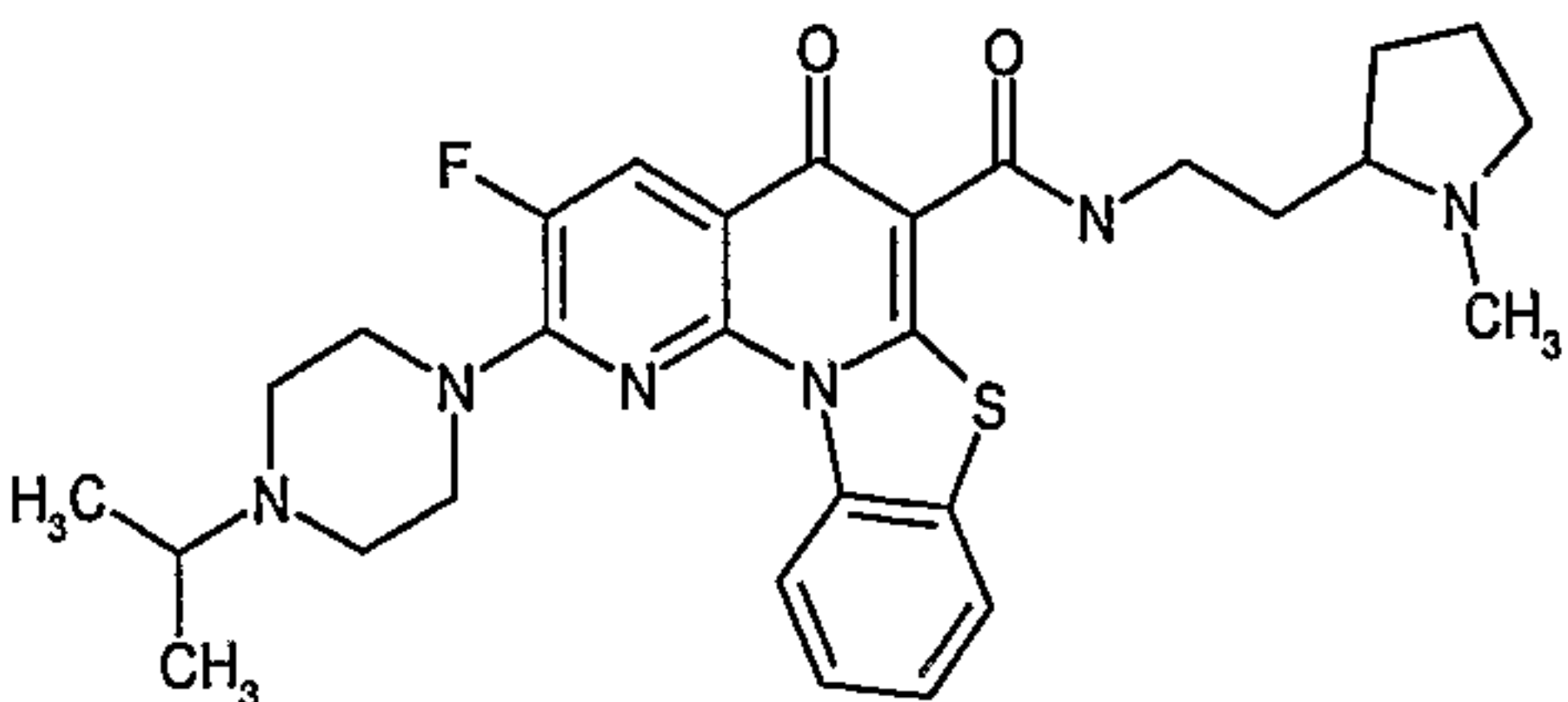
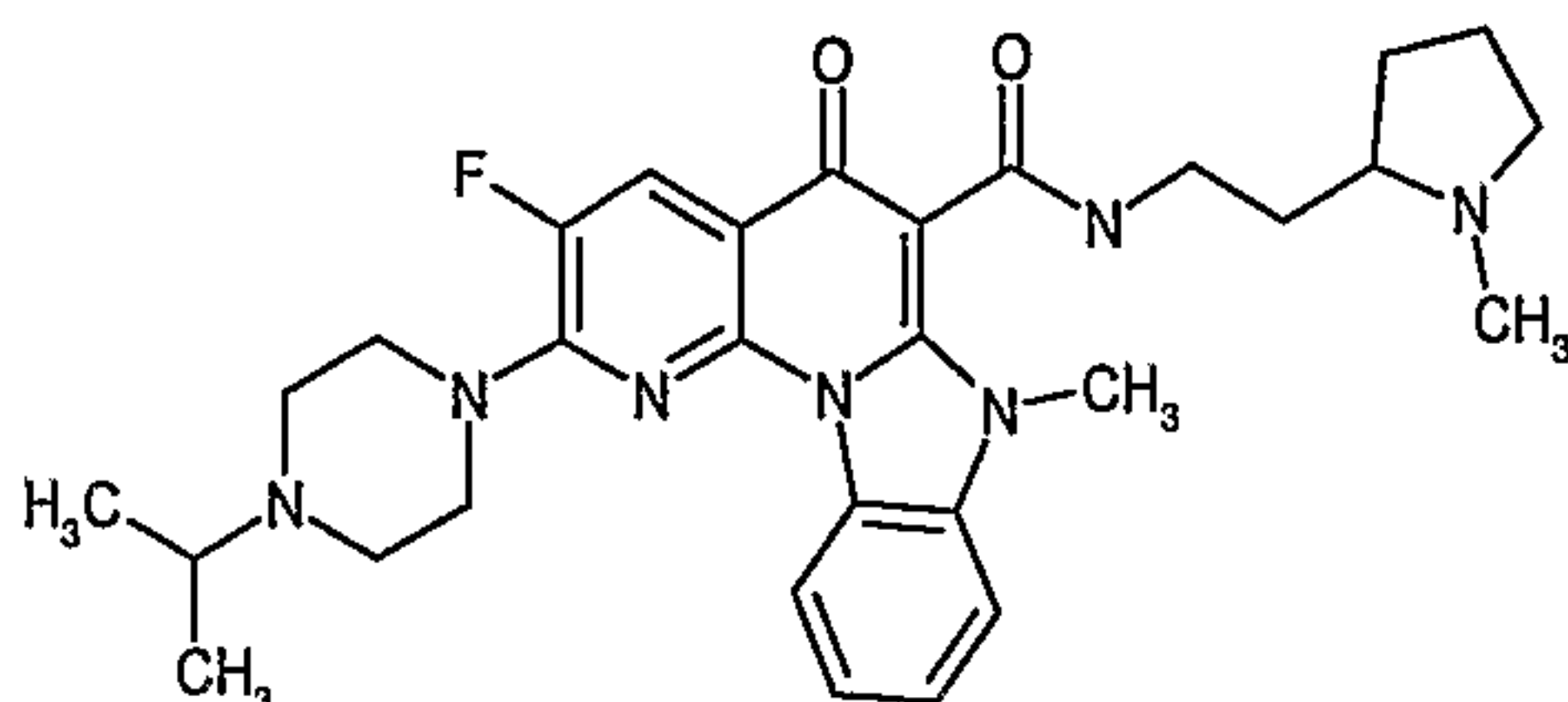
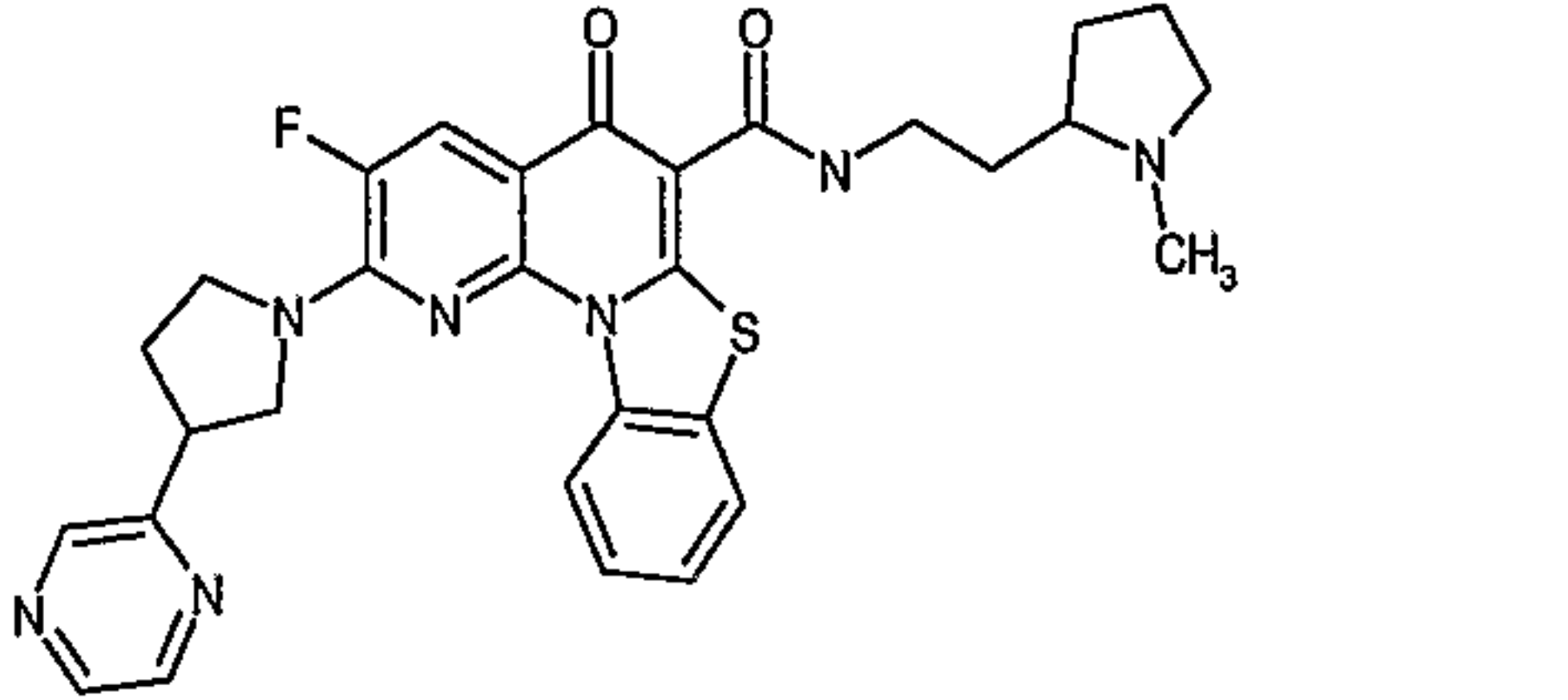
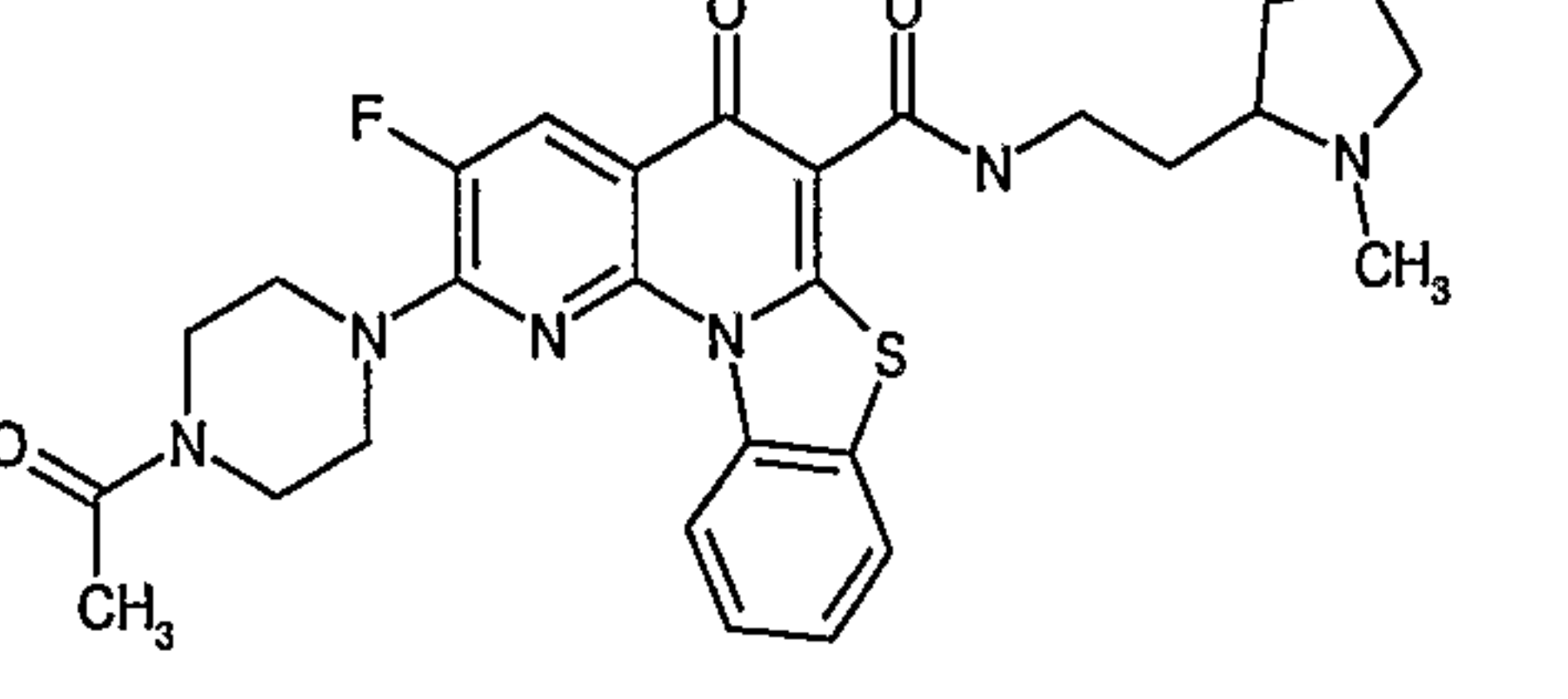
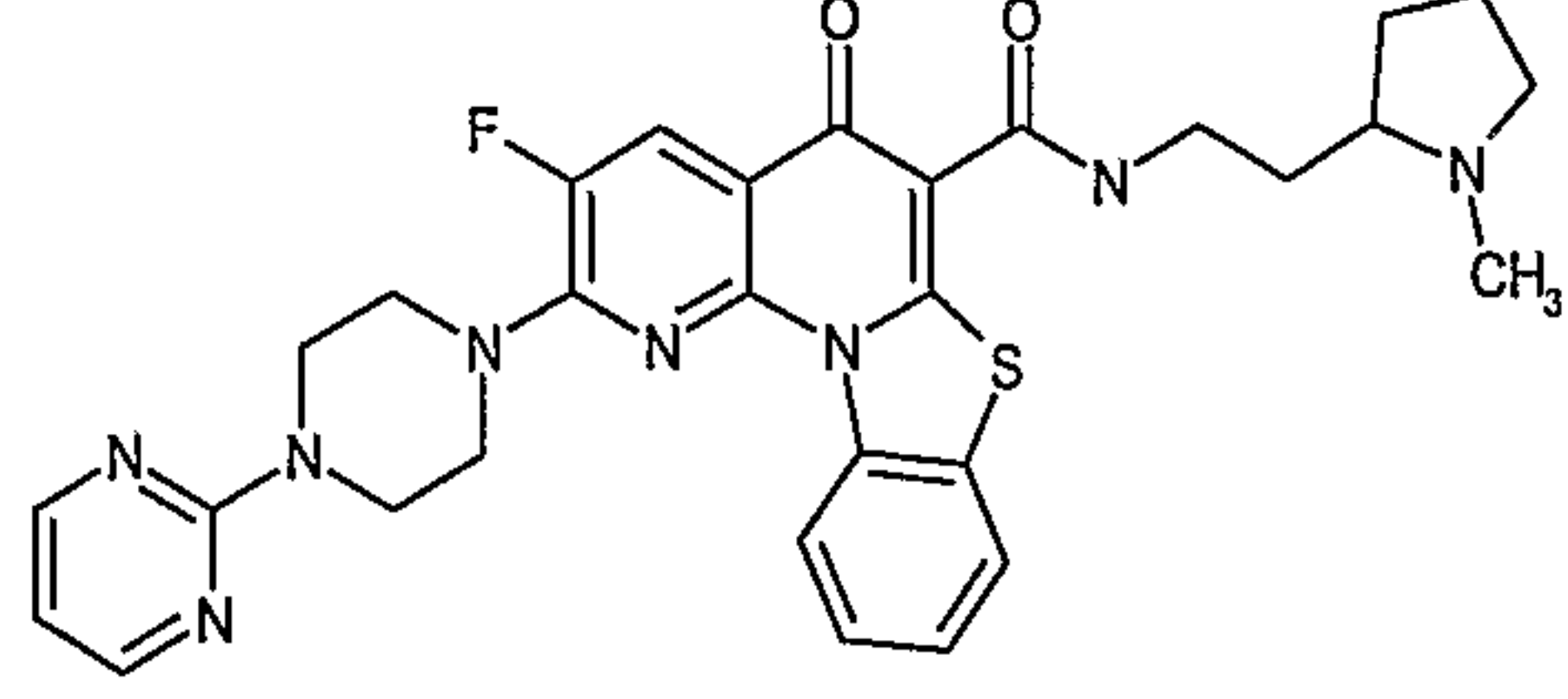
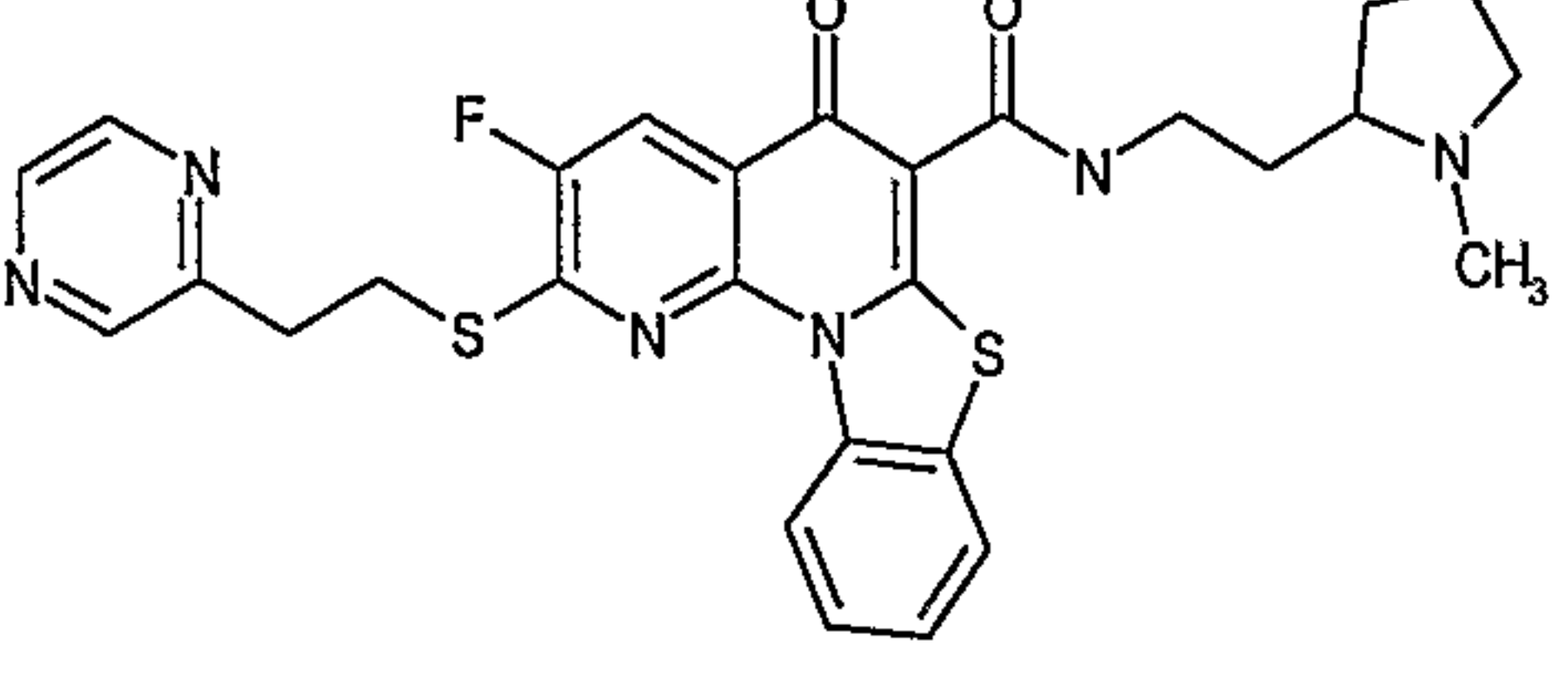
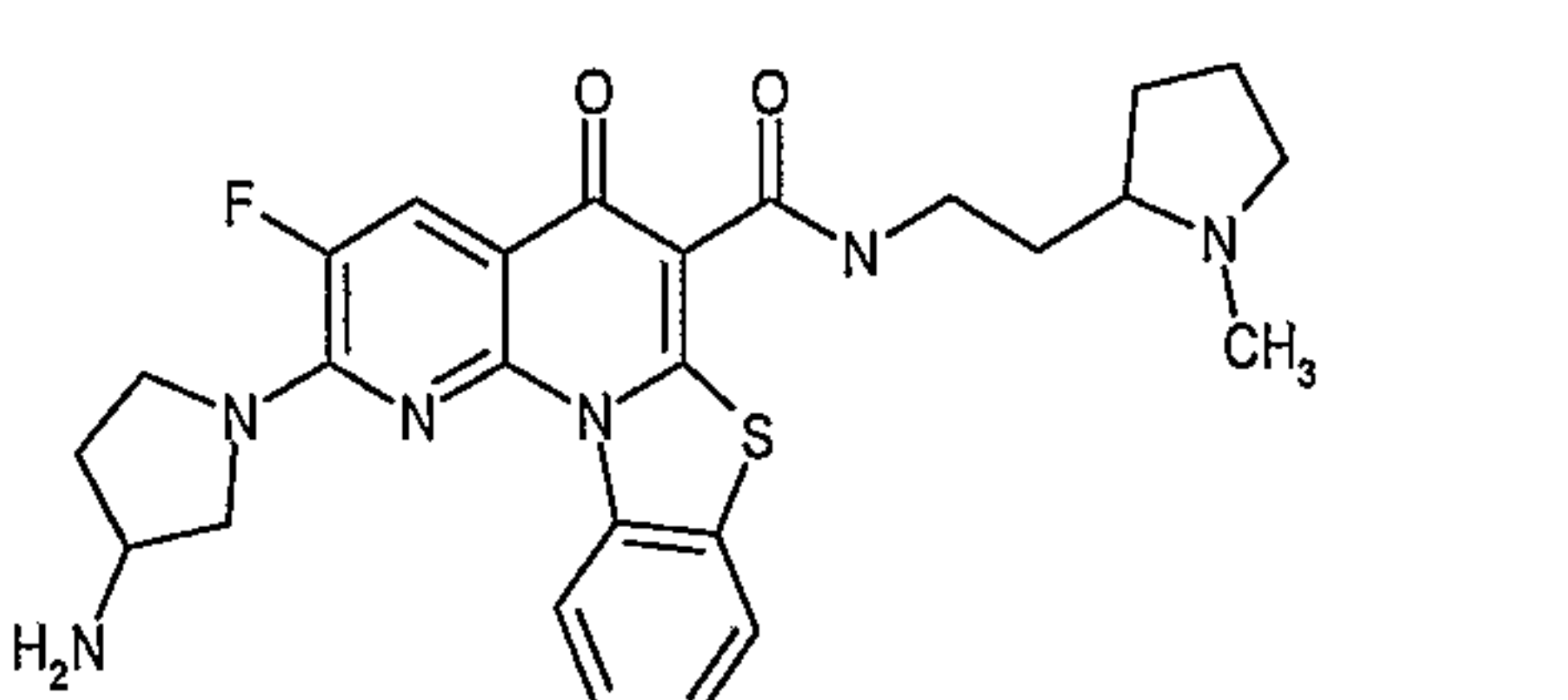
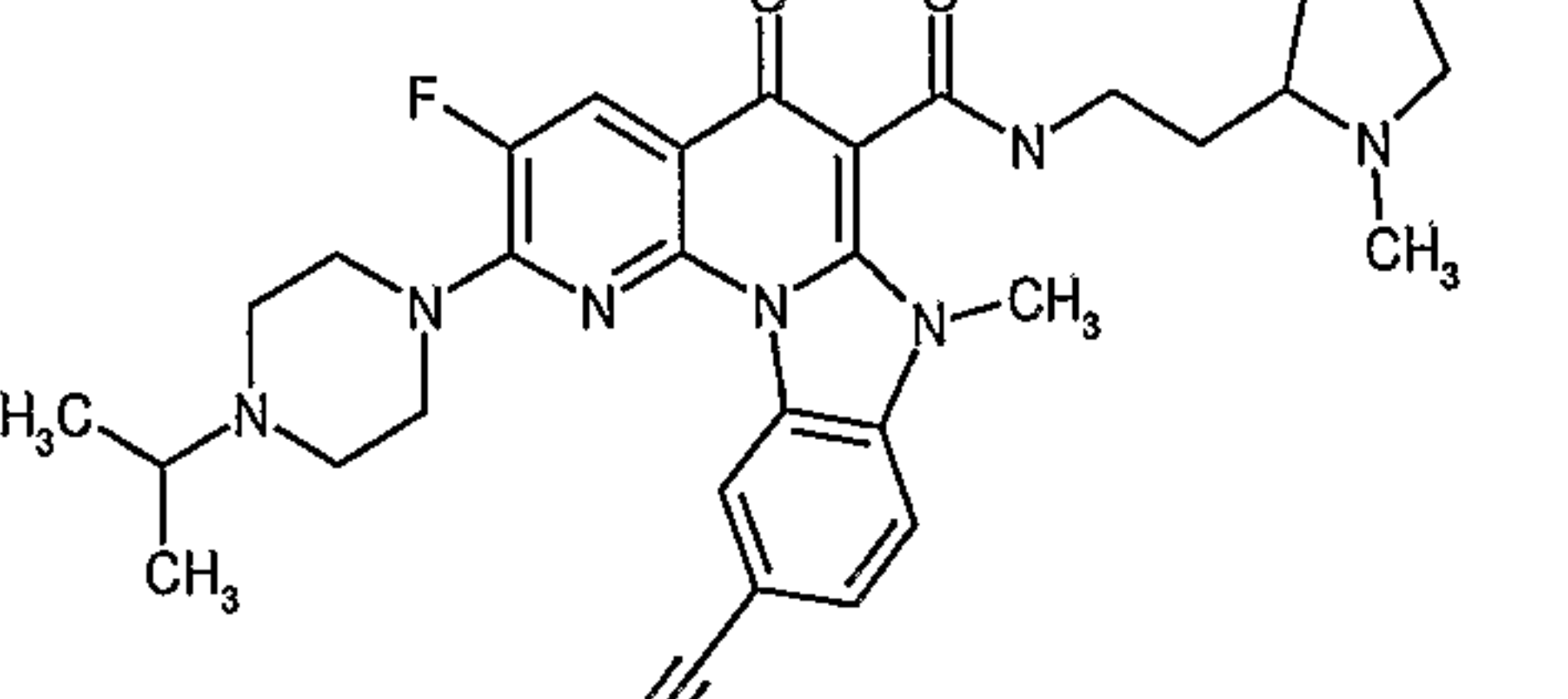
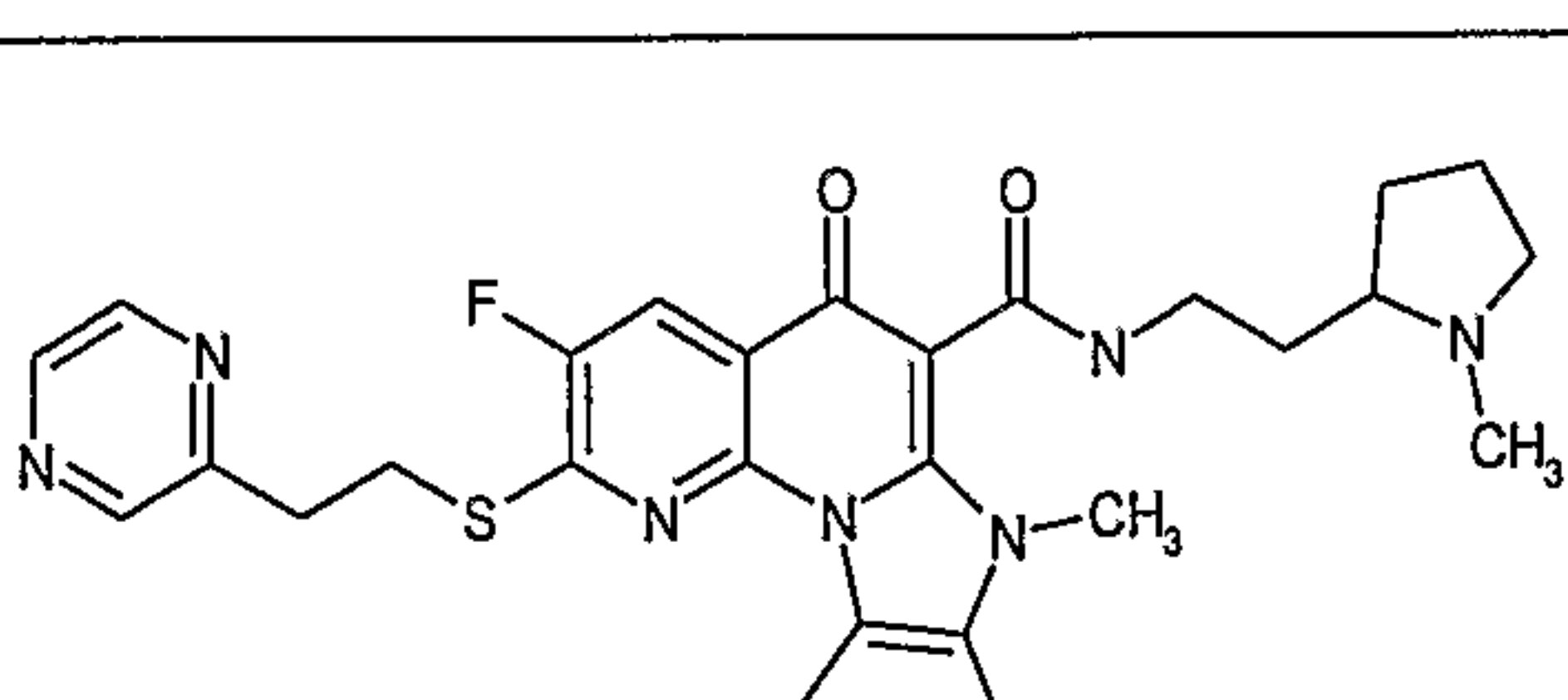
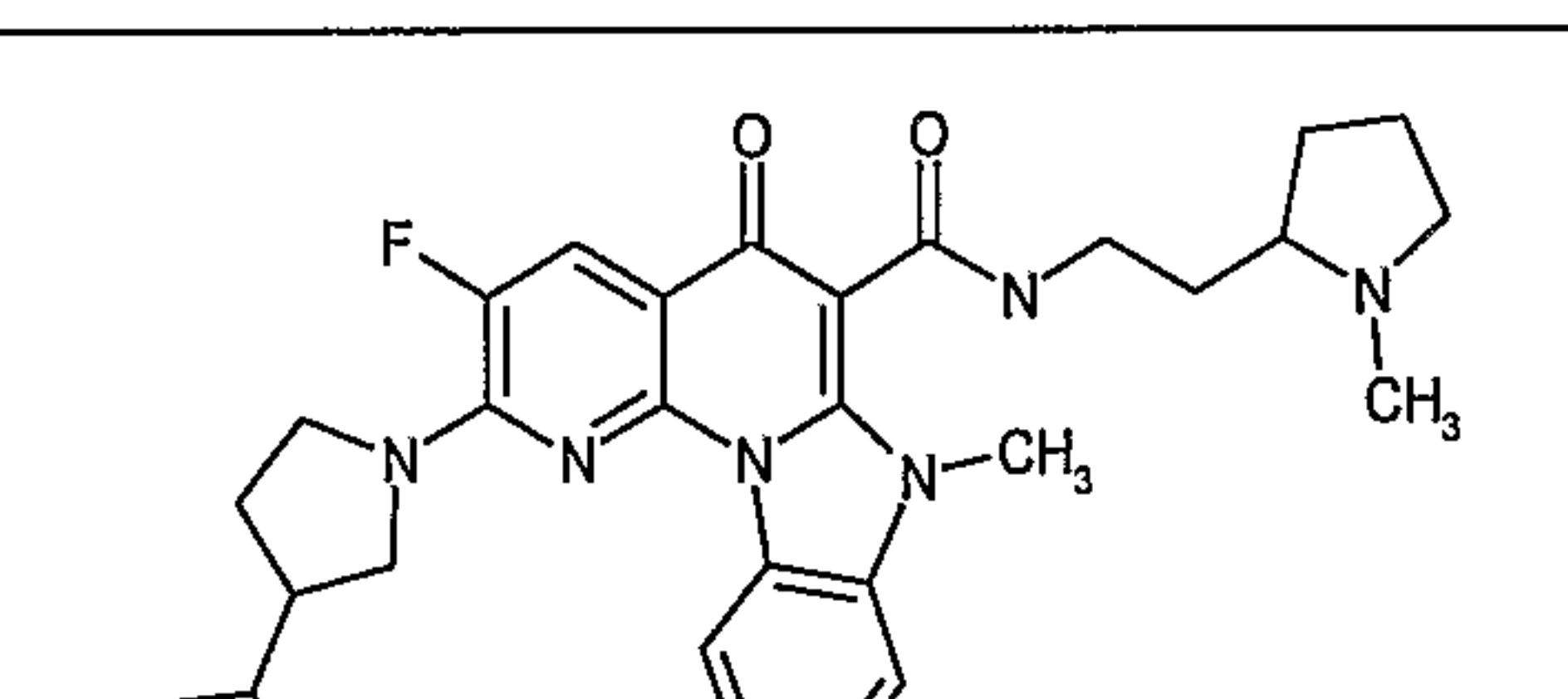
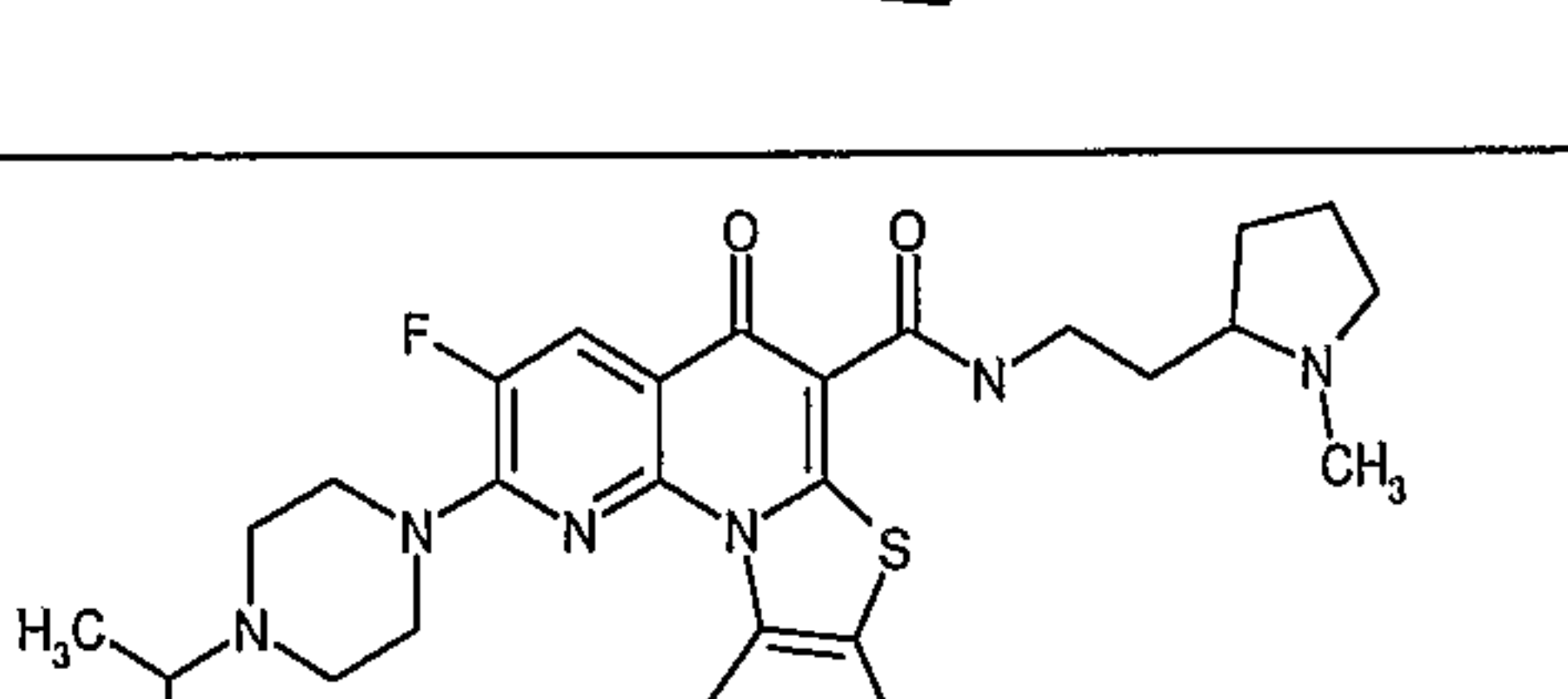
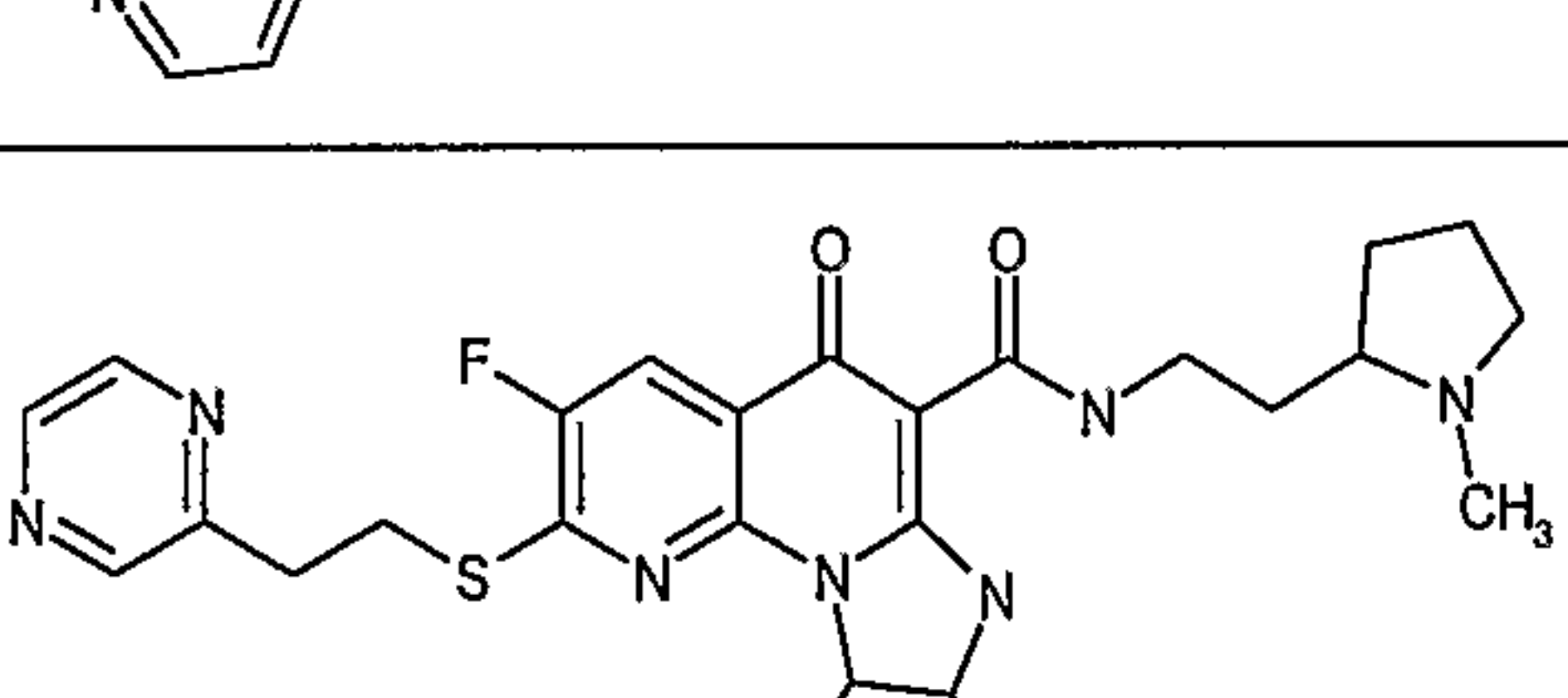
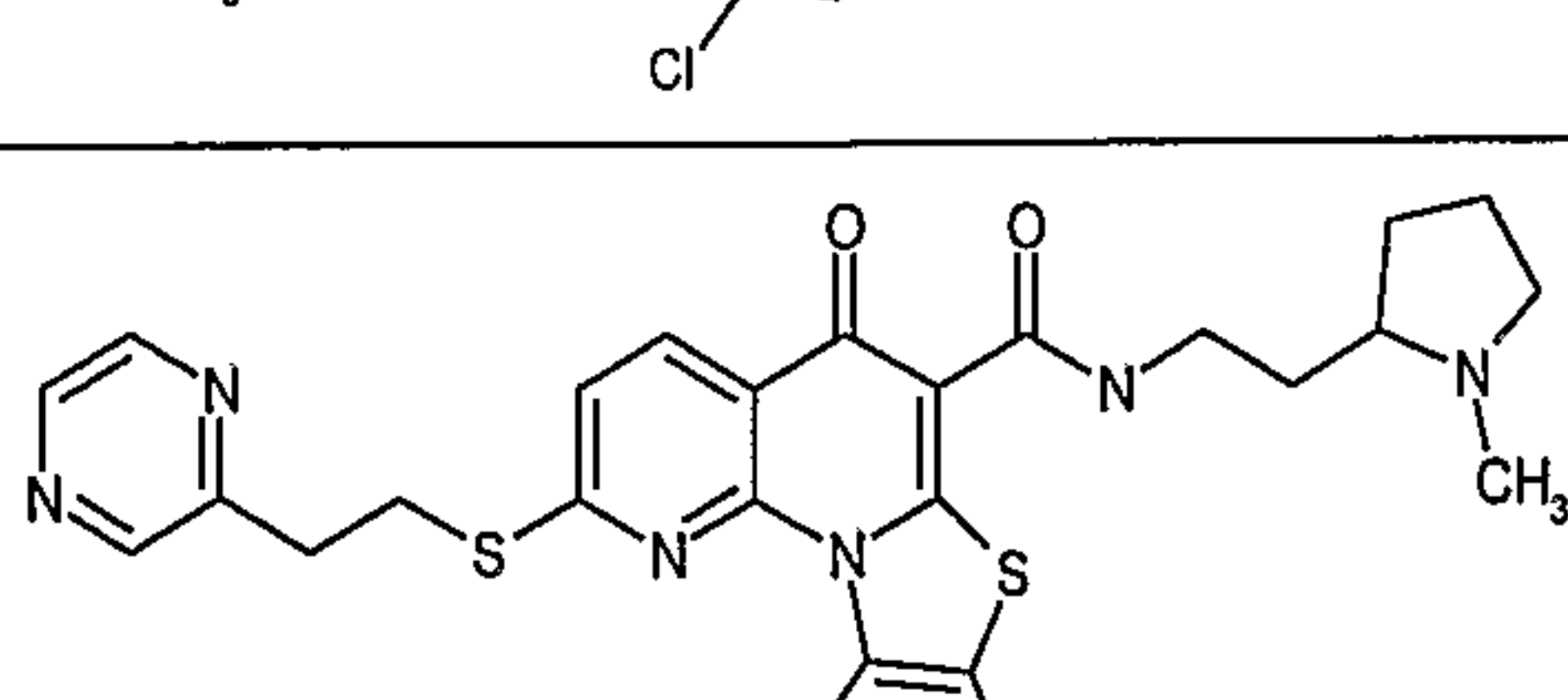
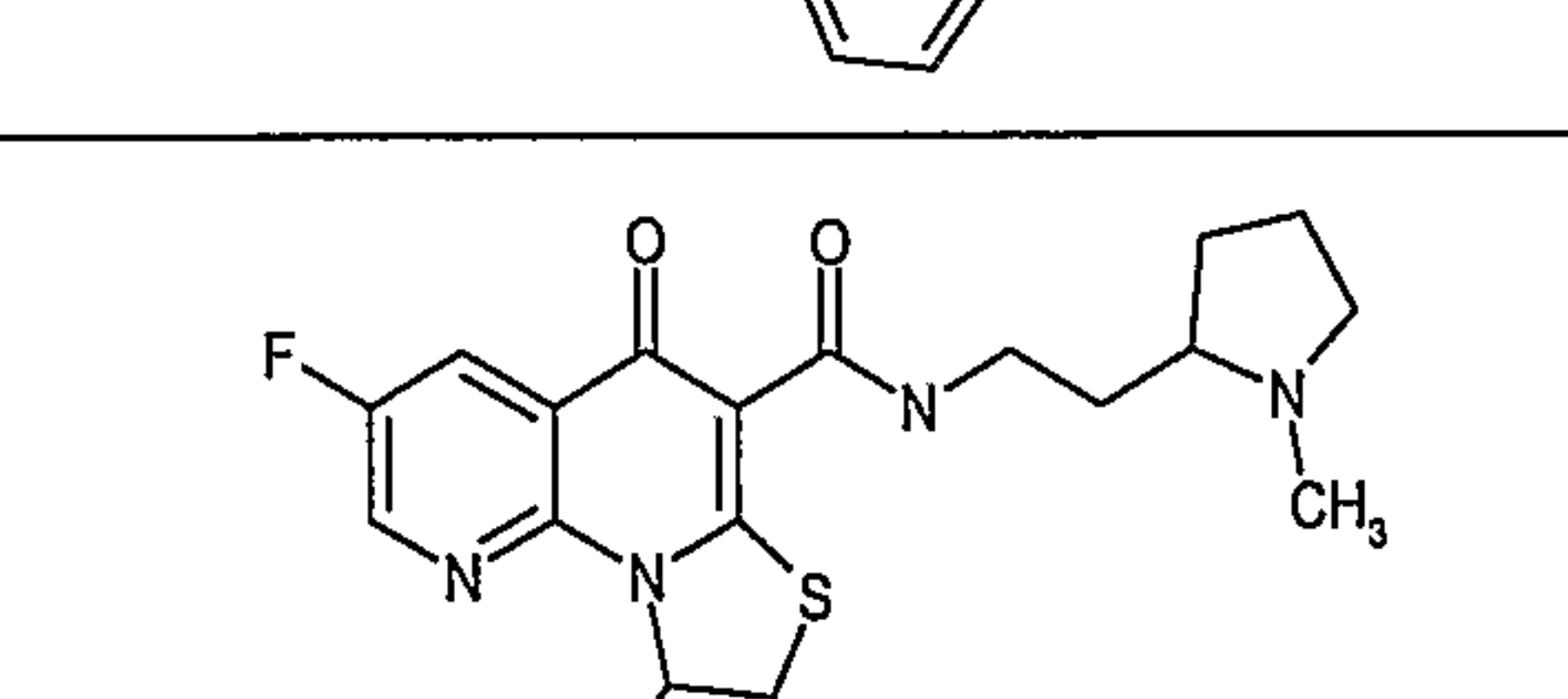
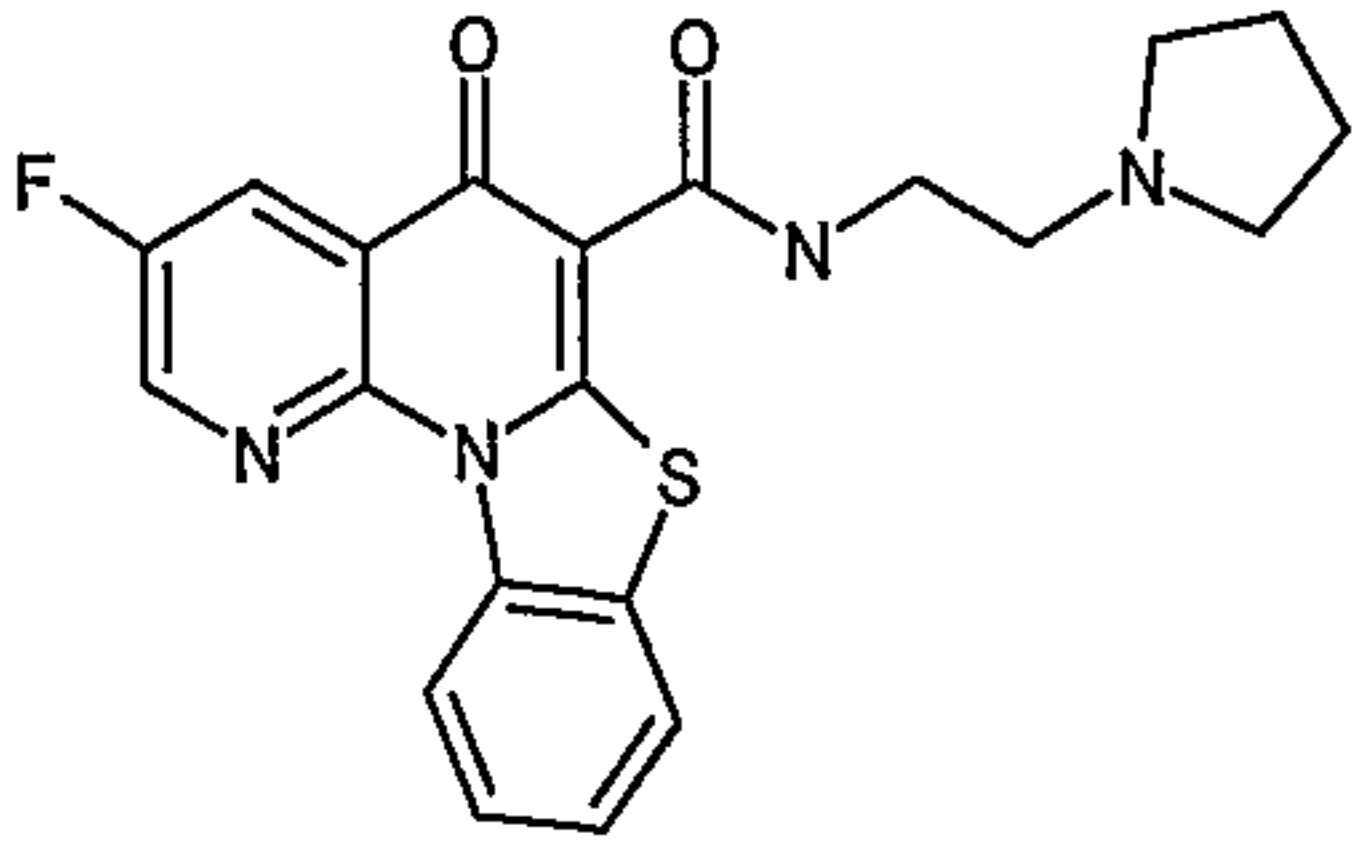
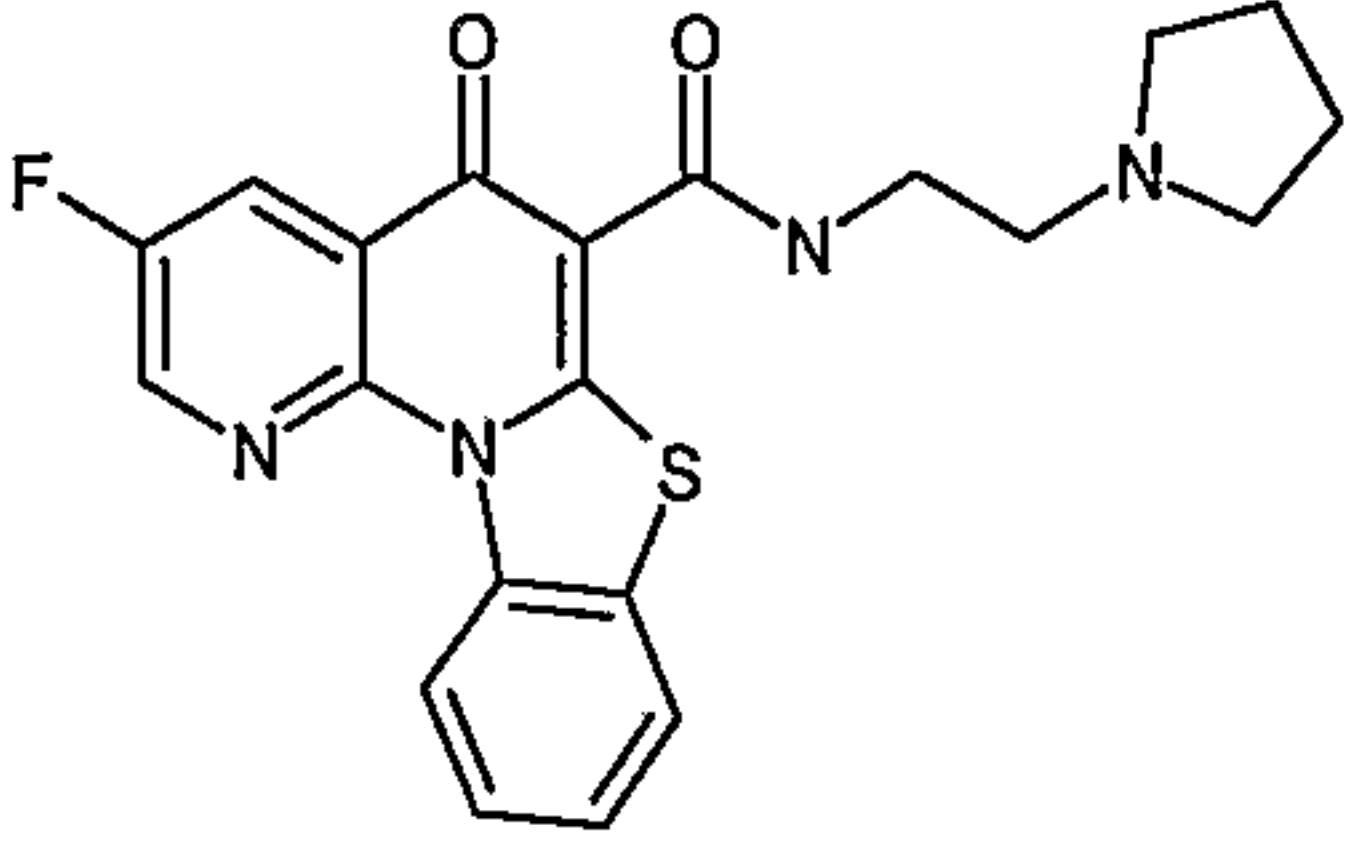
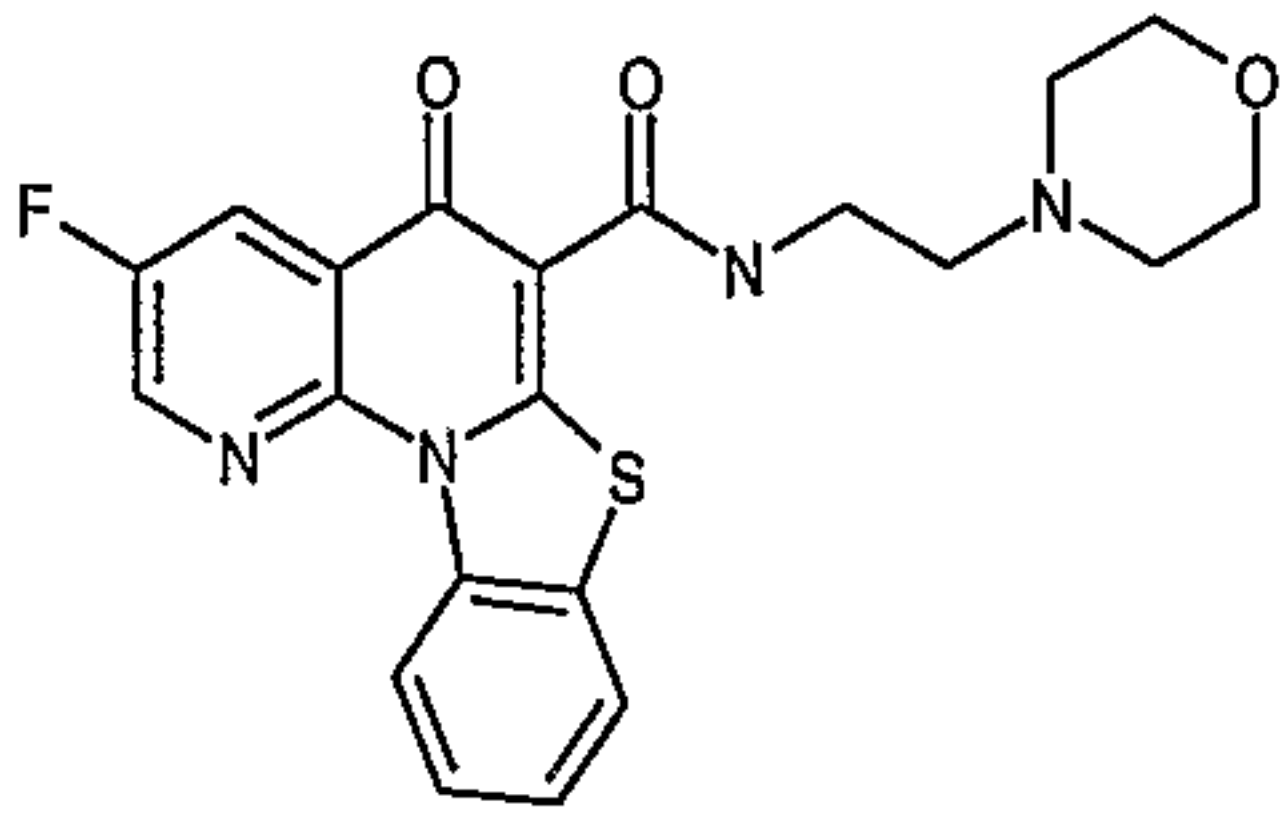
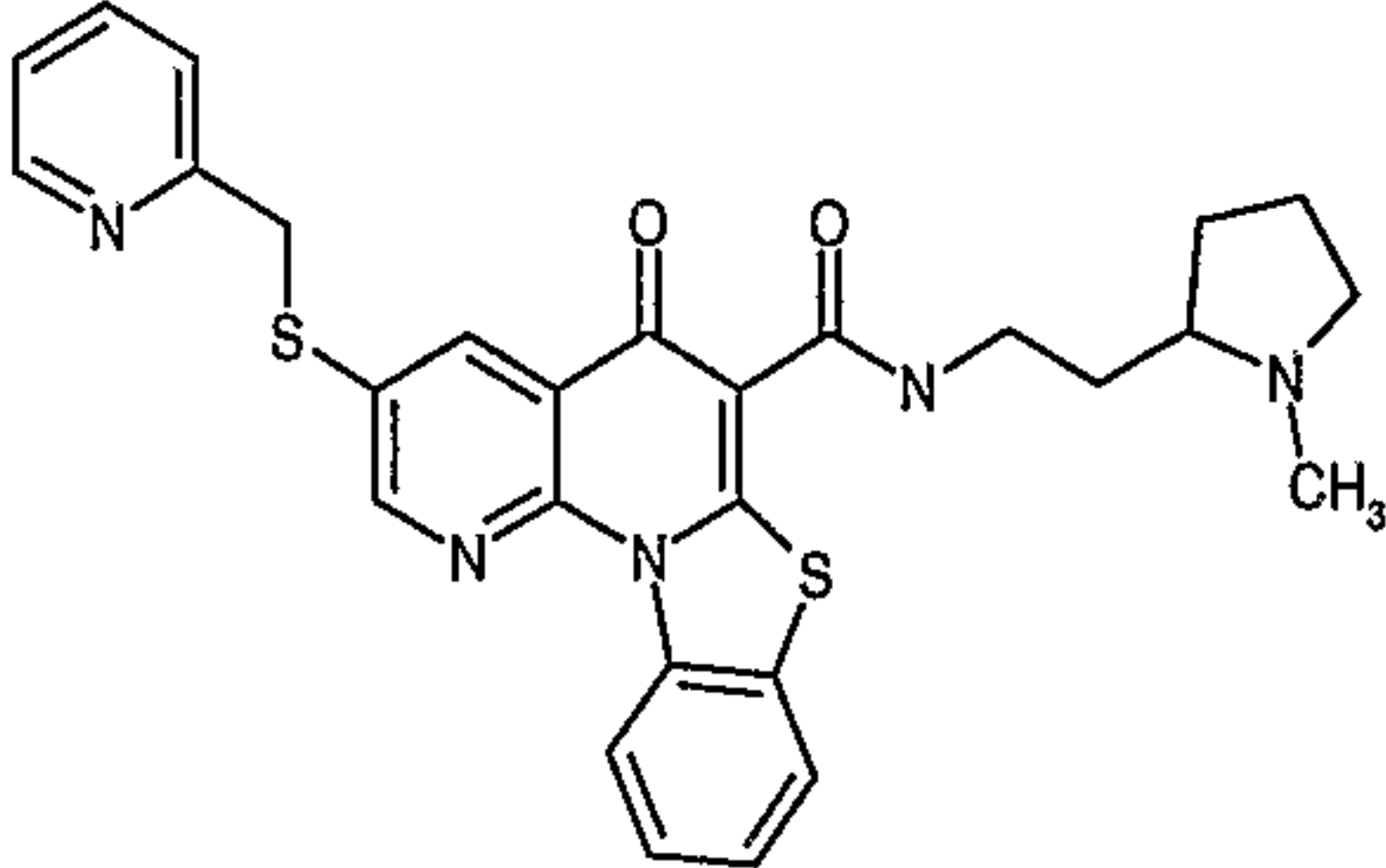
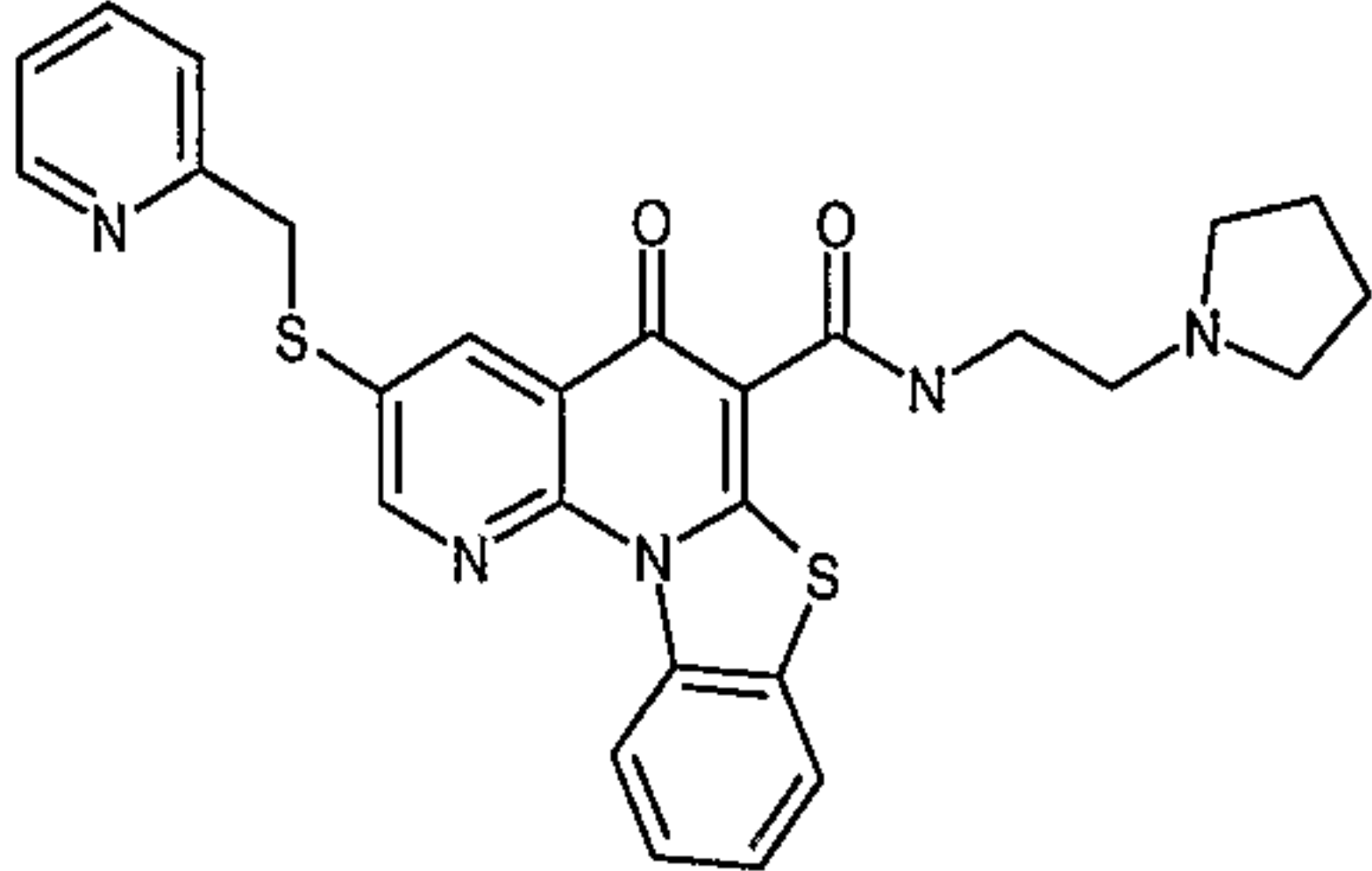
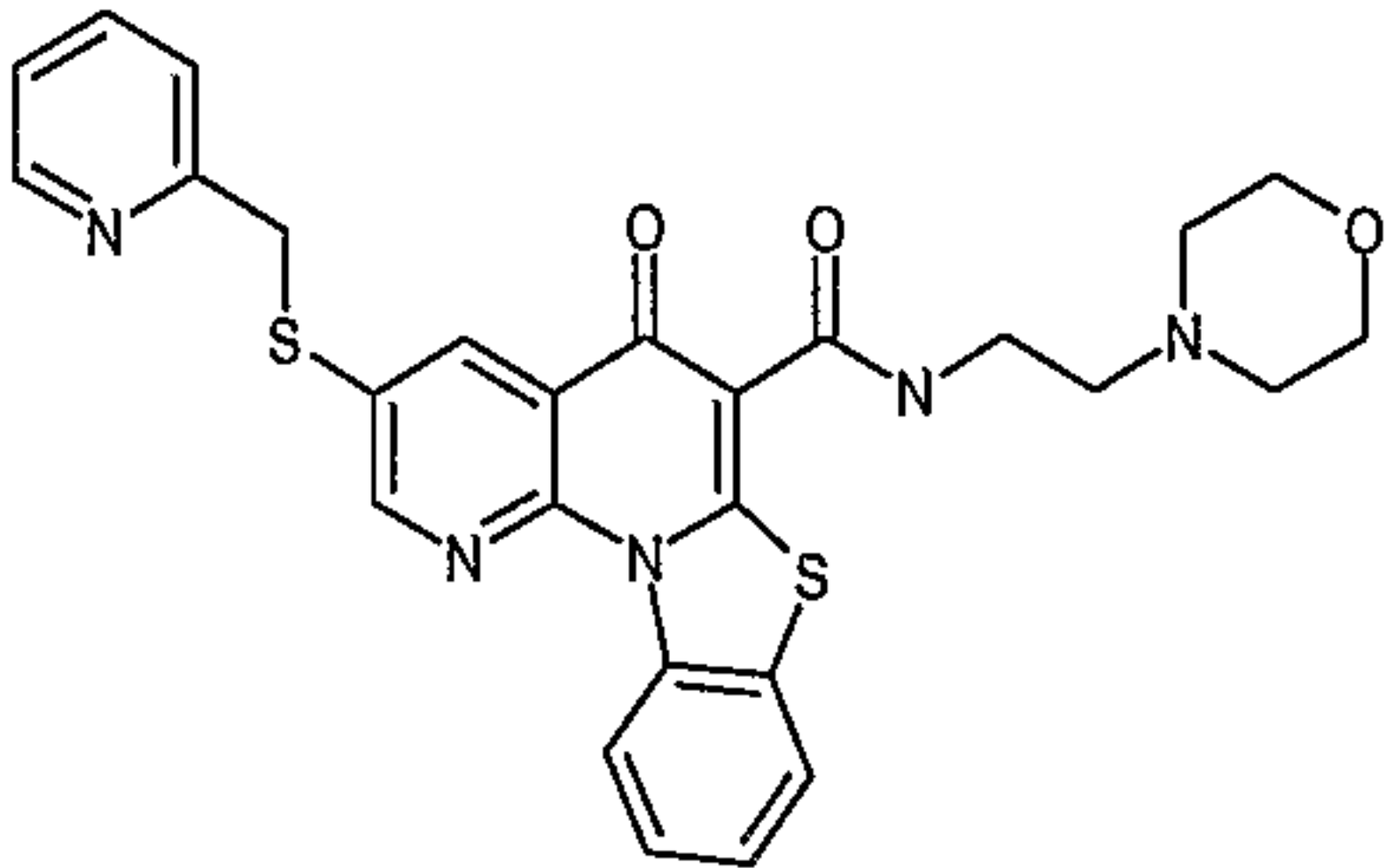
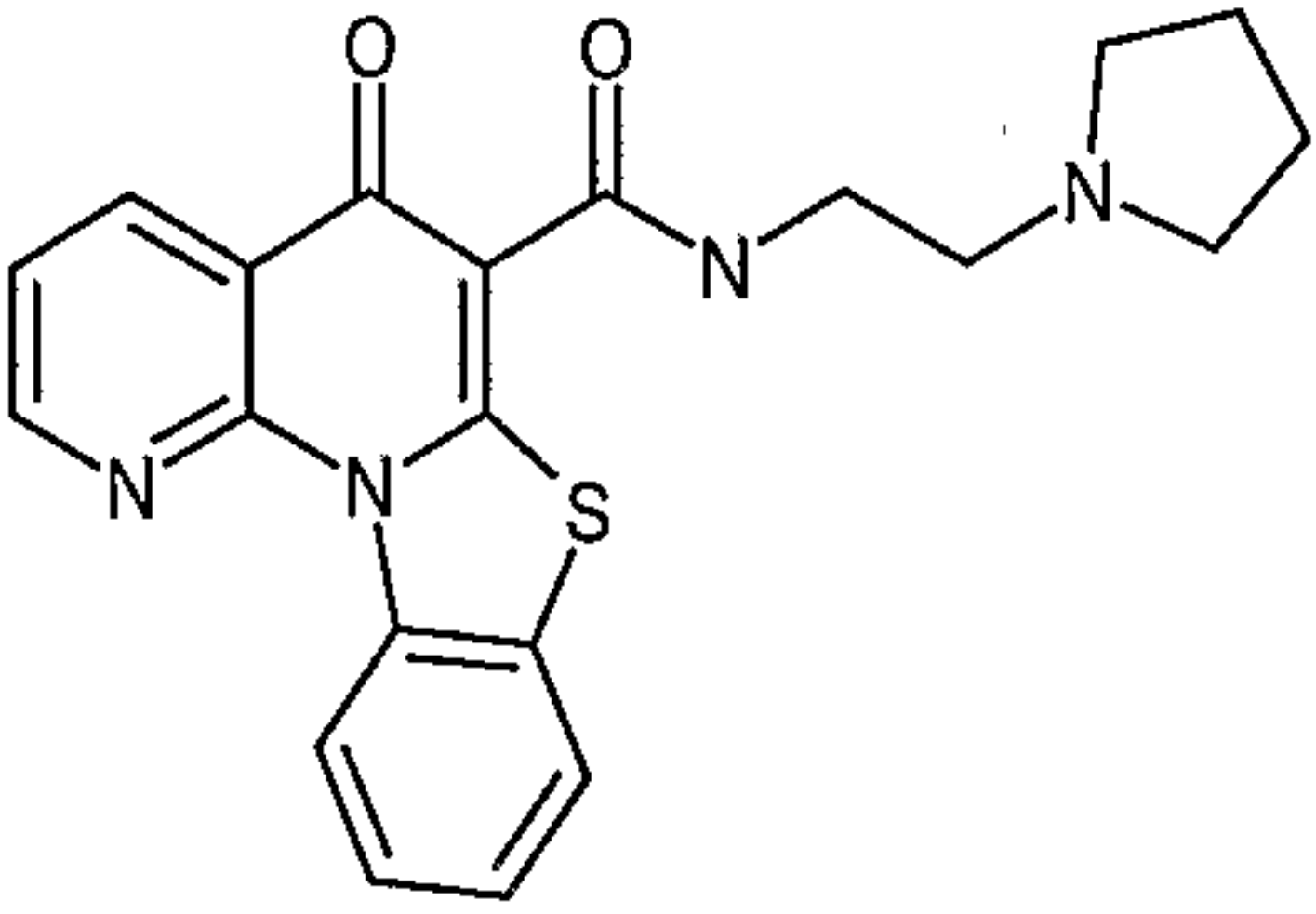
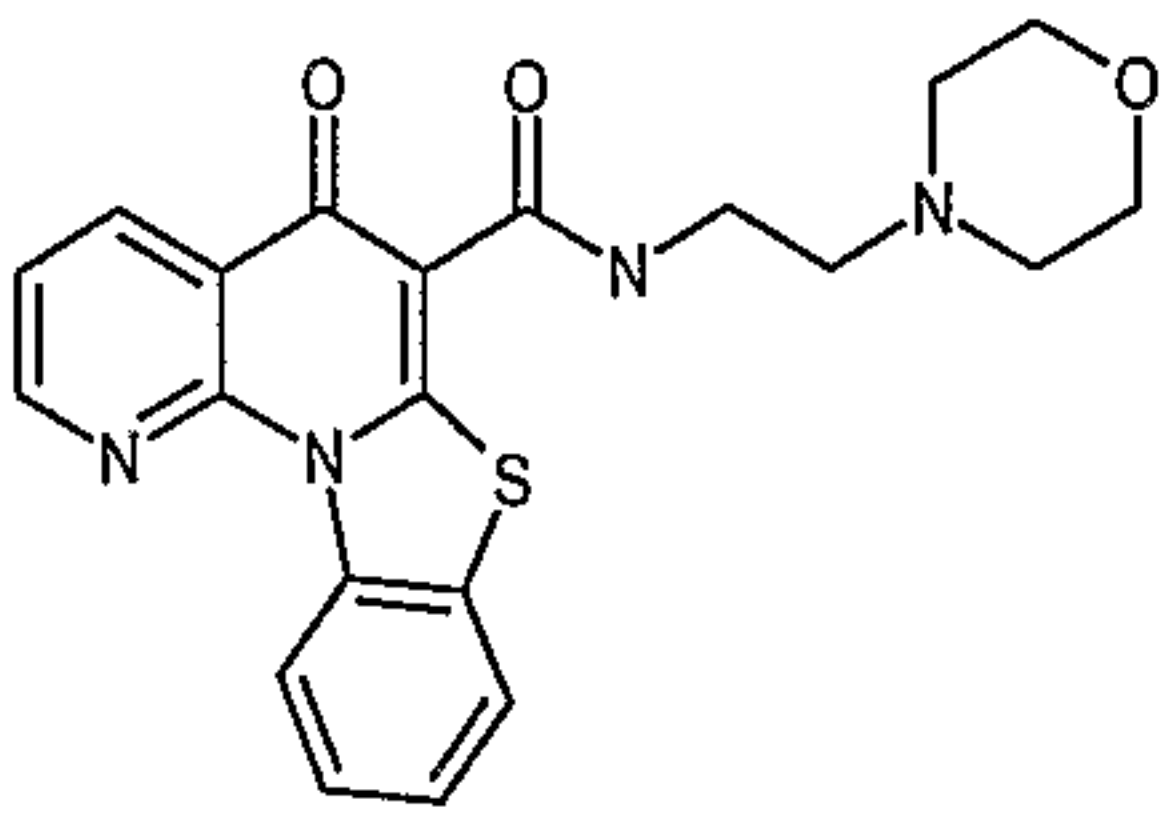
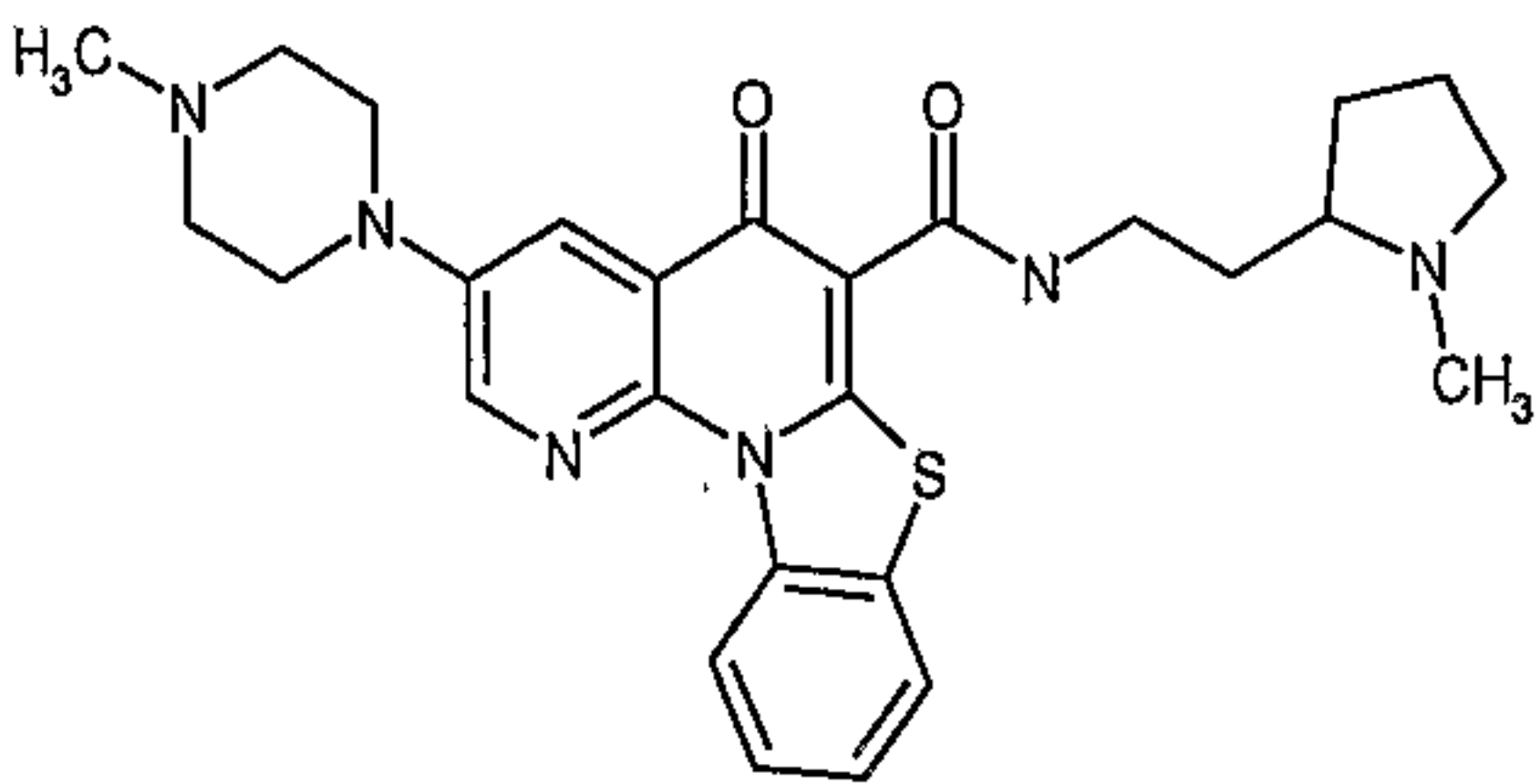
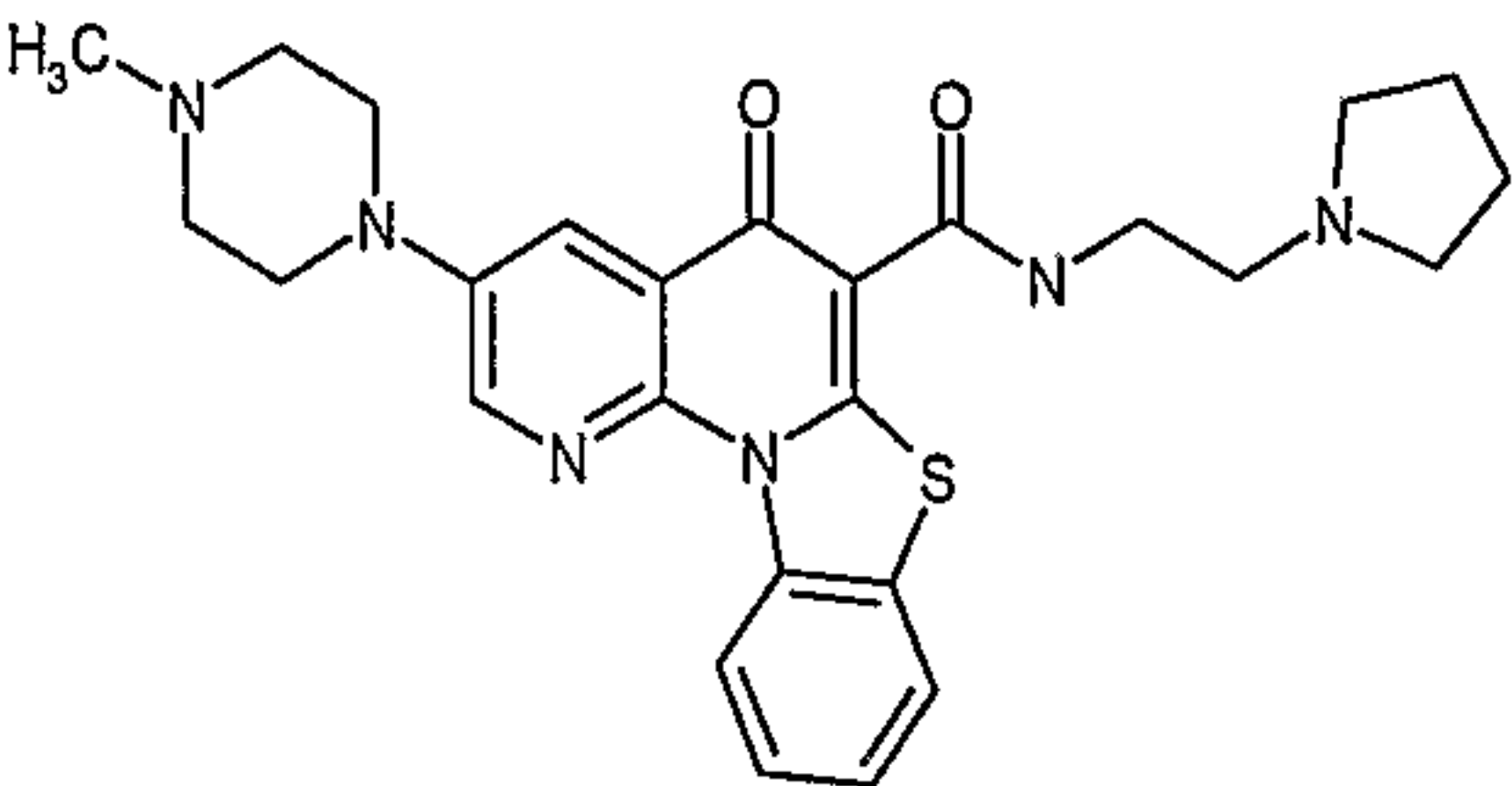
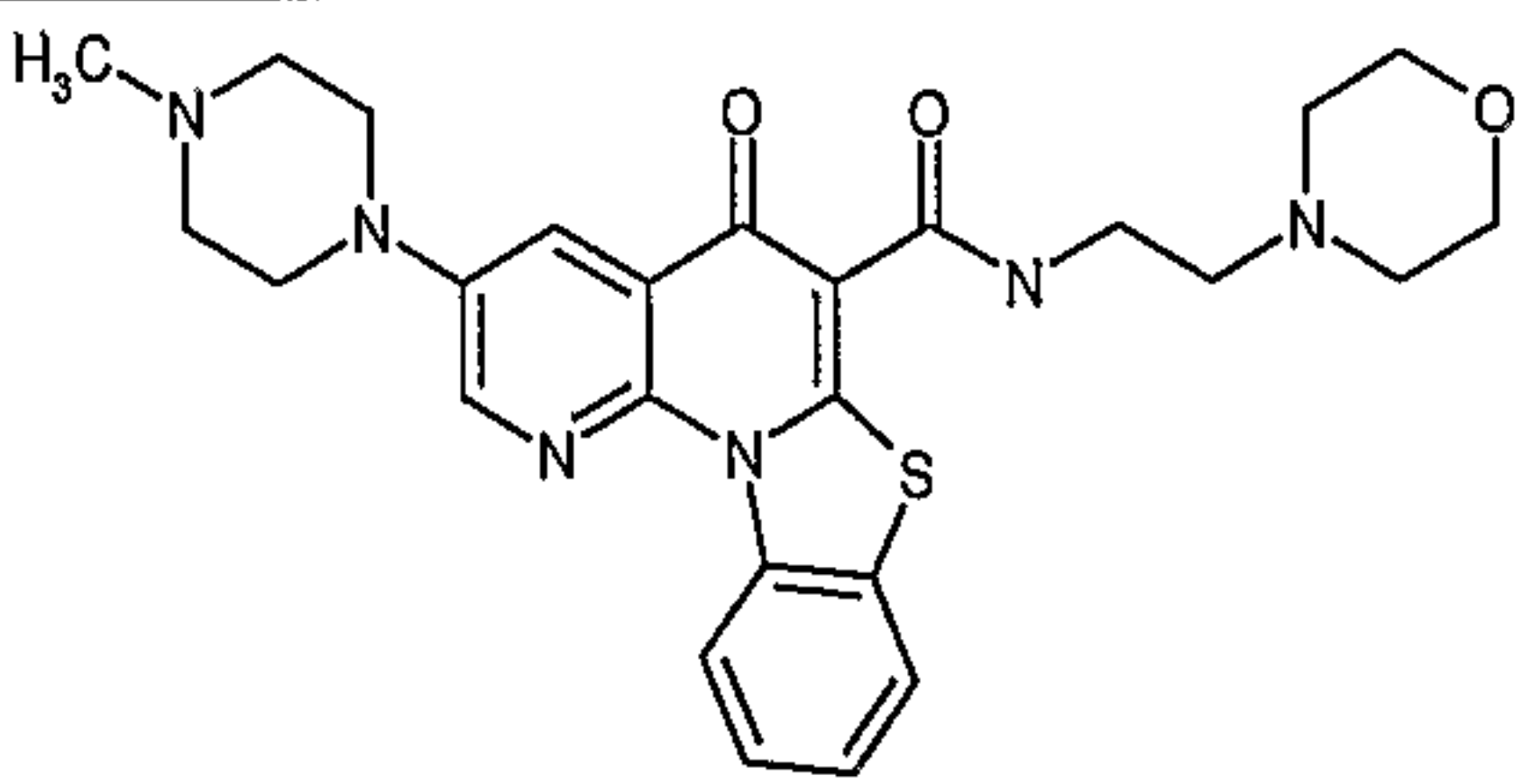
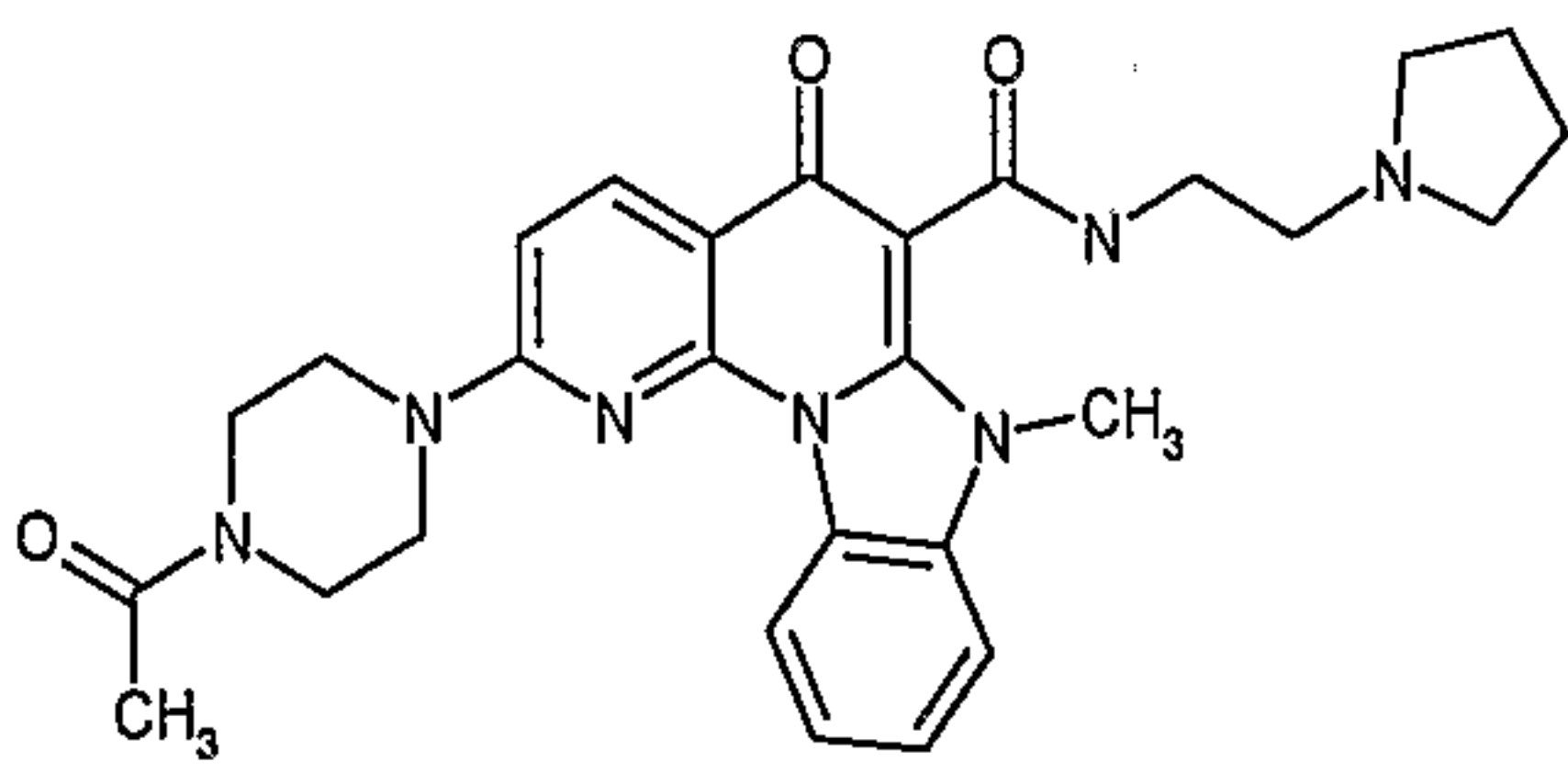
| MOLSTRUCTURE                                                                         | STOP $\mu$ M | HCT-116 MTS $\mu$ M |
|--------------------------------------------------------------------------------------|--------------|---------------------|
|     | 10.5         | 0.7                 |
|     | 8.2          | 0.1                 |
|   | 15           | 2.3                 |
|  | 15           | 0.3                 |
|   | 15           | 0.3                 |
|  | 15           | 0.3                 |
|  | > 15         |                     |

Table 1B

| MOLSTRUCTURE                                                                         | MOLSTRUCTURE                                                                          |
|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
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| MOLSTRUCTURE                                                                        | MOLSTRUCTURE                                                                          |
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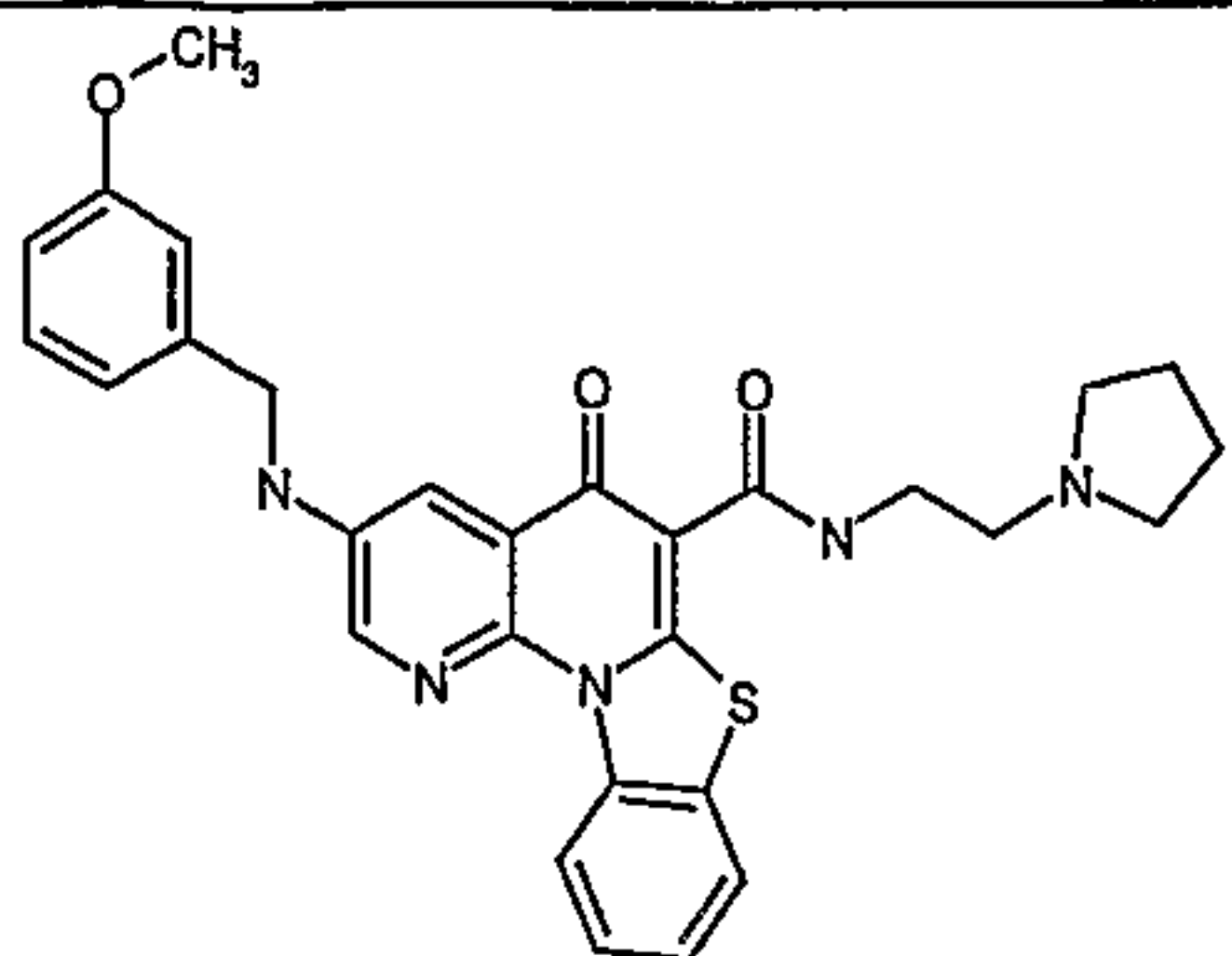
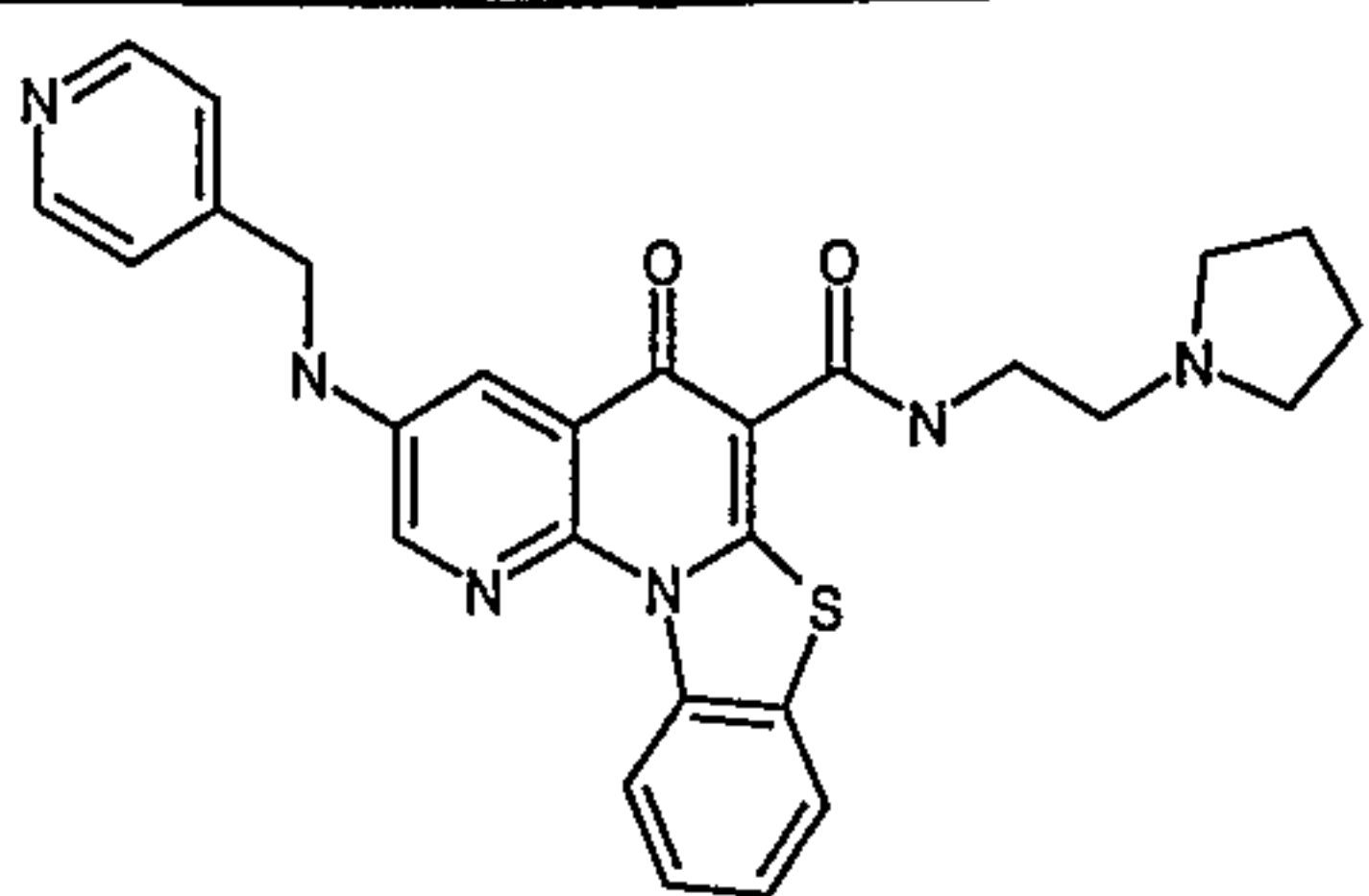
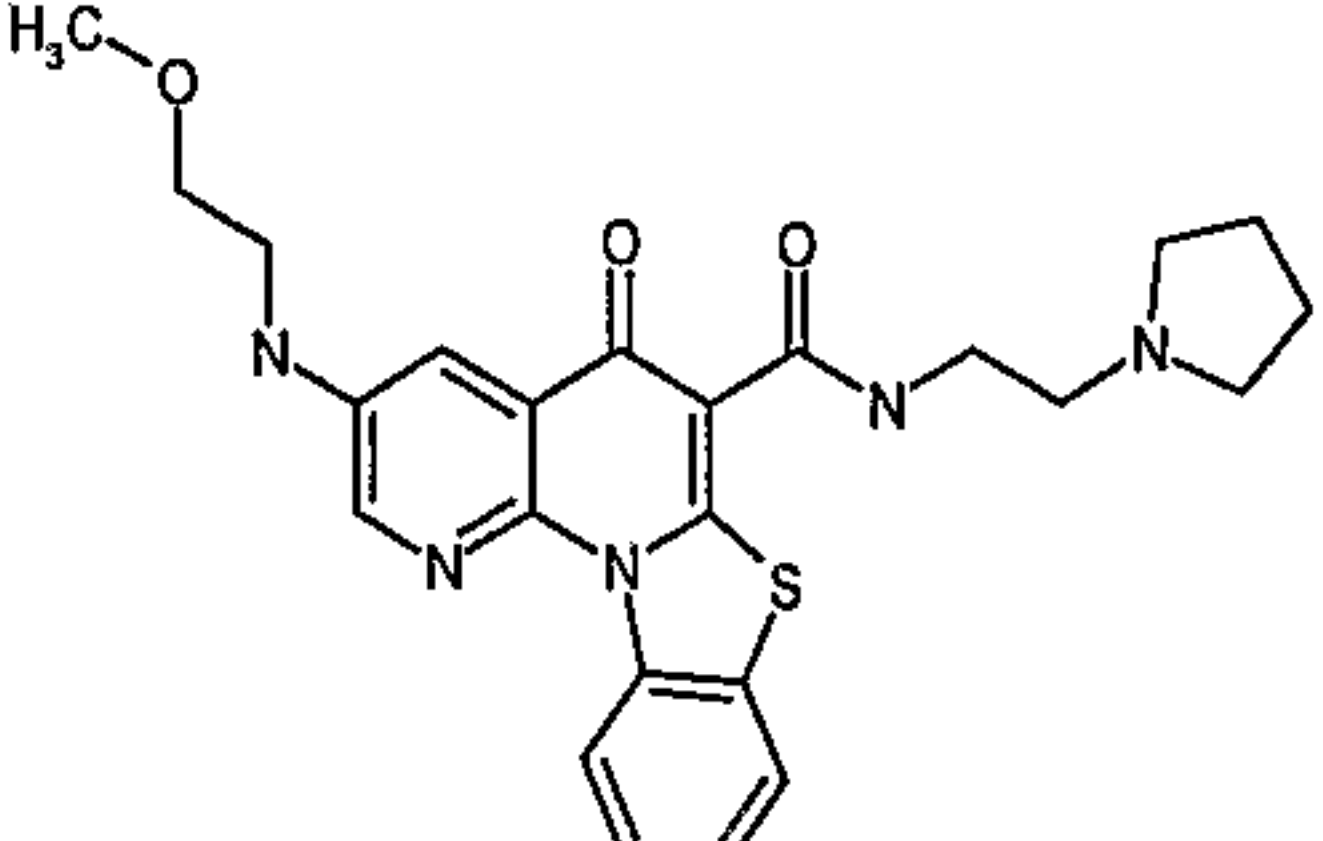
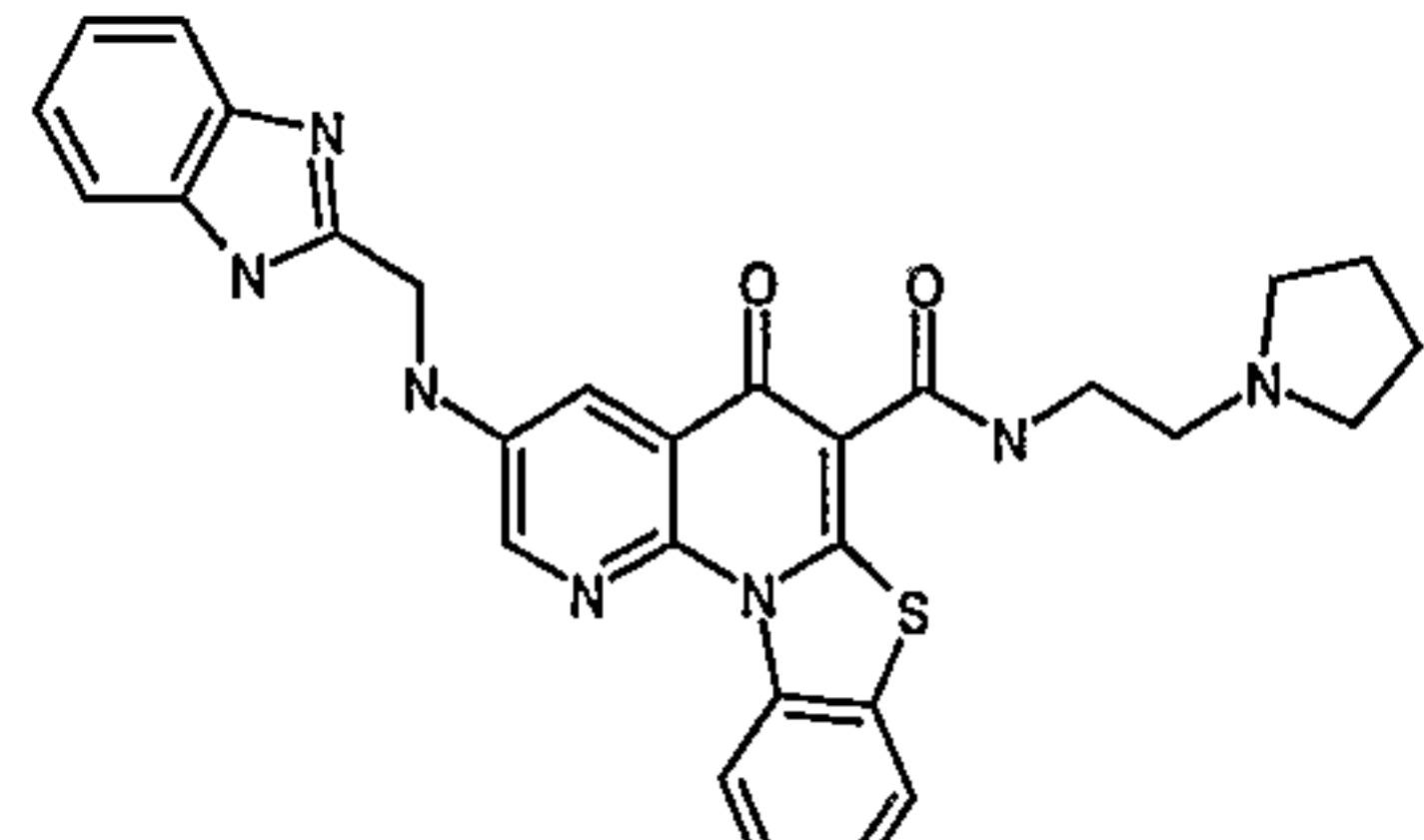
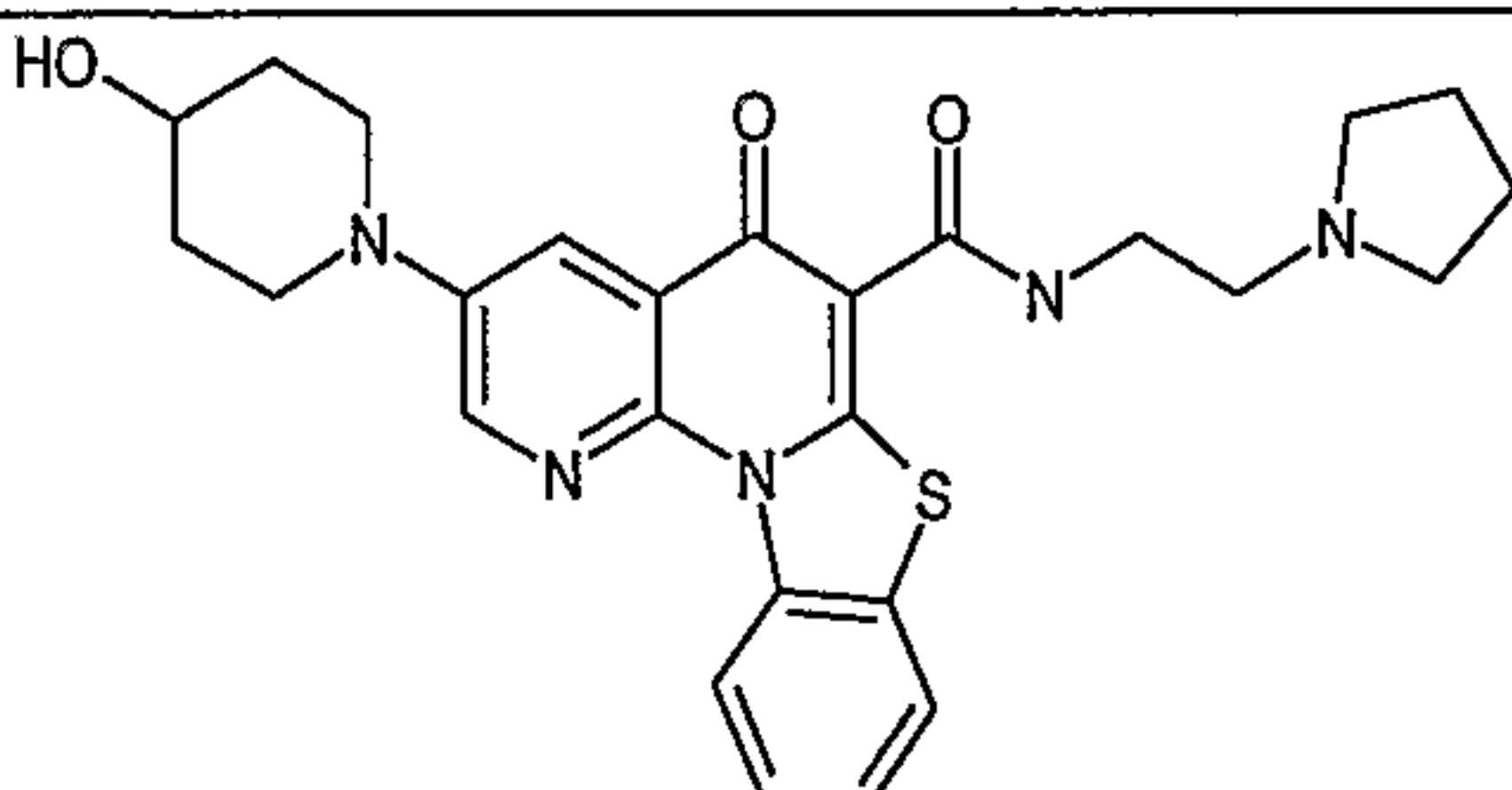
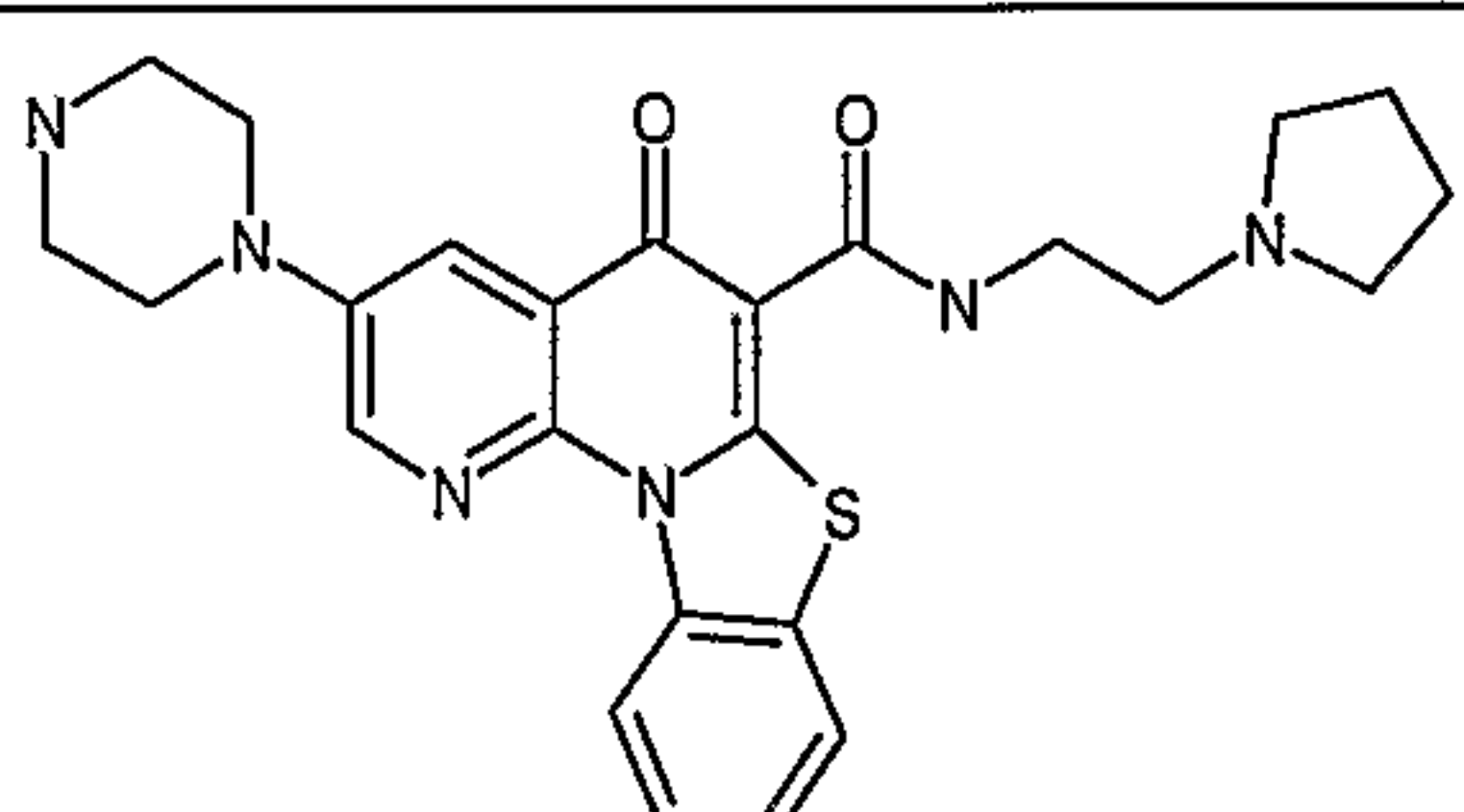
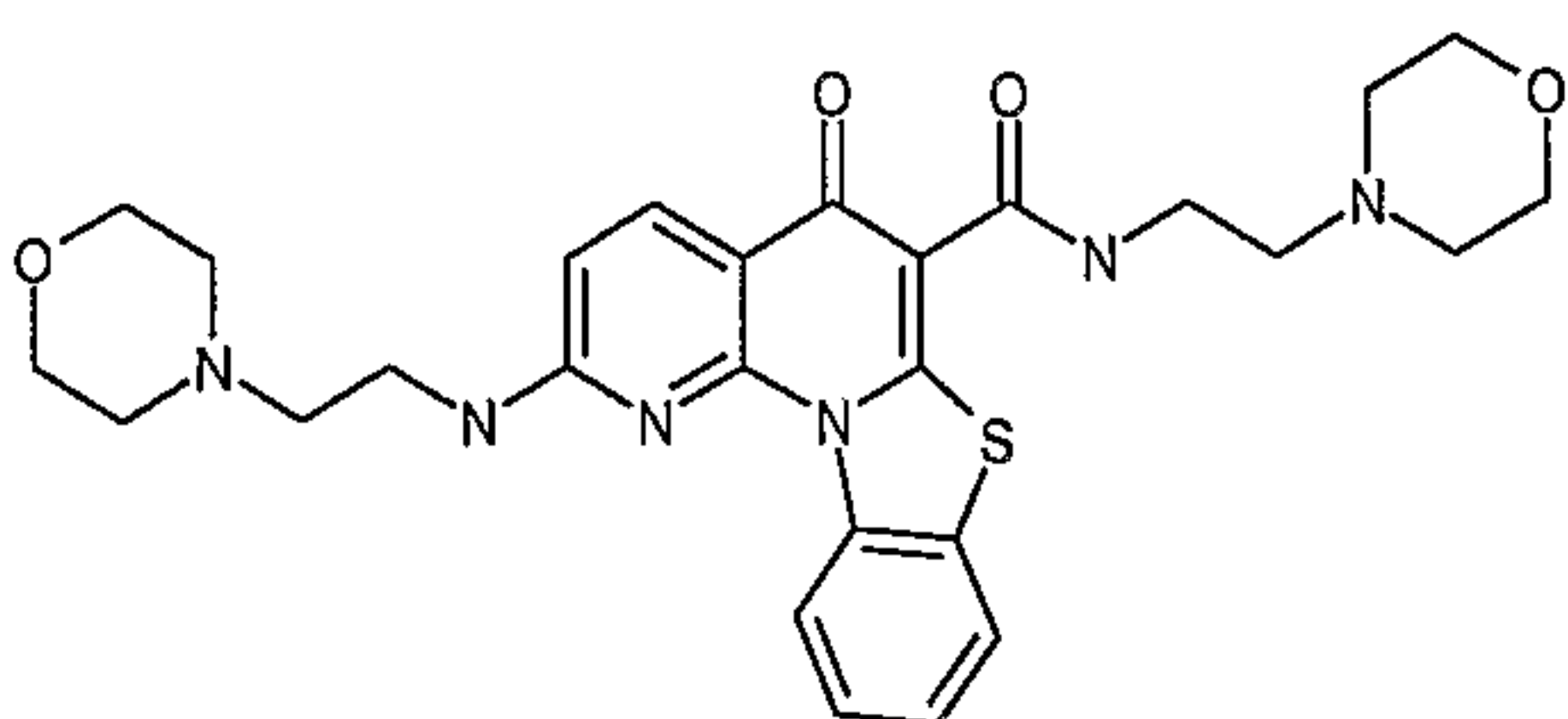
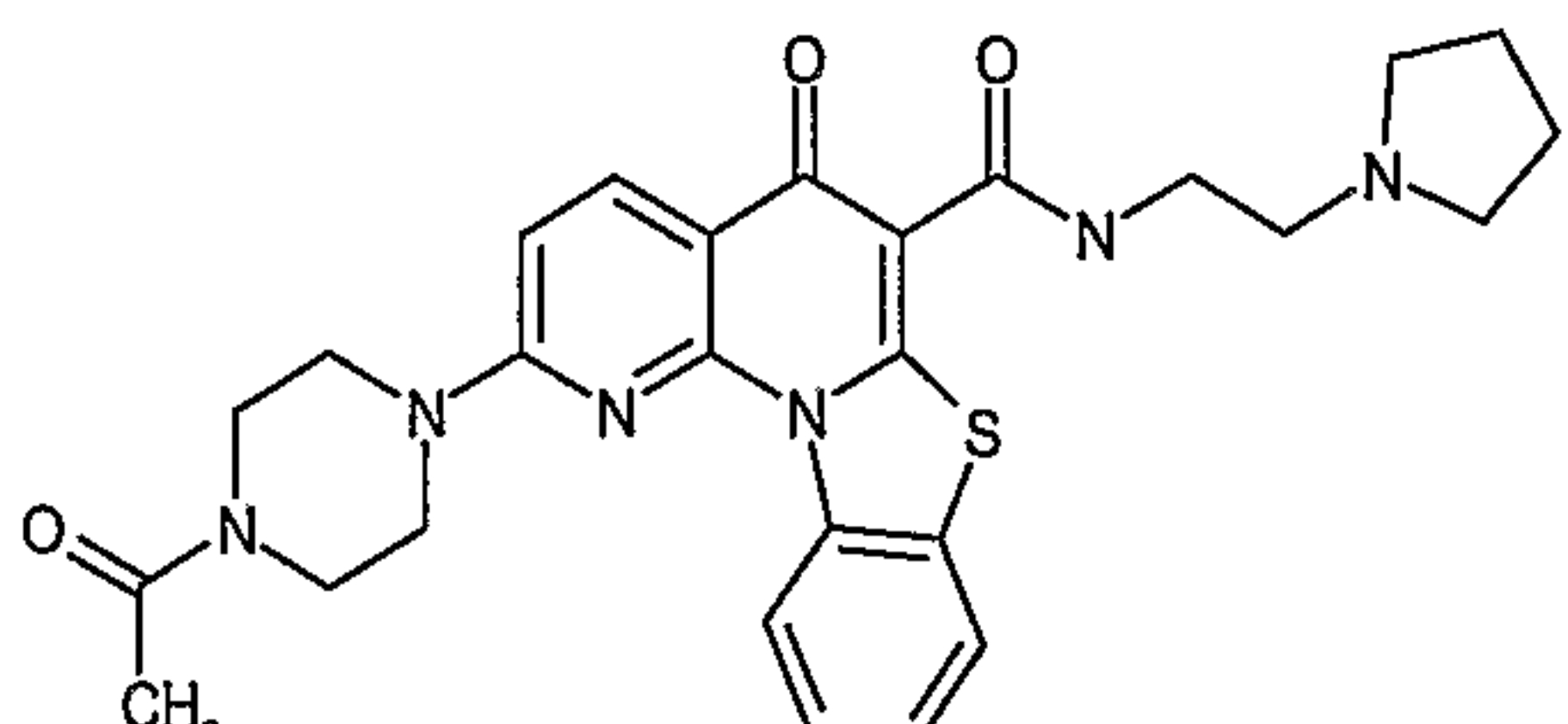
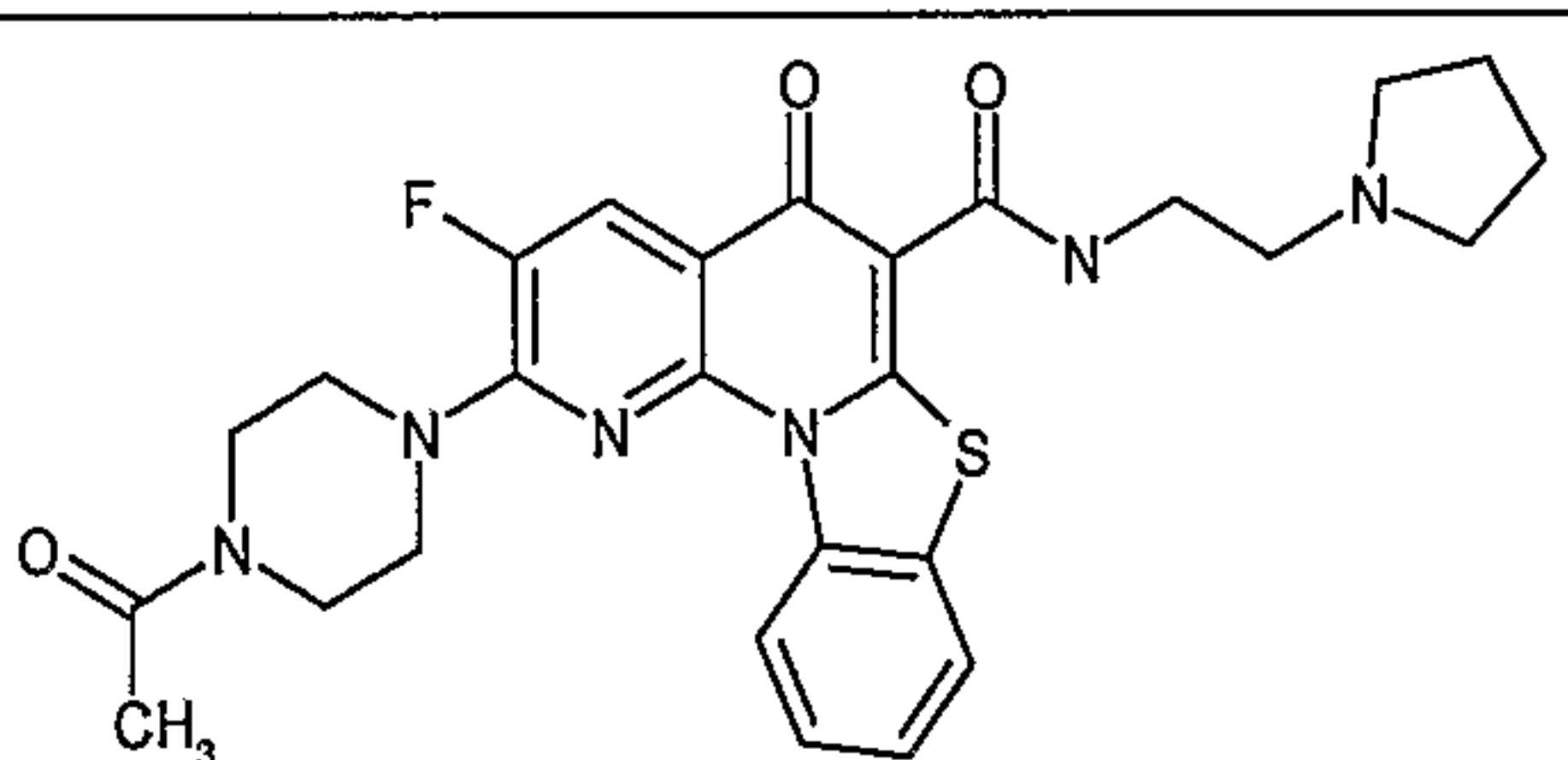
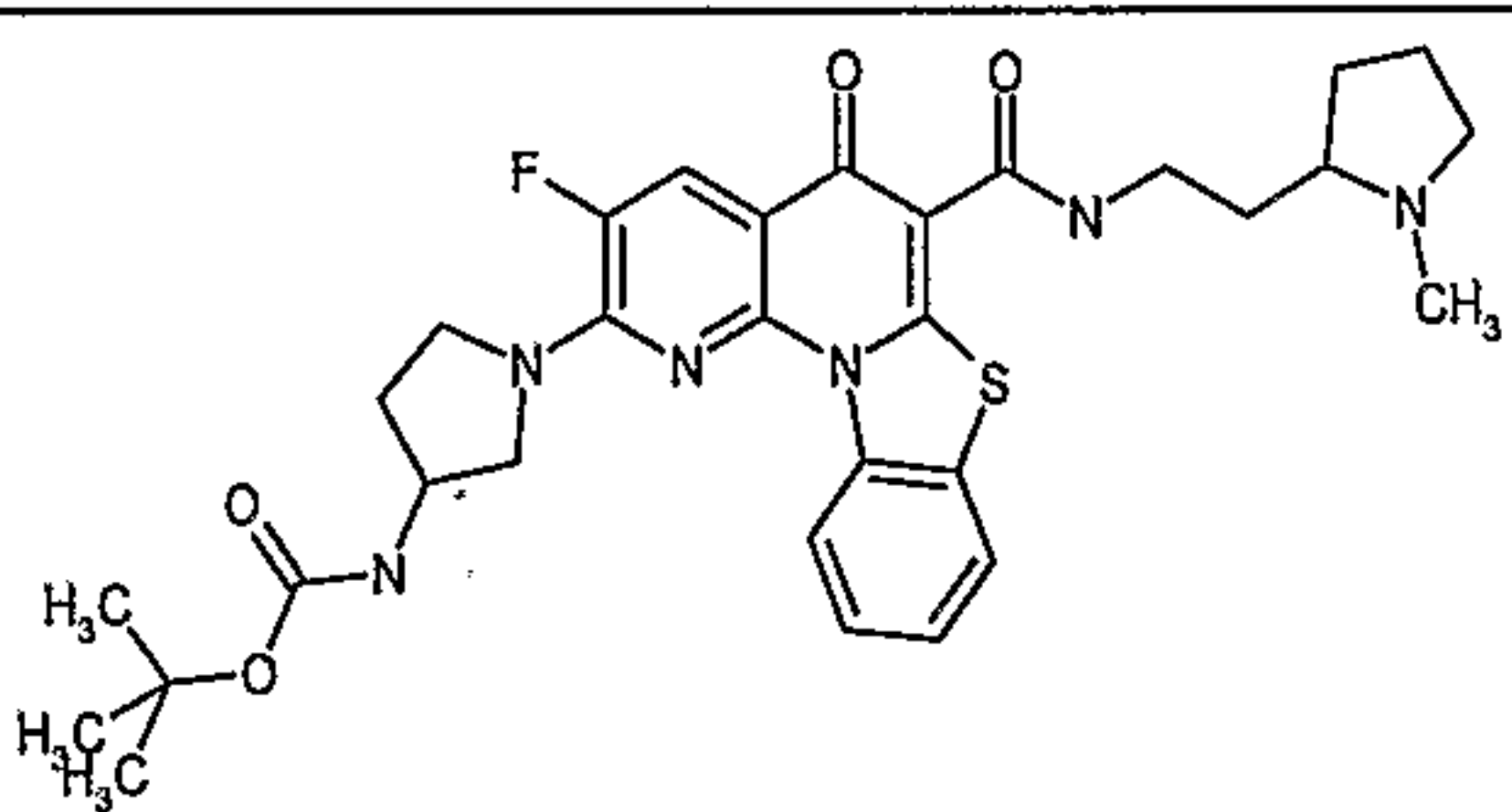
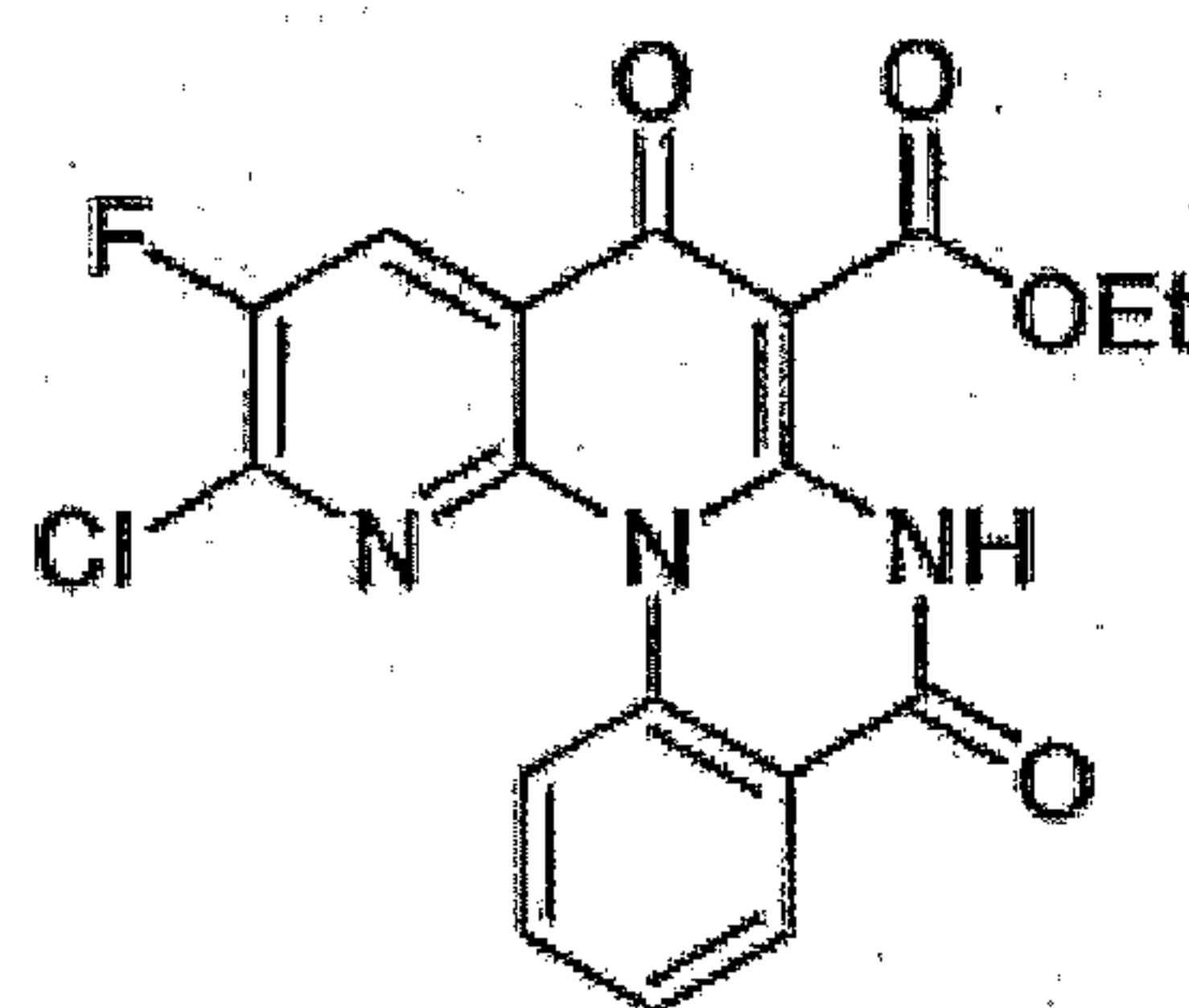
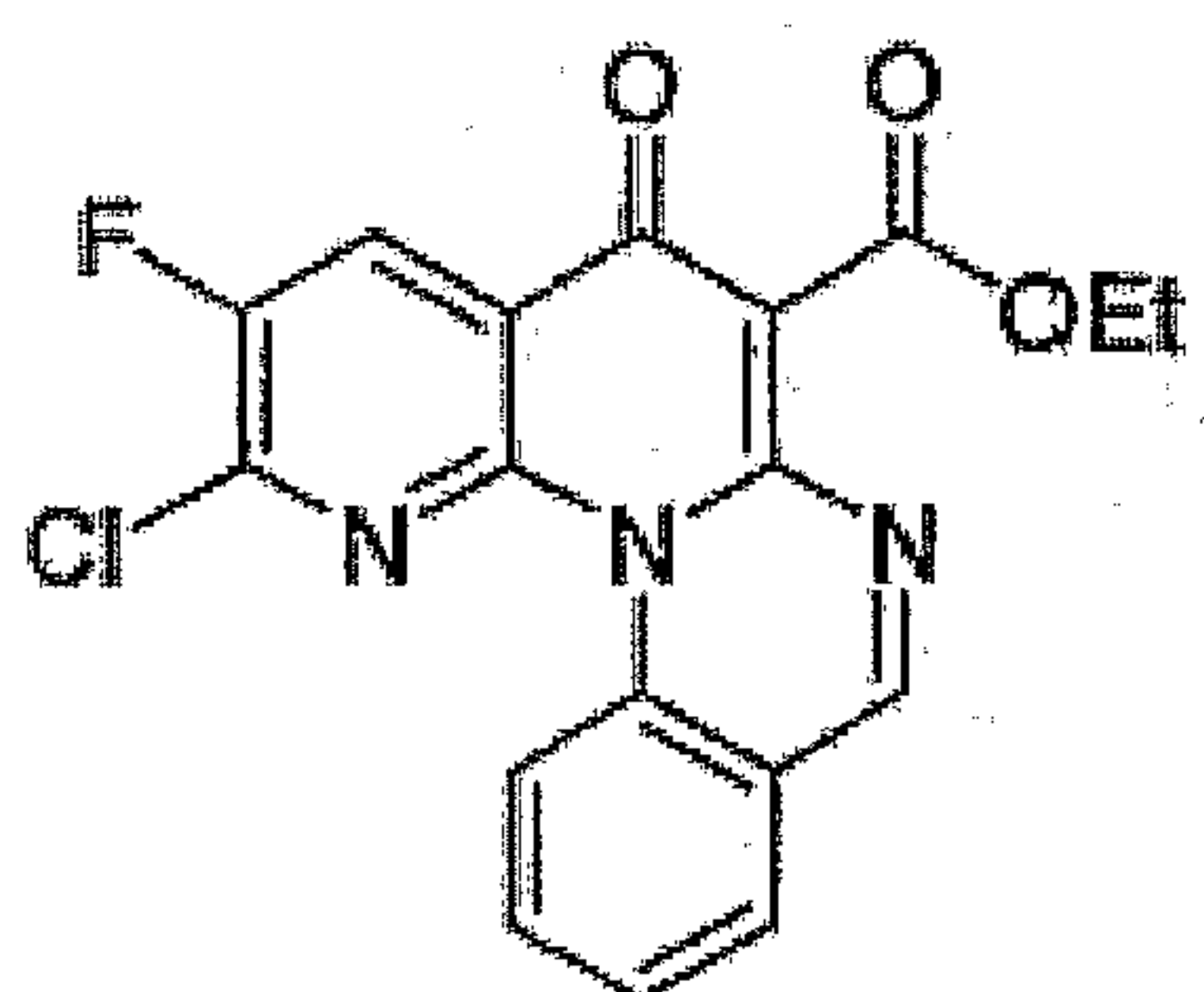
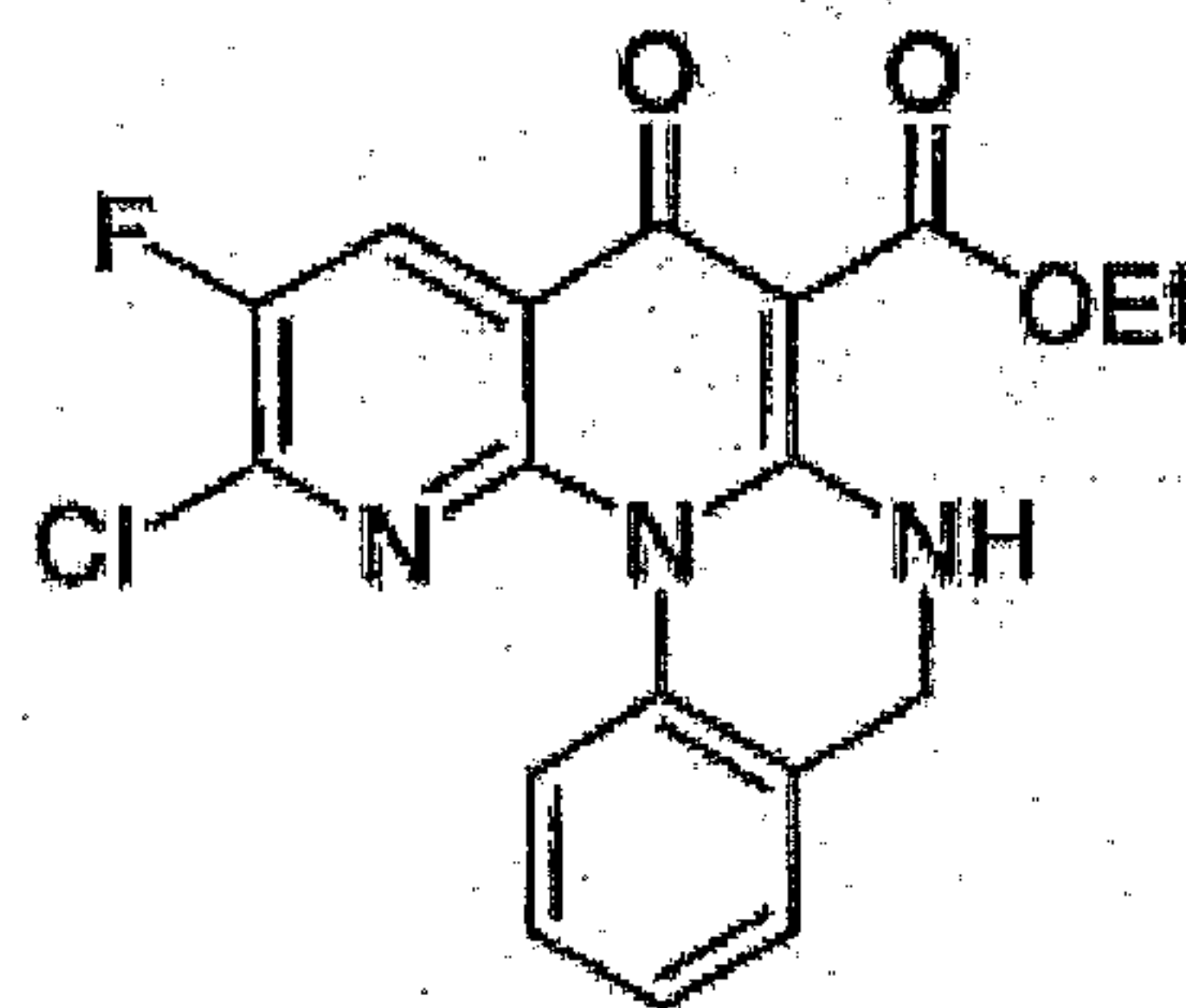
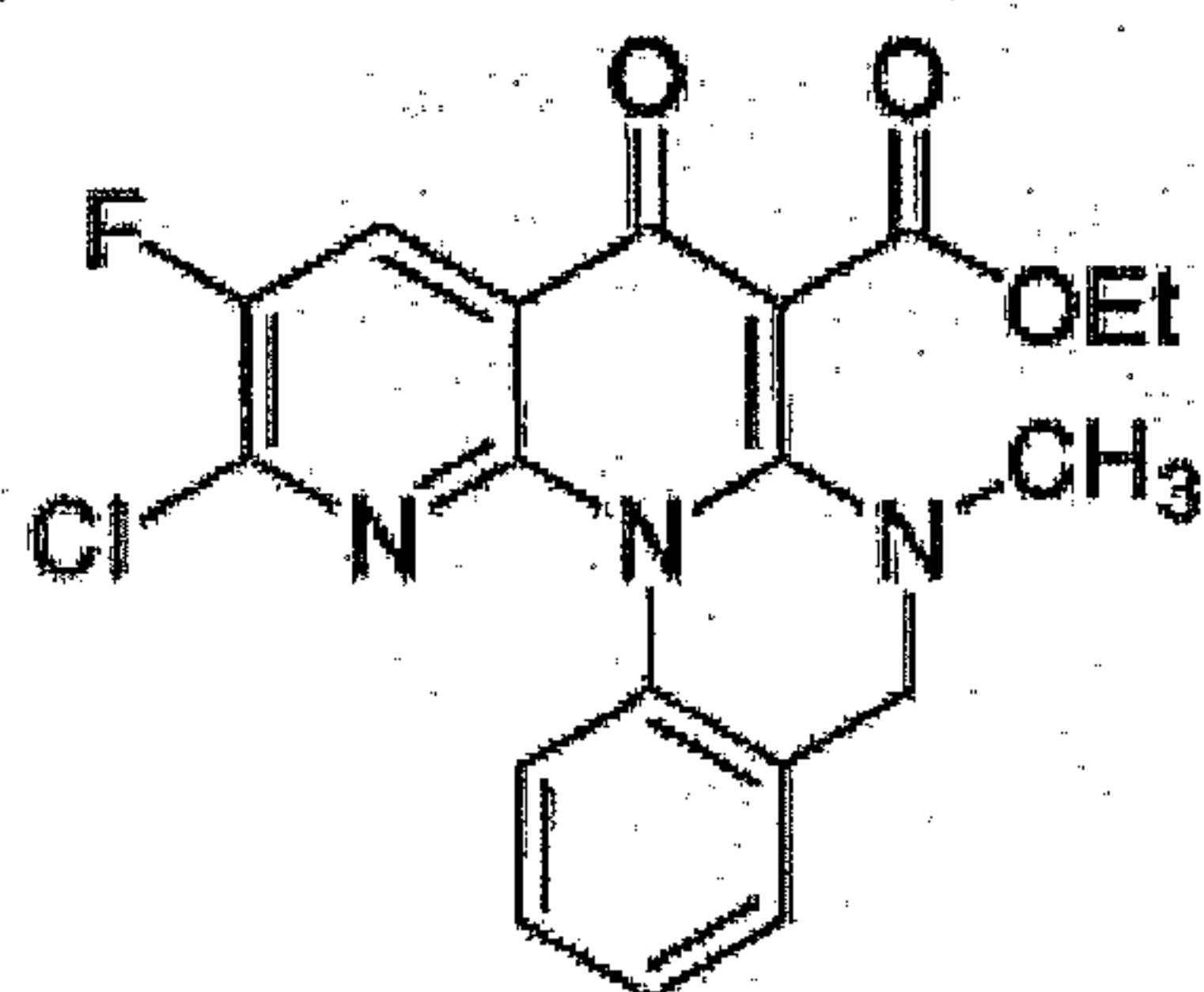
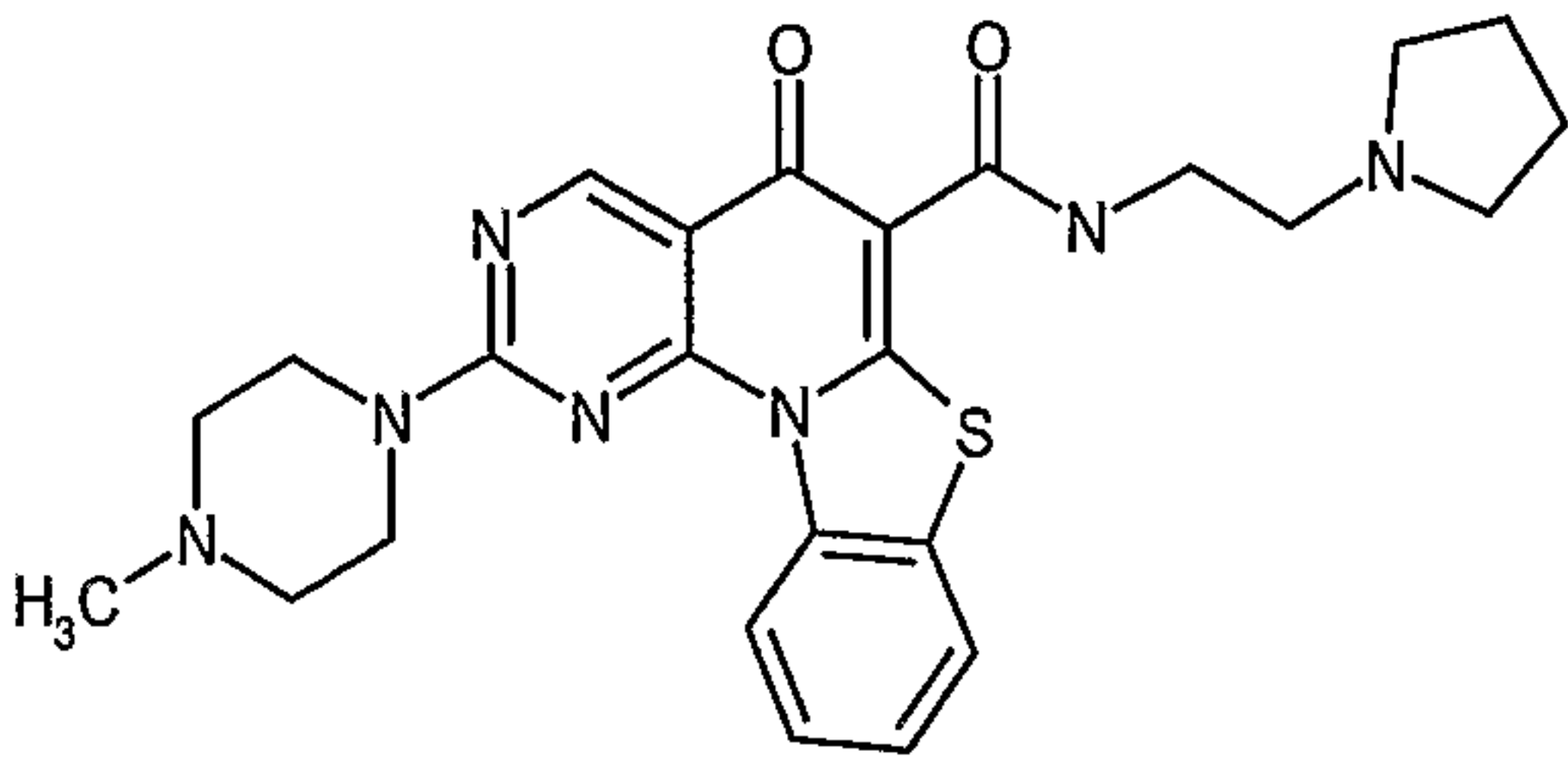
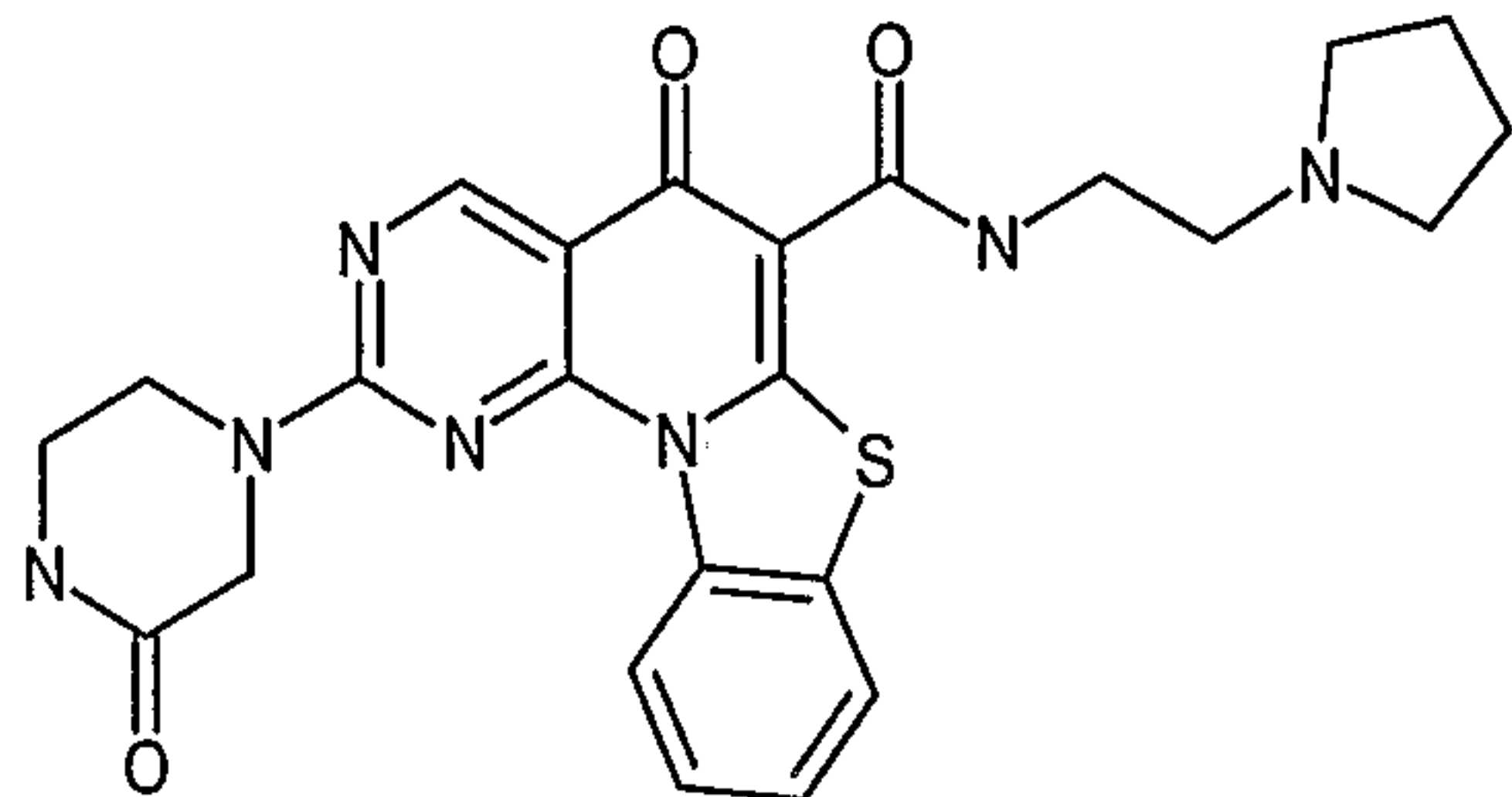
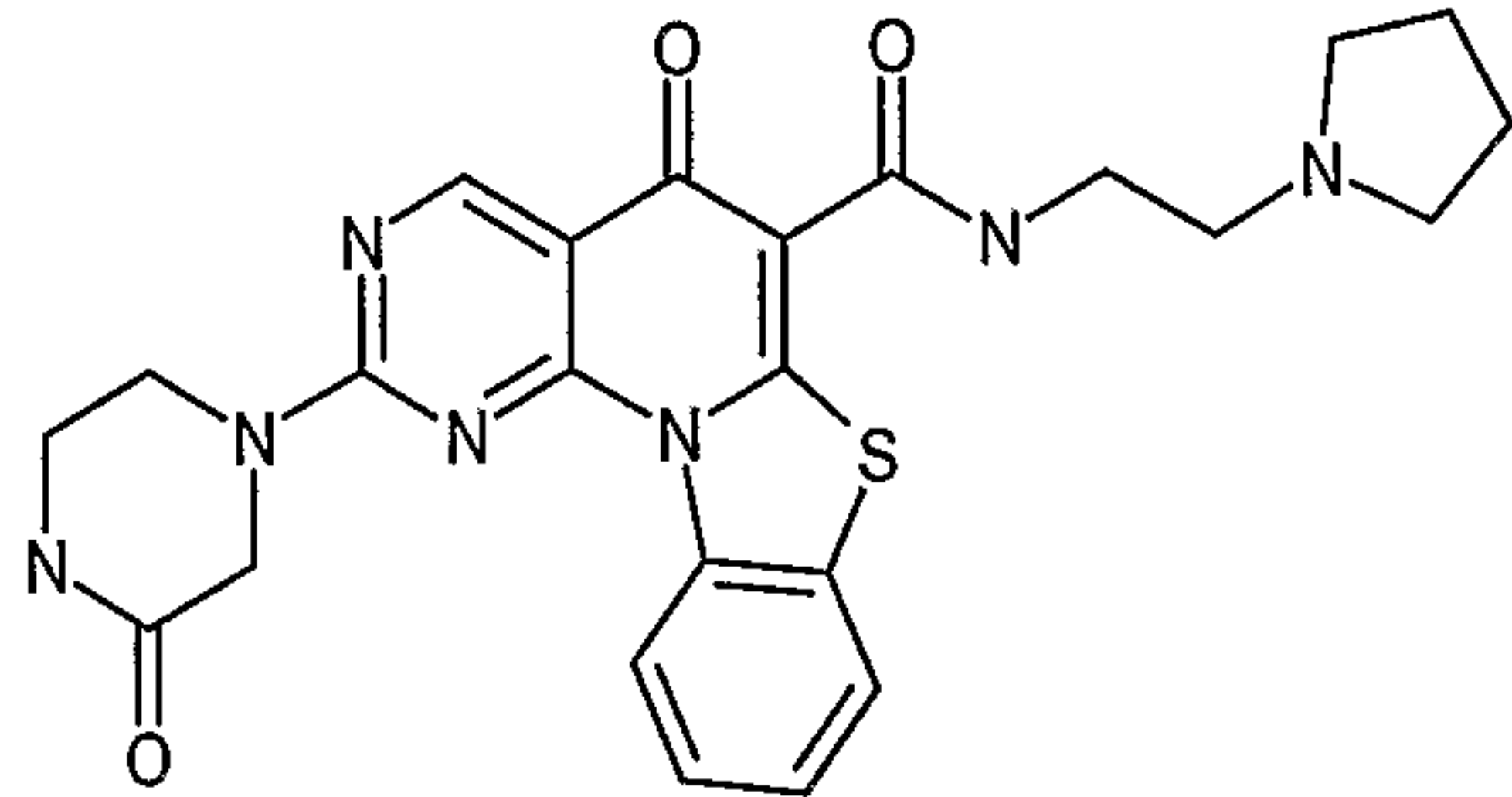
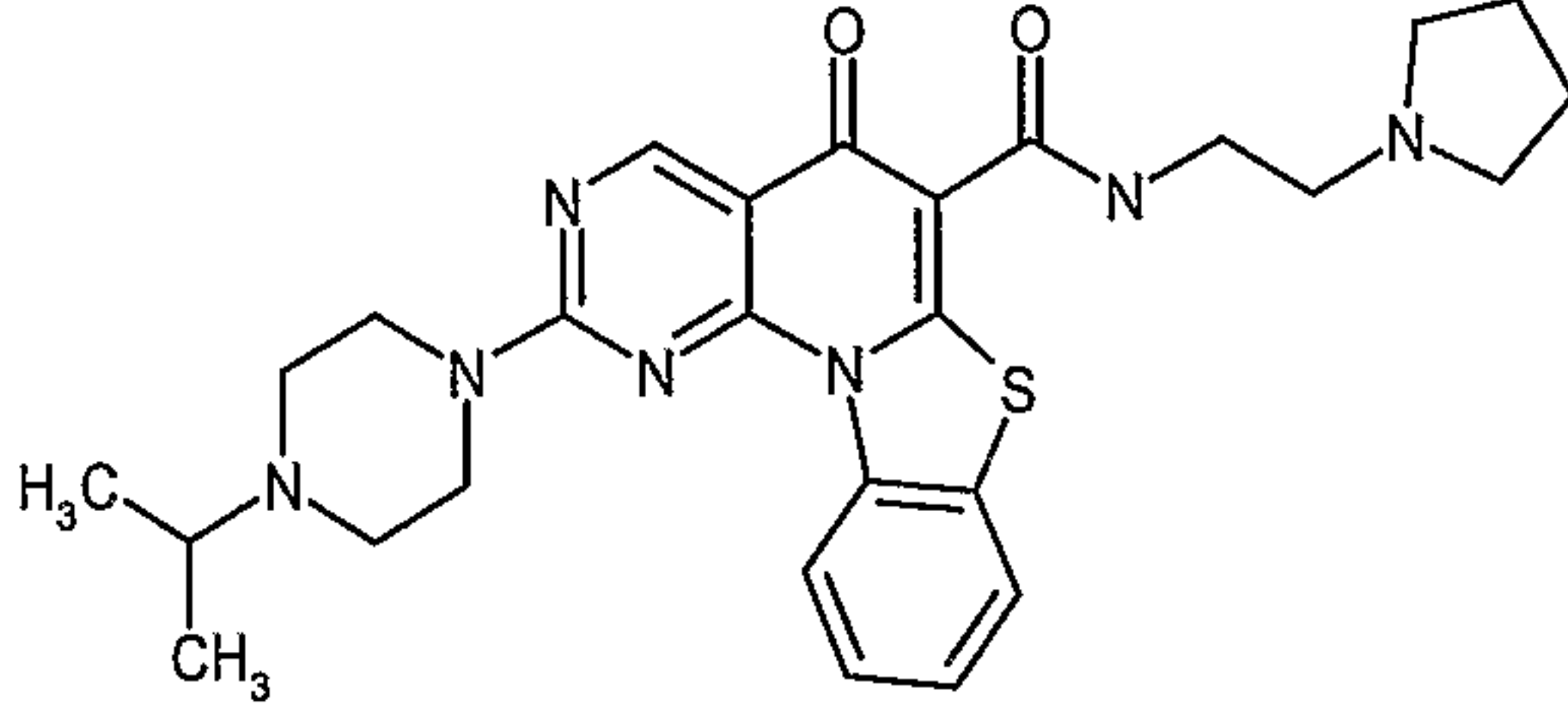
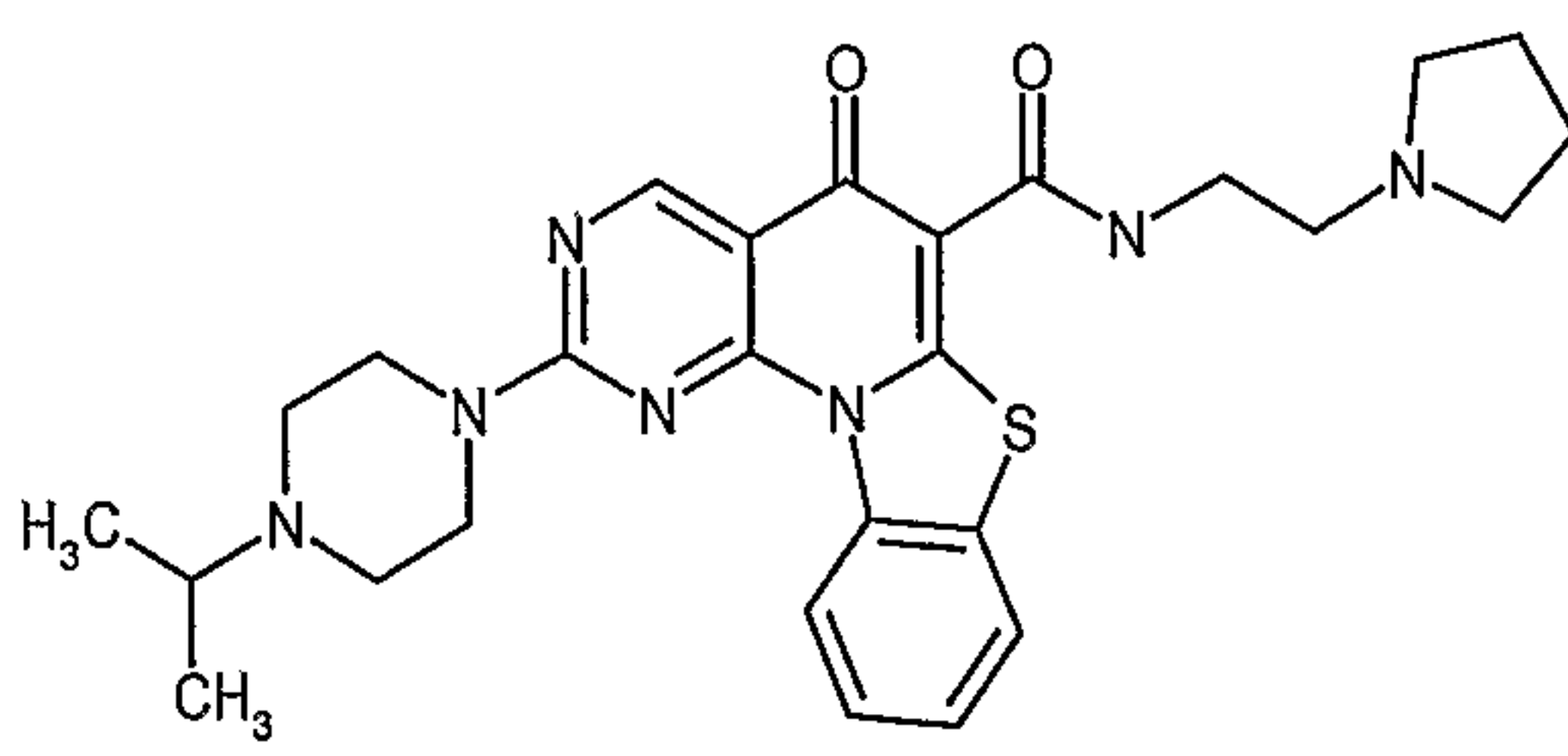
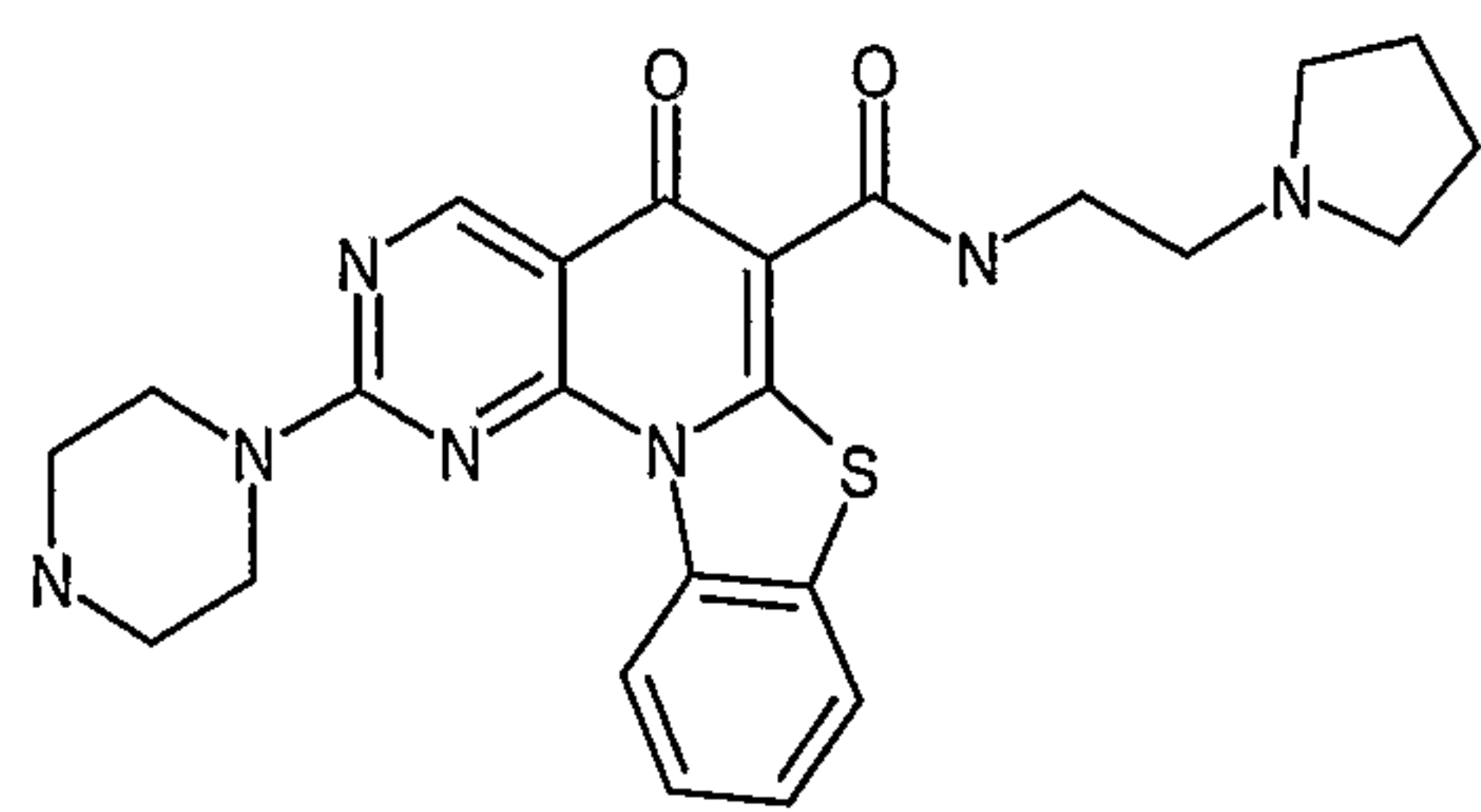
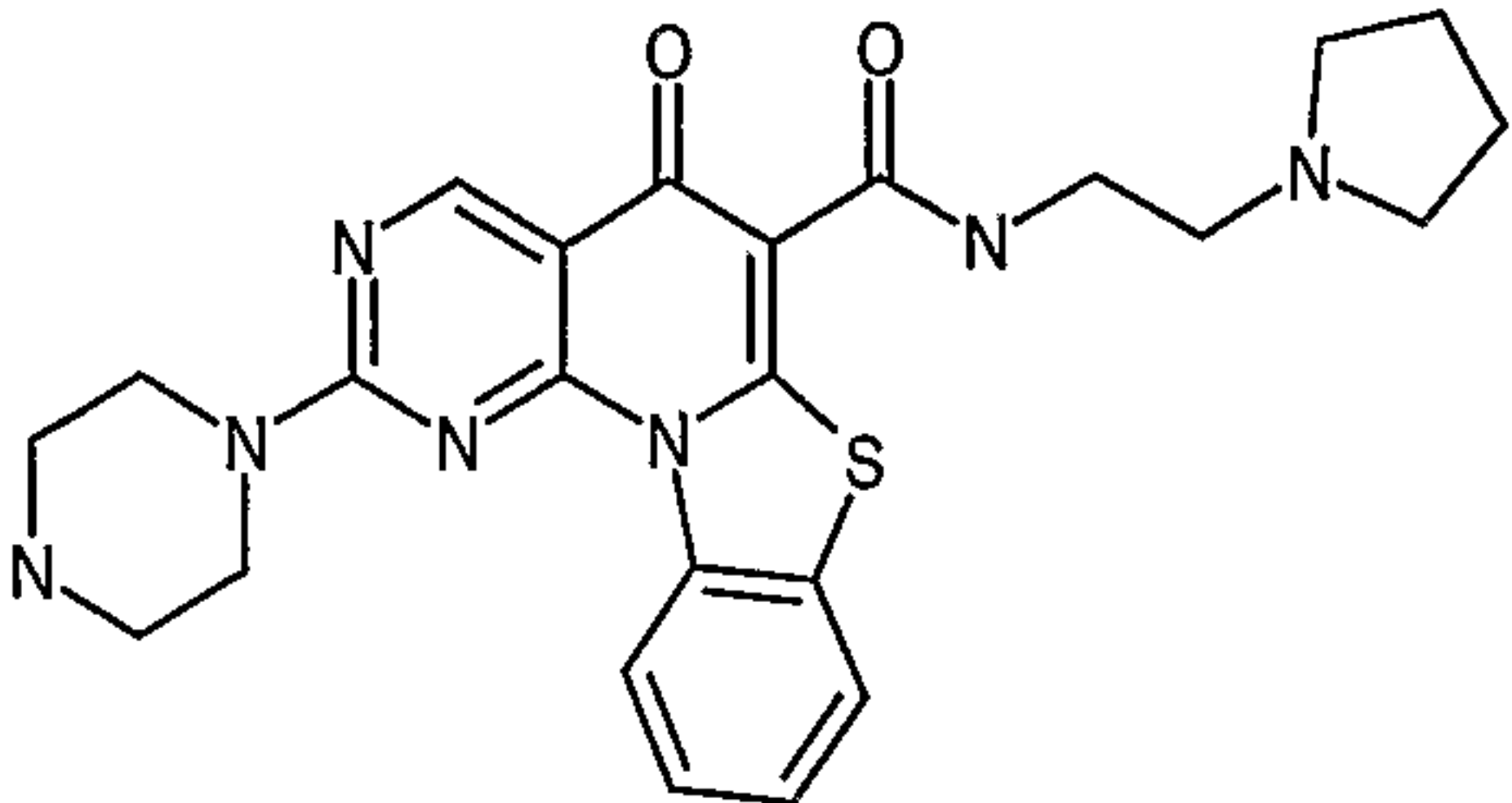
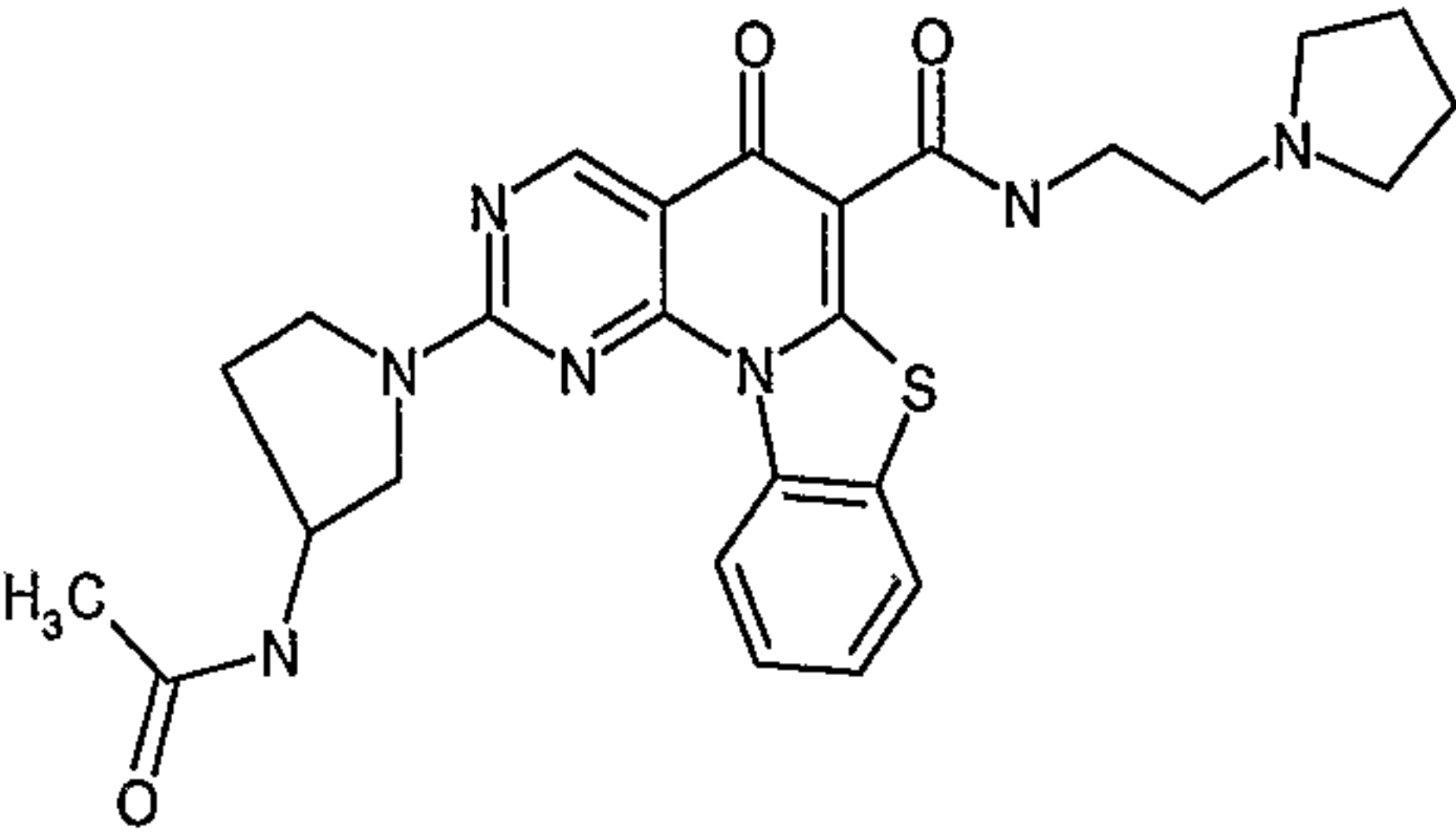
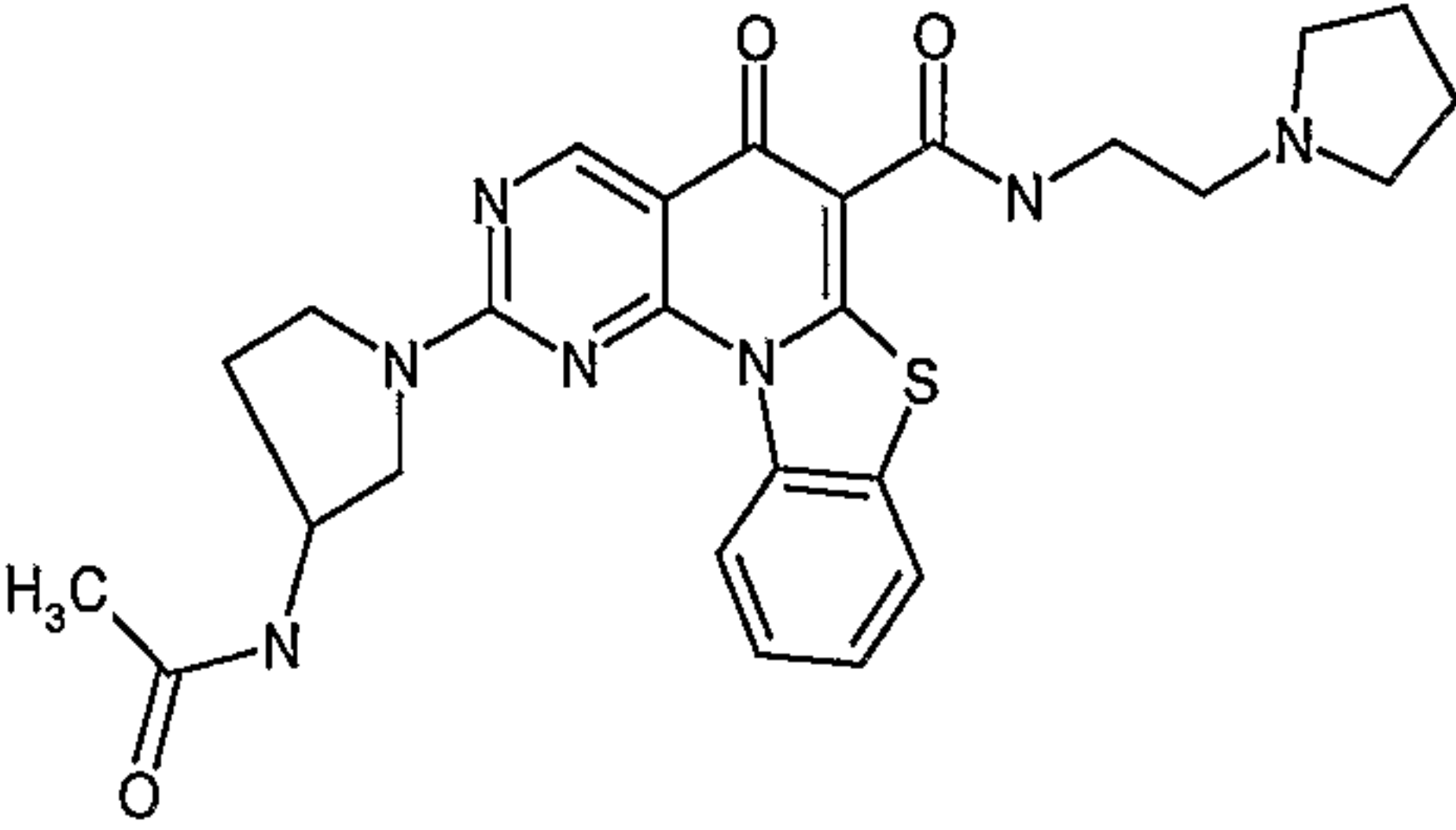
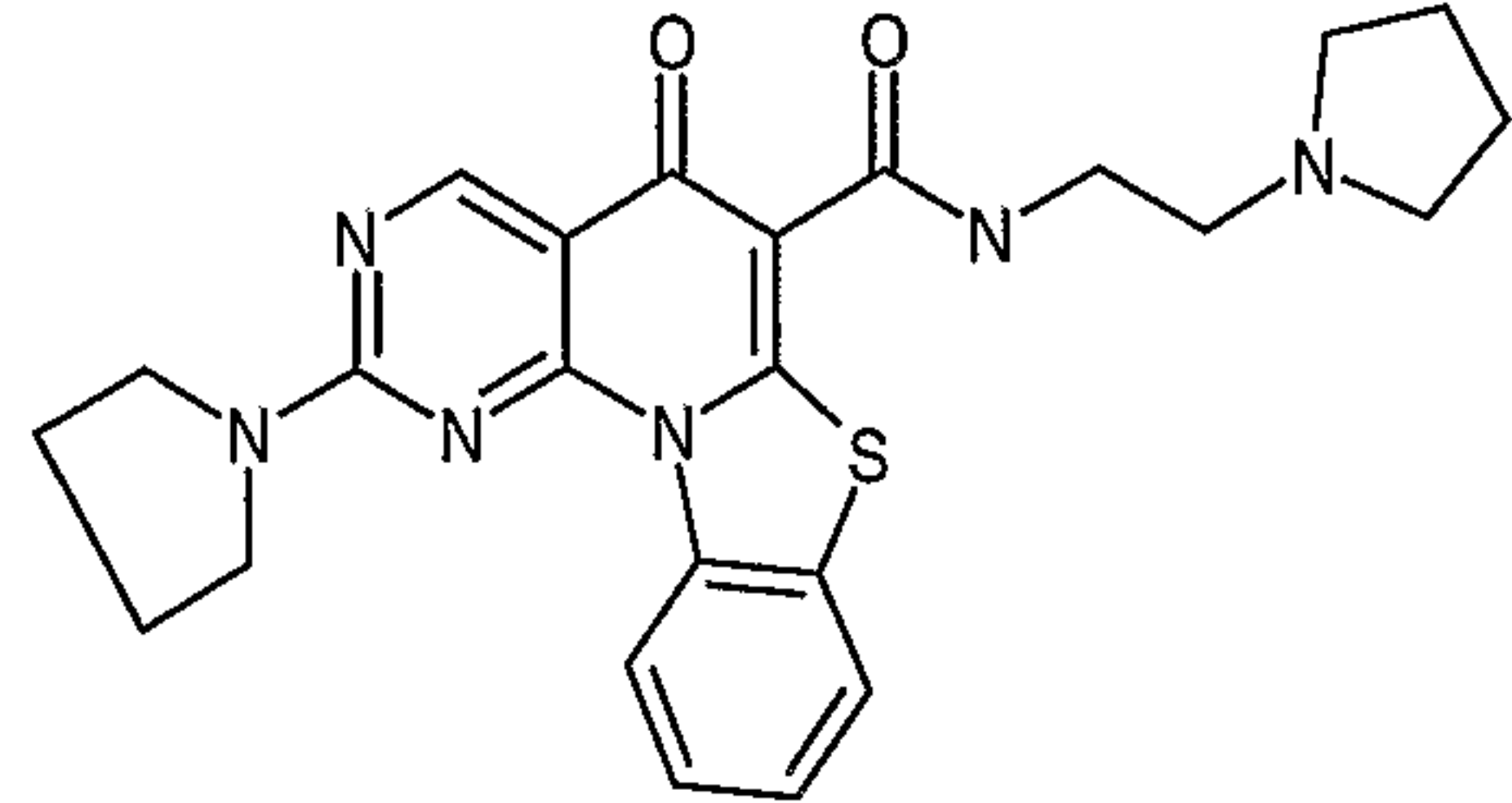
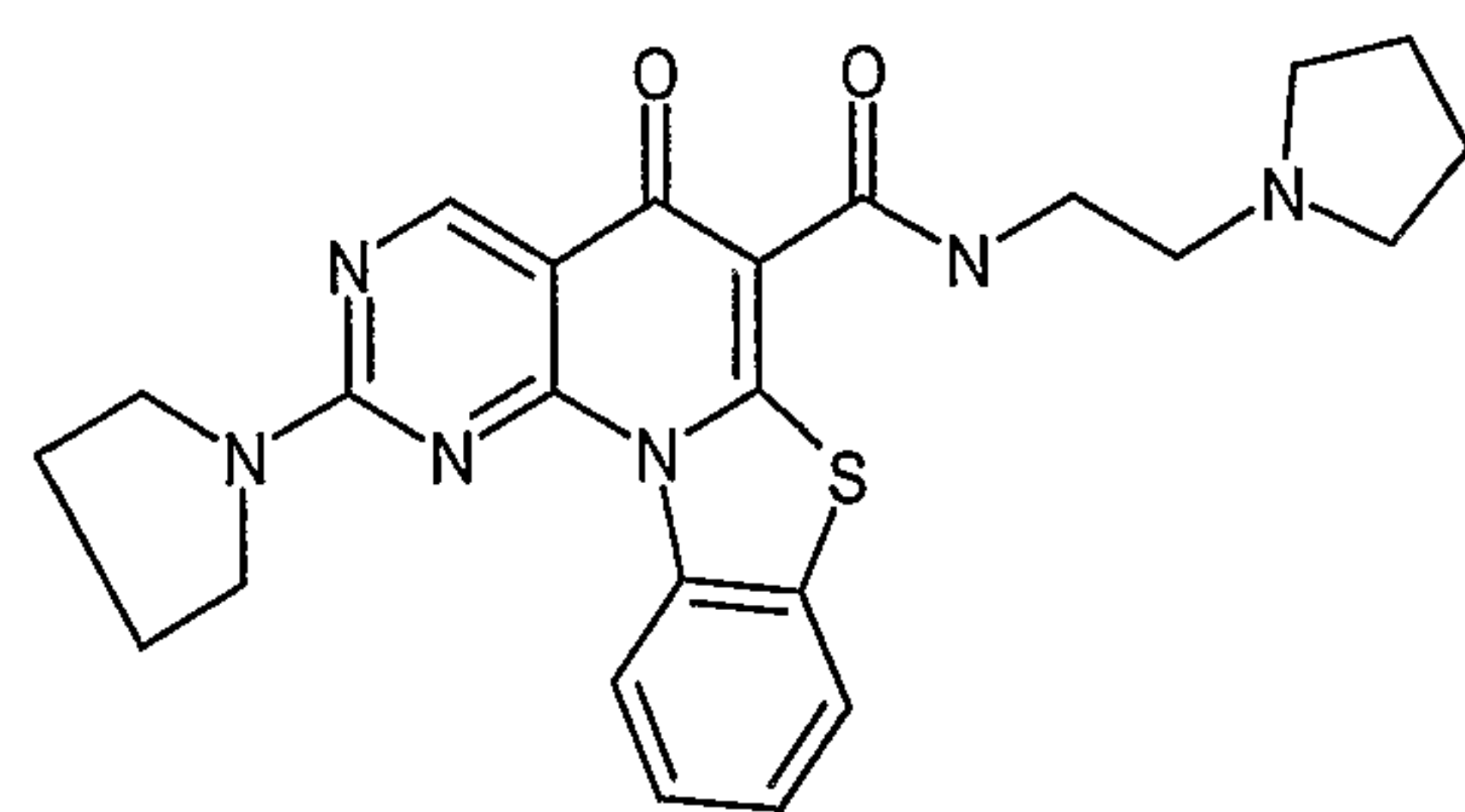
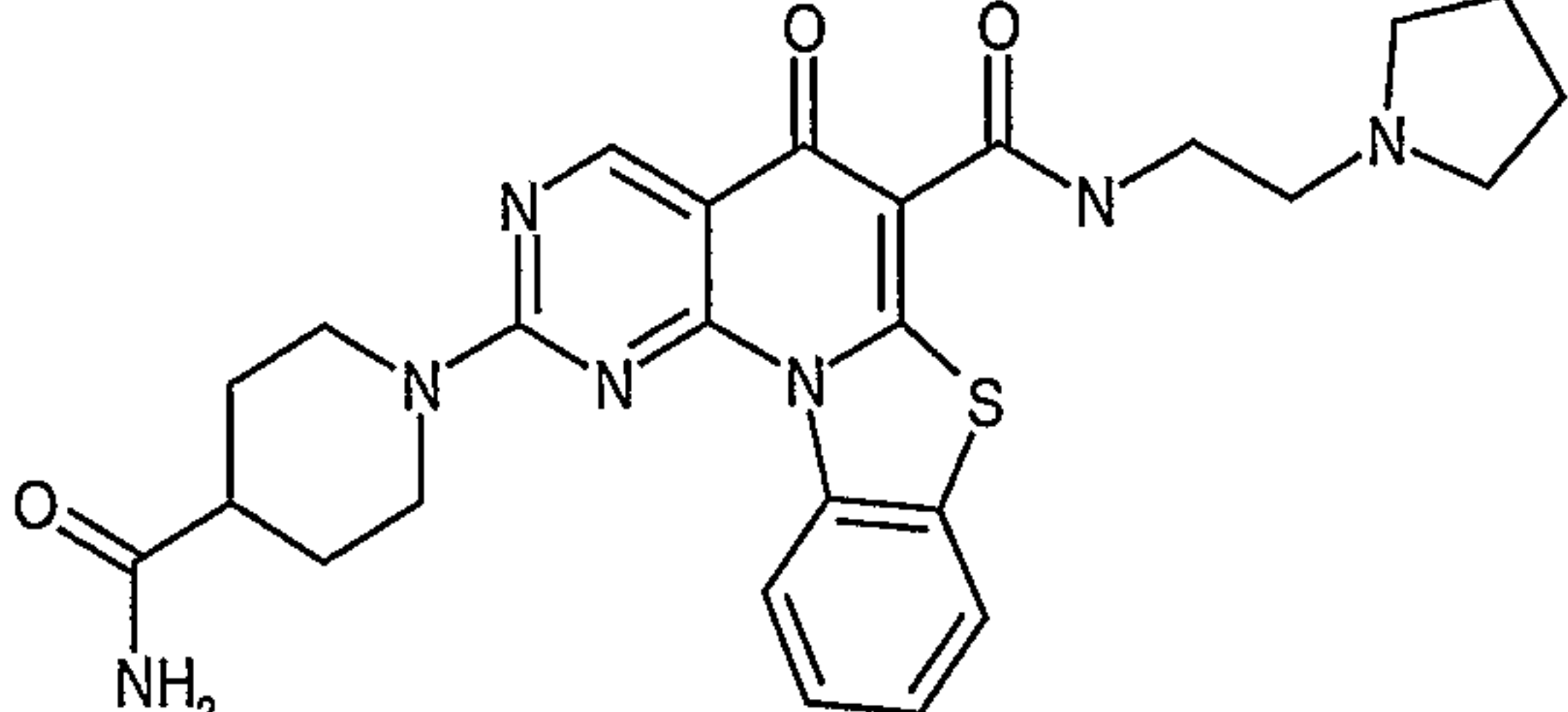
| MOLSTRUCTURE                                                                         | MOLSTRUCTURE                                                                          |
|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|
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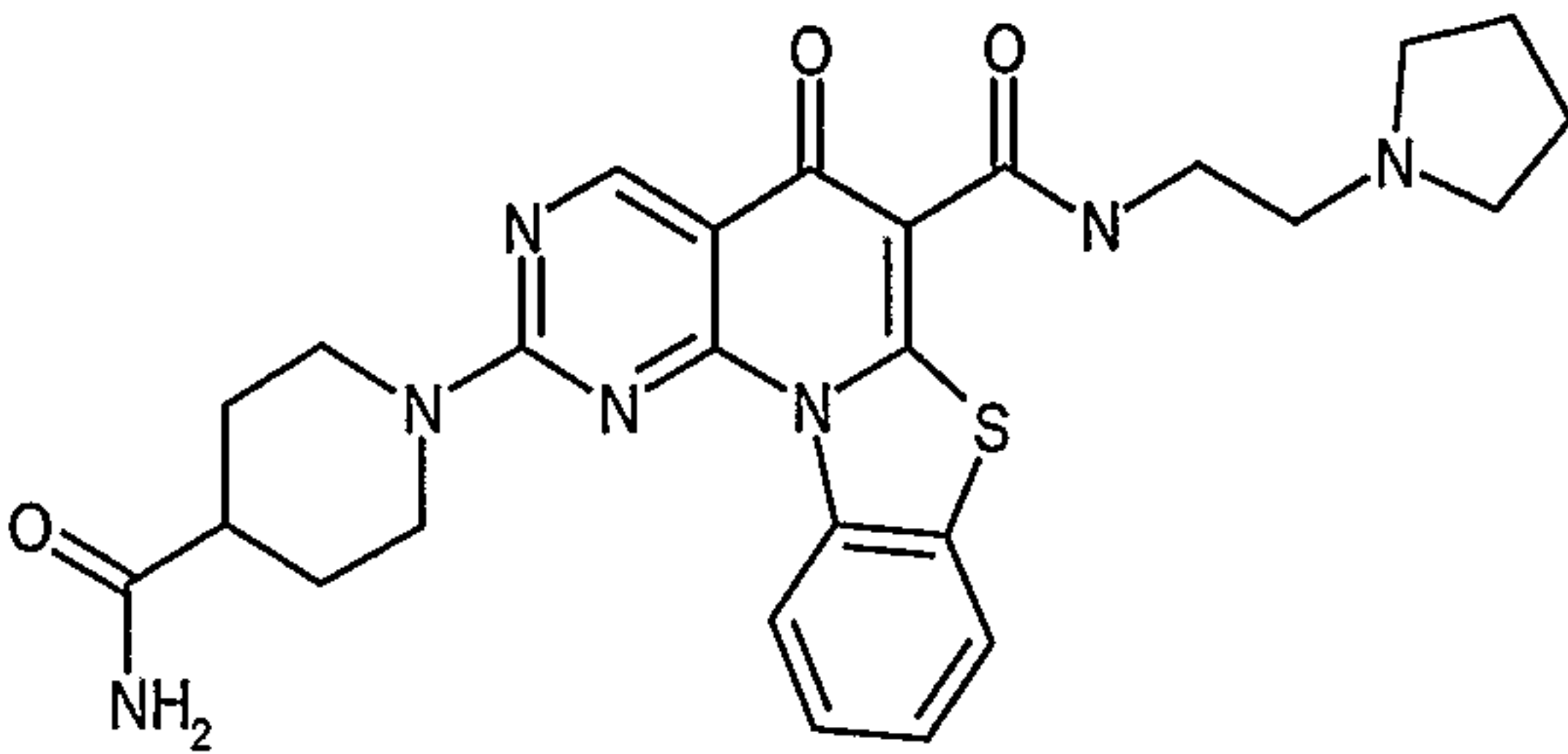
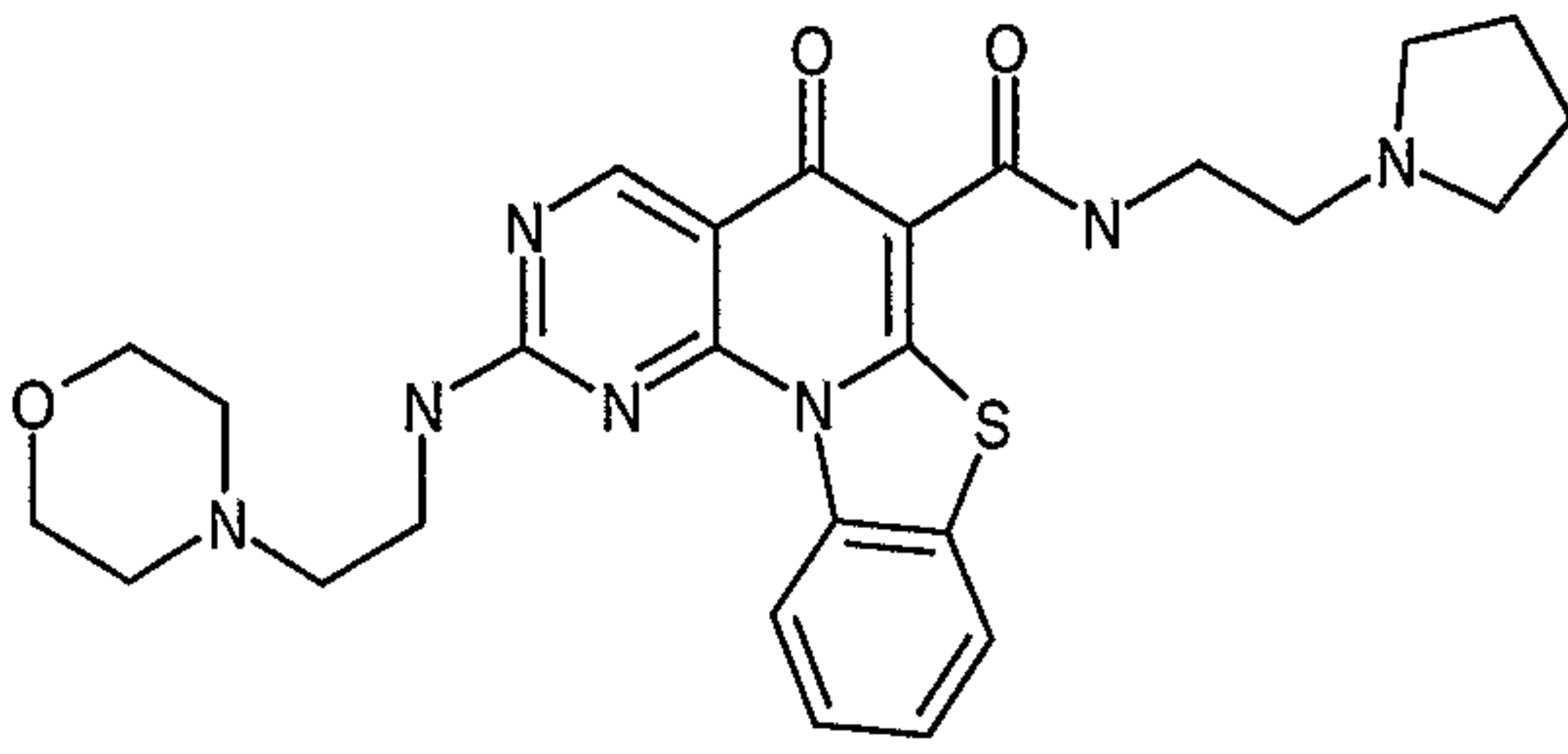
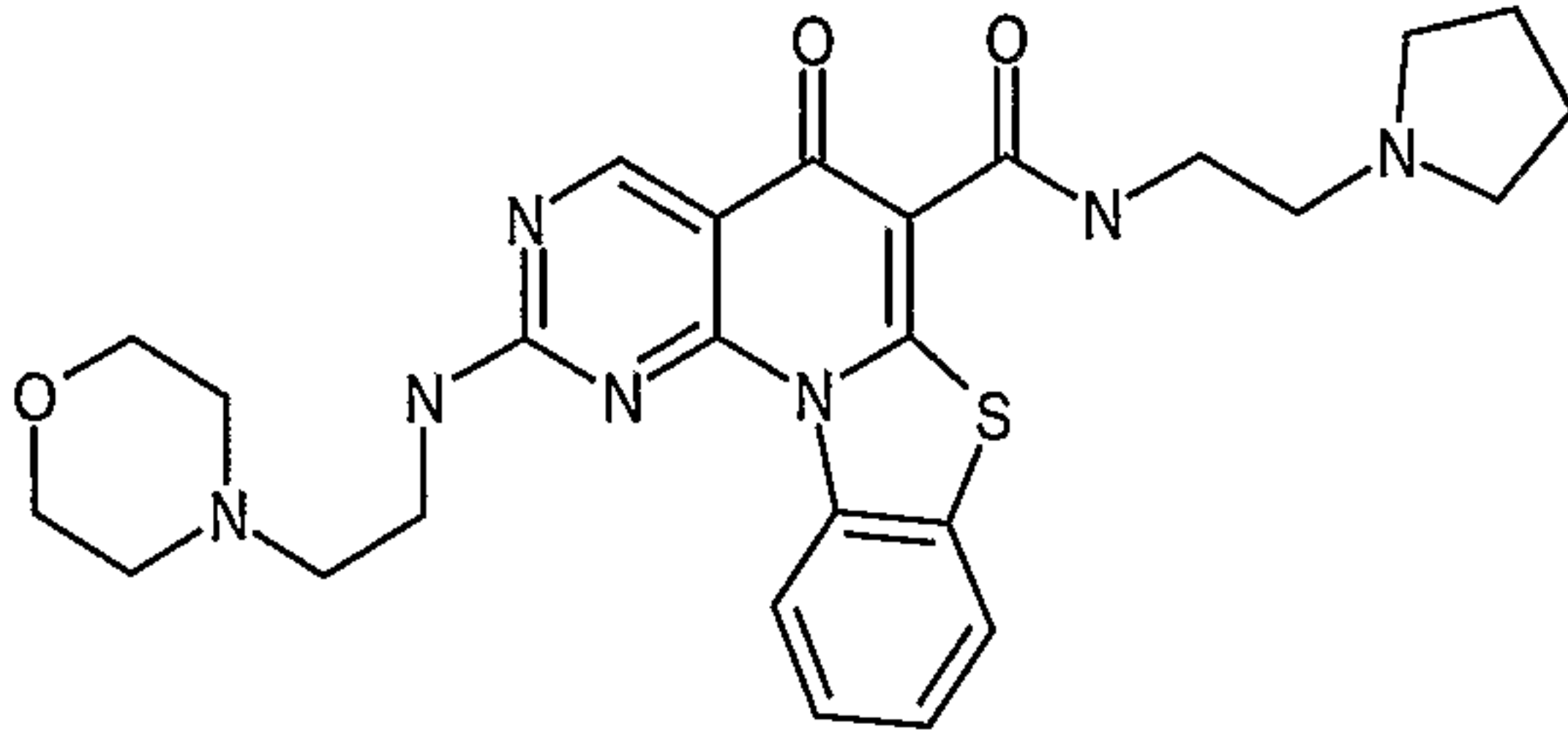
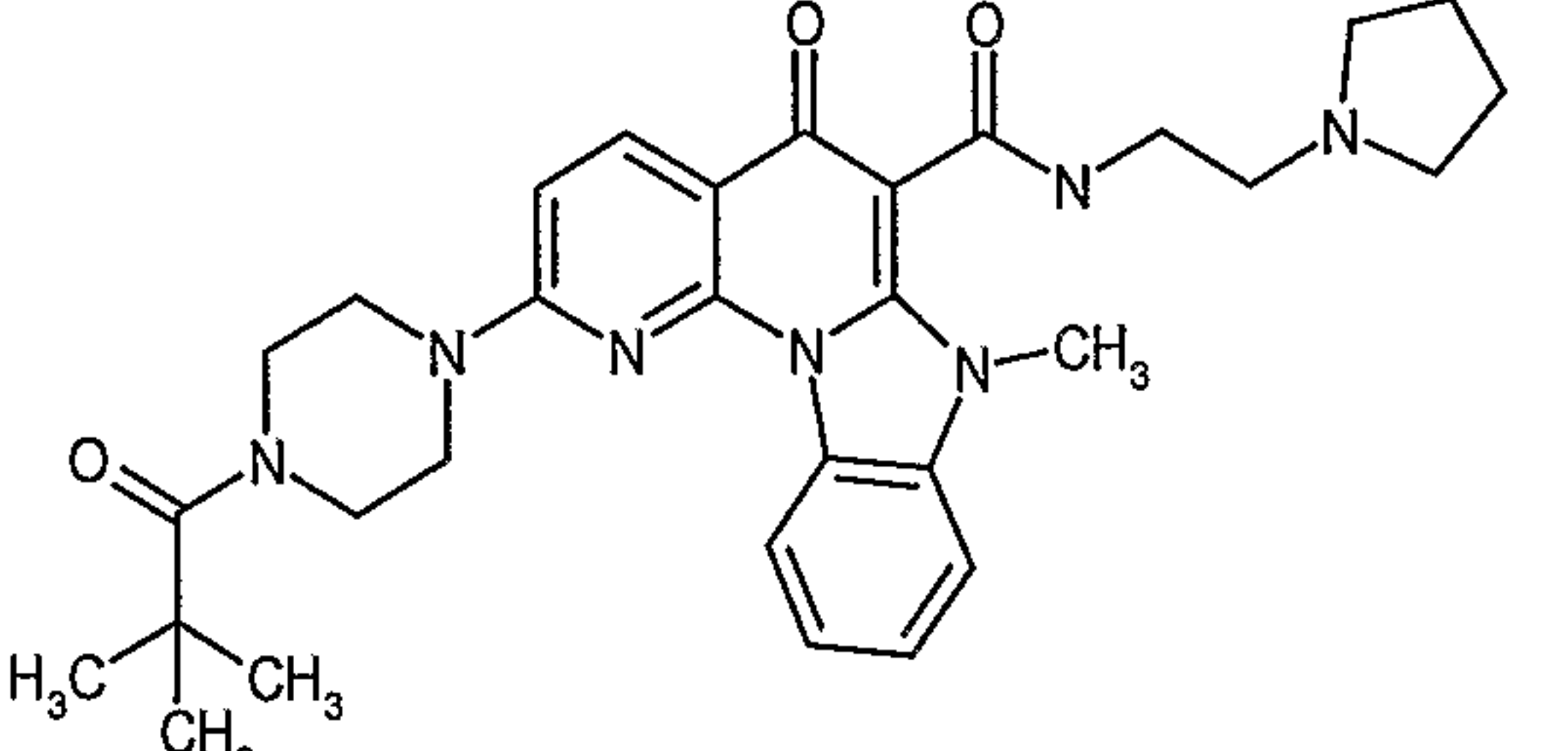
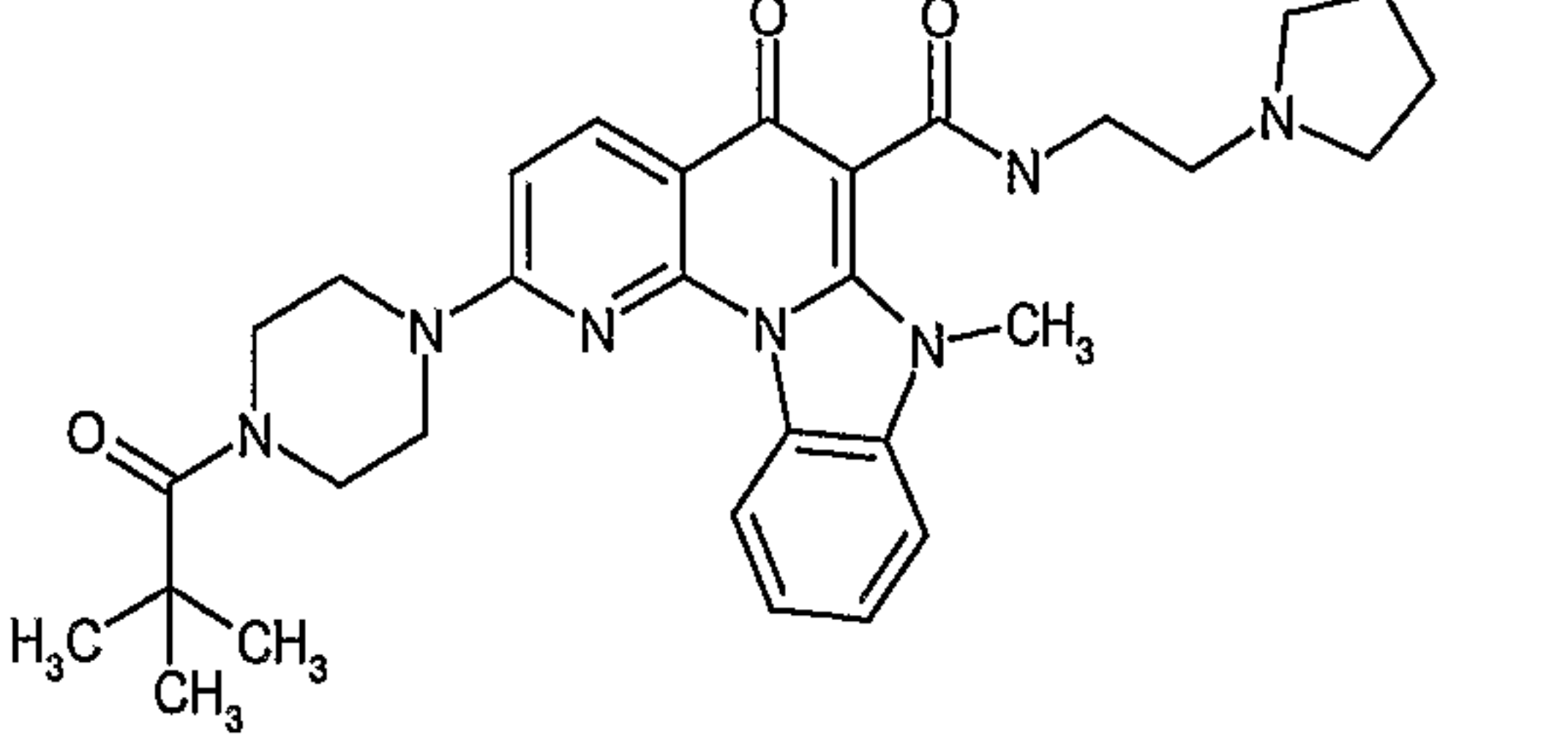
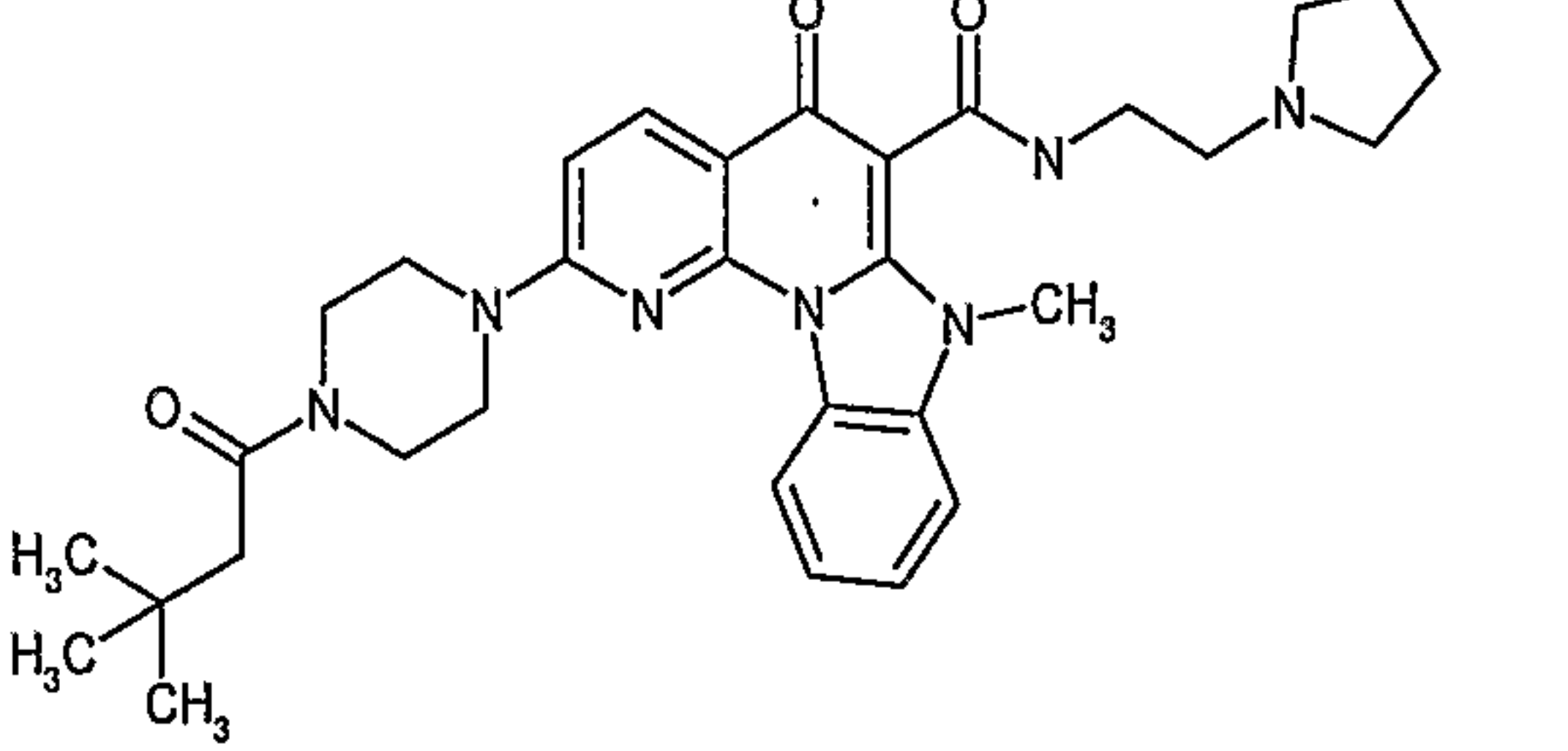
Table 1C



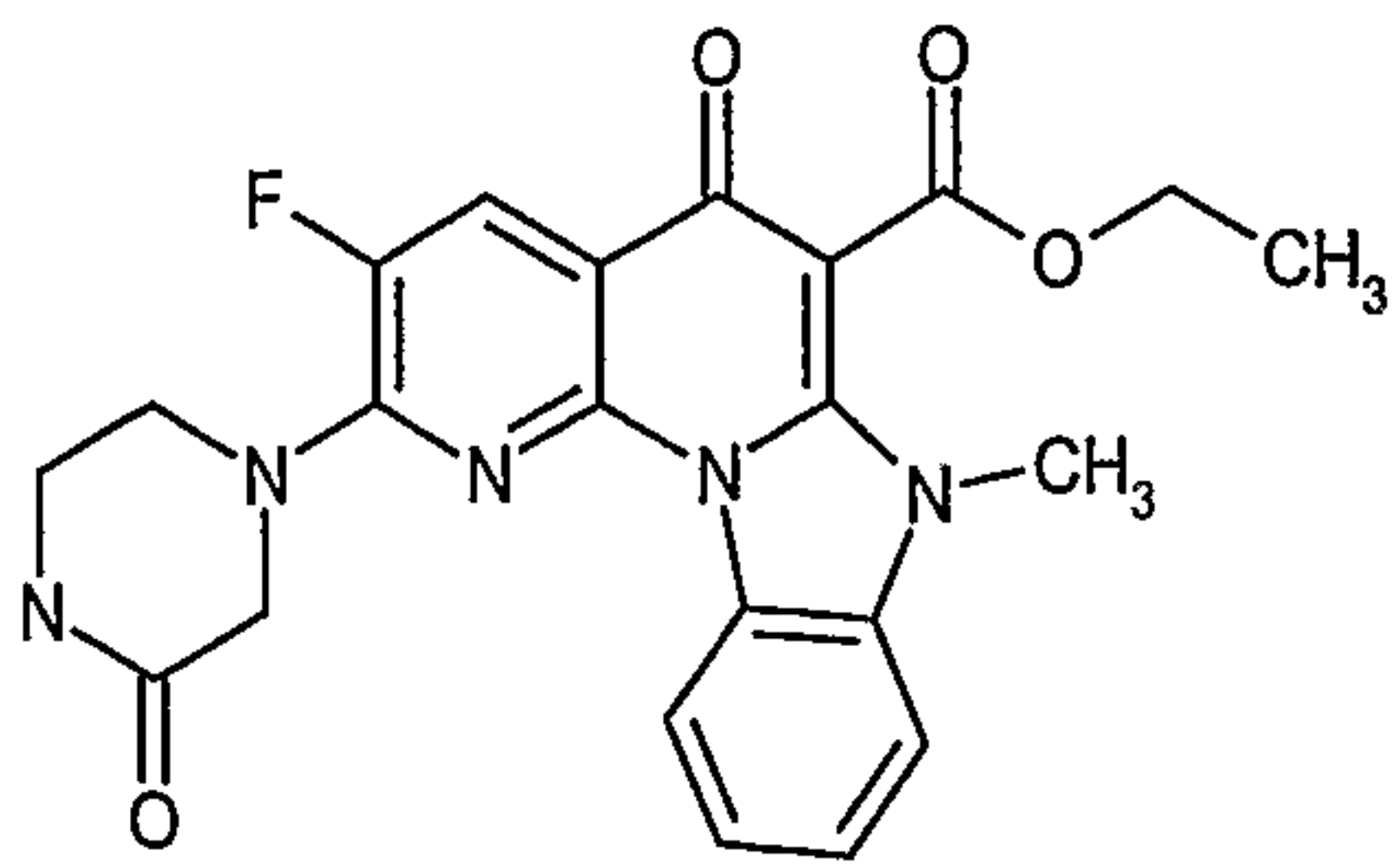
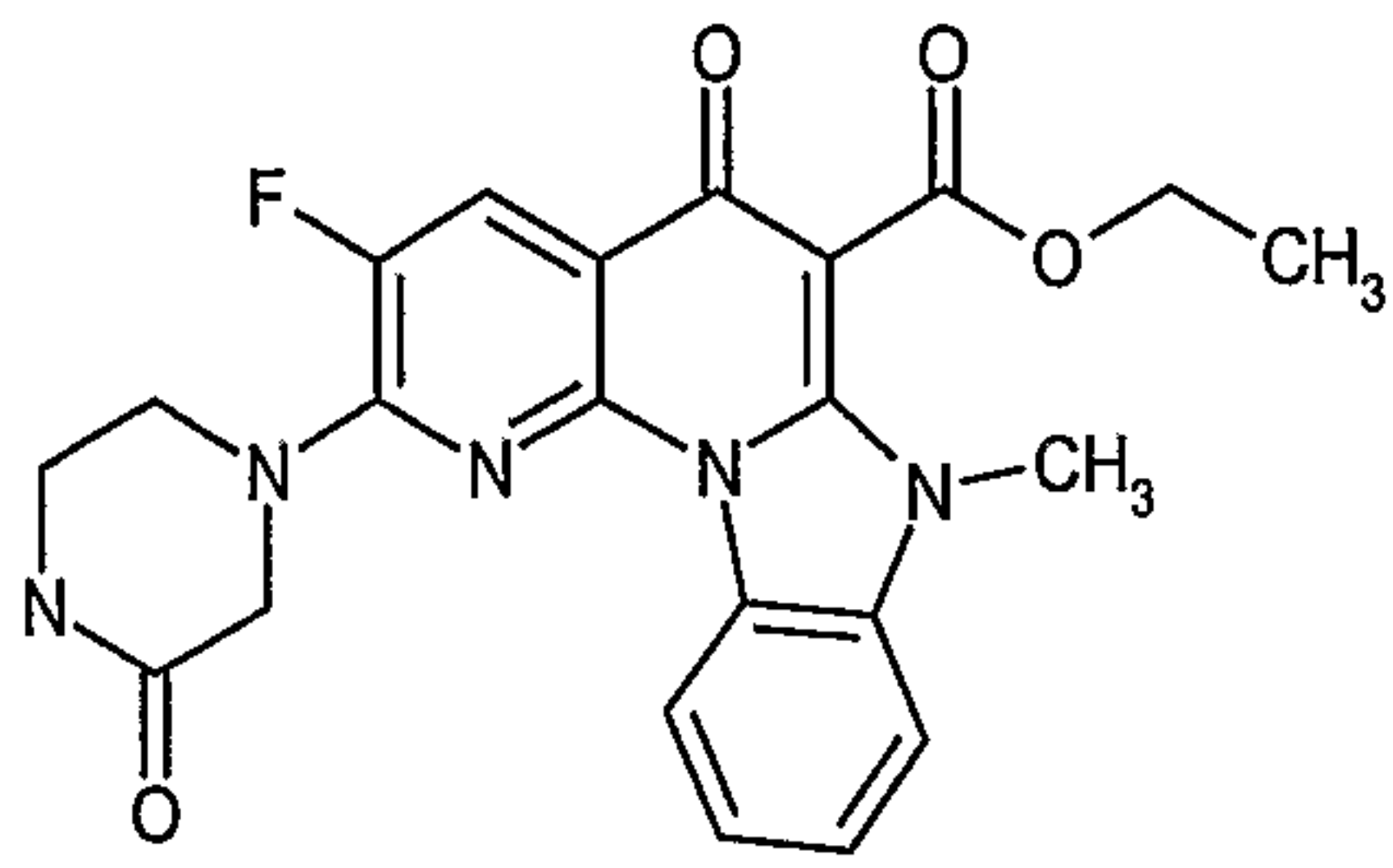
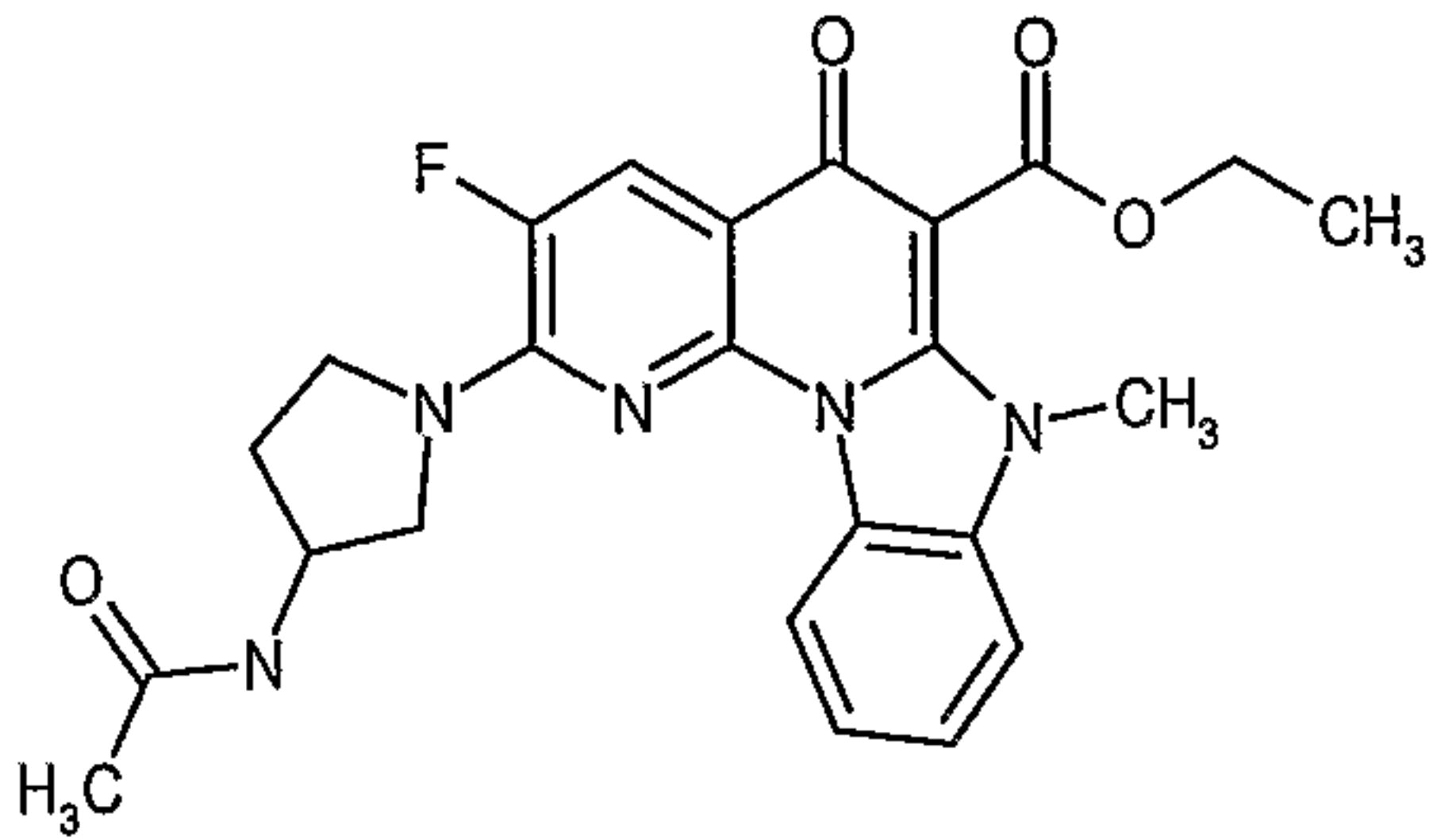
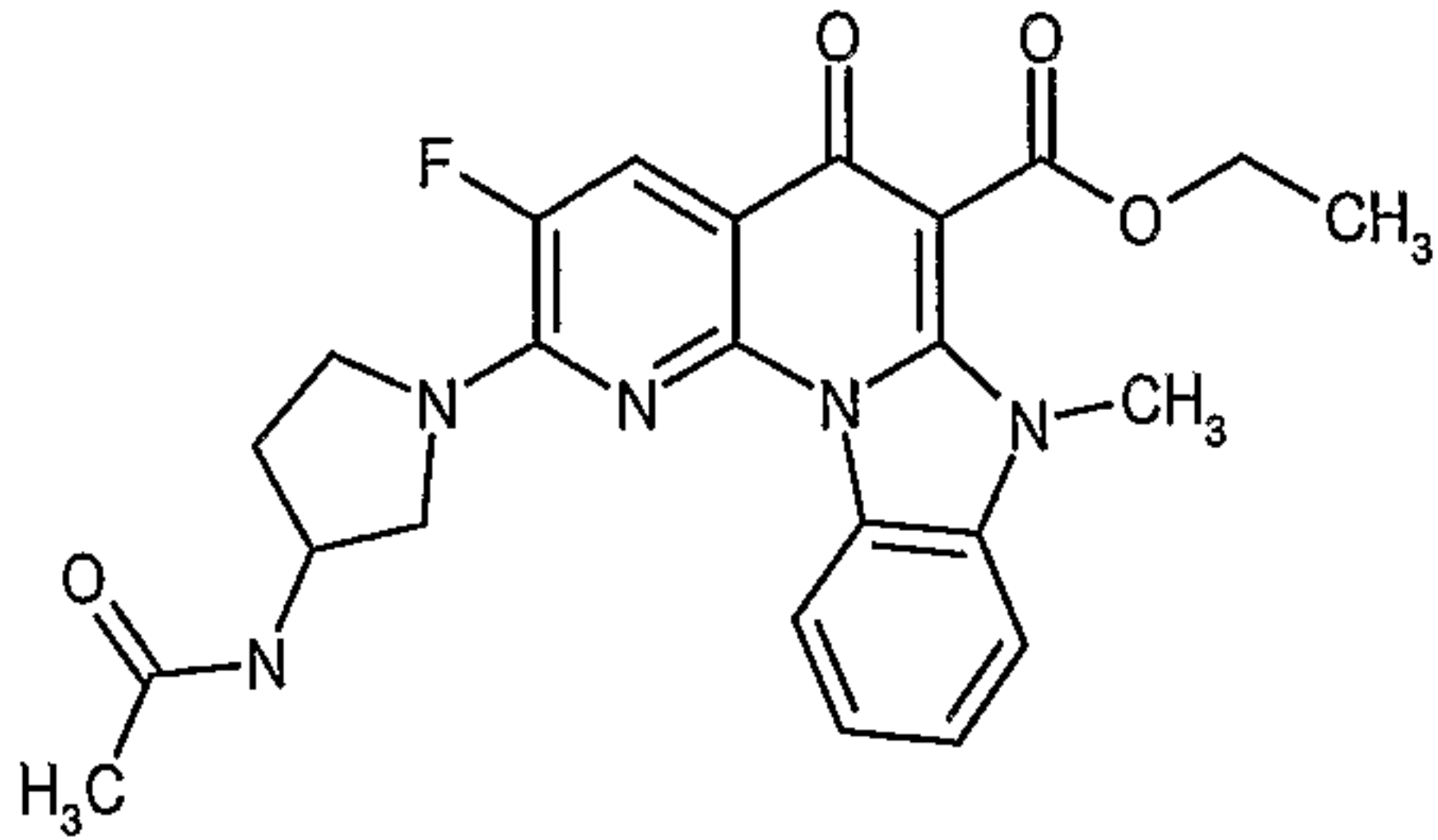
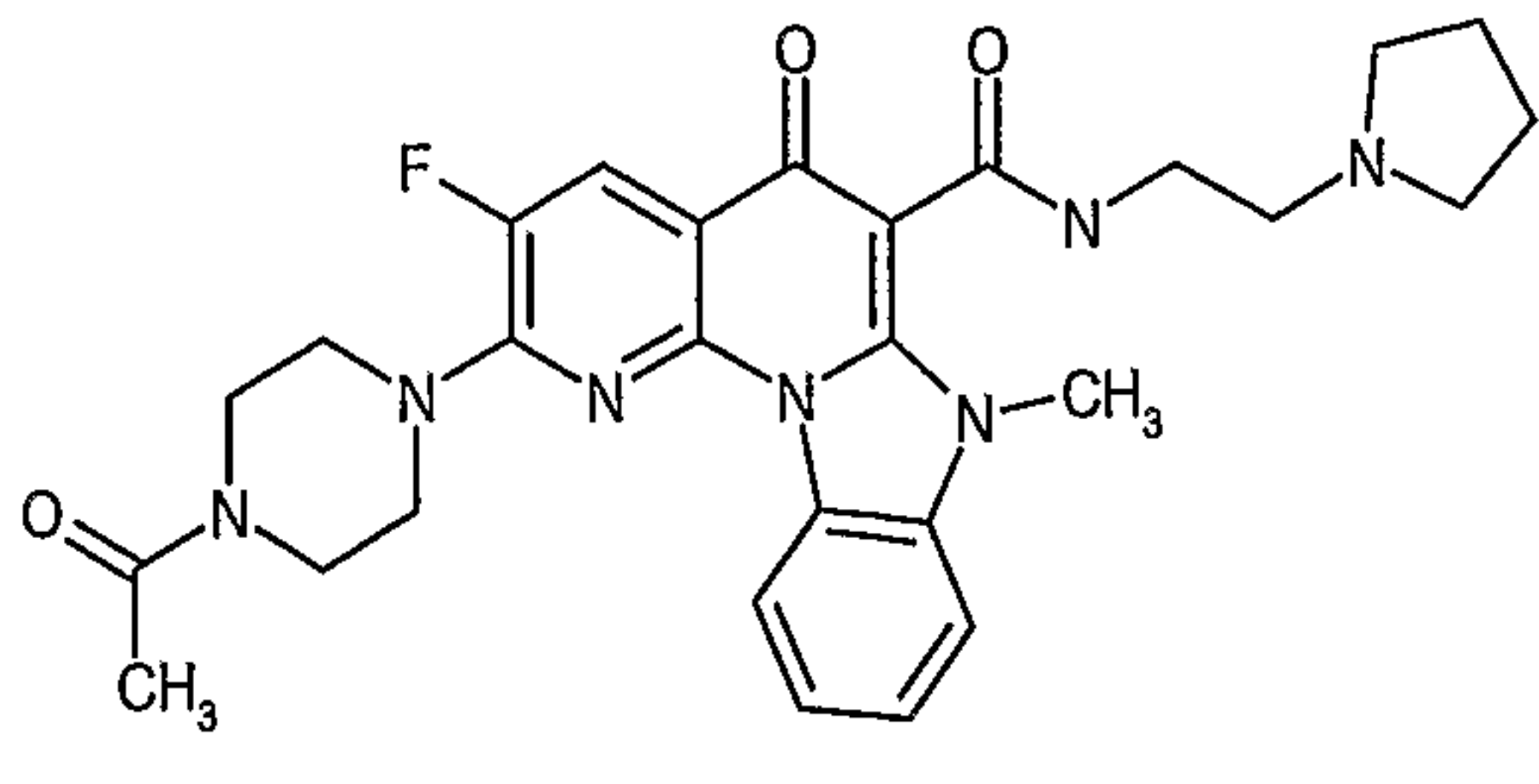
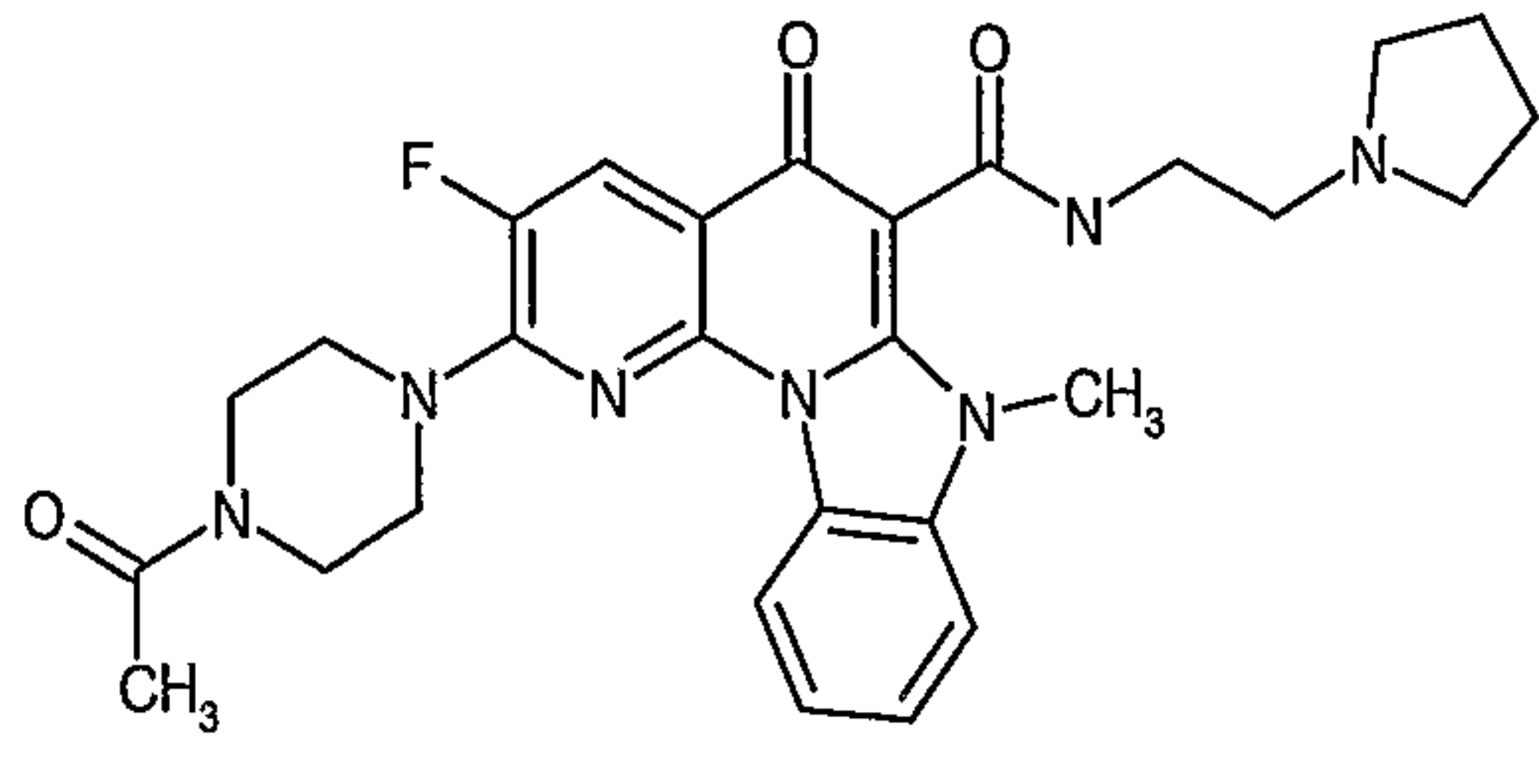
| MOLSTRUCTURE | M CELL  | M DATA |
|--------------|---------|--------|
|              | HCT-116 | 0.03   |
|              | Miapaca | 0.02   |
|              | HCT-116 | 0.02   |

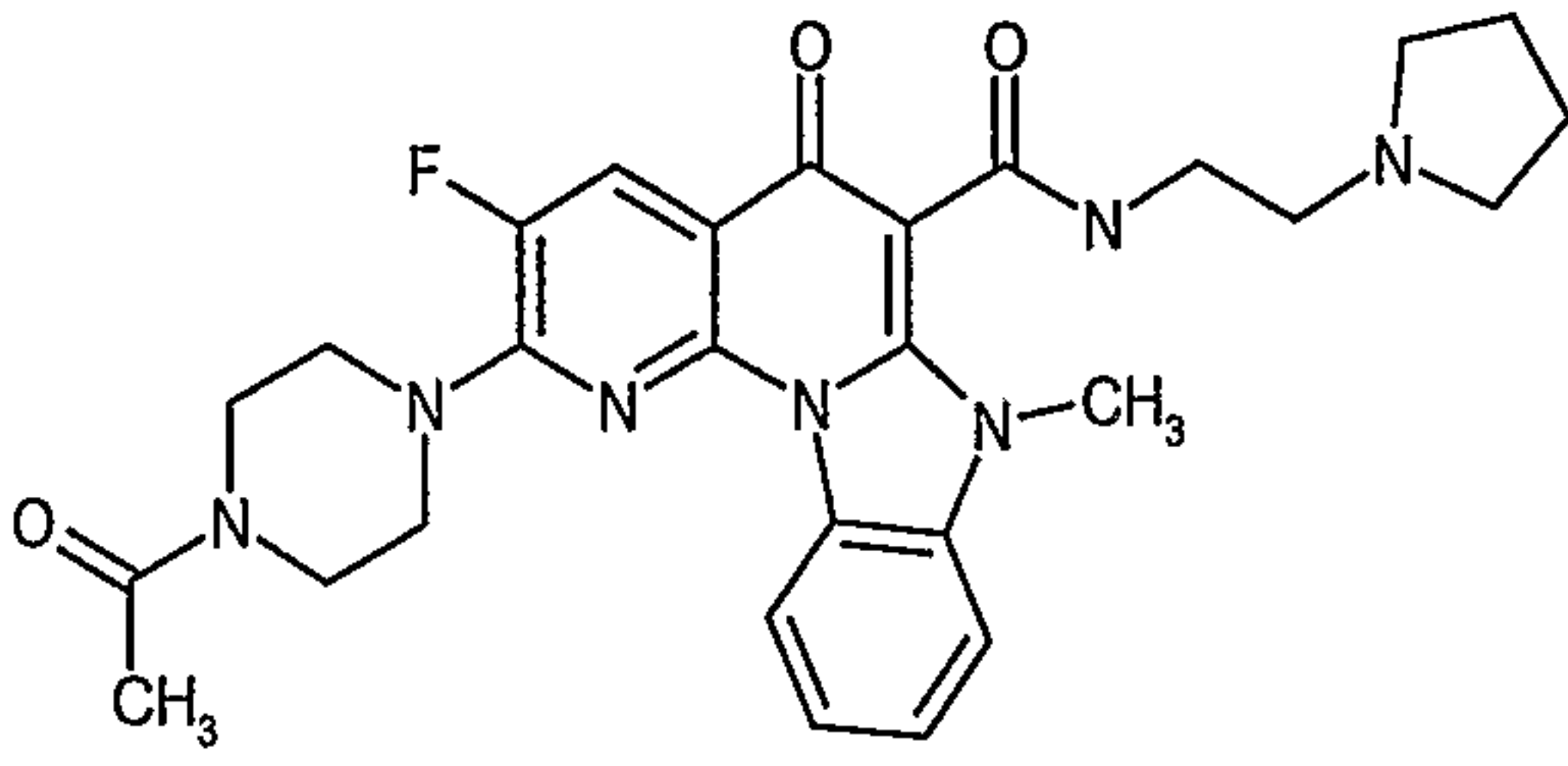
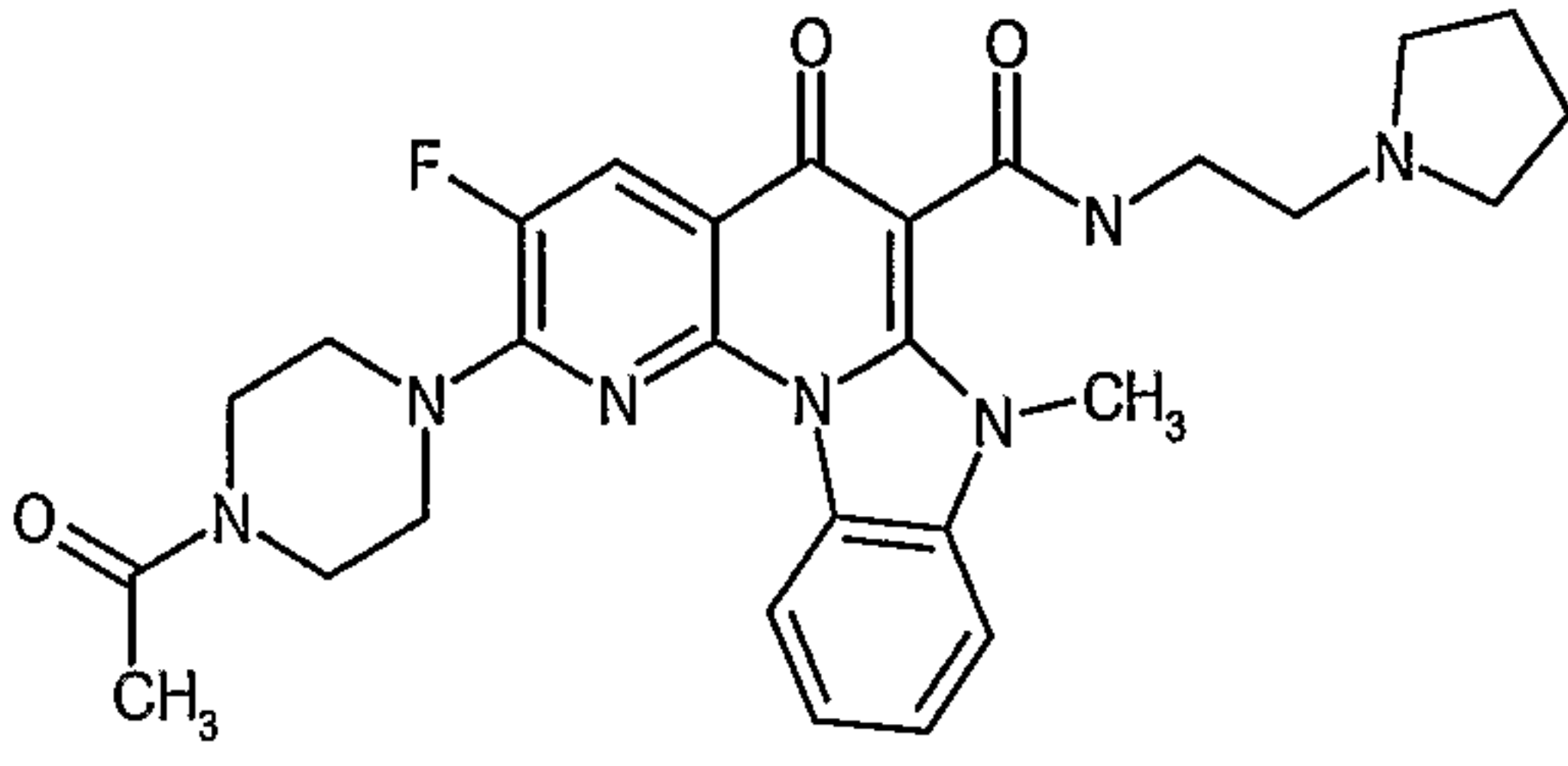
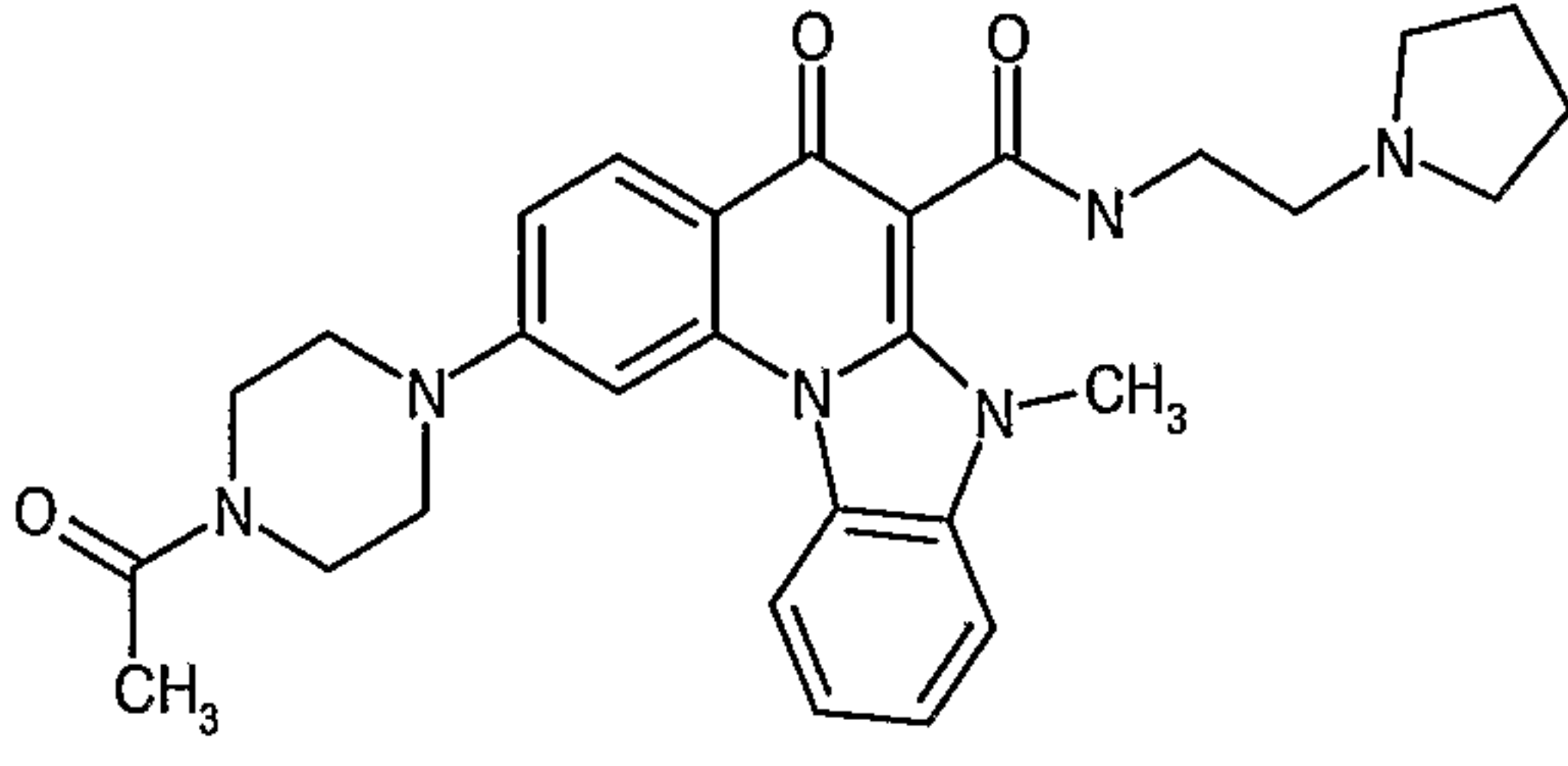
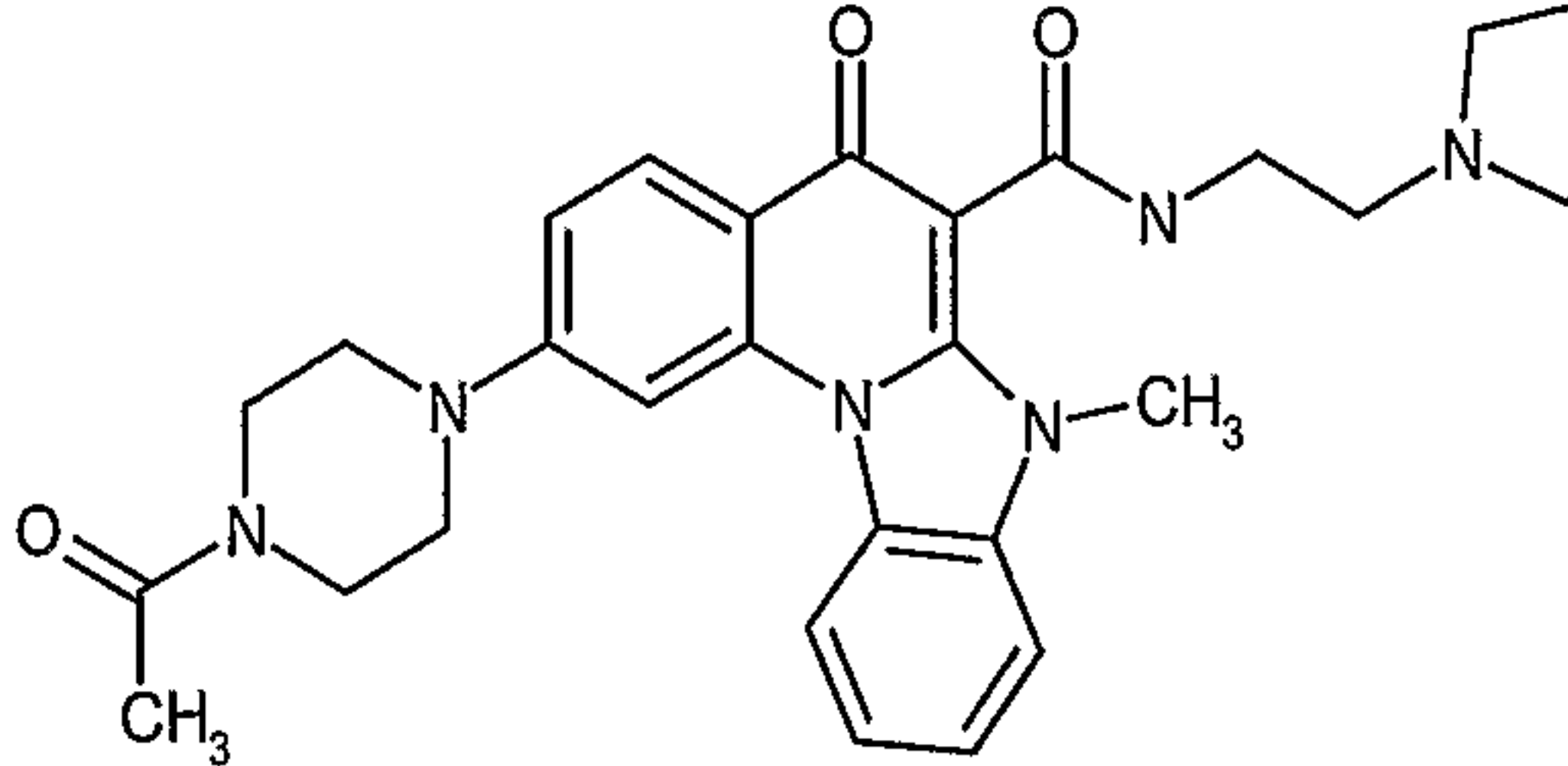
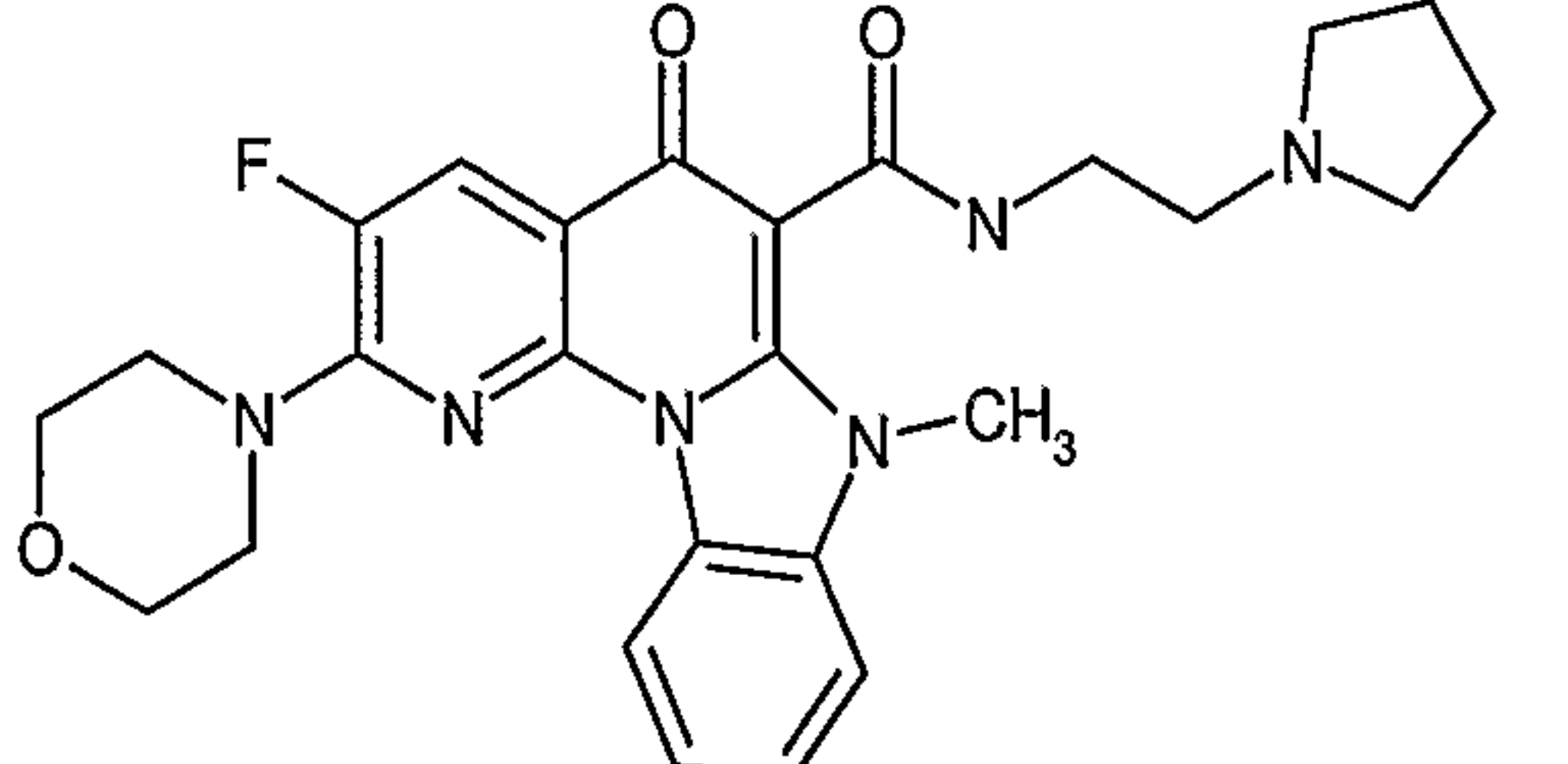
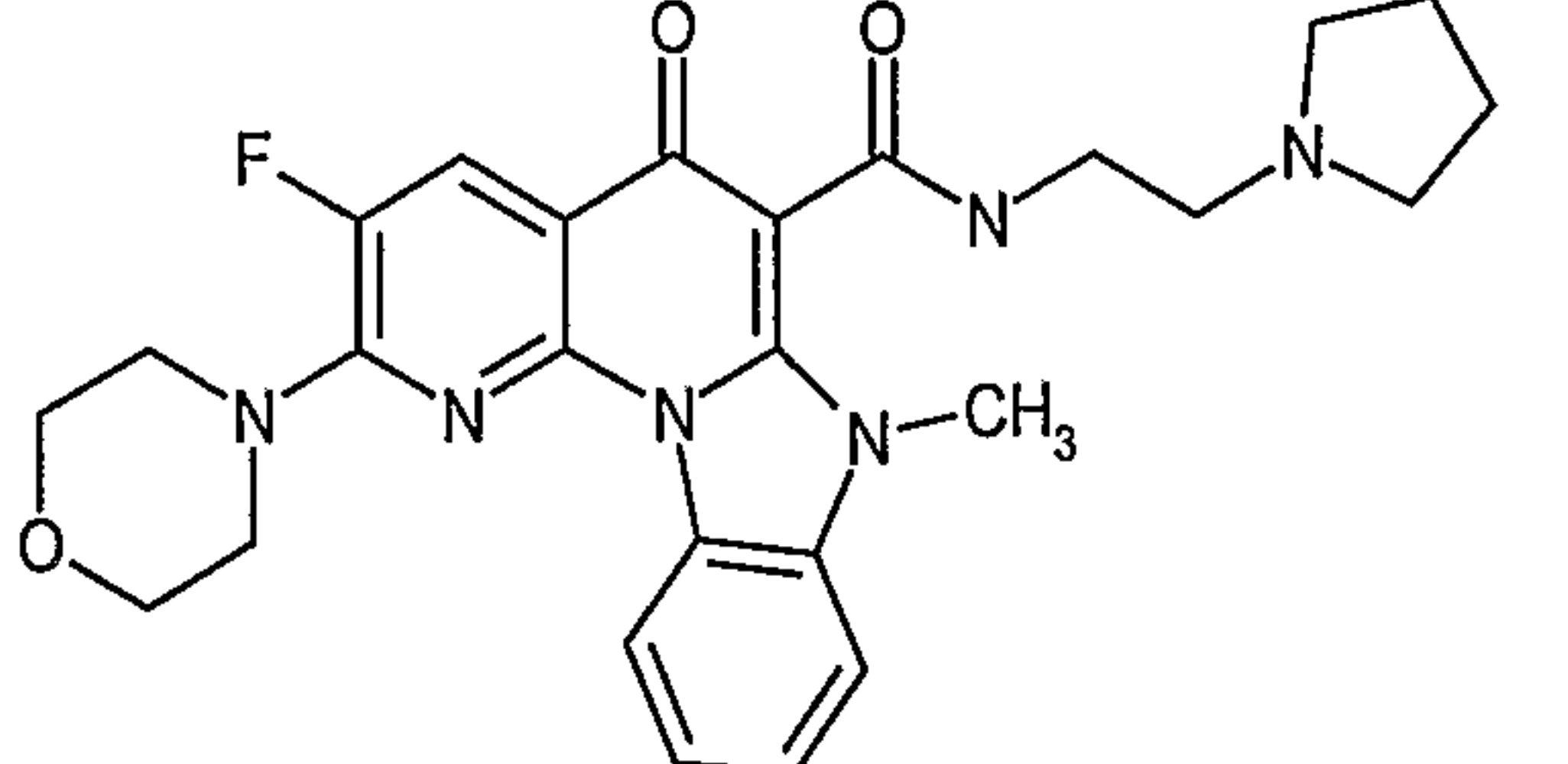
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | <0.01  |
|   | HCT-116 | 0.16   |
|  | Miapaca | 0.17   |
|  | HCT-116 | <0.01  |
|  | Miapaca | <0.01  |
|   | HCT-116 | <0.01  |

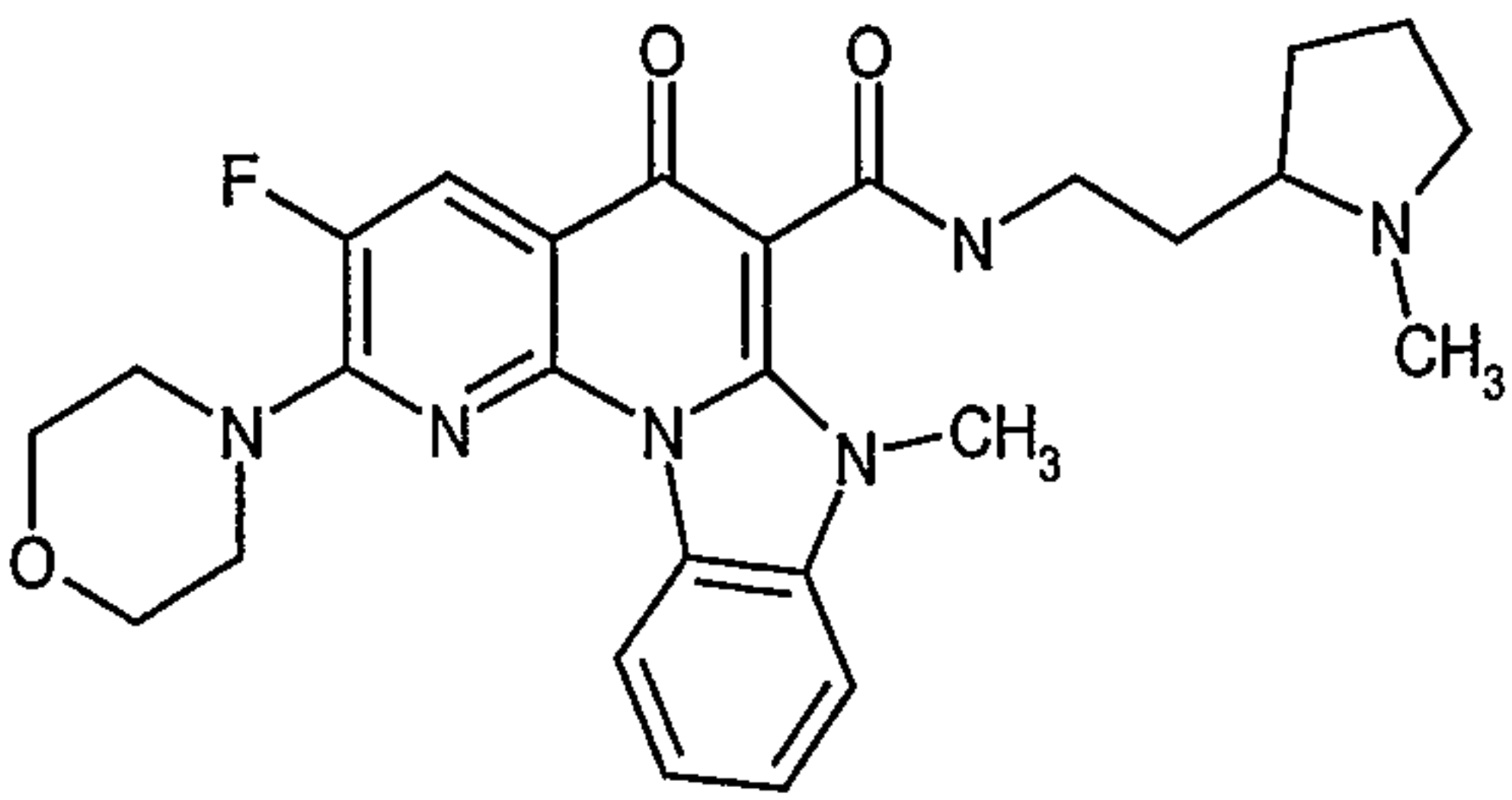
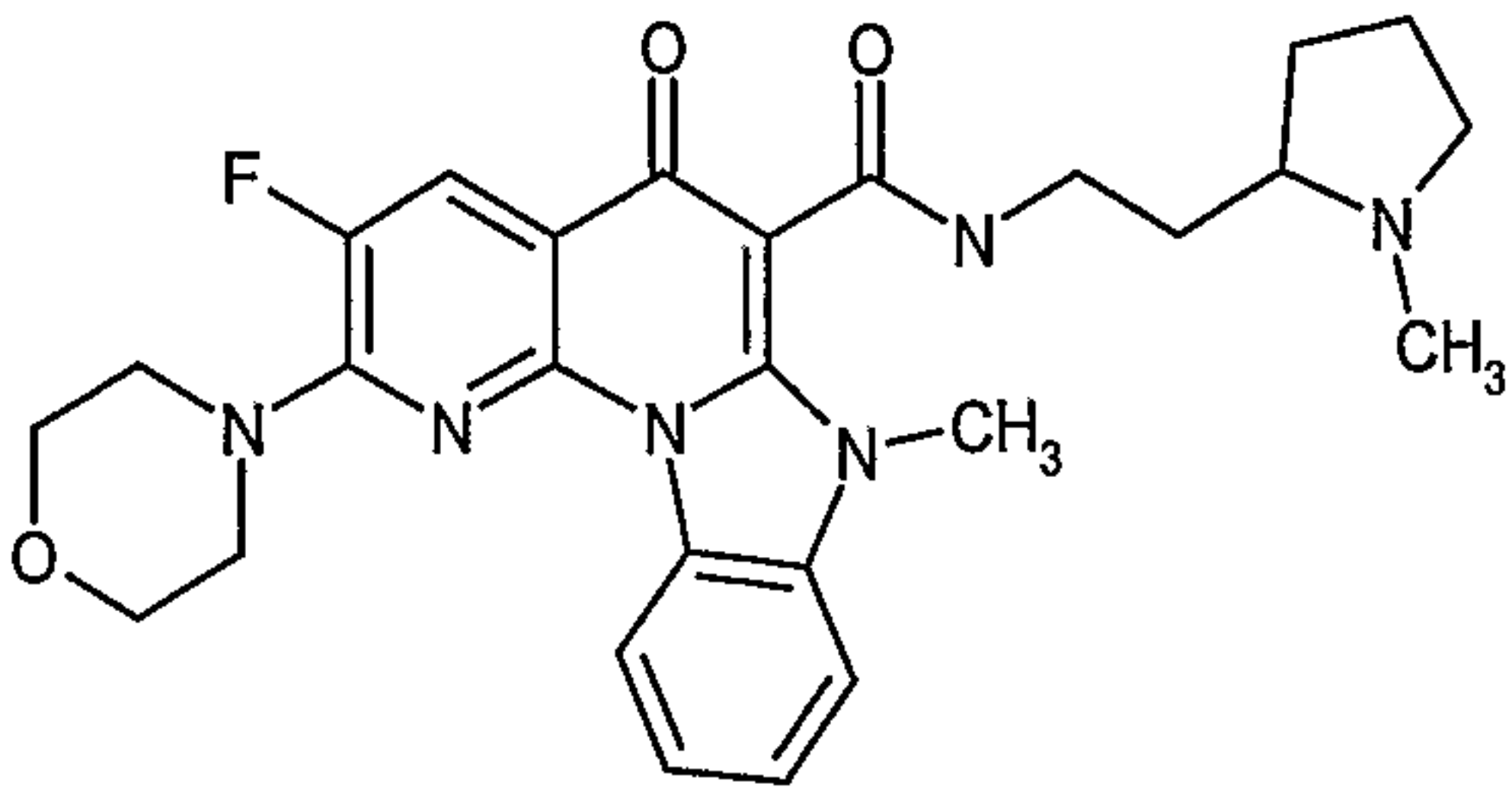
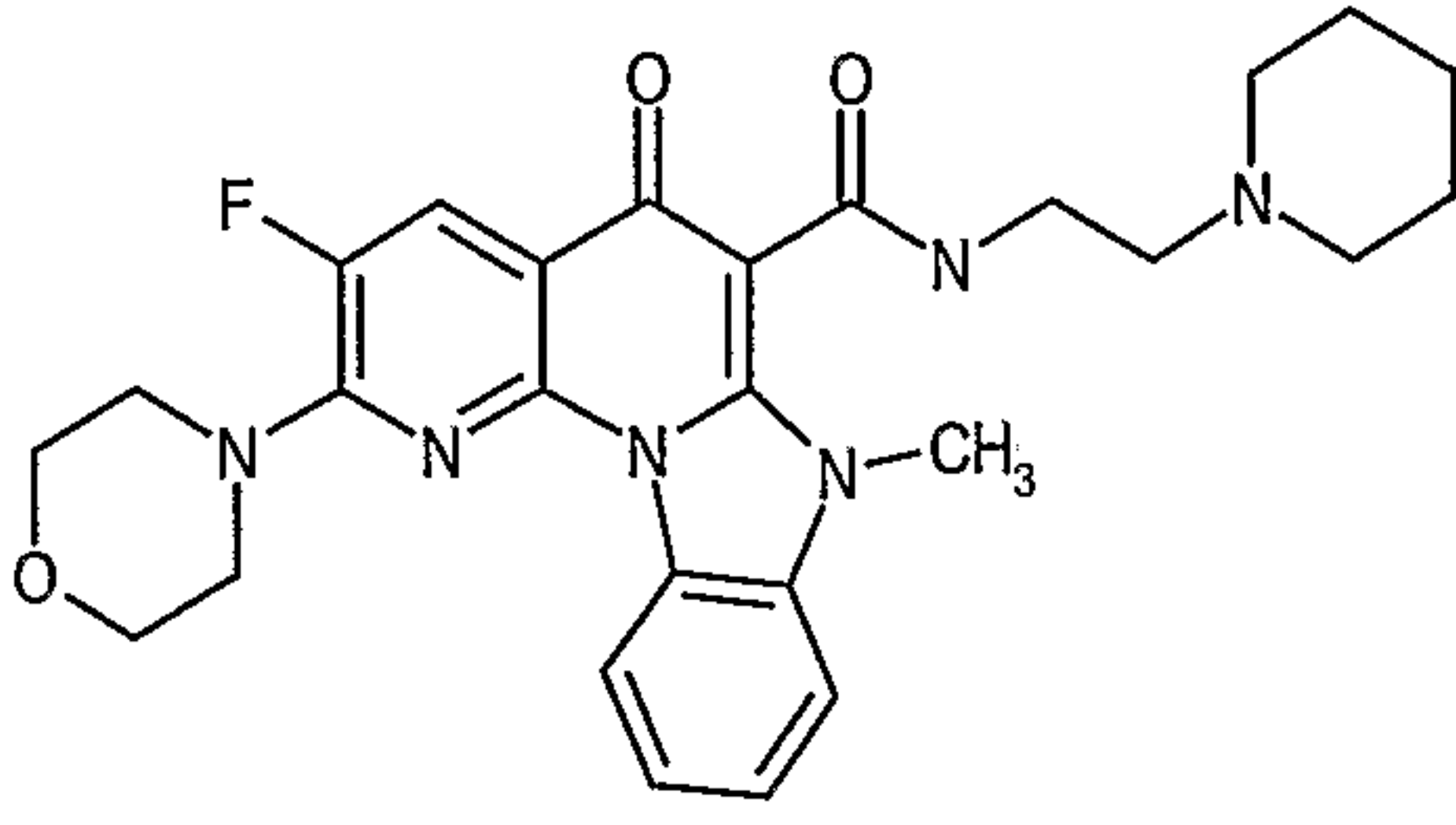
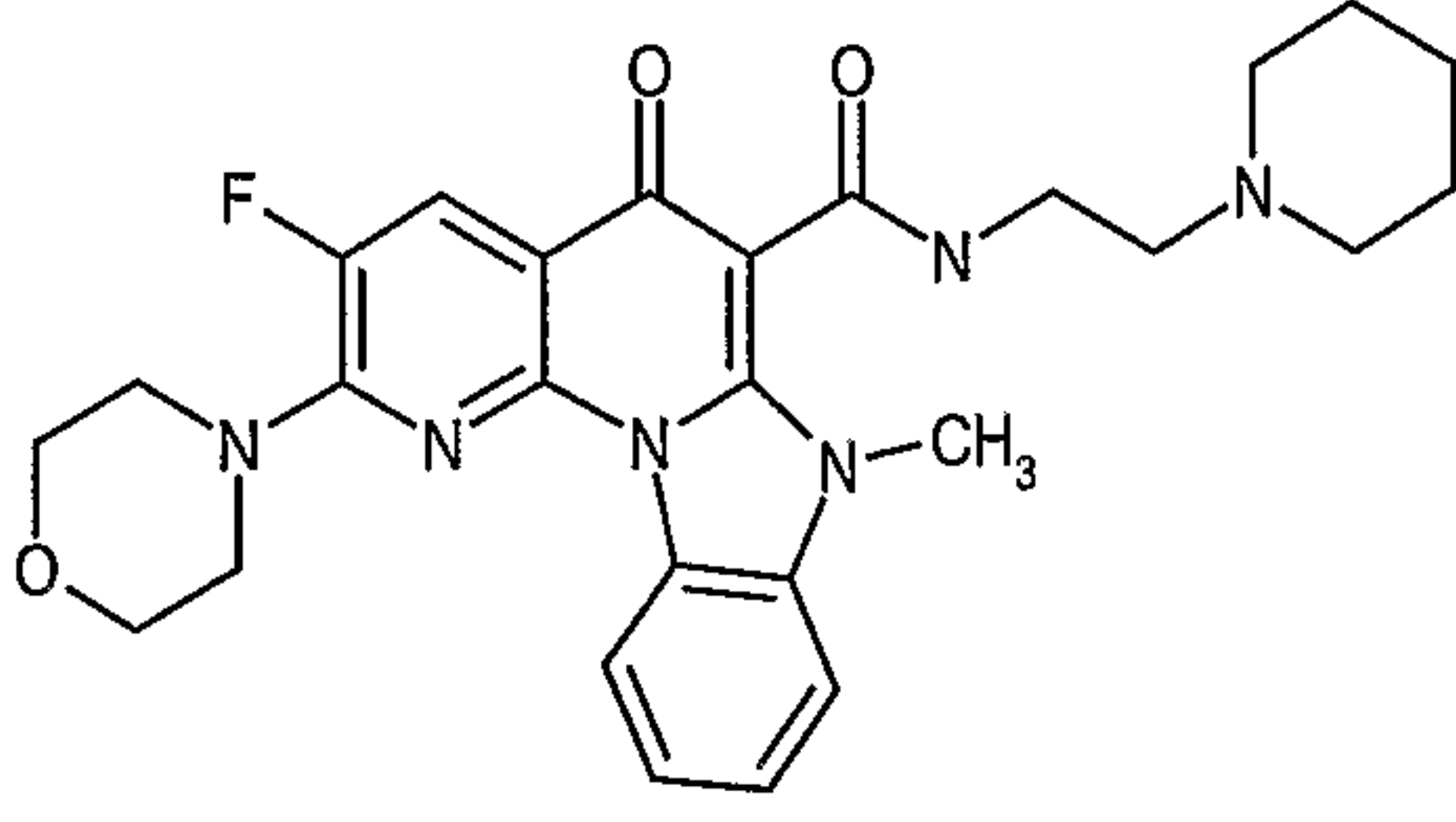
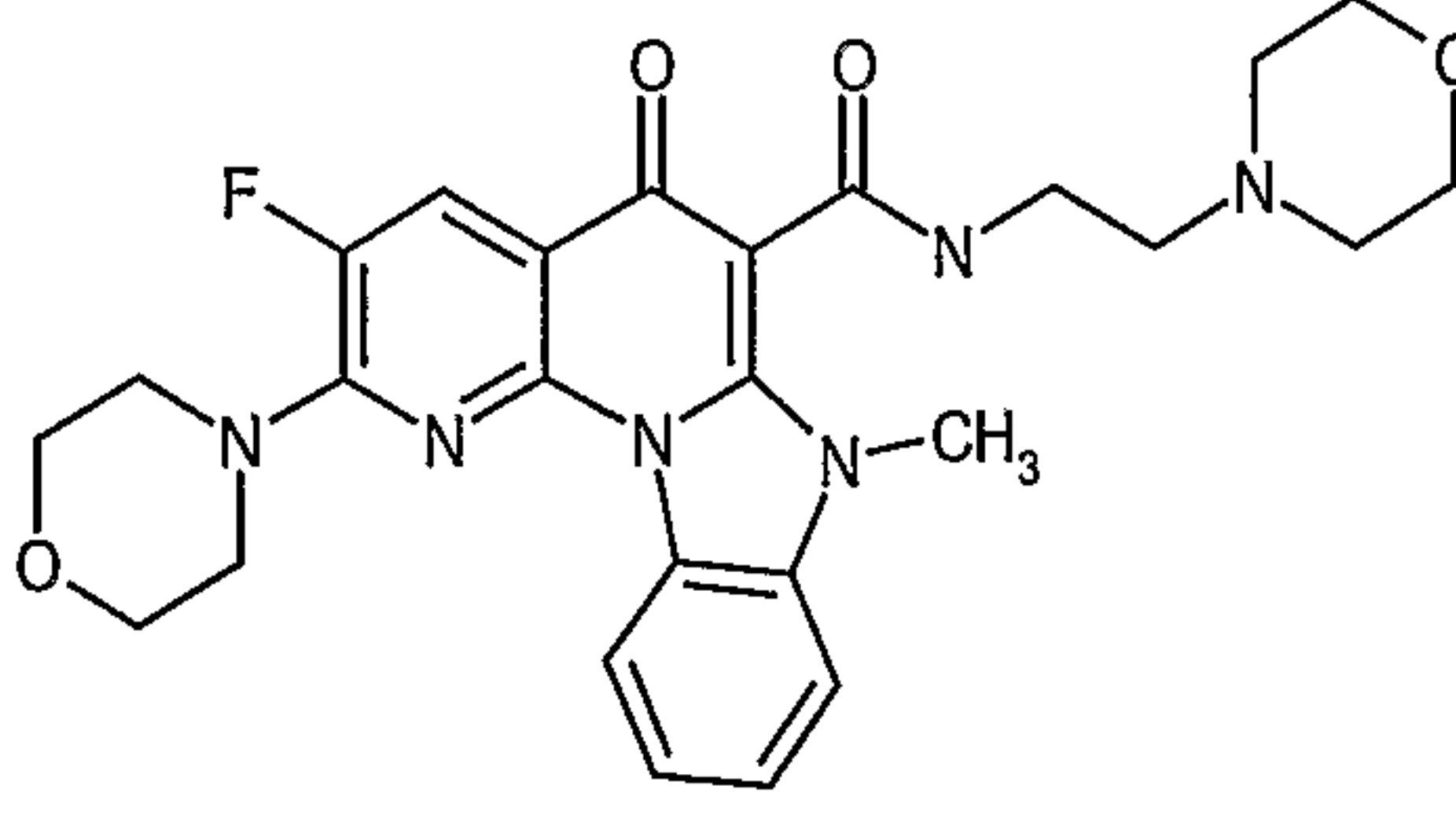
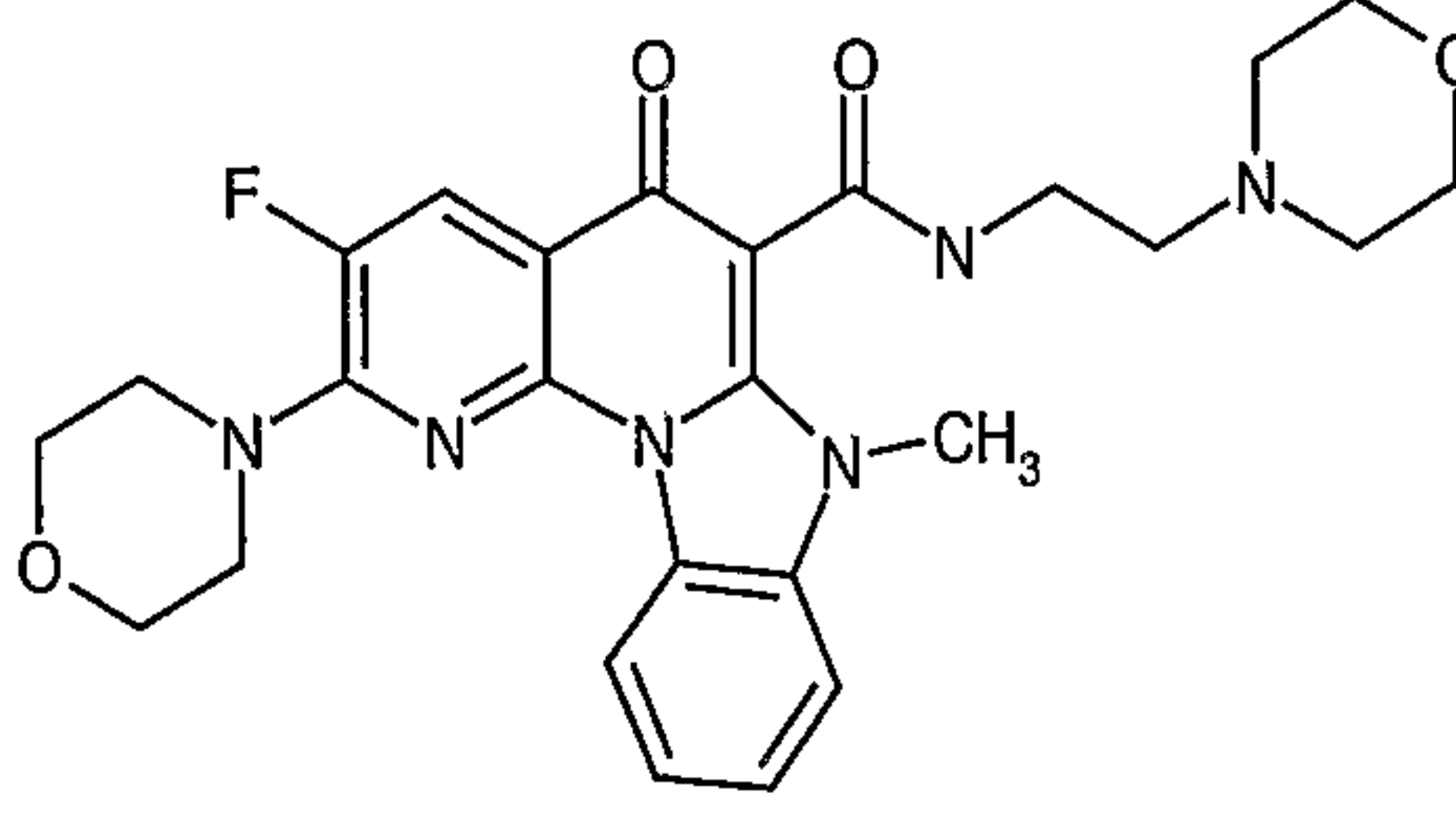
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | Miapaca | <0.01  |
|    | HCT-116 | 0.03   |
|   | Miapaca | 0.02   |
|   | HCT-116 | 0.25   |
|  | MiaPaca | 0.1    |
|  | HCT-116 | 0.06   |

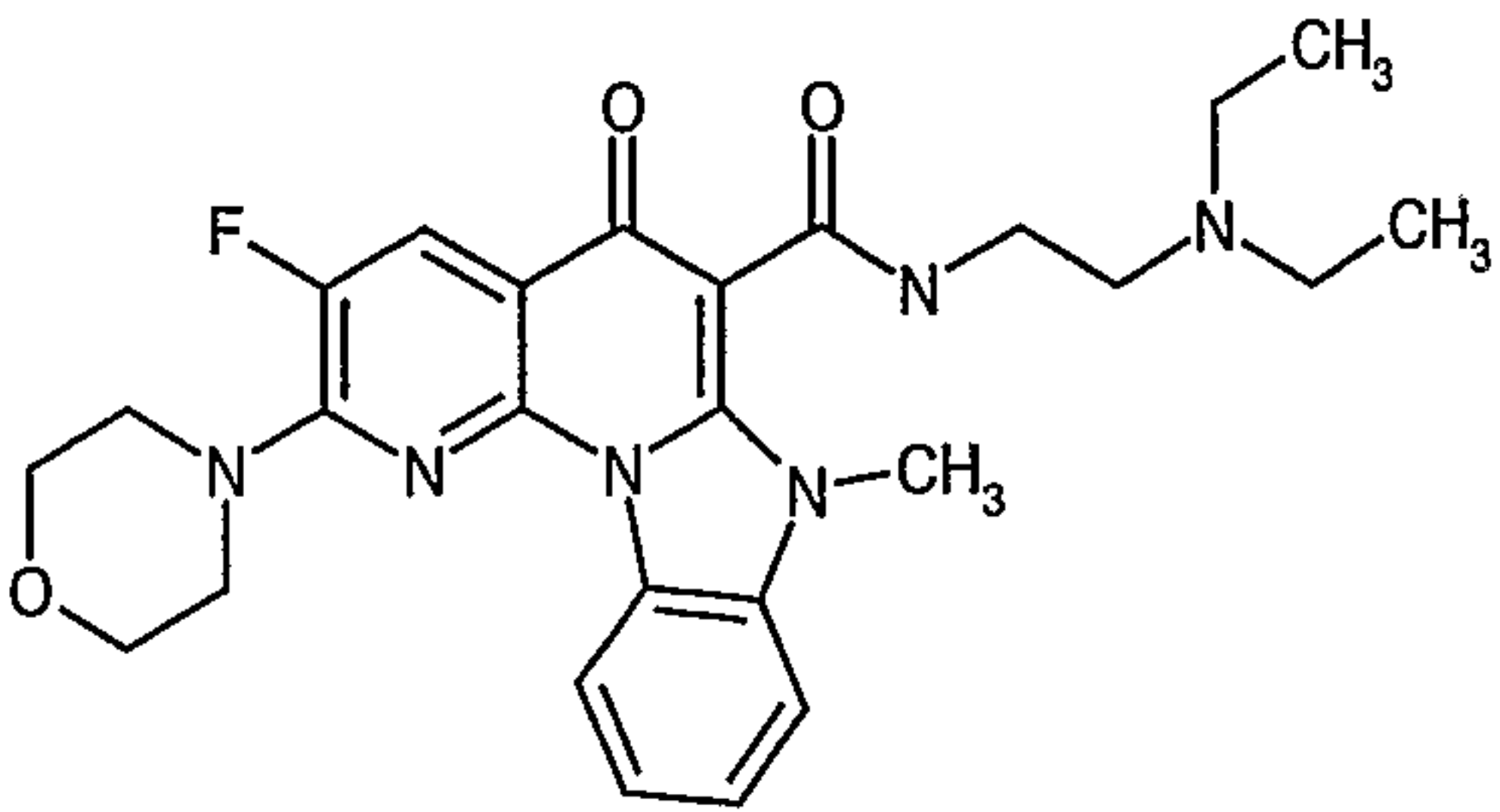
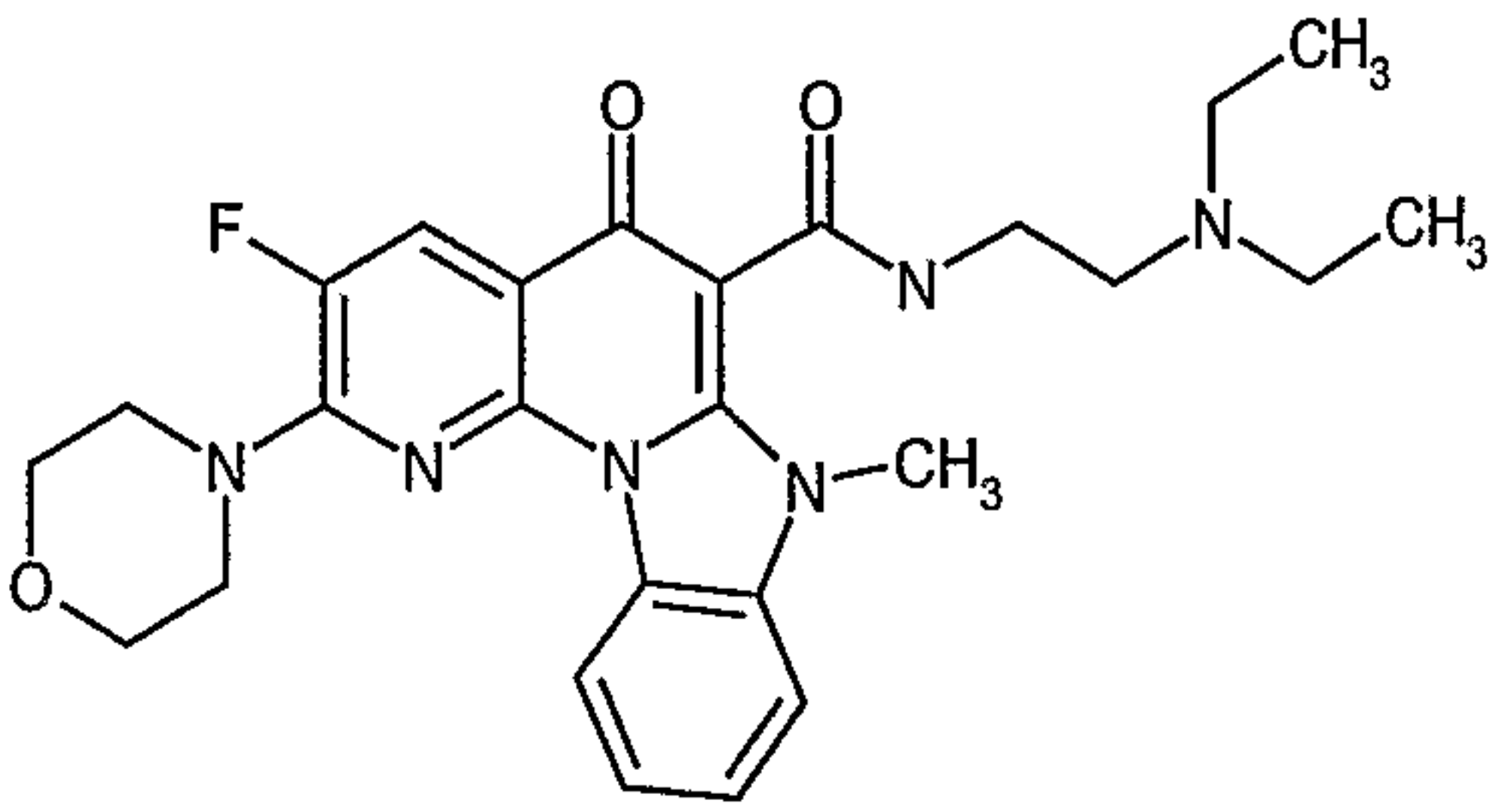
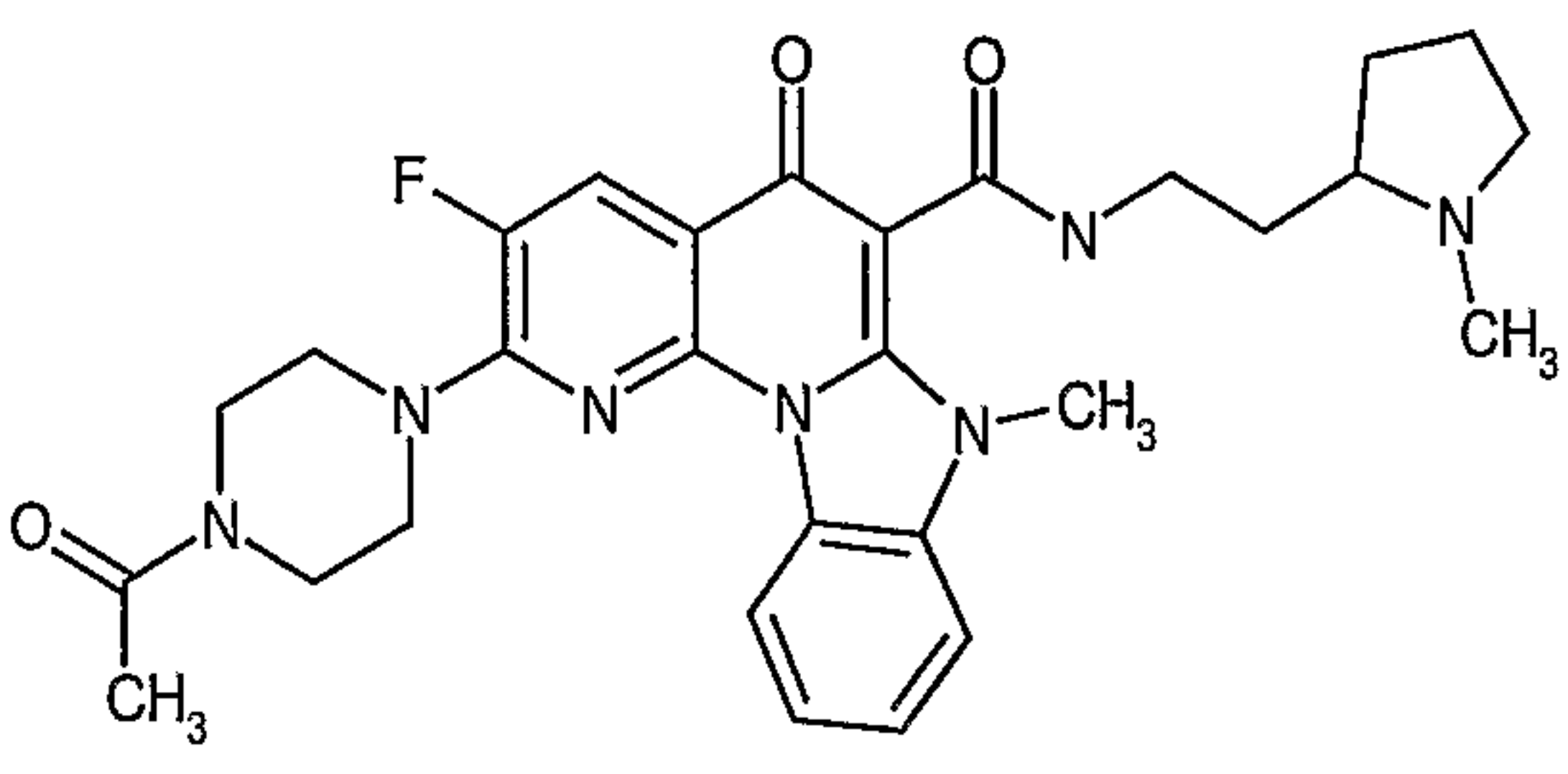
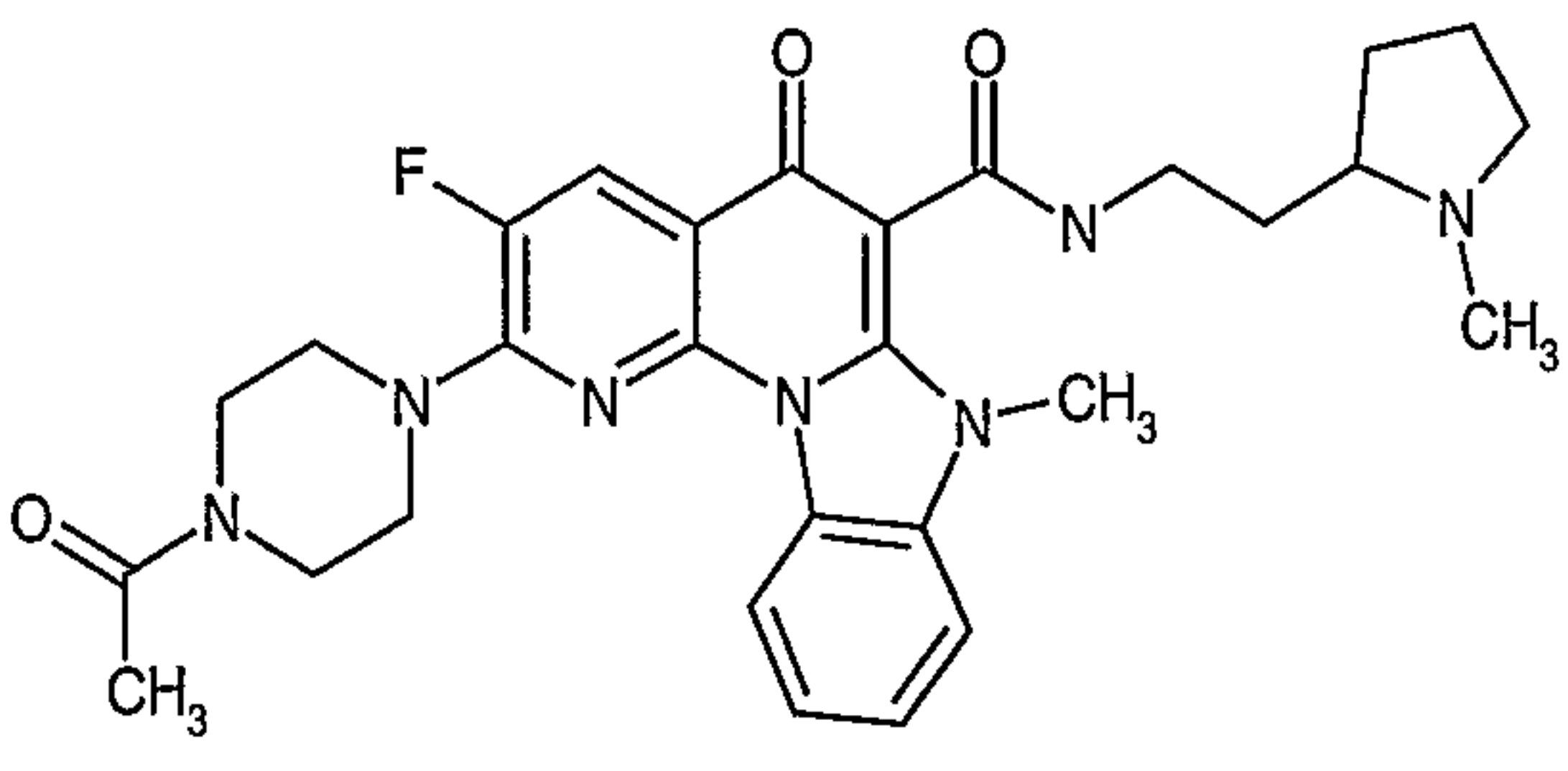
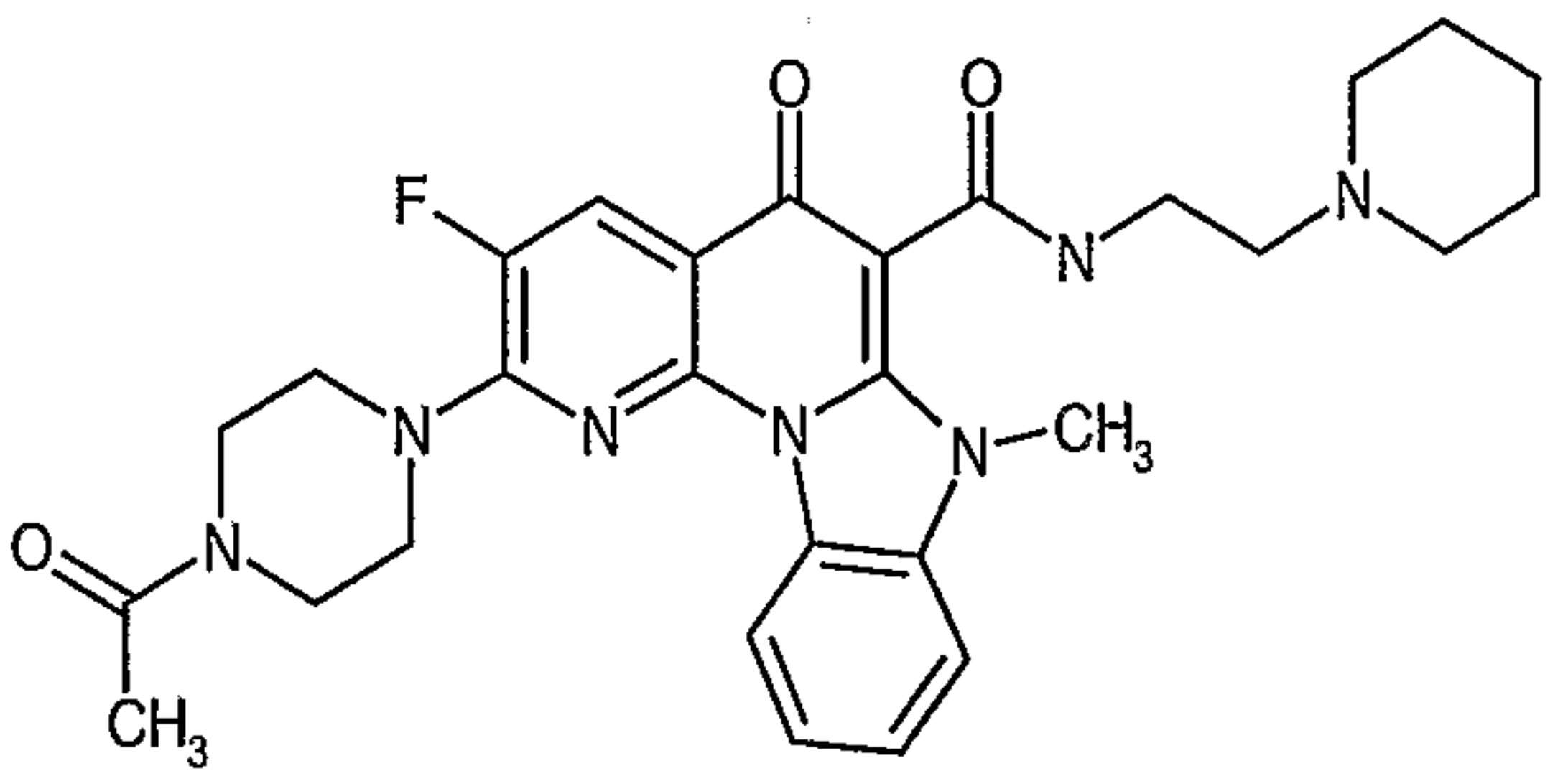
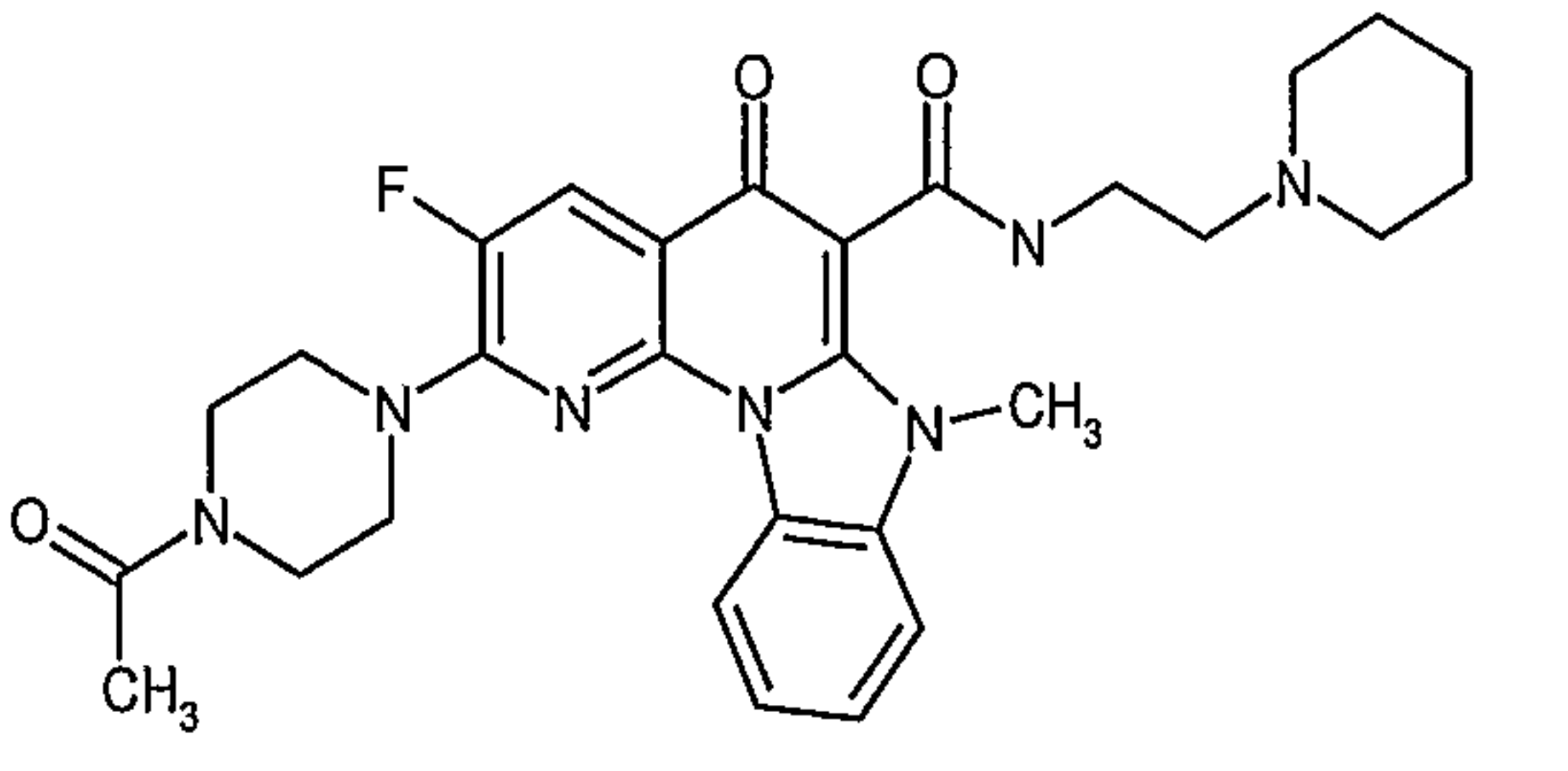
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | 0.03   |
|   | HCT-116 | 0.03   |
|  | Miapaca | 0.02   |
|  | HCT-116 | 0.32   |
|  | Miapaca | 0.24   |
|  | HCT-116 | 0.18   |

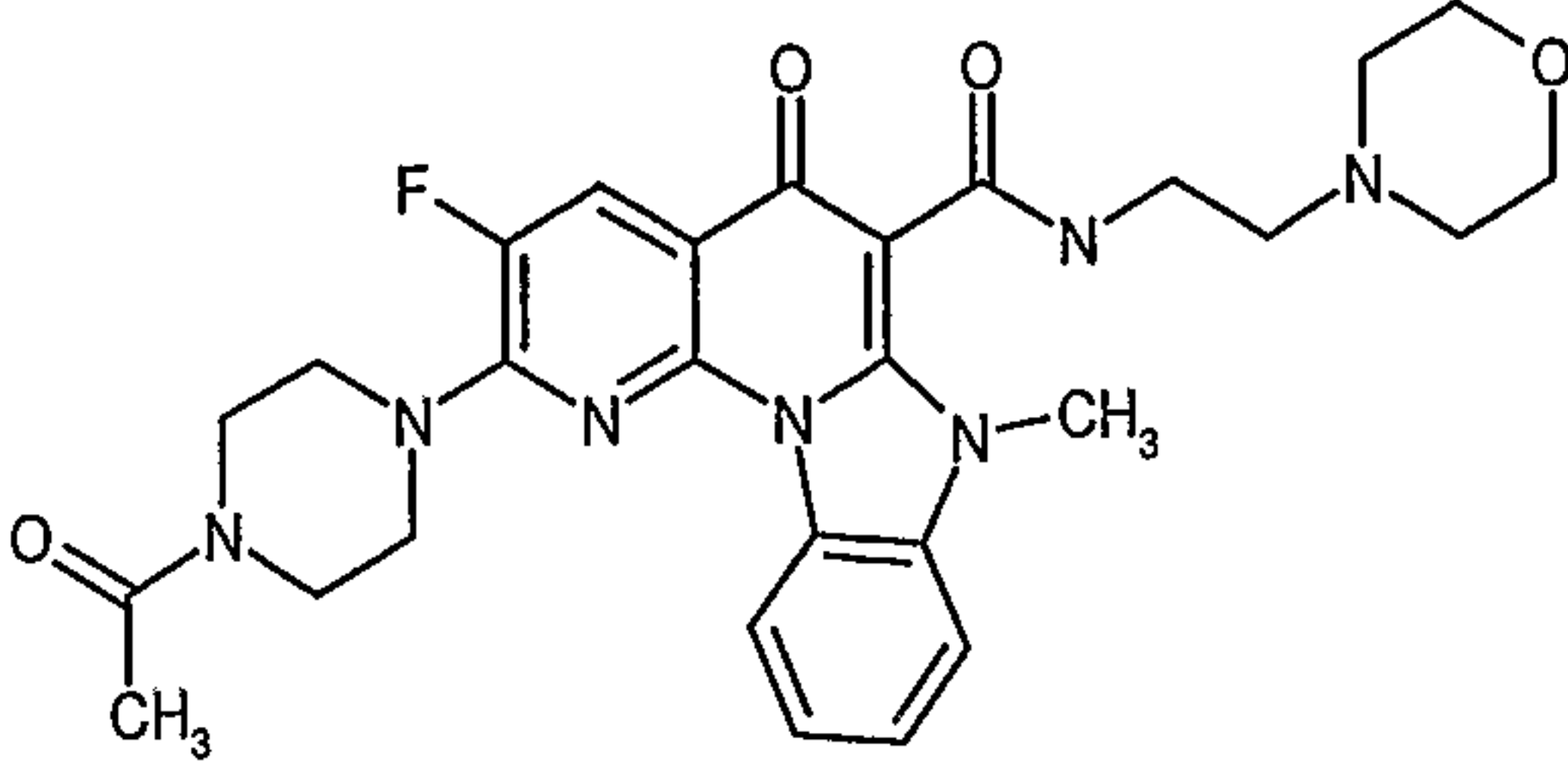
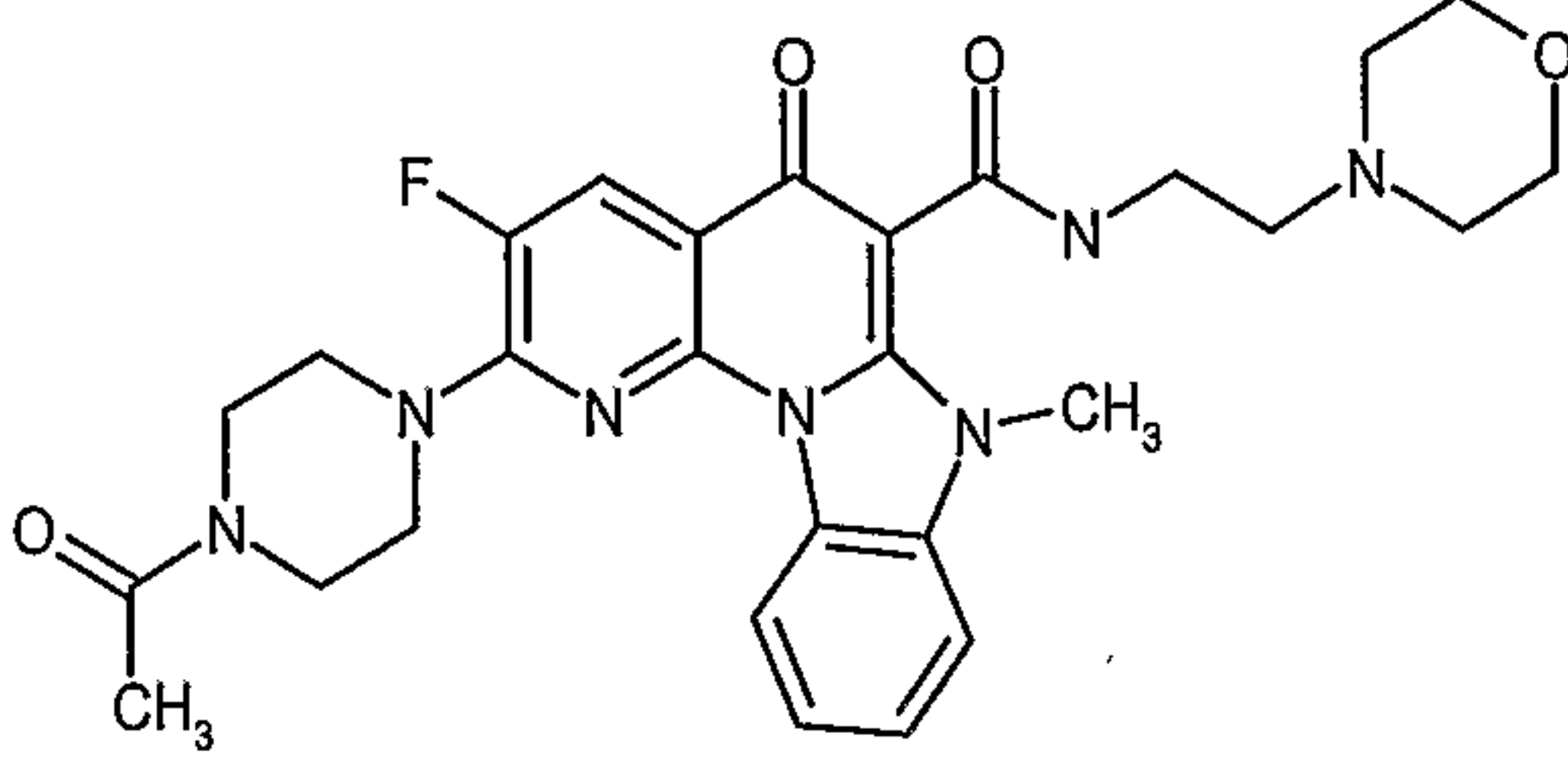
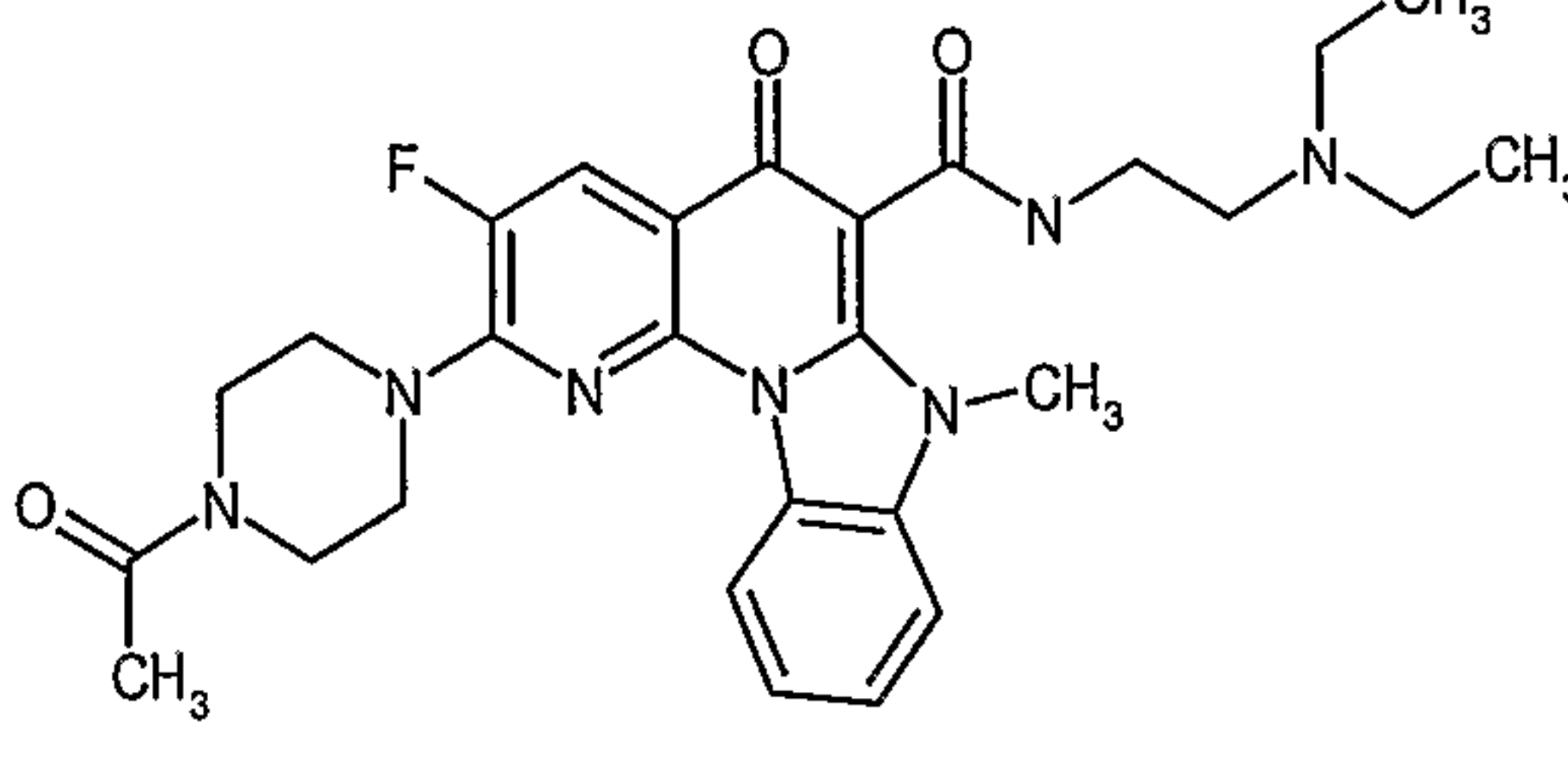
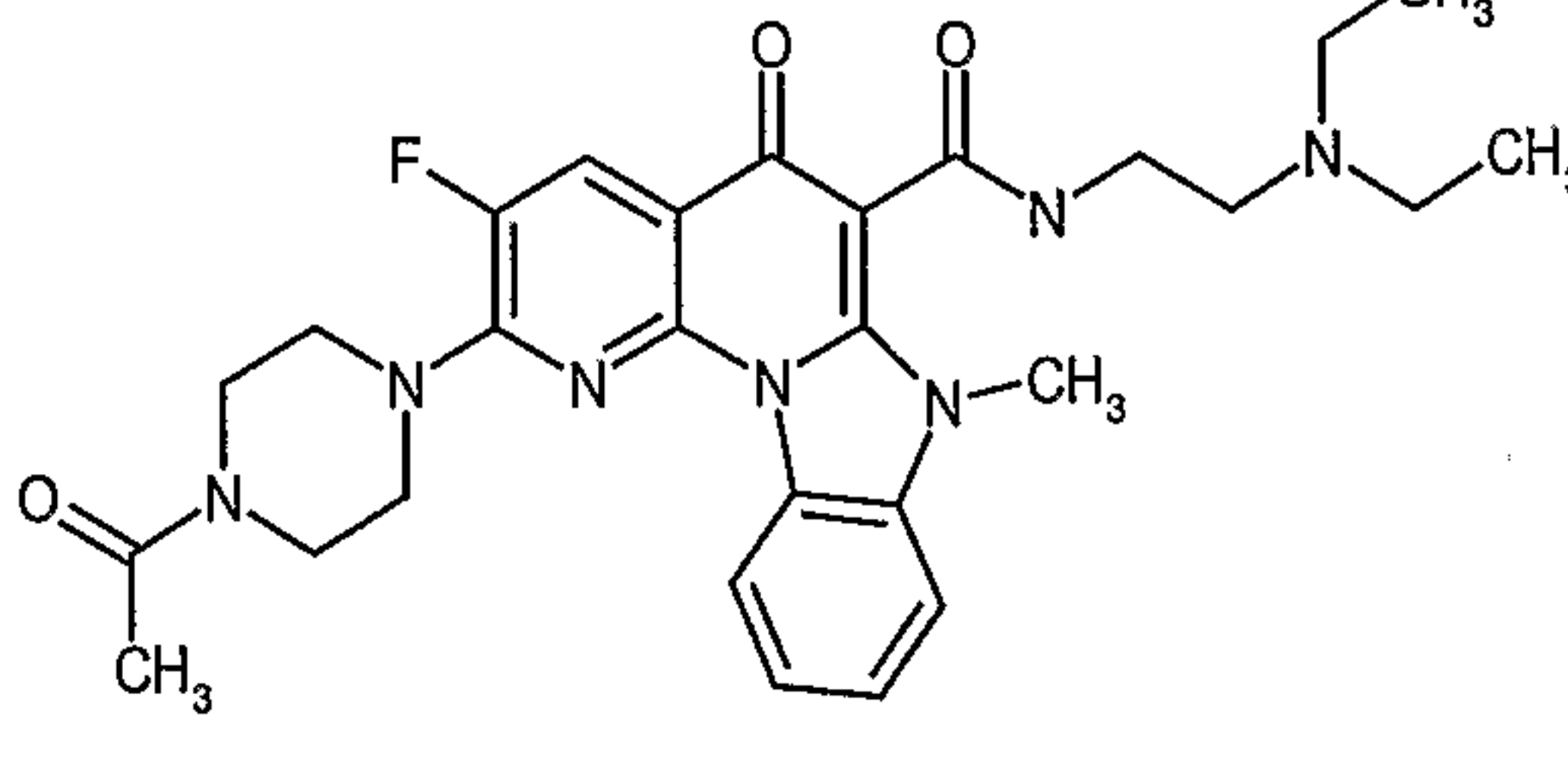
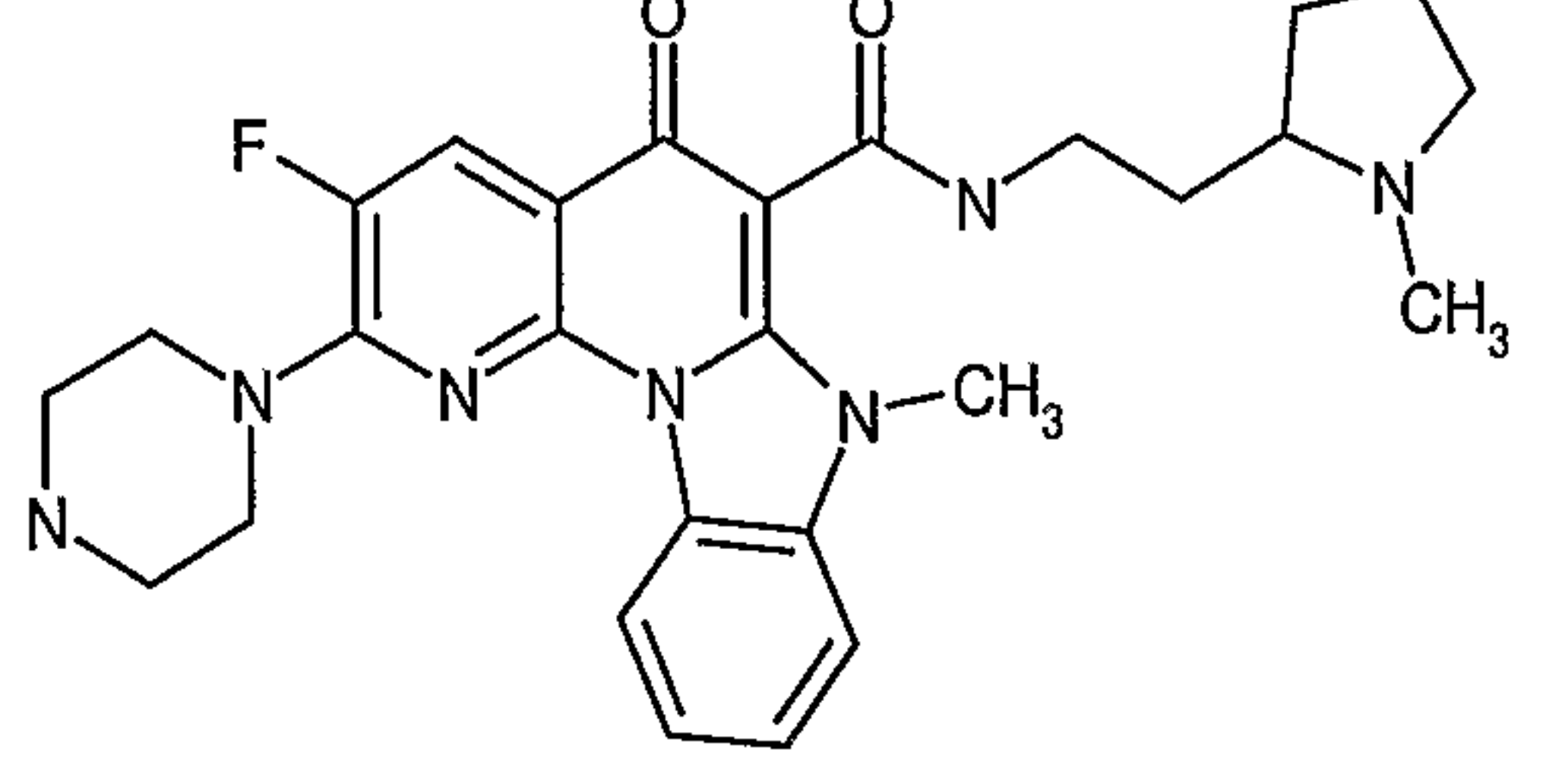
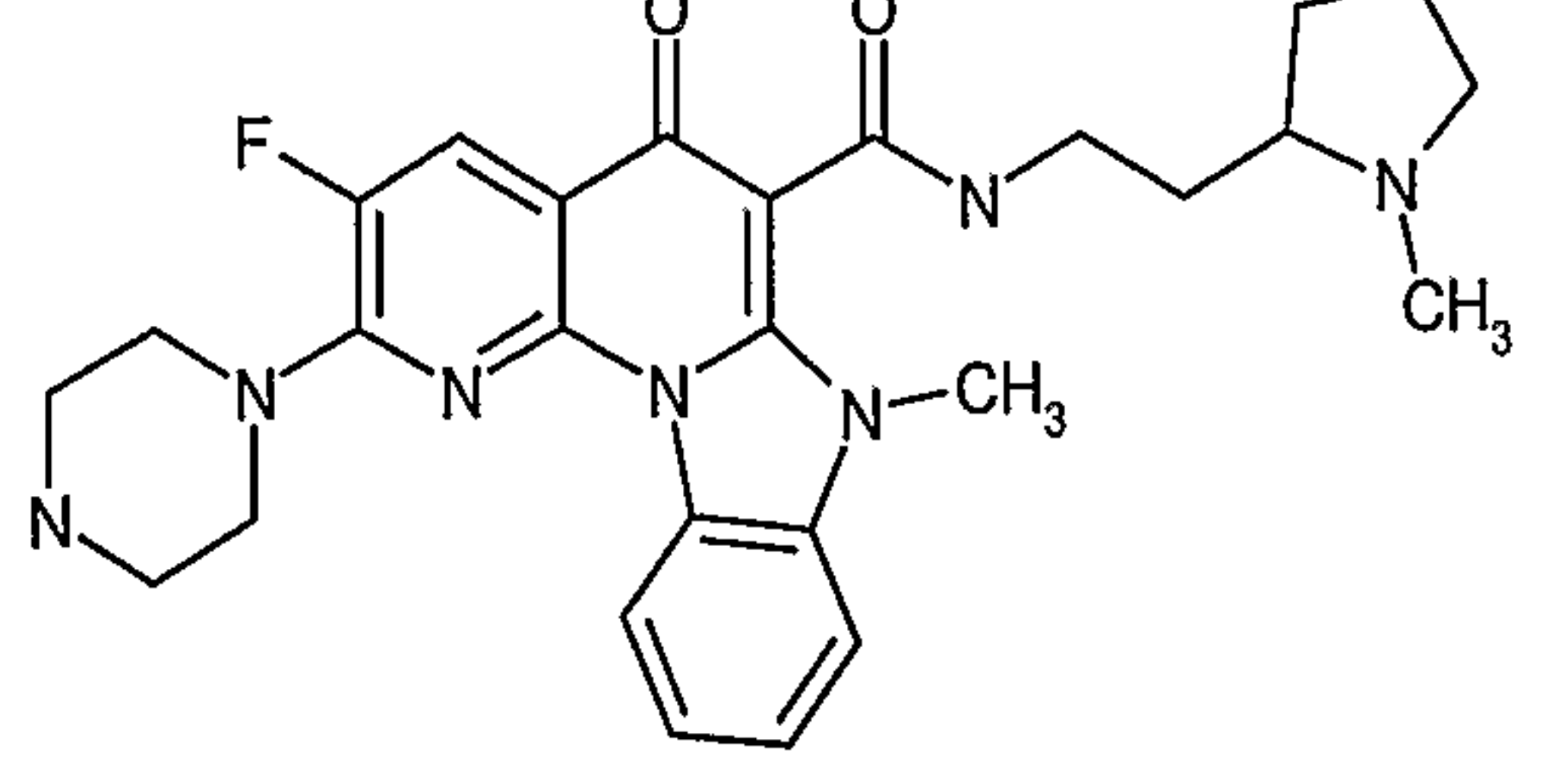
28

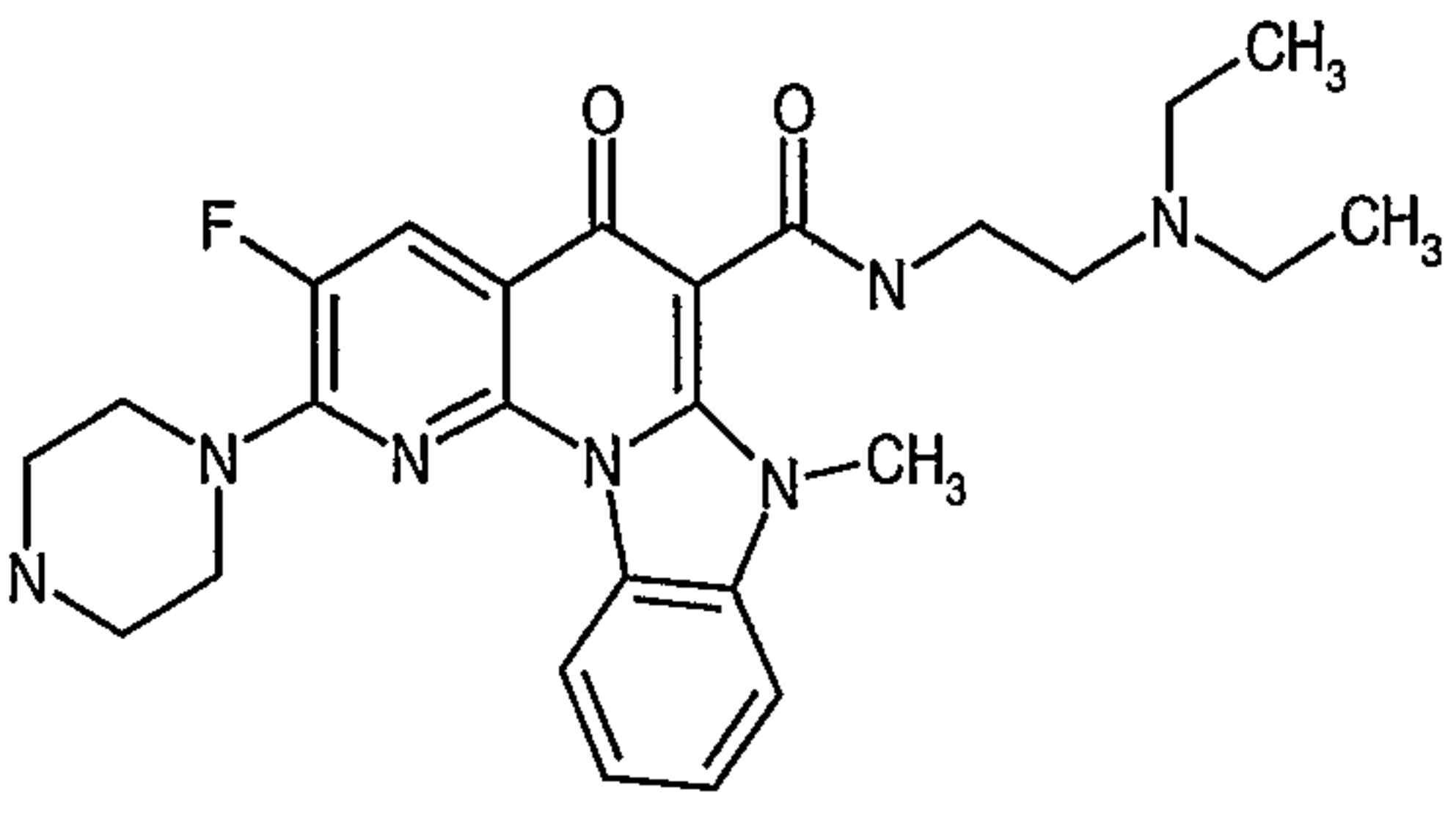
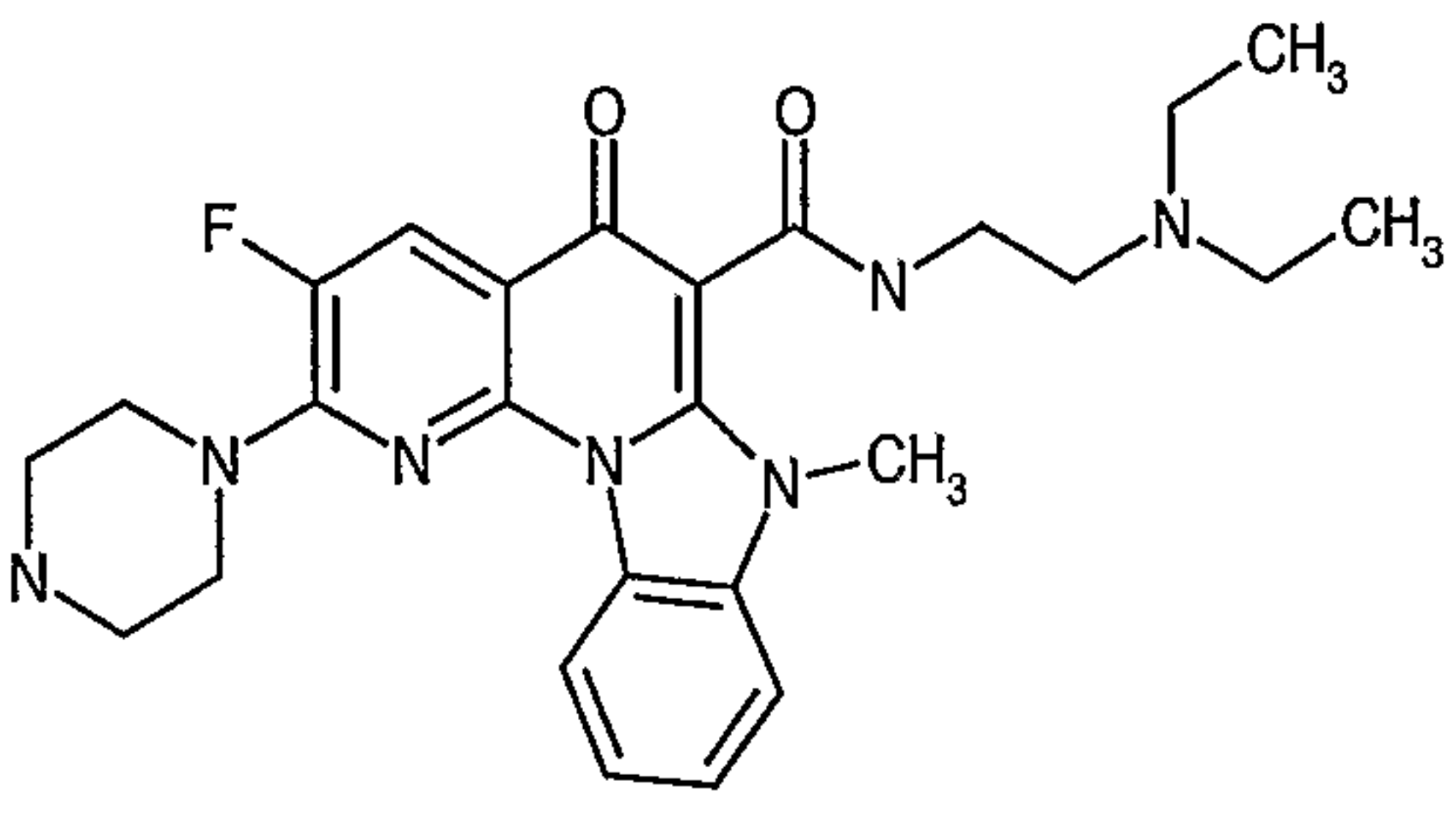
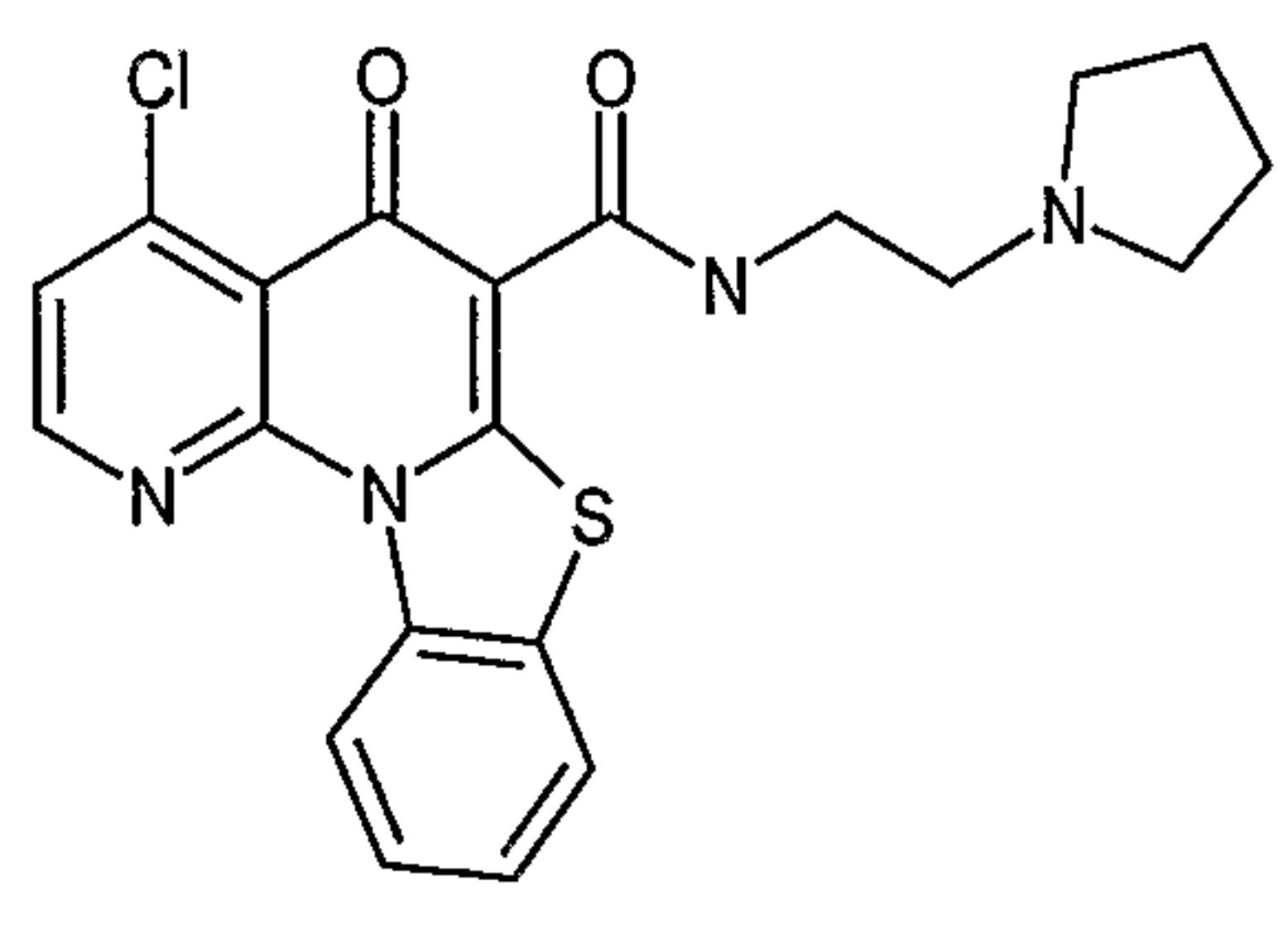
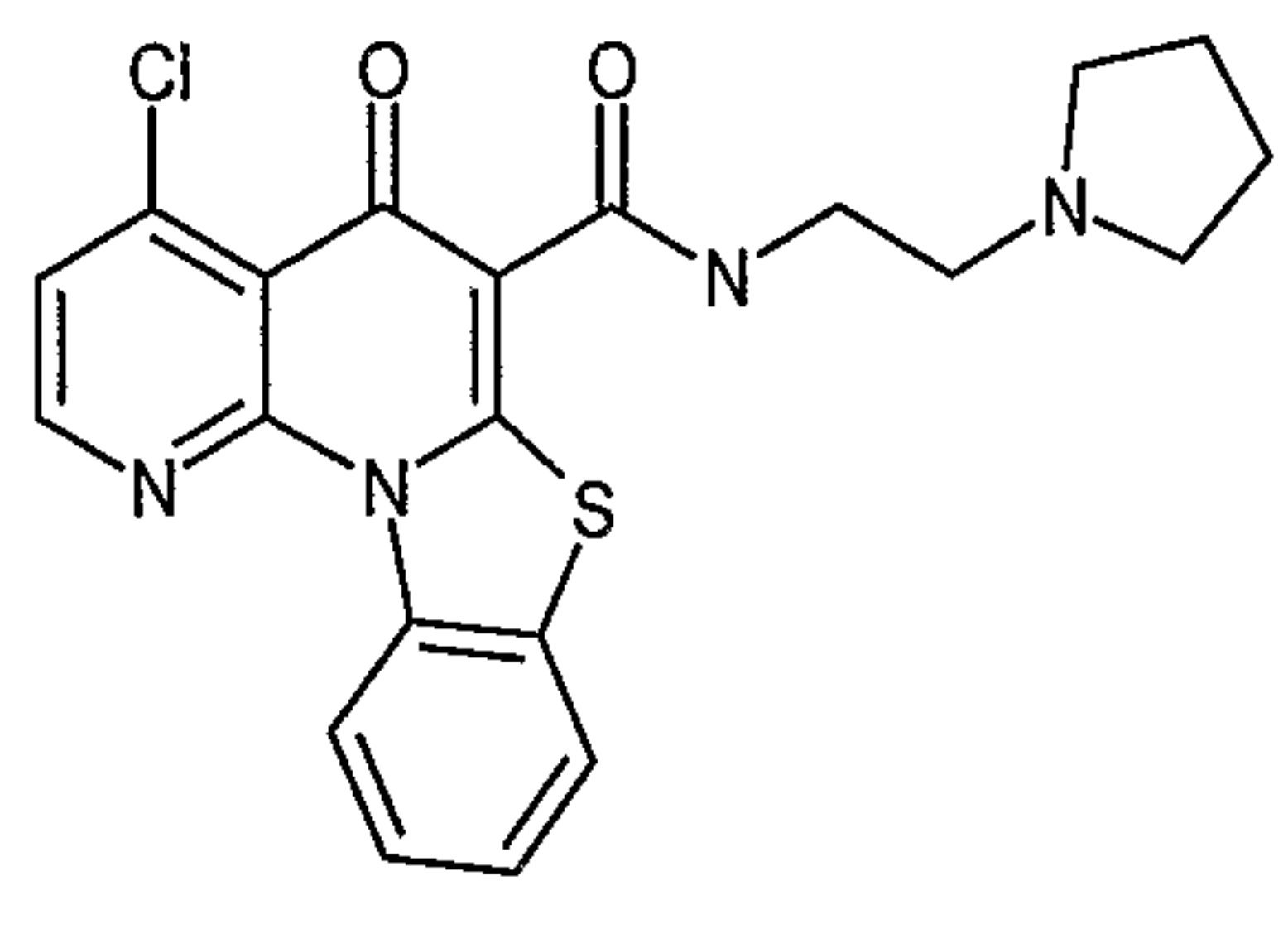
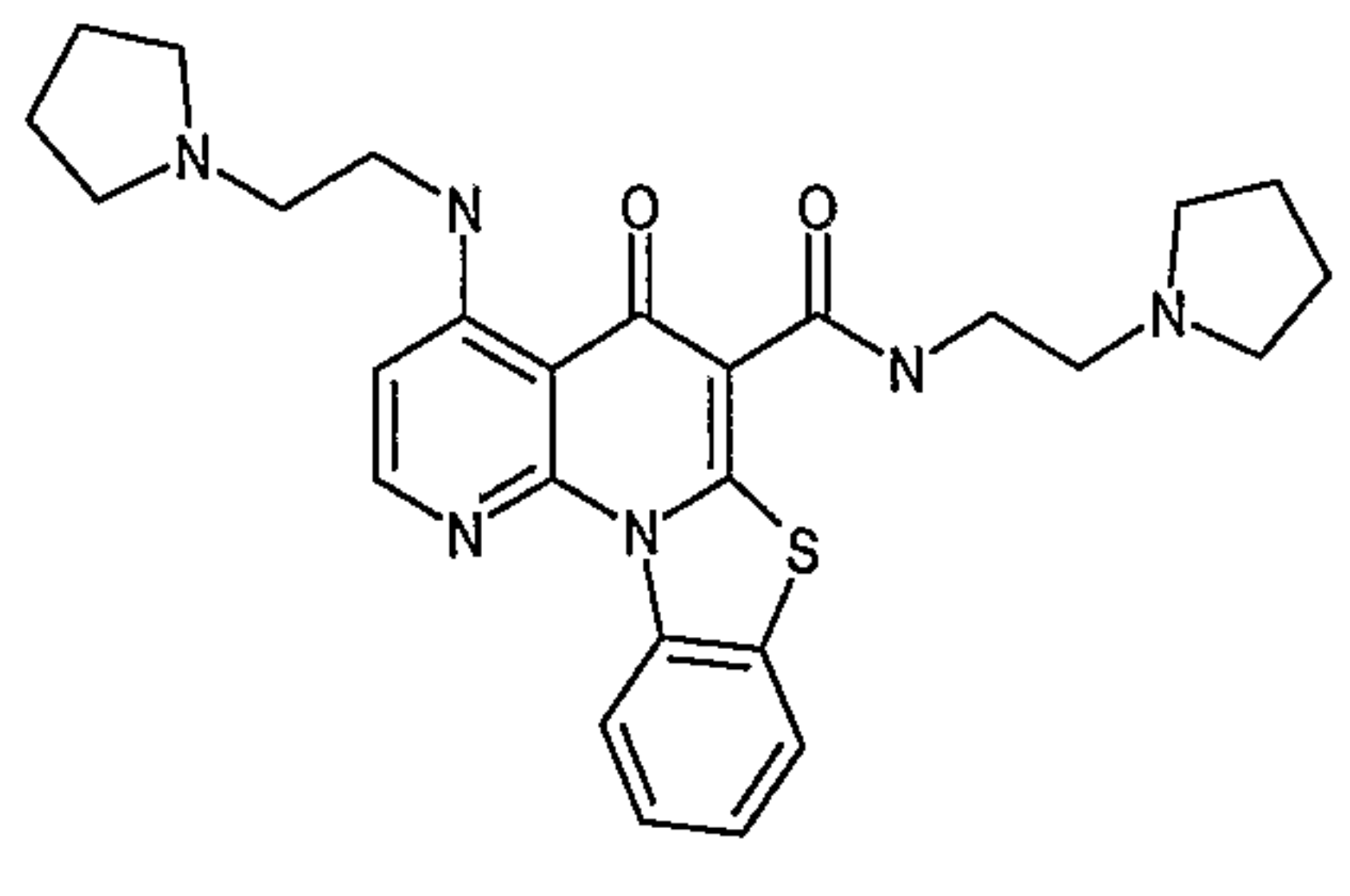
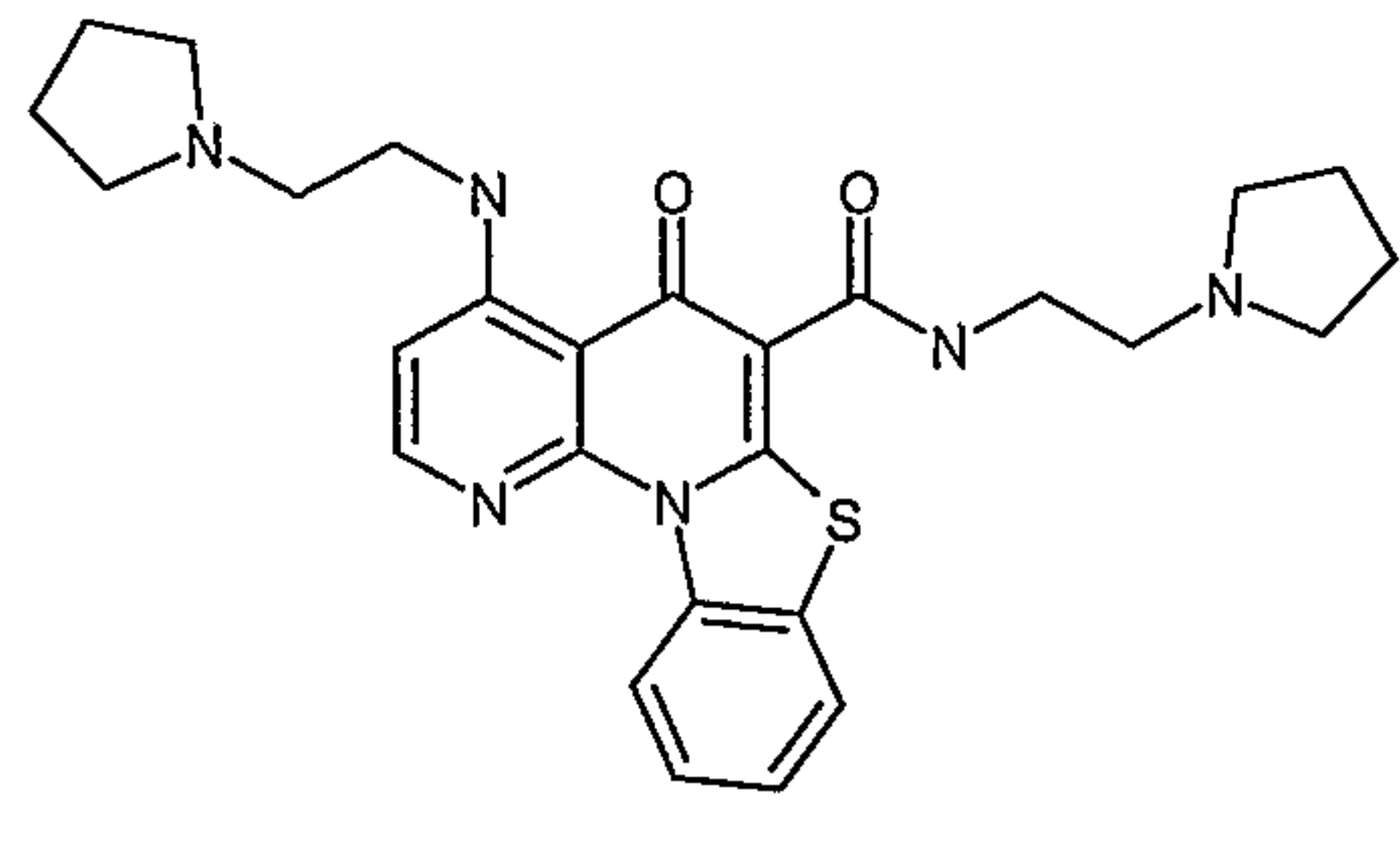
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | HCT-116 | 3.00   |
|    | Miapaca | 2.45   |
|   | HCT-116 | >10    |
|   | Miapaca | >10    |
|  | HCT-116 | 0.11   |
|  | HCT-116 | 0.1    |

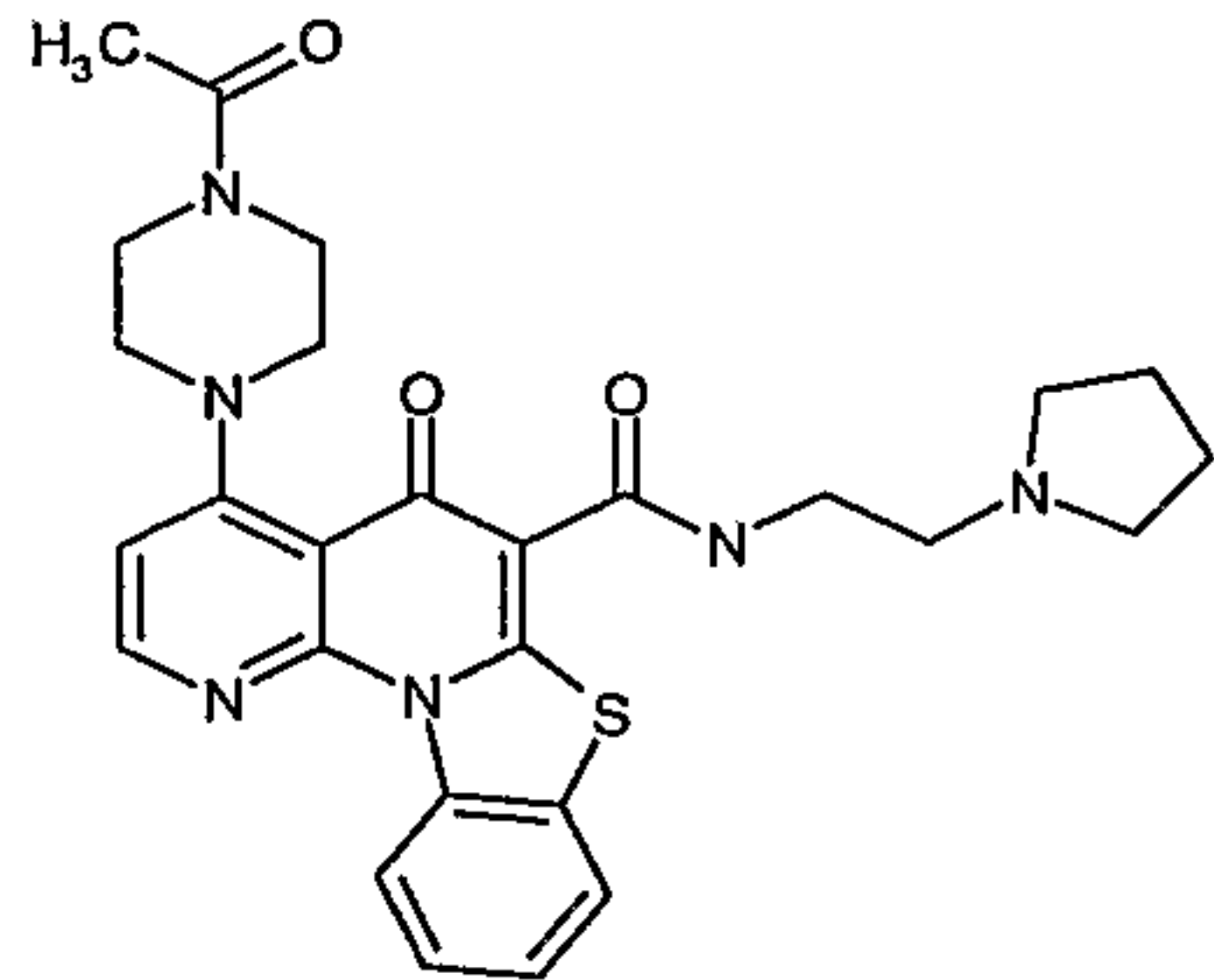
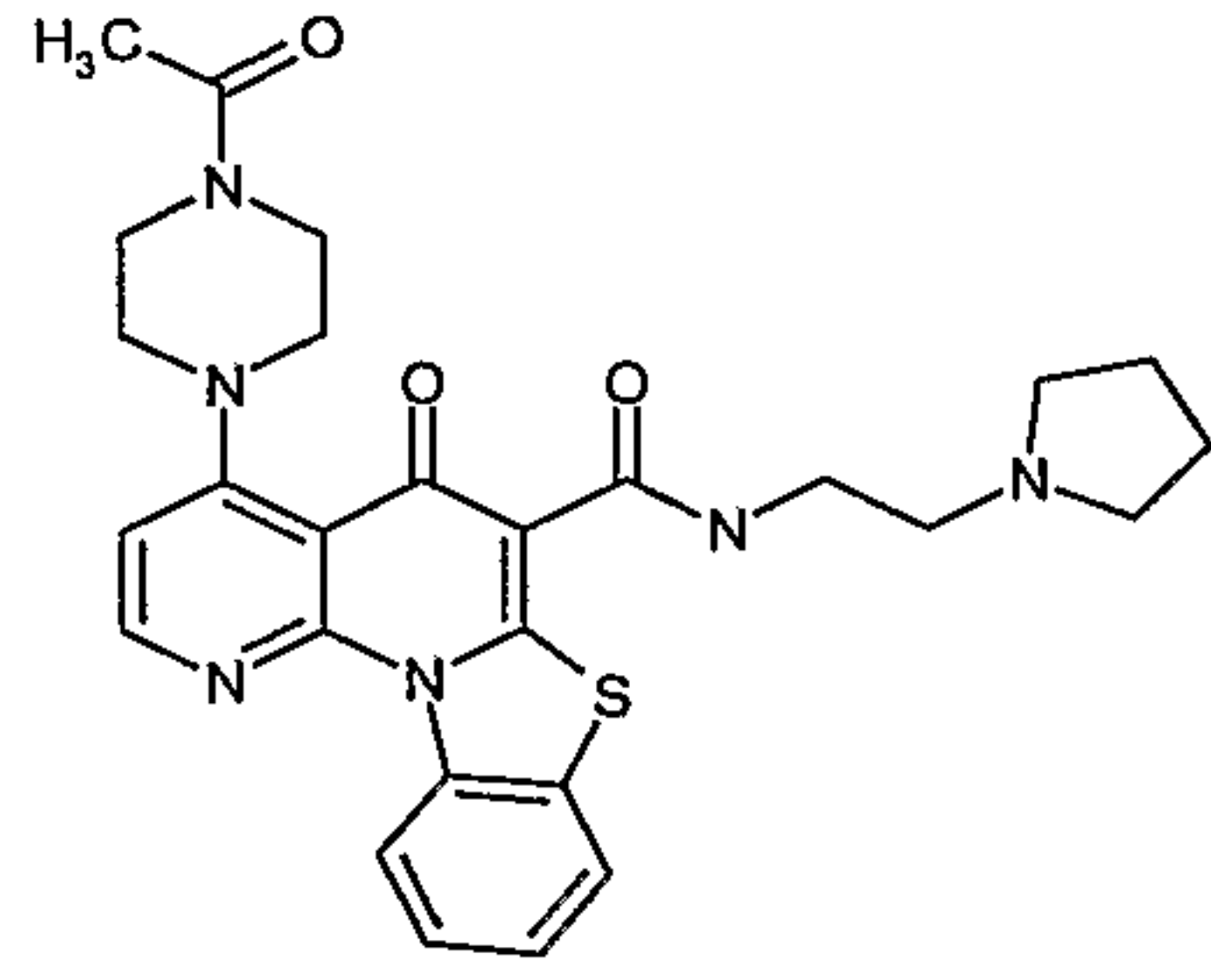
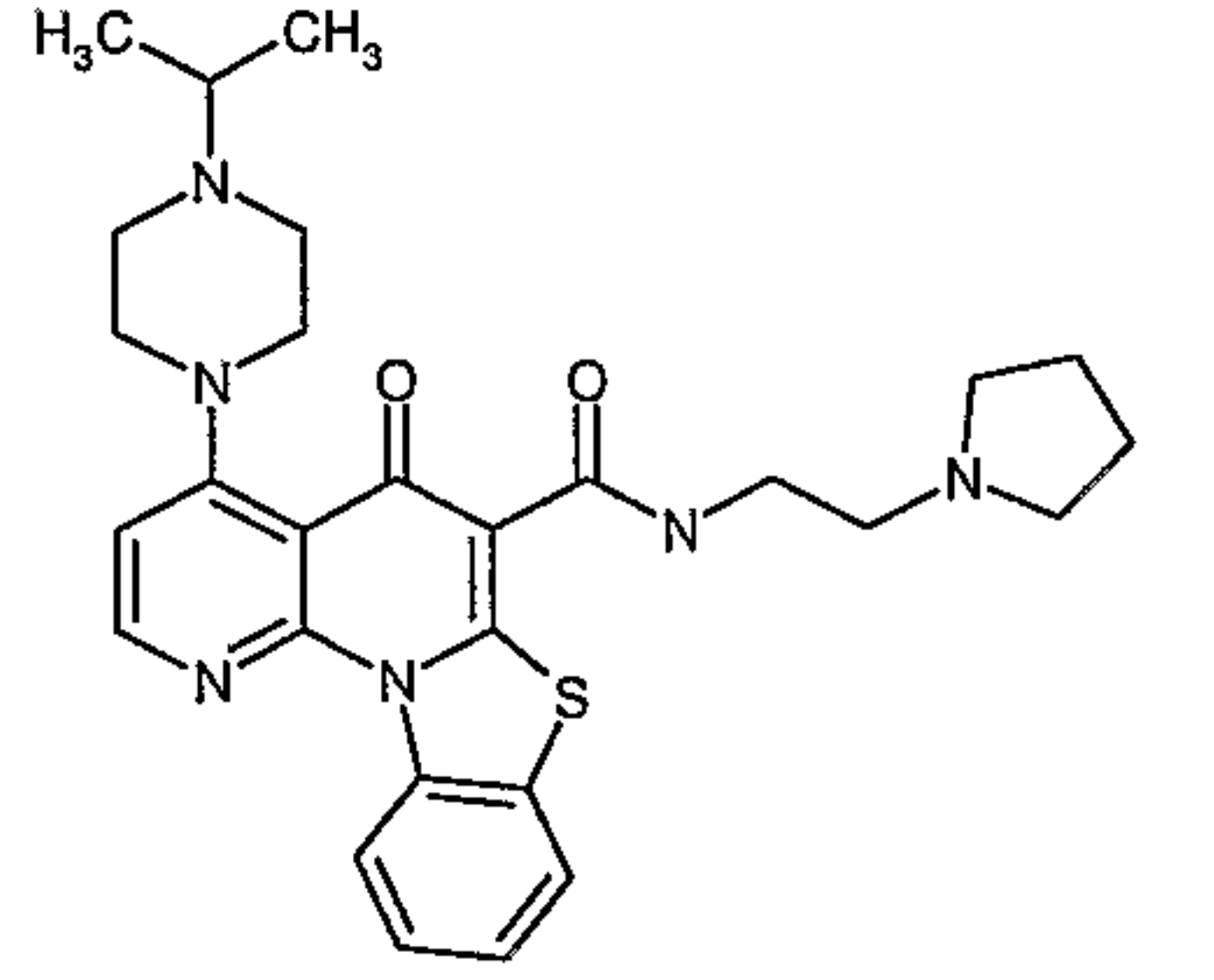
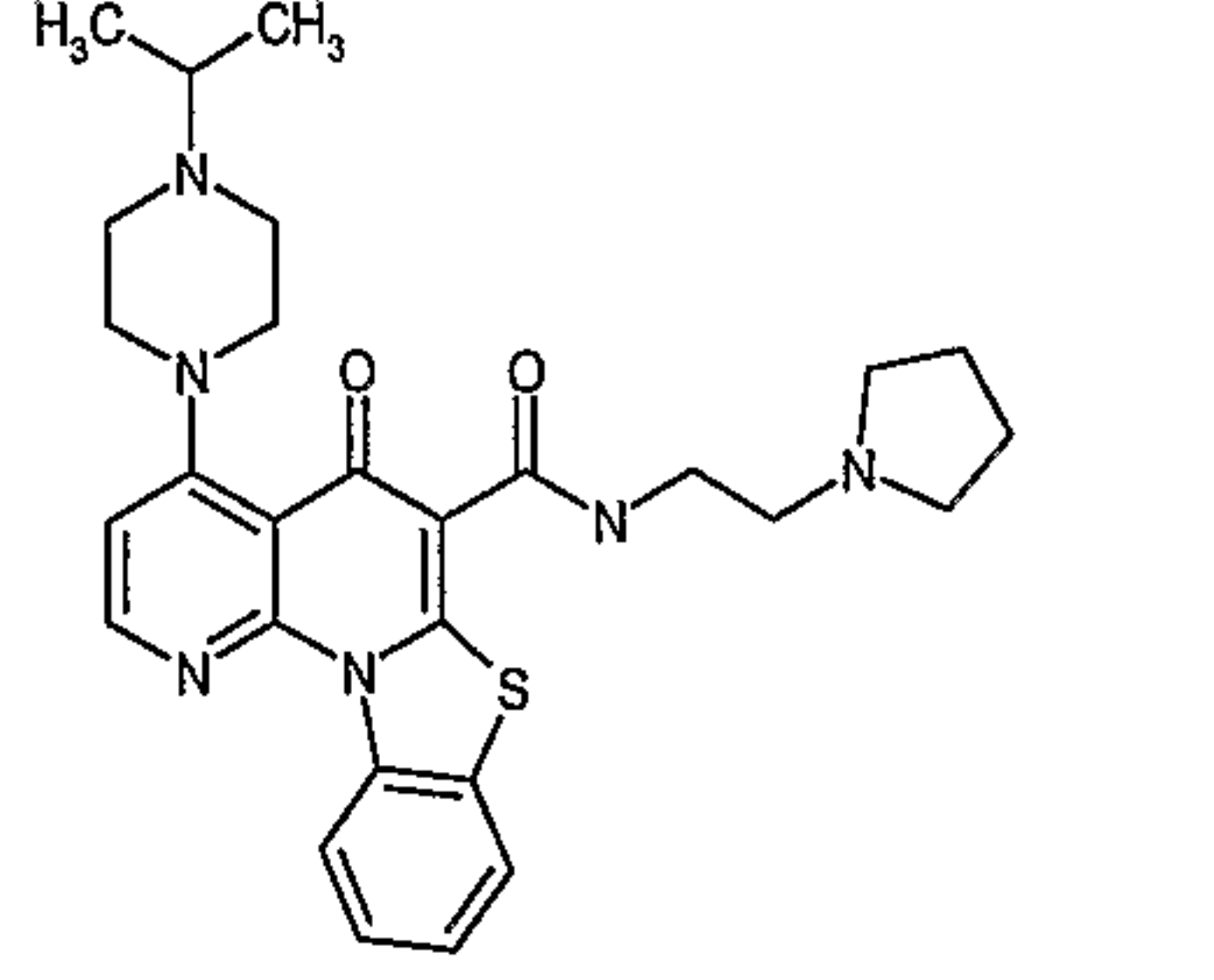
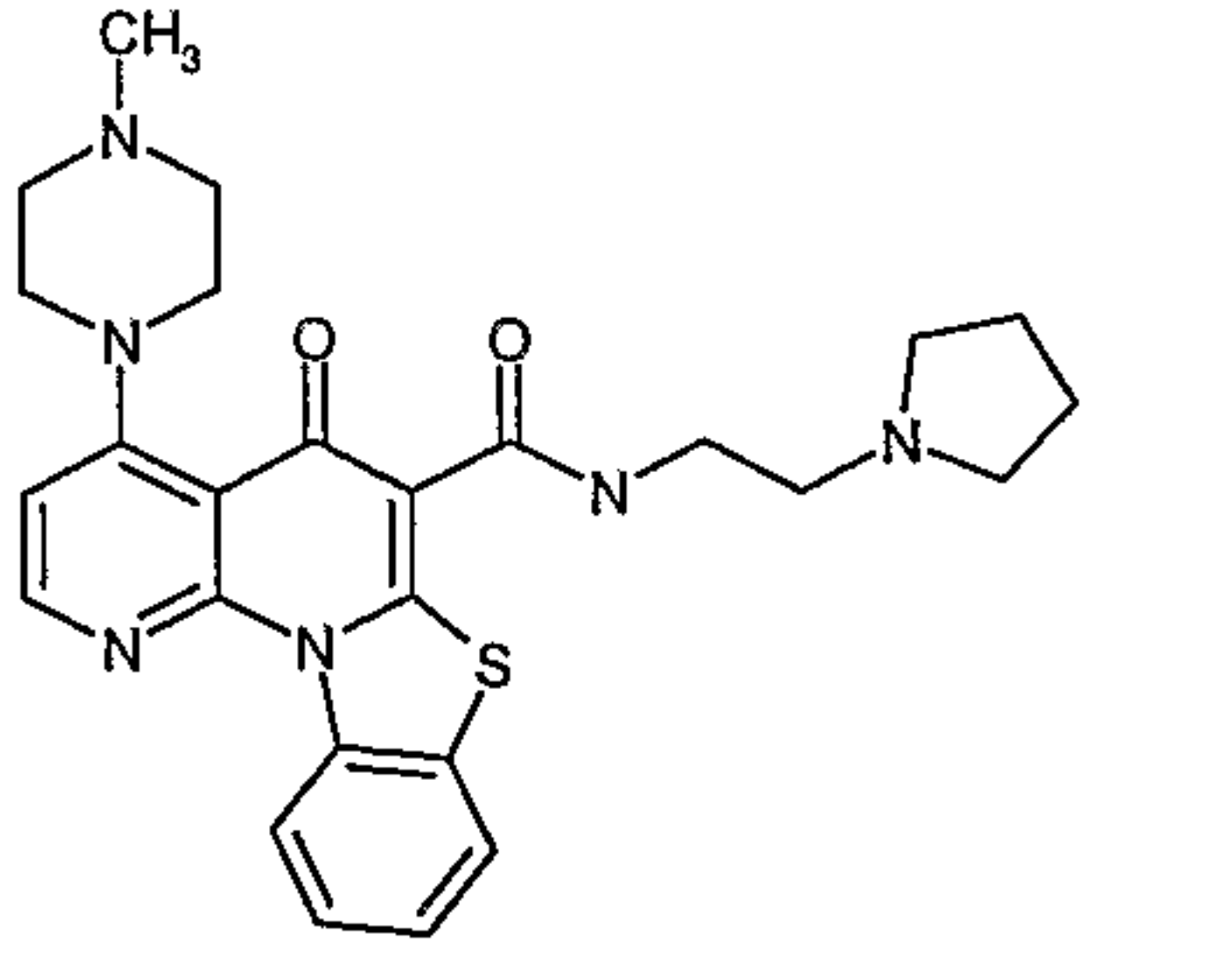
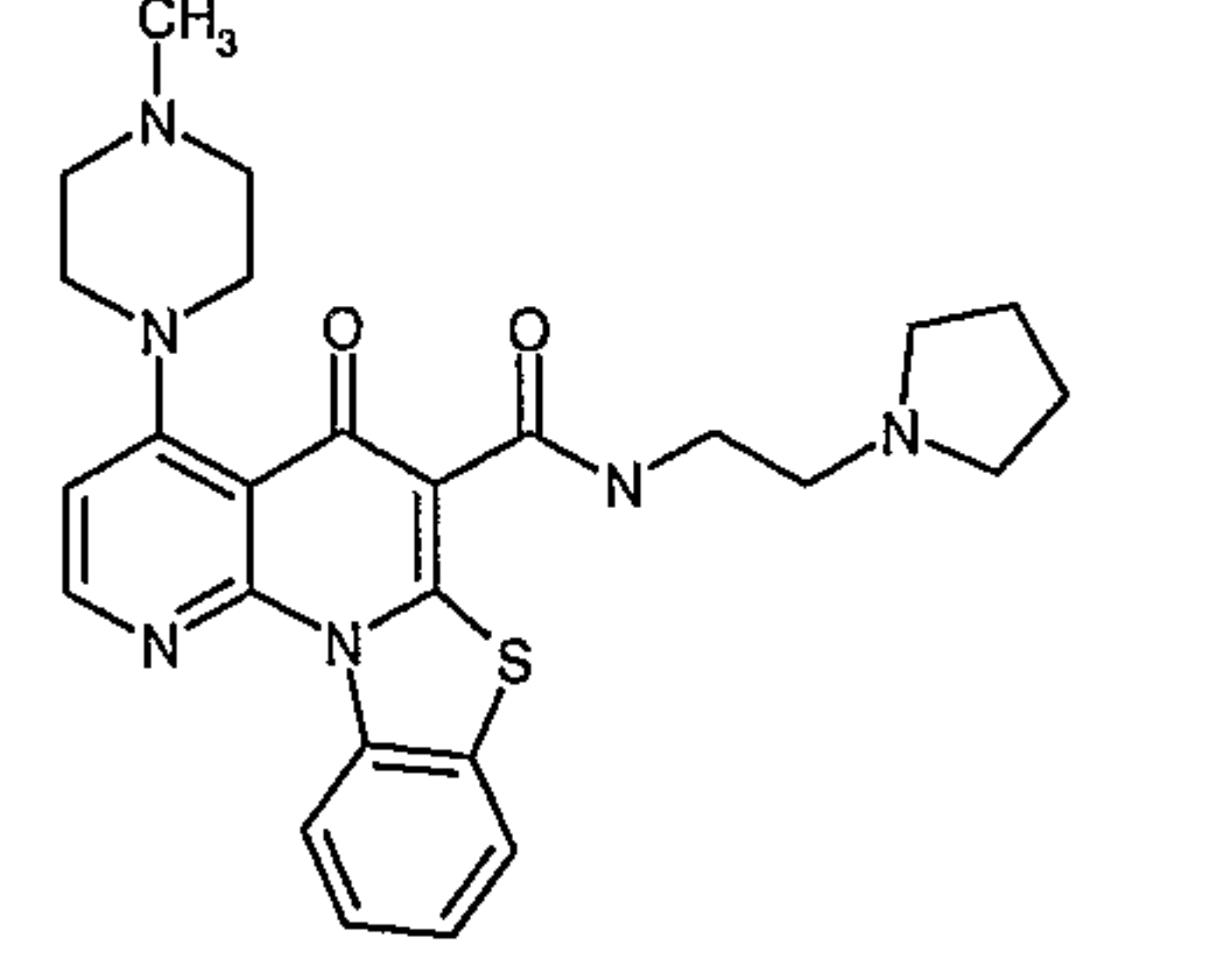
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | 0.03   |
|   | HCT-116 | 0.1    |
|  | HCT-116 | 4.20   |
|  | Miapaca | 3.00   |
|  | HCT-116 | 0.17   |
|  | Miapaca | 0.13   |

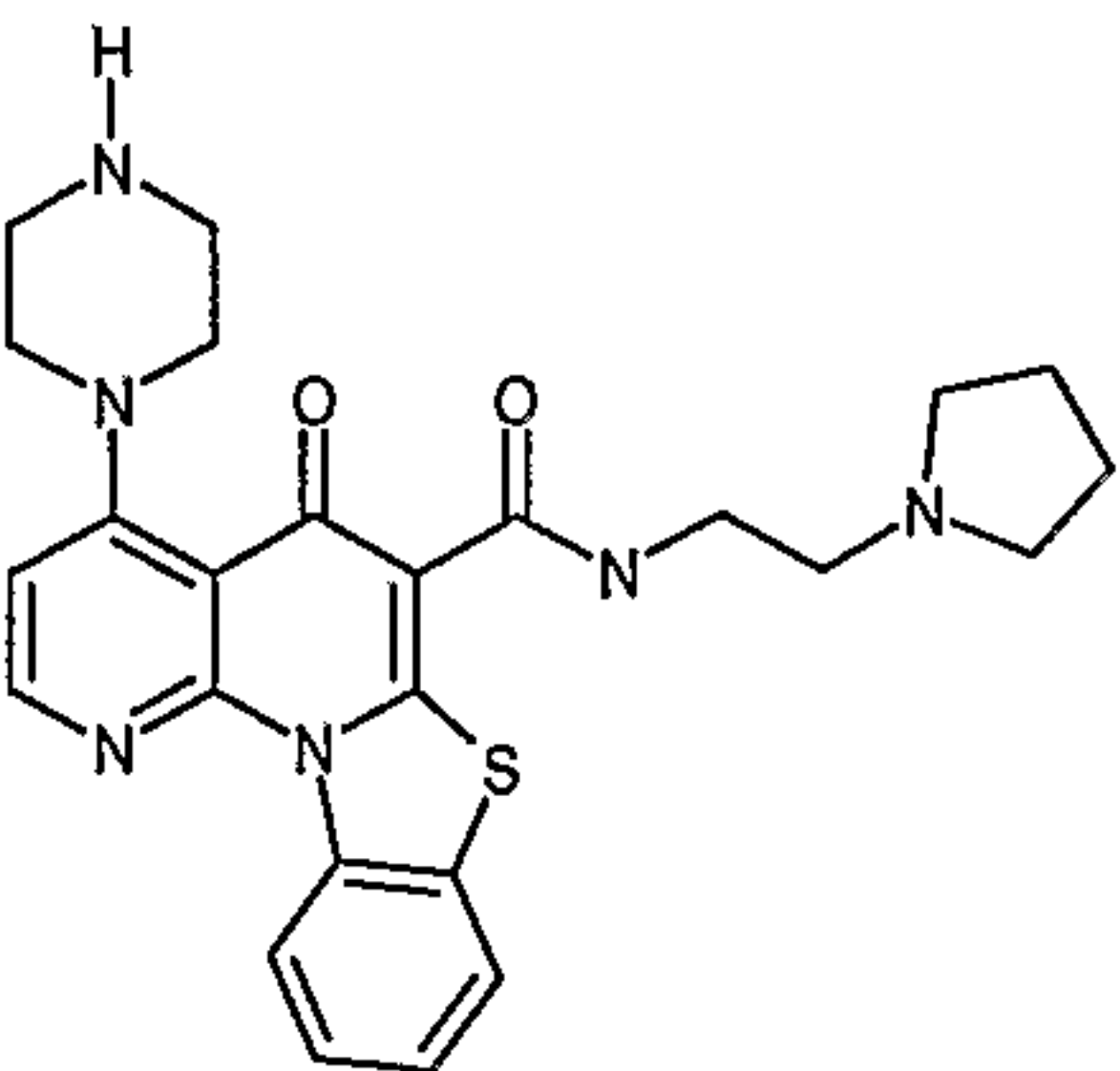
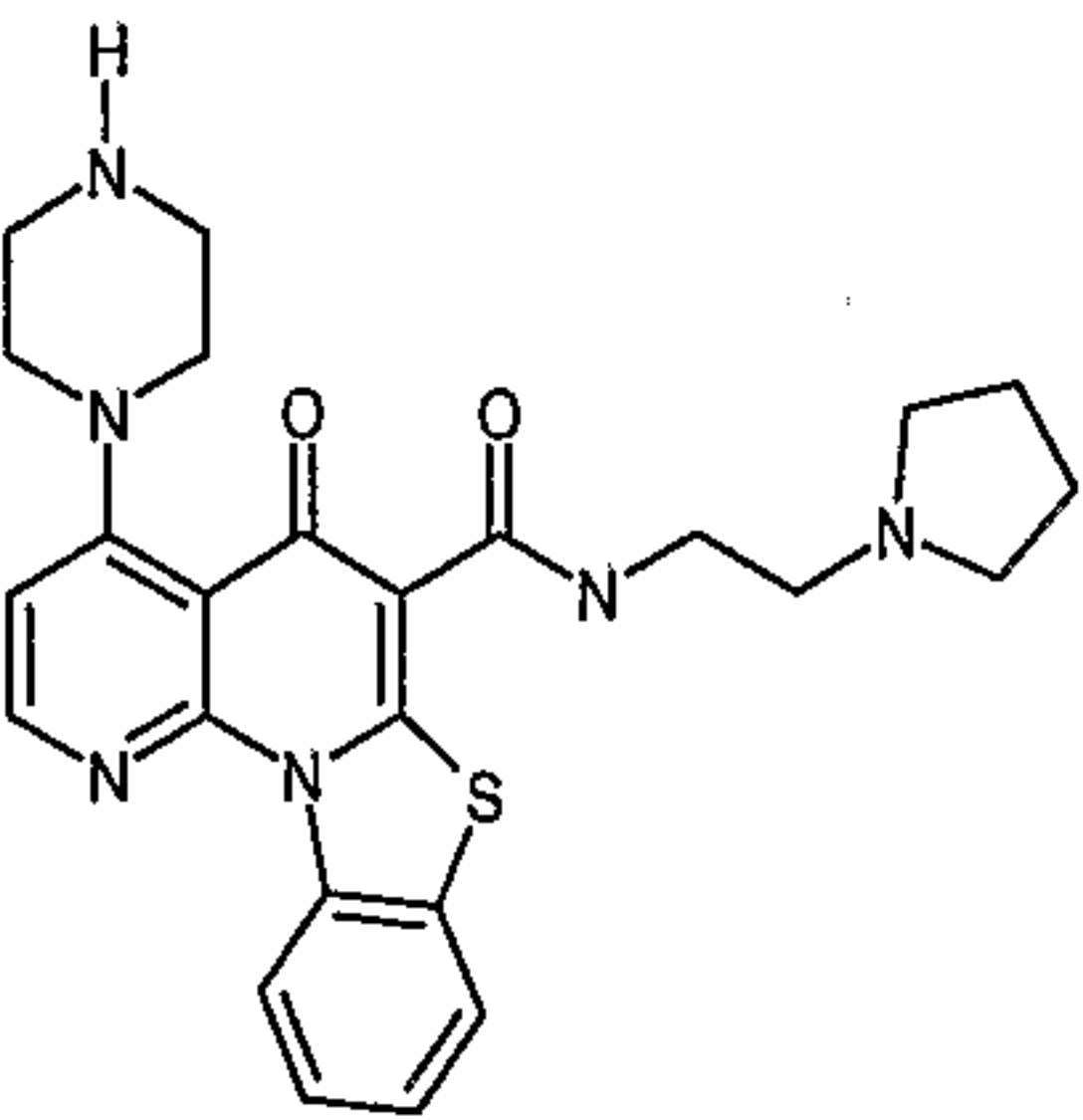
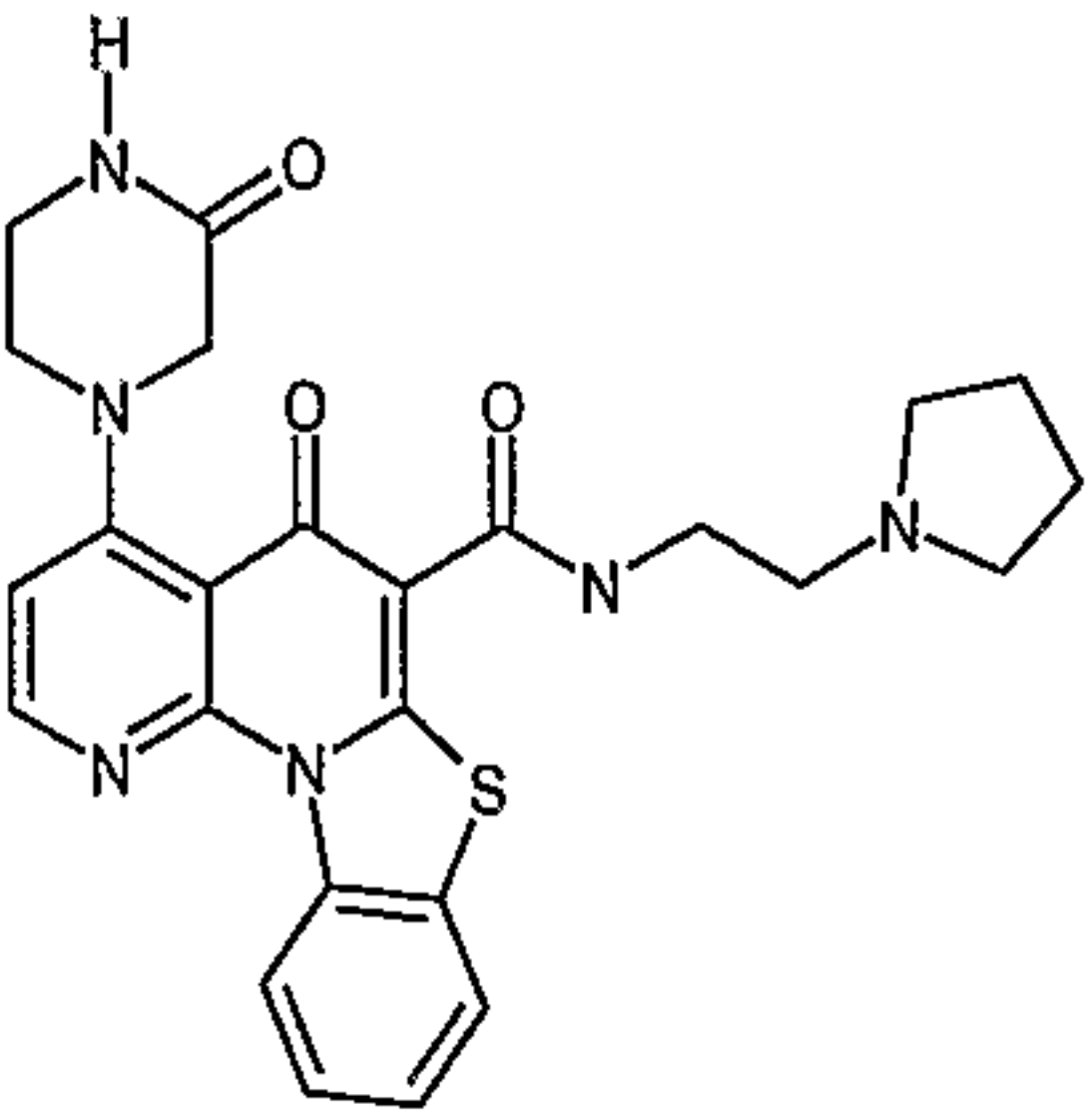
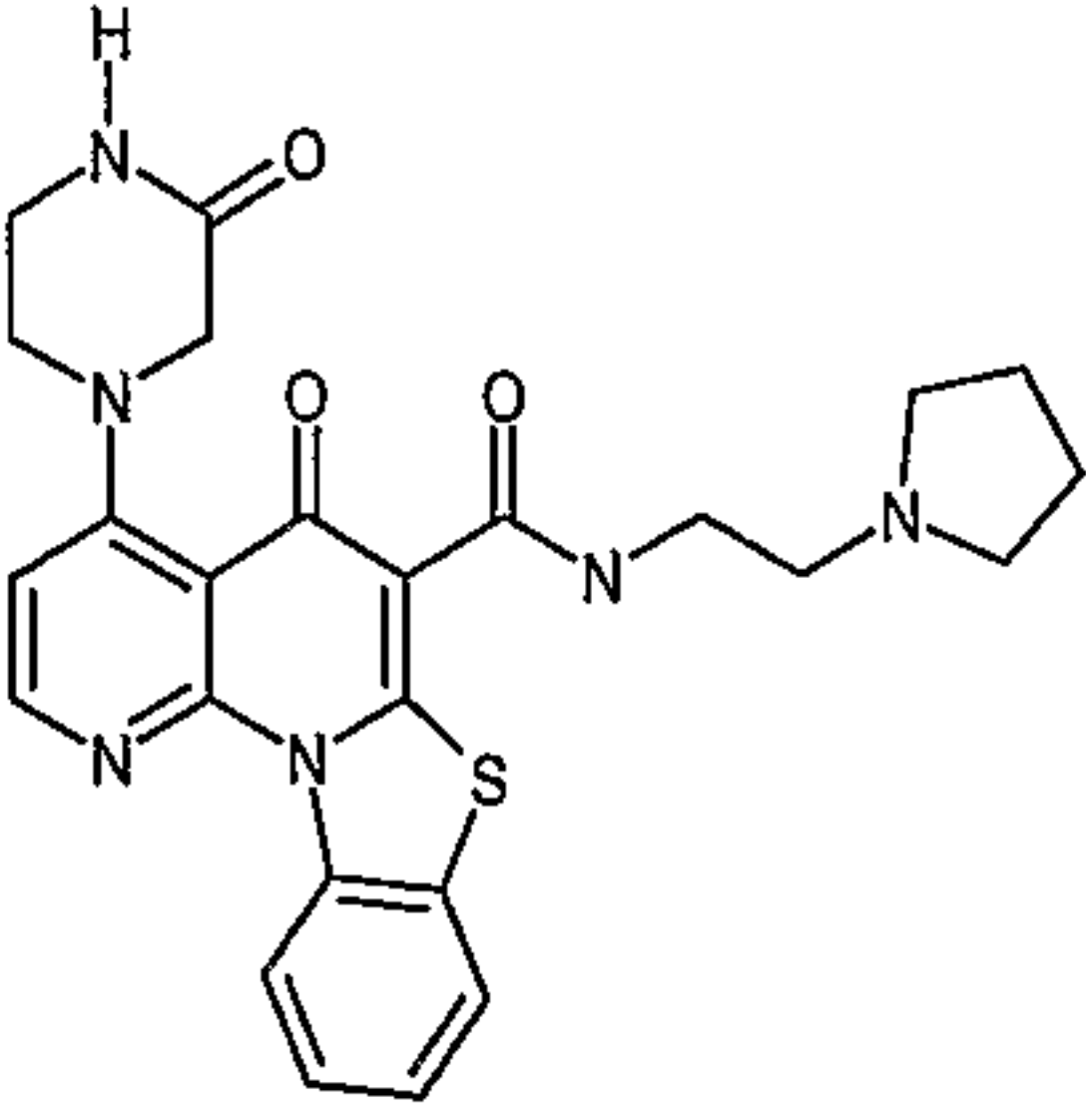
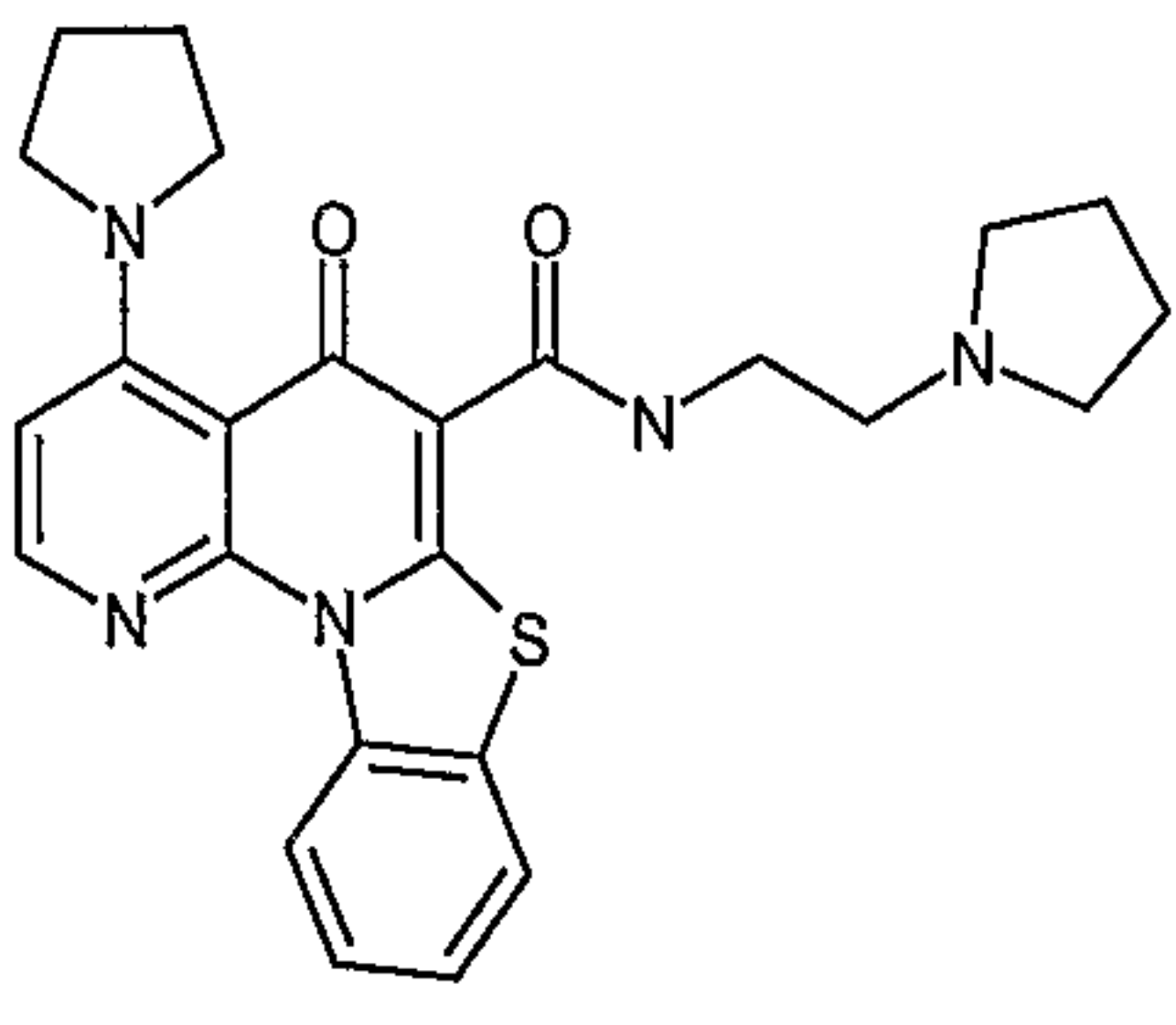
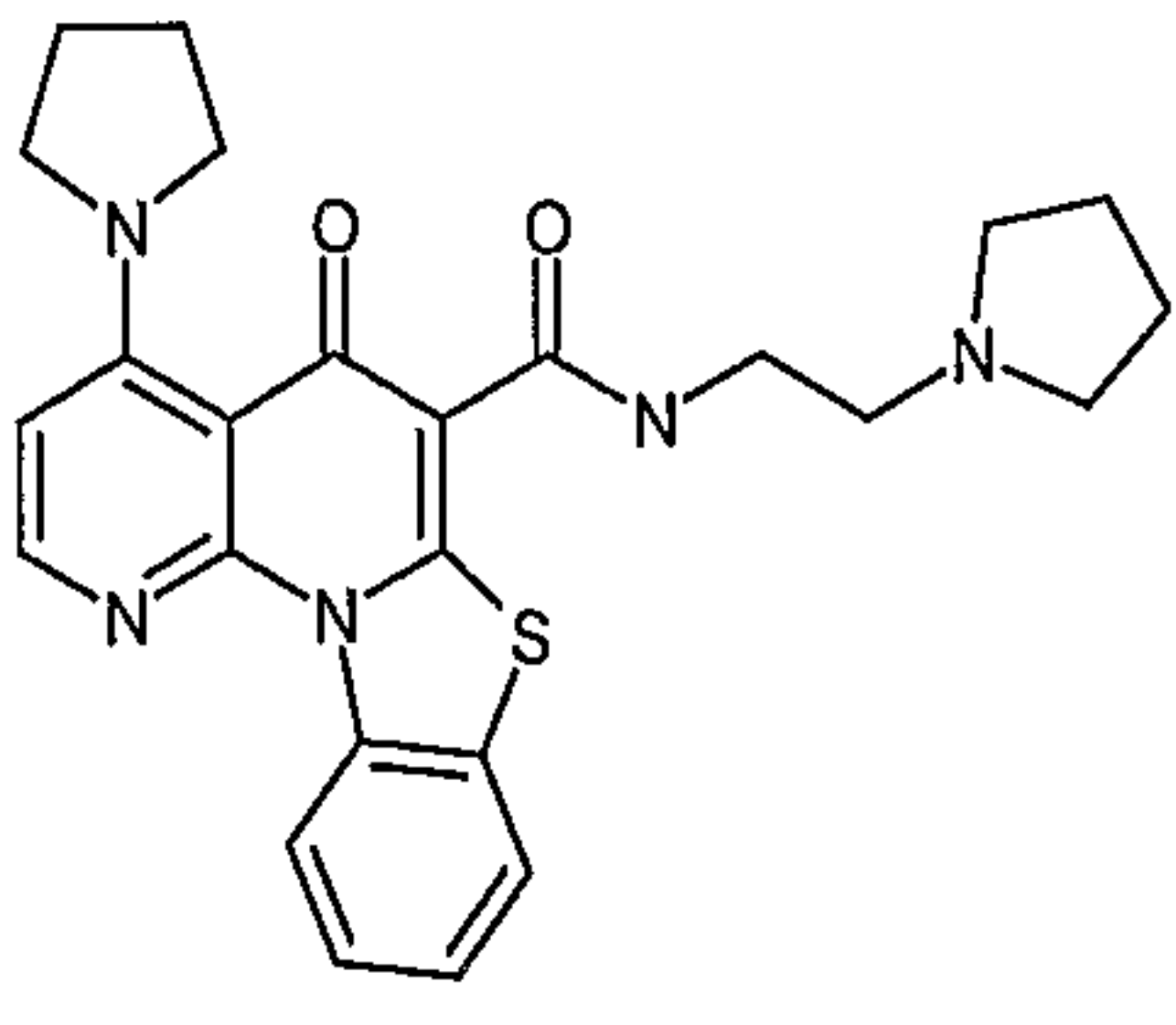
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|   | HCT-116 | 0.28   |
|  | Miapaca | 0.3    |
|  | HCT-116 | 0.28   |
|  | Miapaca | 0.18   |
|  | HCT-116 | 1.8    |
|  | Miapaca | 1.55   |

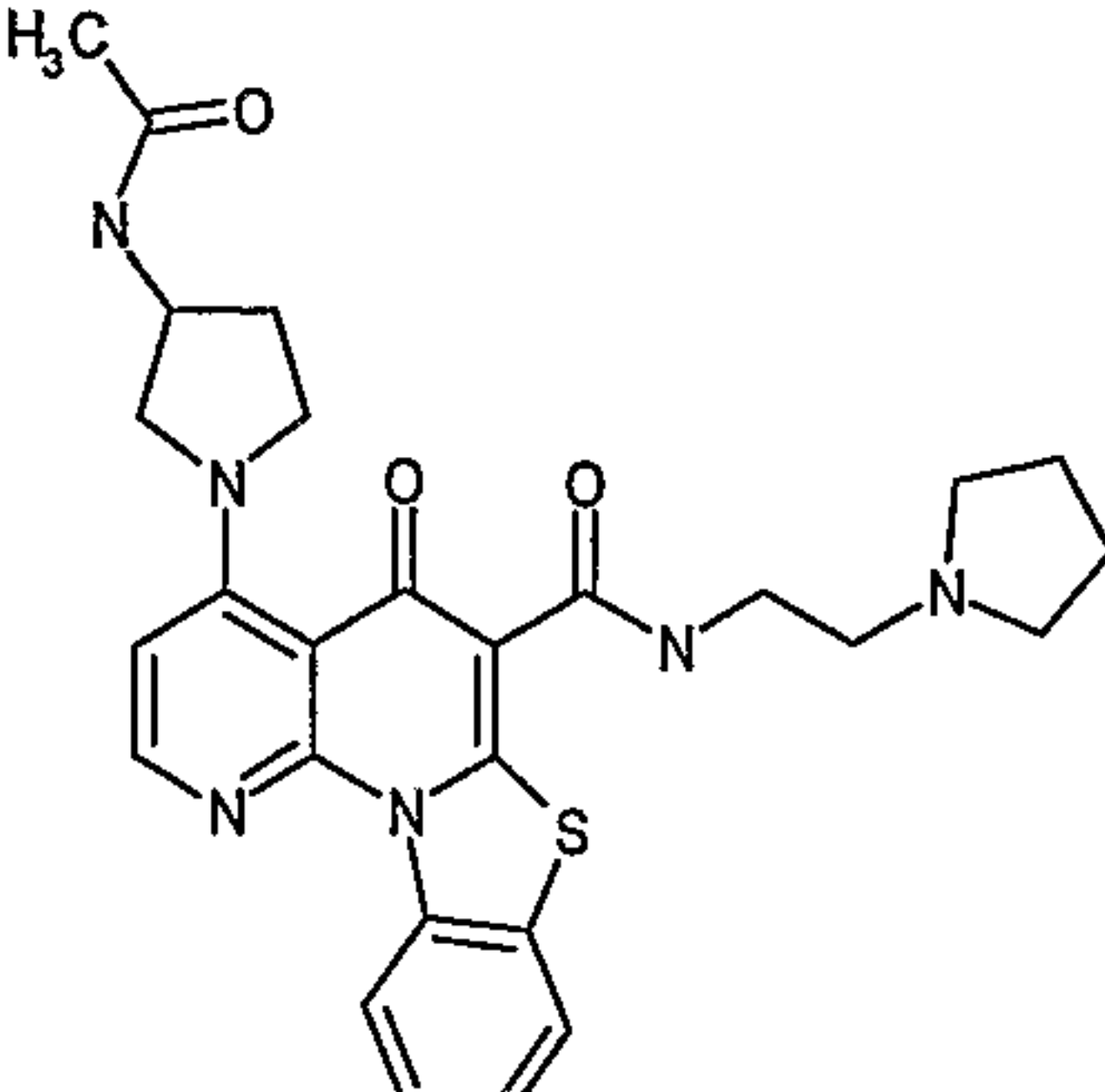
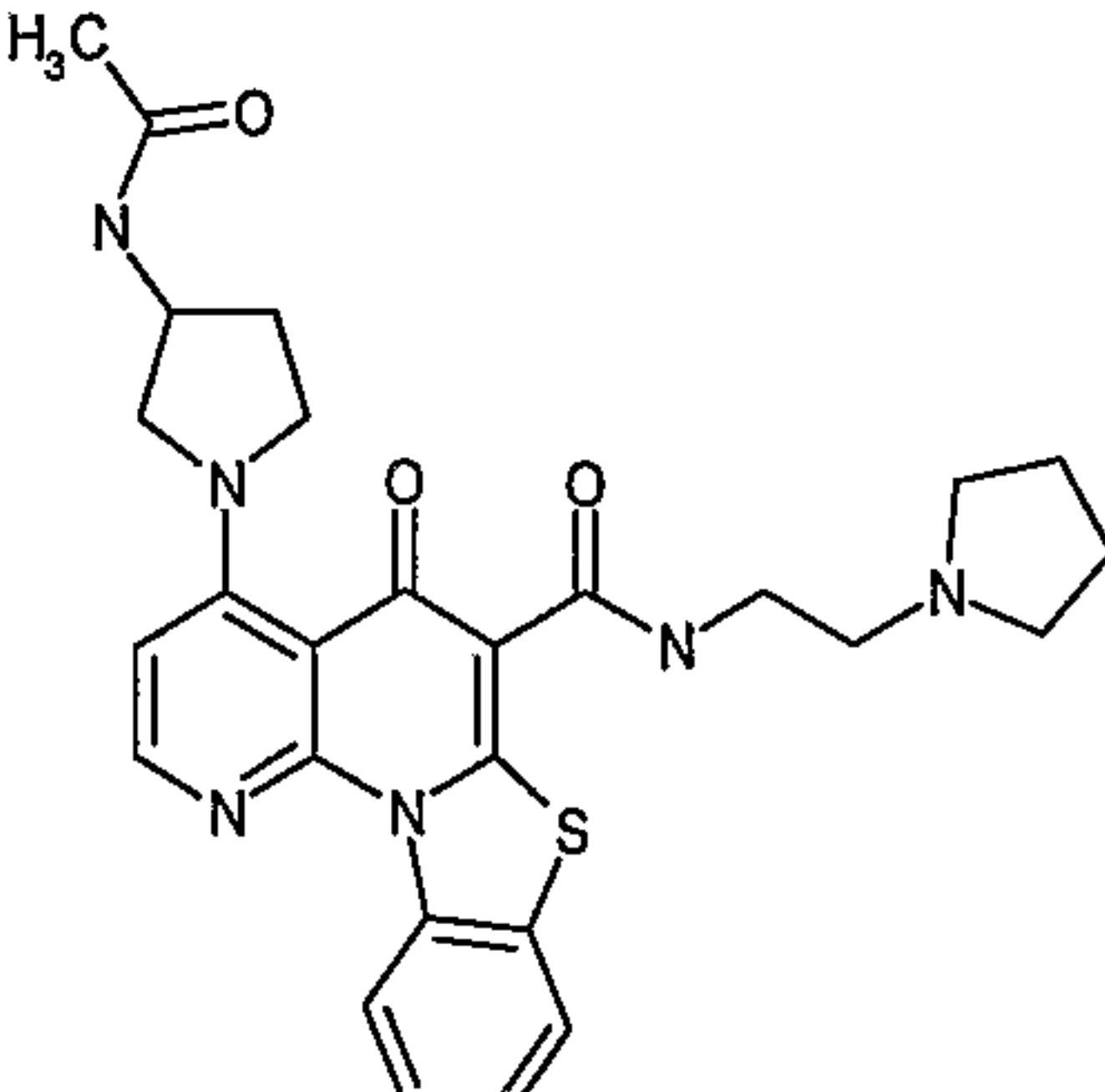
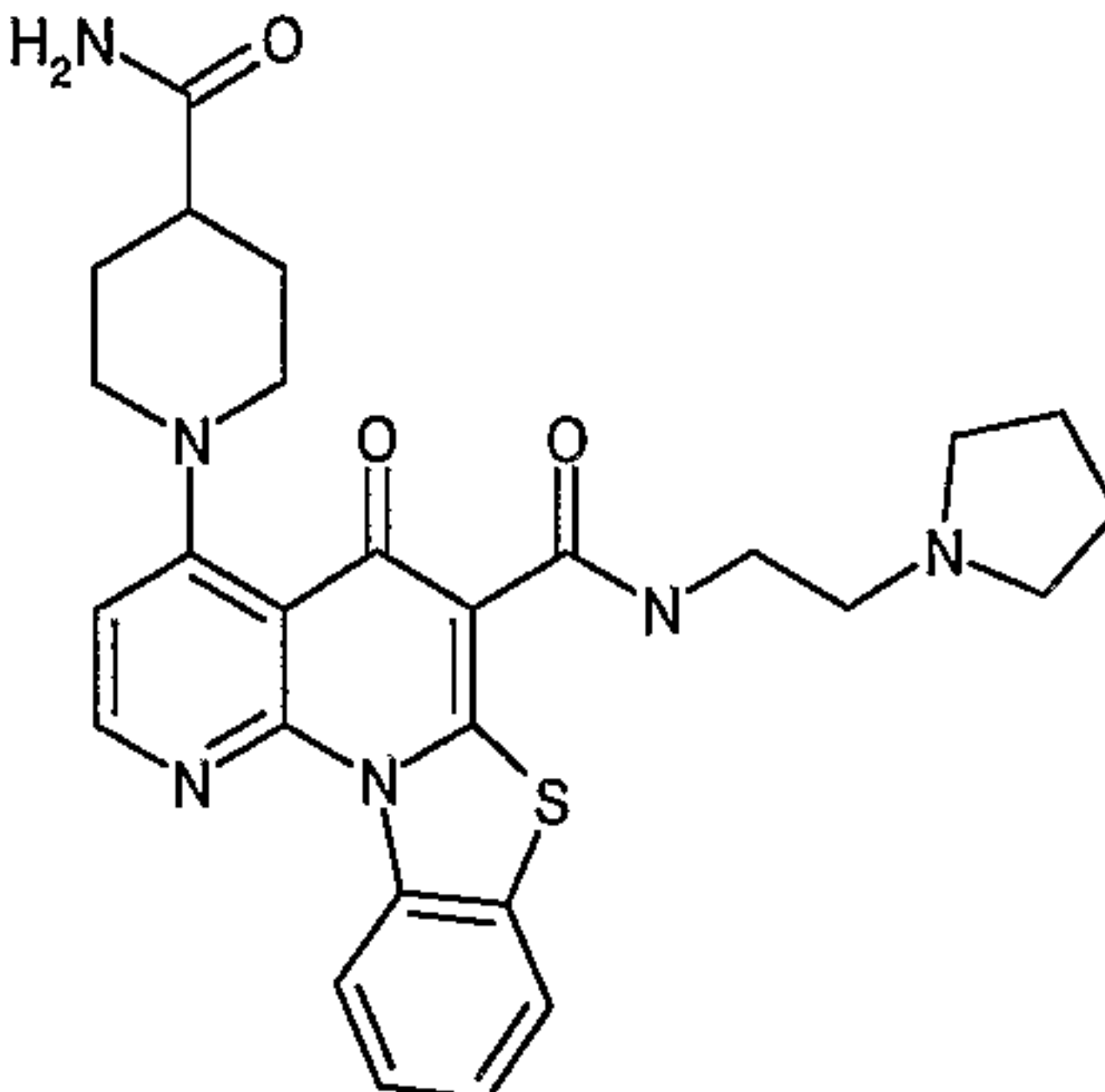
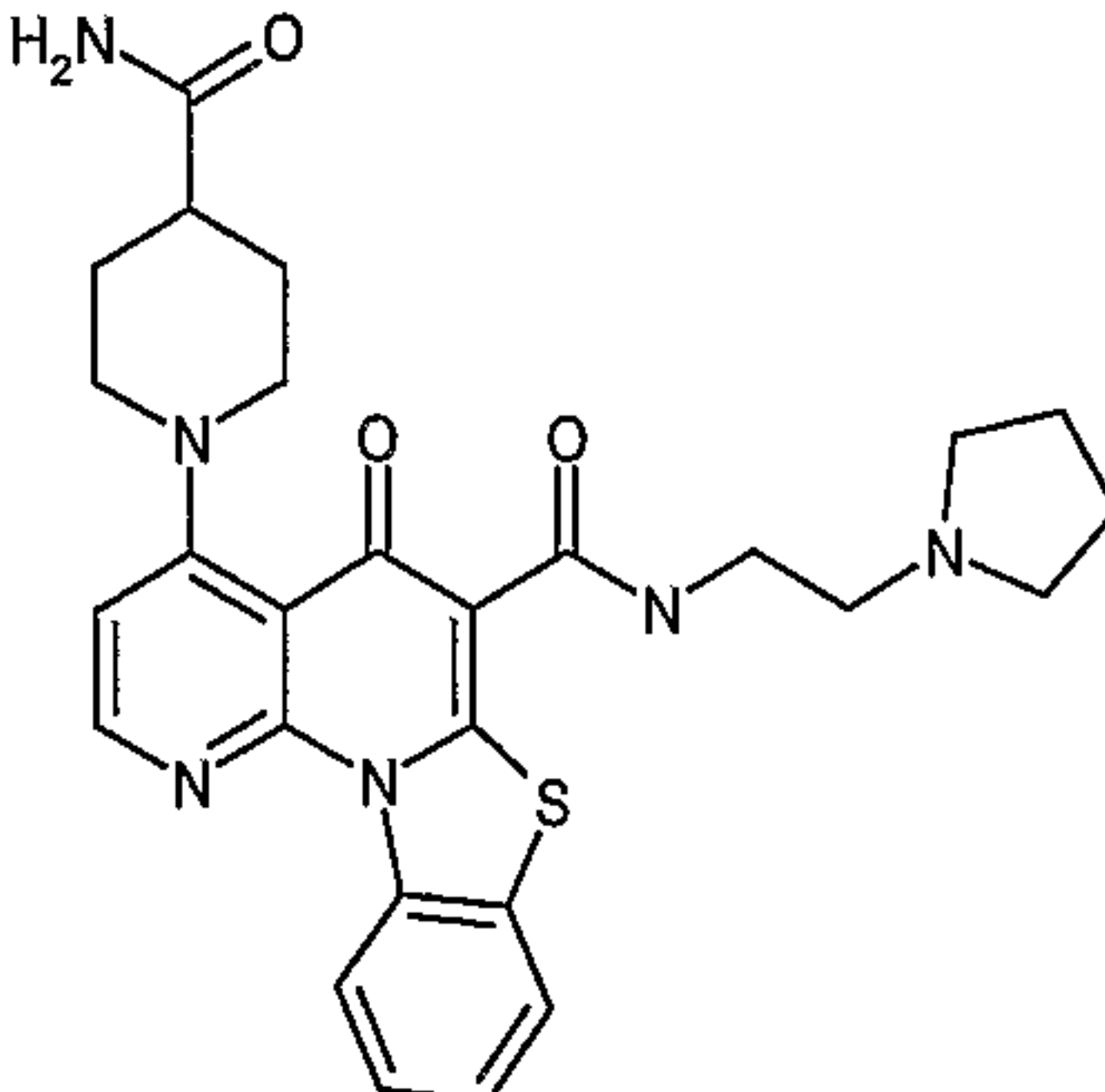
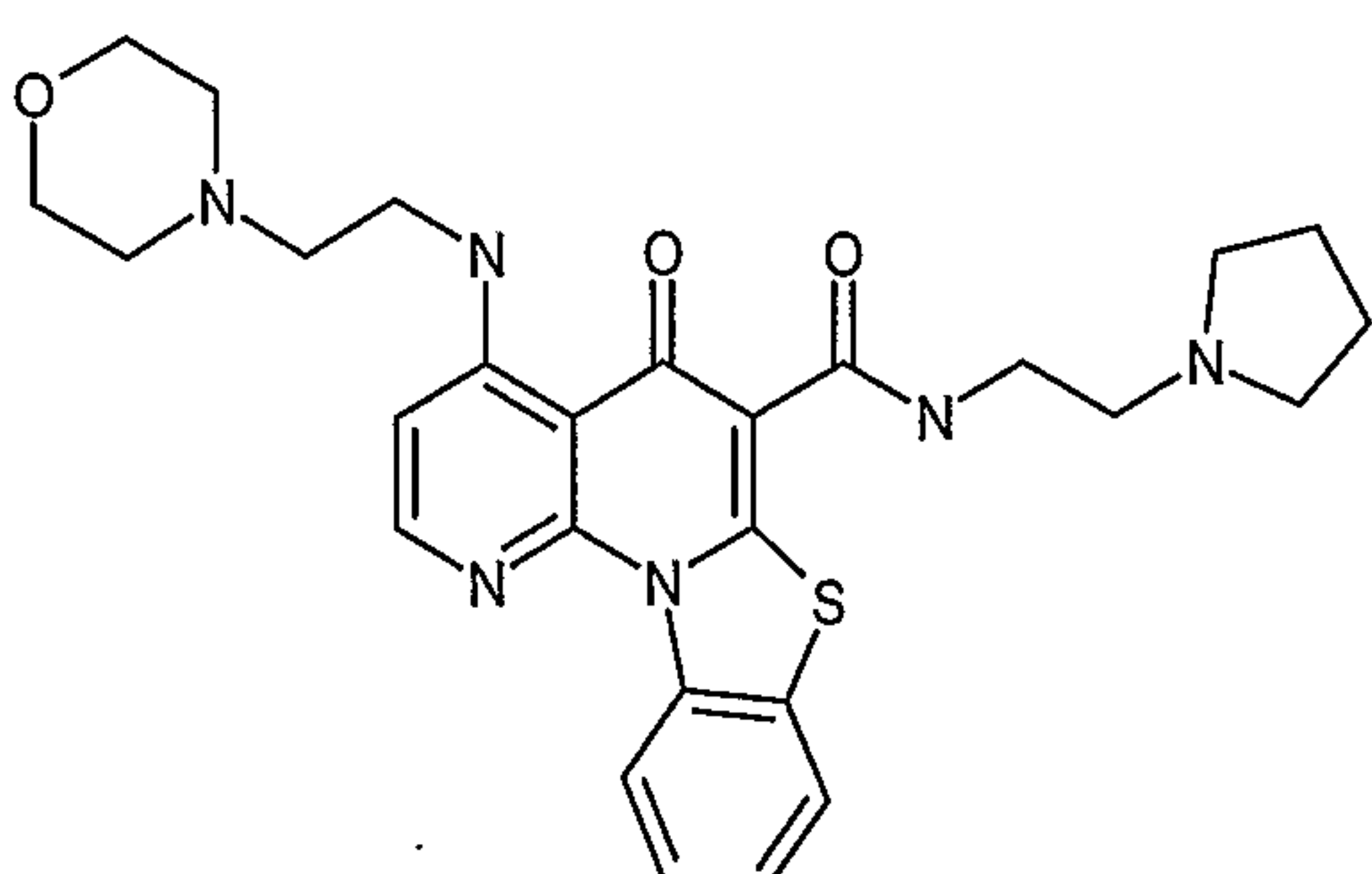
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 0.31   |
|   | Miapaca | 0.16   |
|  | HCT-116 | 0.27   |
|  | Miapaca | 0.15   |
|  | HCT-116 | 0.04   |
|  | Miapaca | 0.12   |

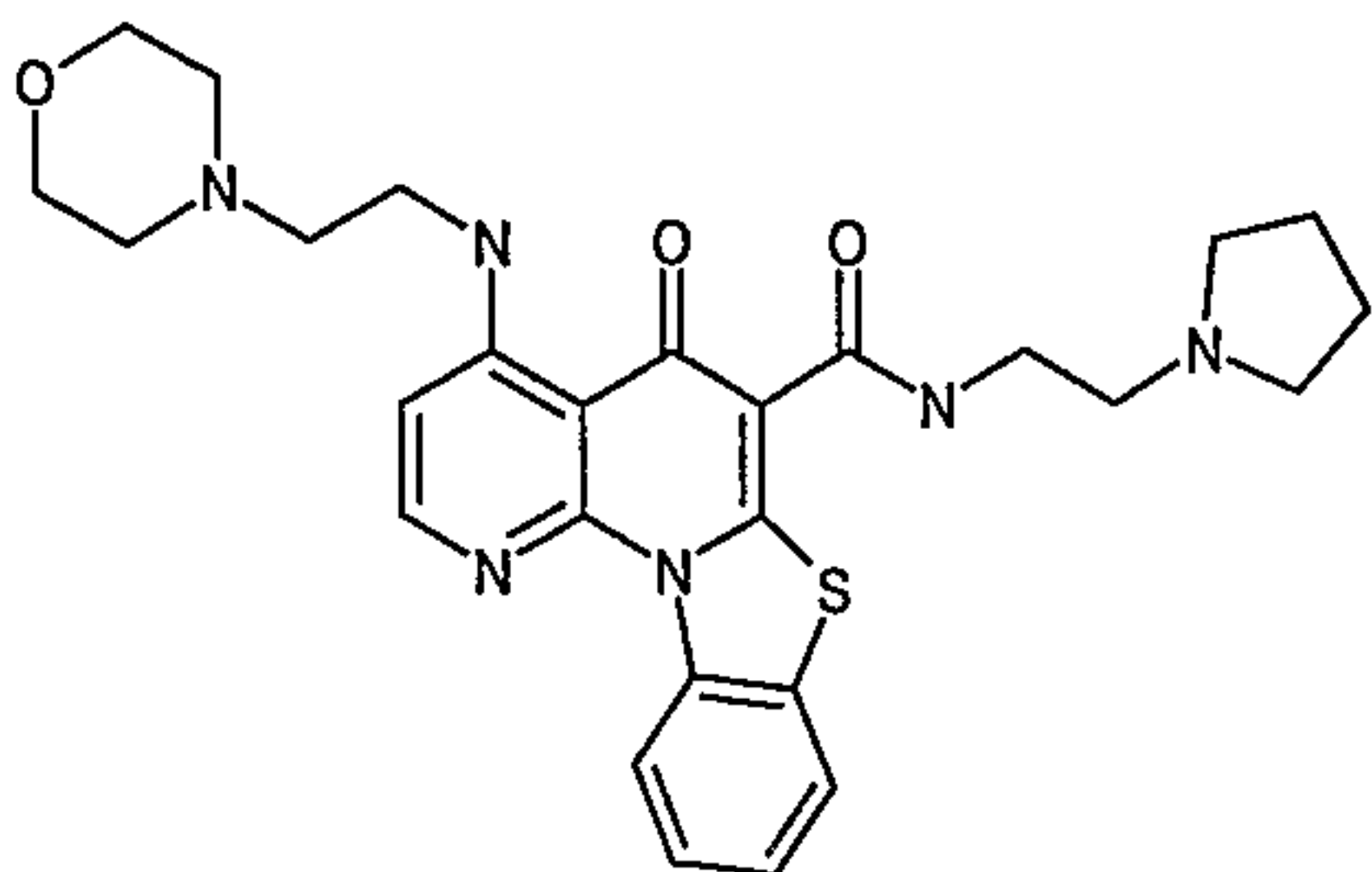
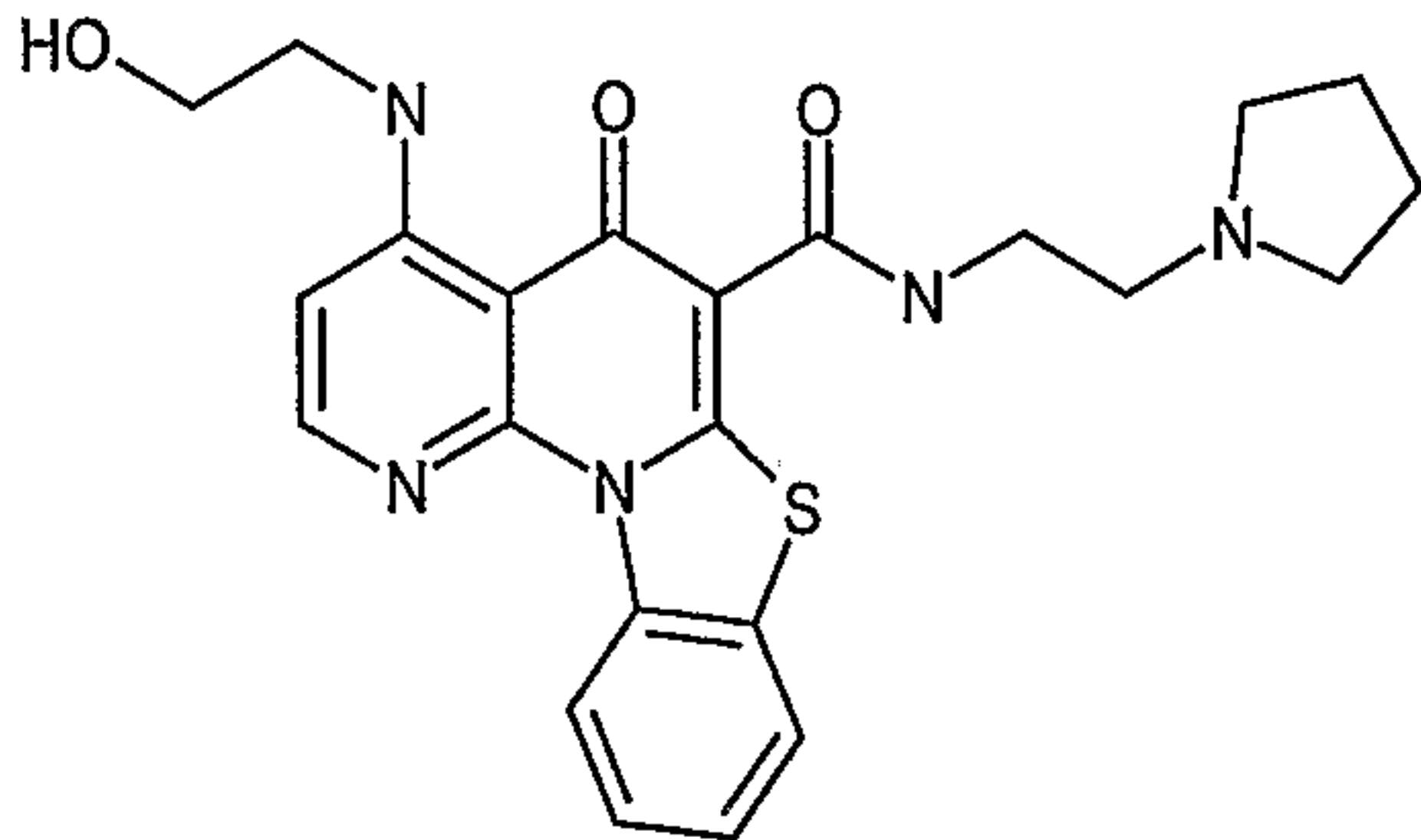
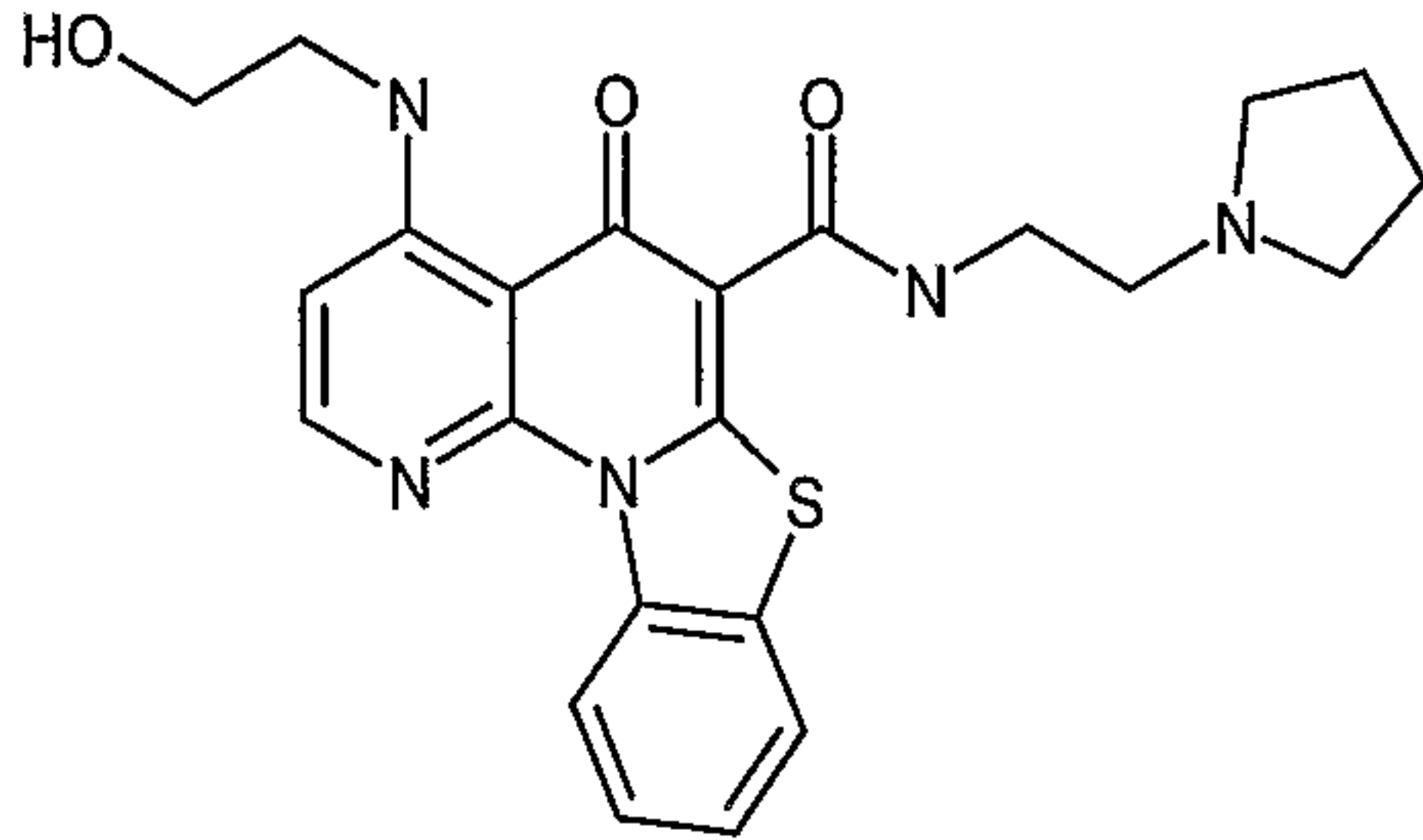
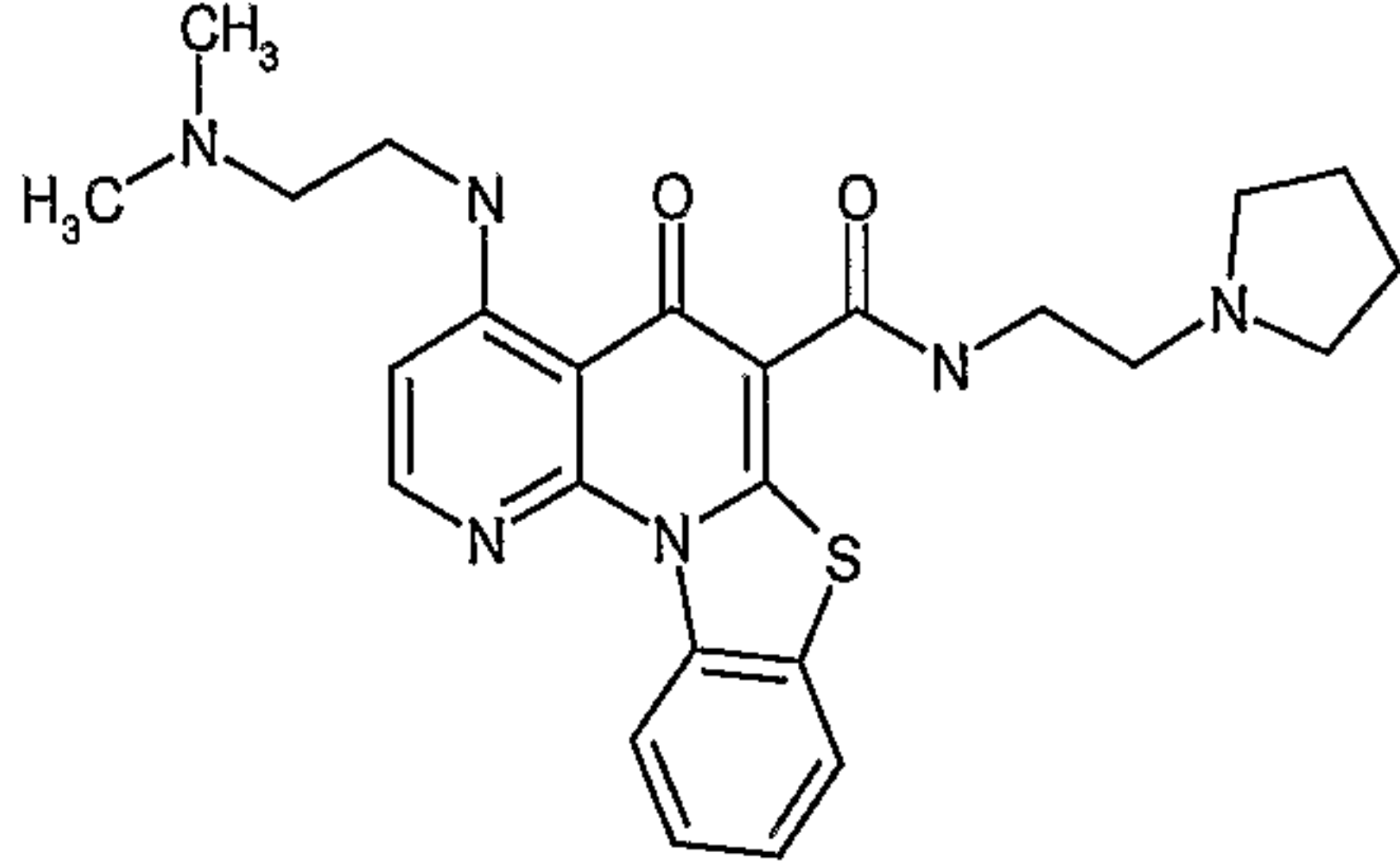
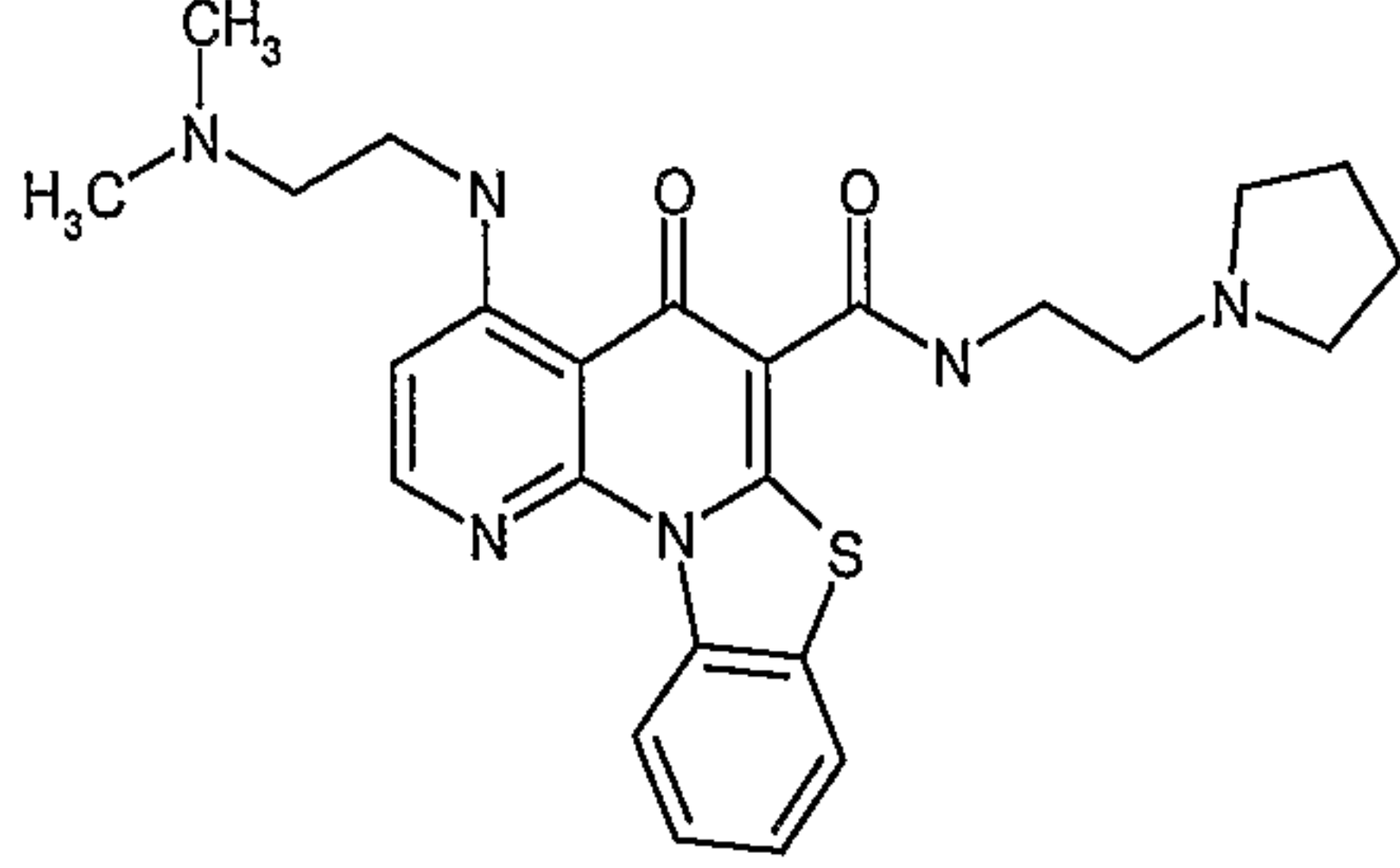
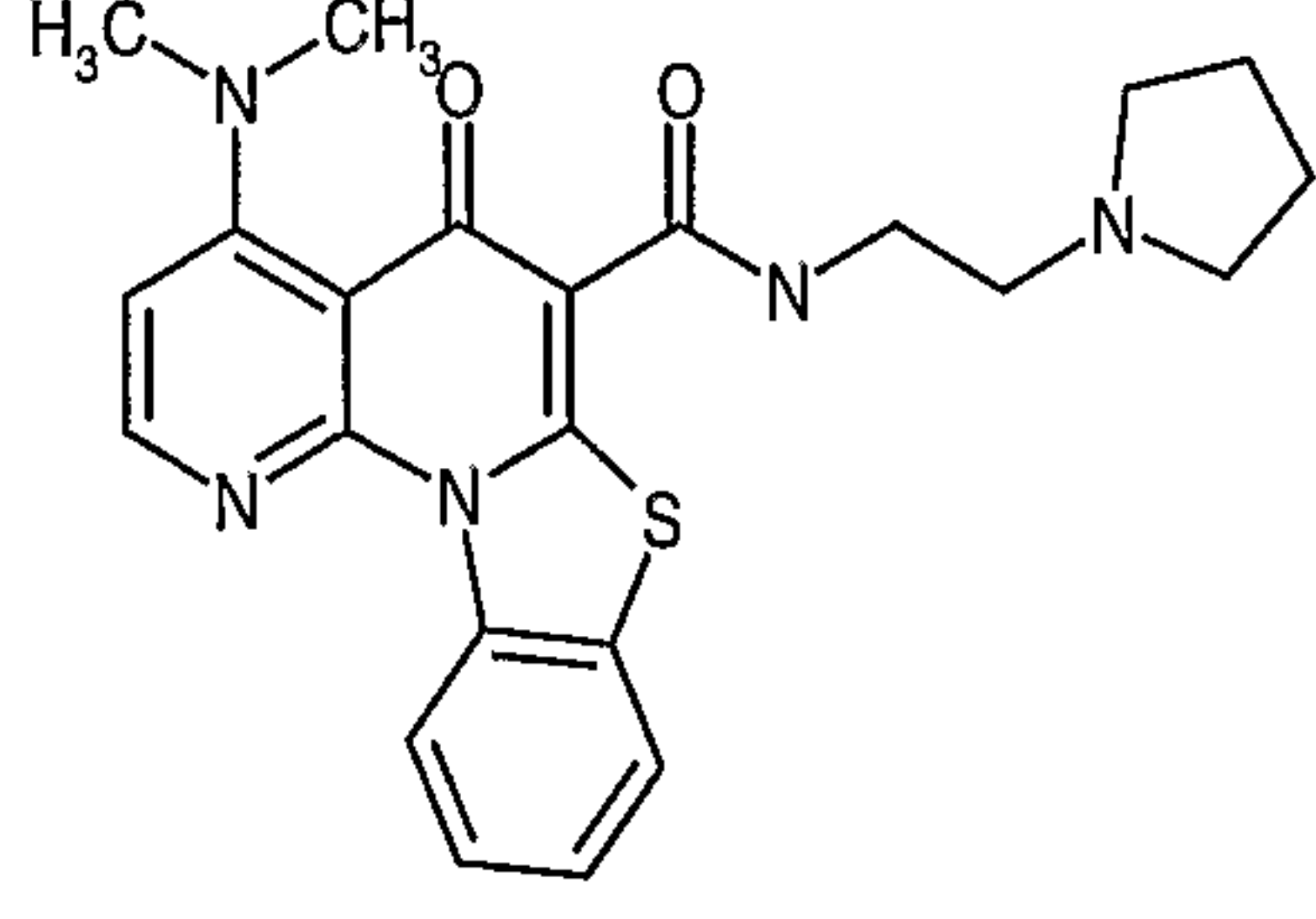
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 0.09   |
|   | Miapaca | 0.2    |
|  | HCT-116 | 0.1    |
|  | Miapaca | 0.34   |
|  | HCT-116 | 0.03   |
|  | Miapaca | 0.08   |

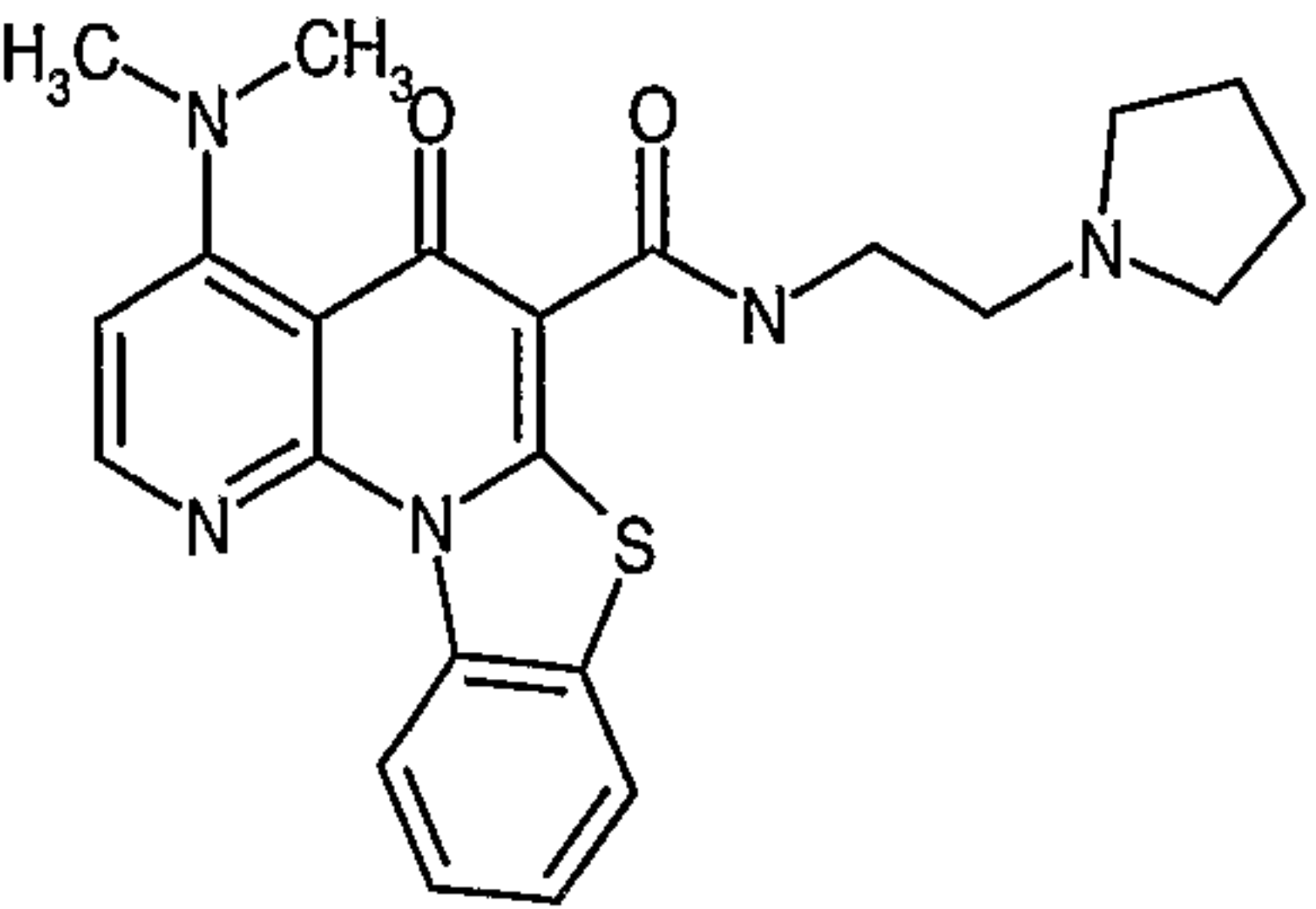
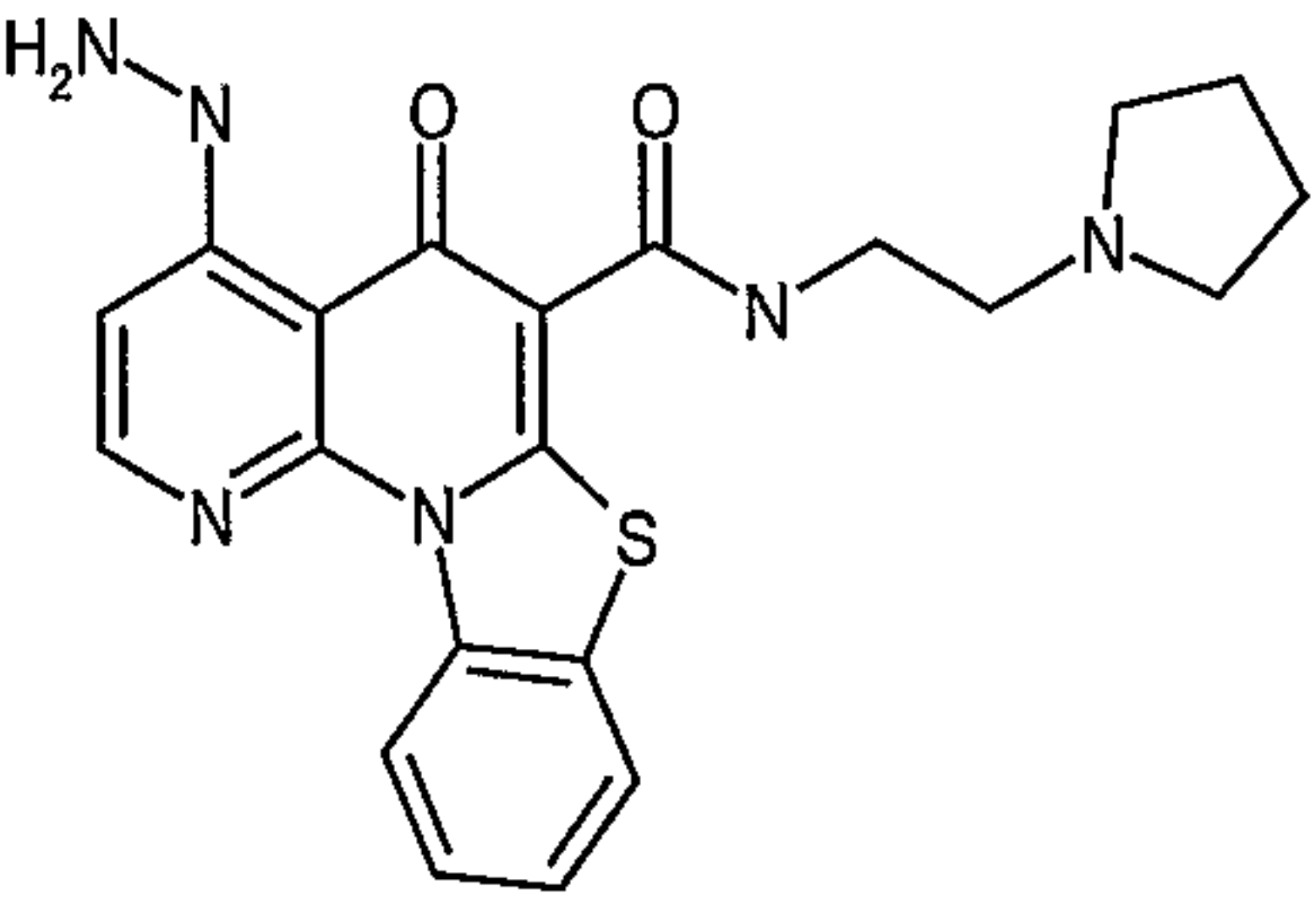
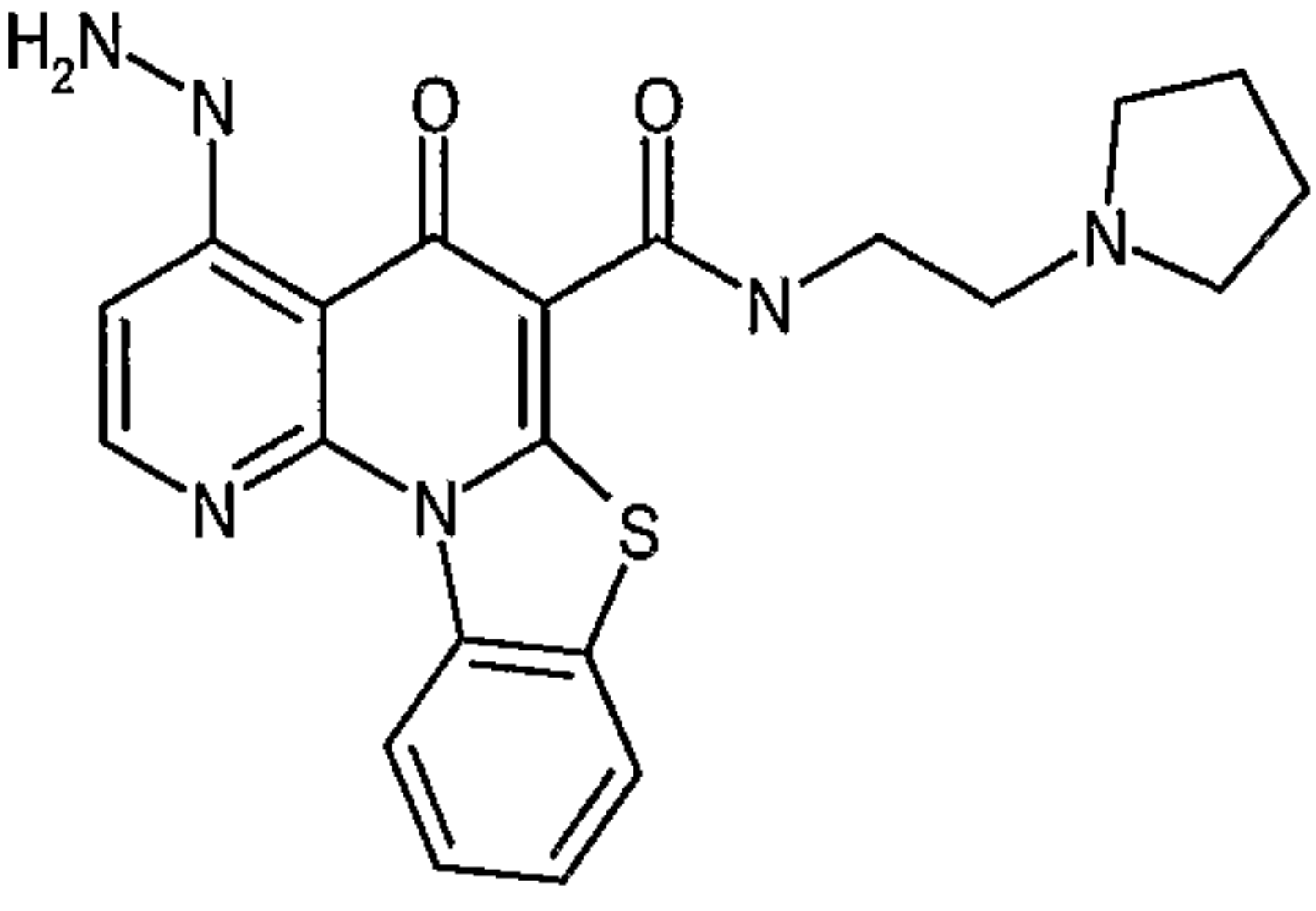
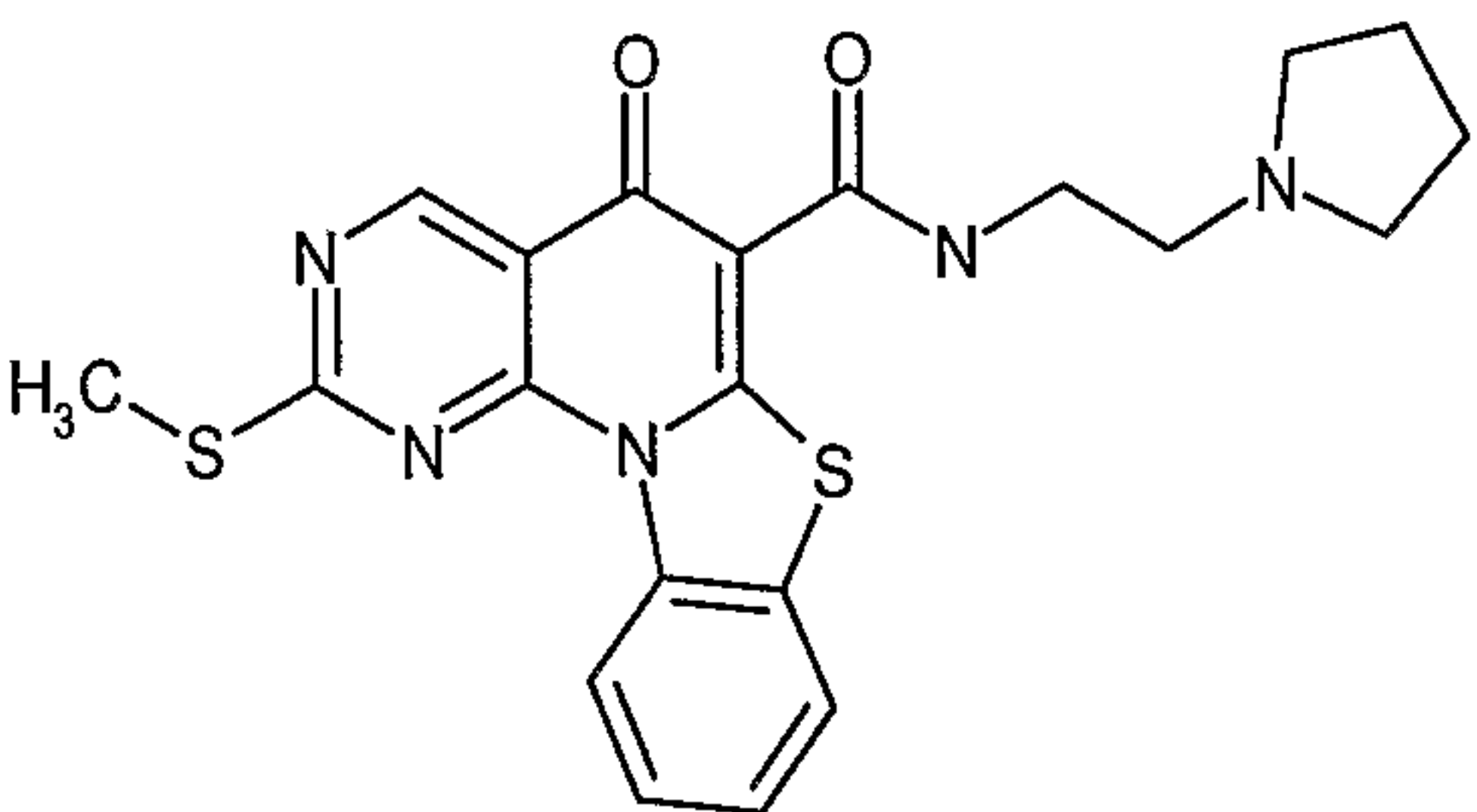
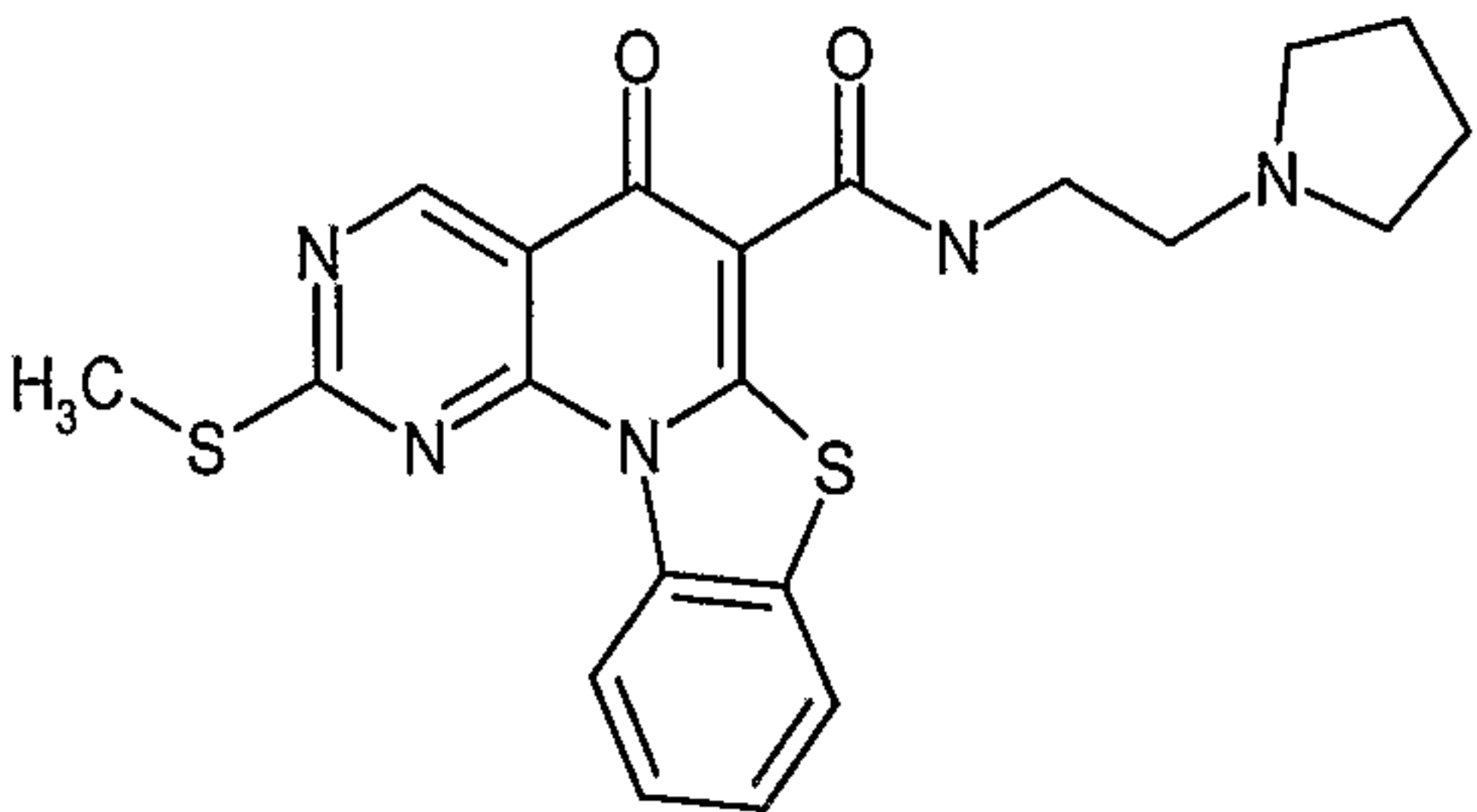
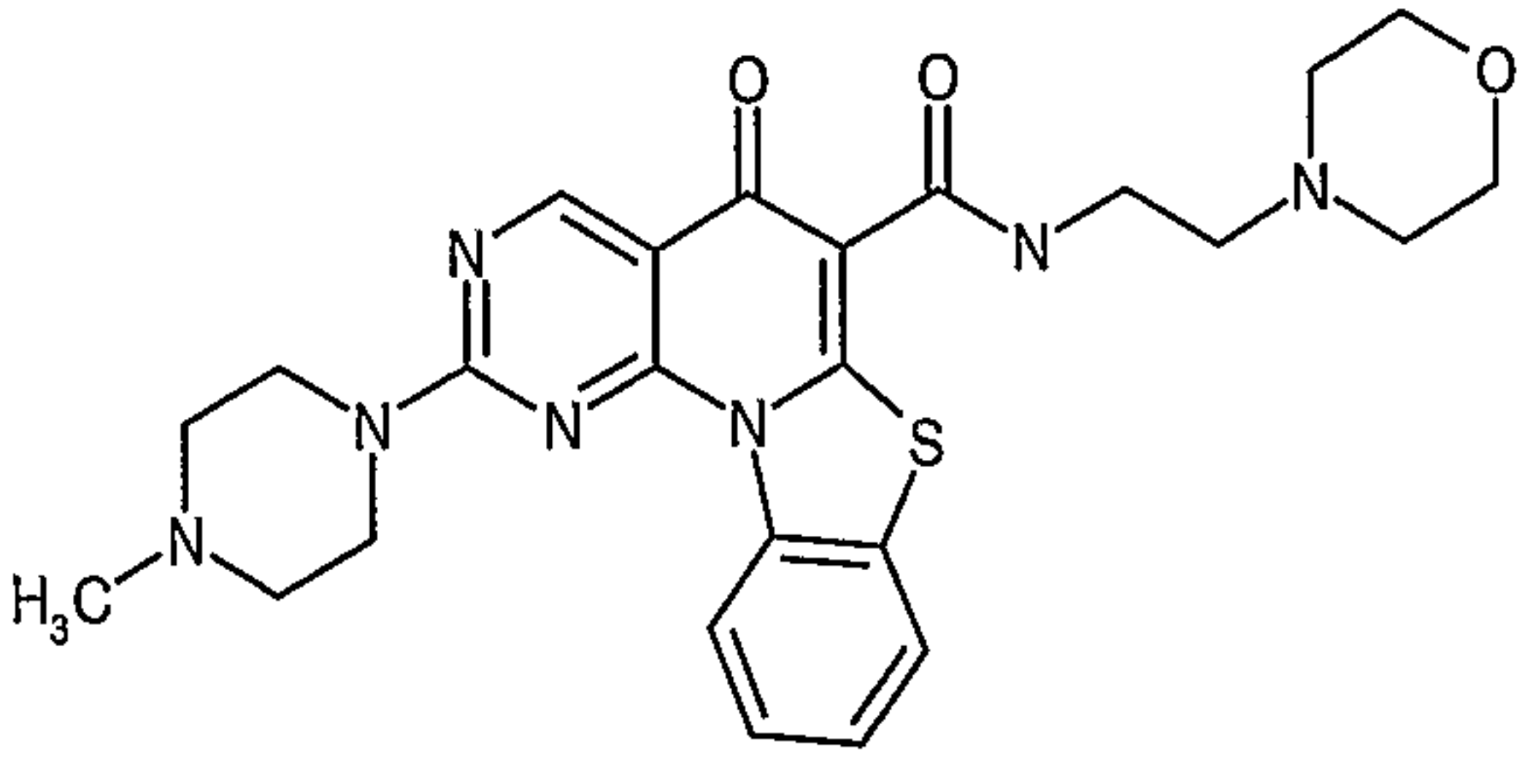
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|   | HCT-116 | 0.03   |
|  | Miapaca | 0.03   |
|  | HCT-116 | 1.75   |
|  | Miapaca | 1      |
|  | HCT-116 | 0.03   |
|  | Miapaca | 0.02   |

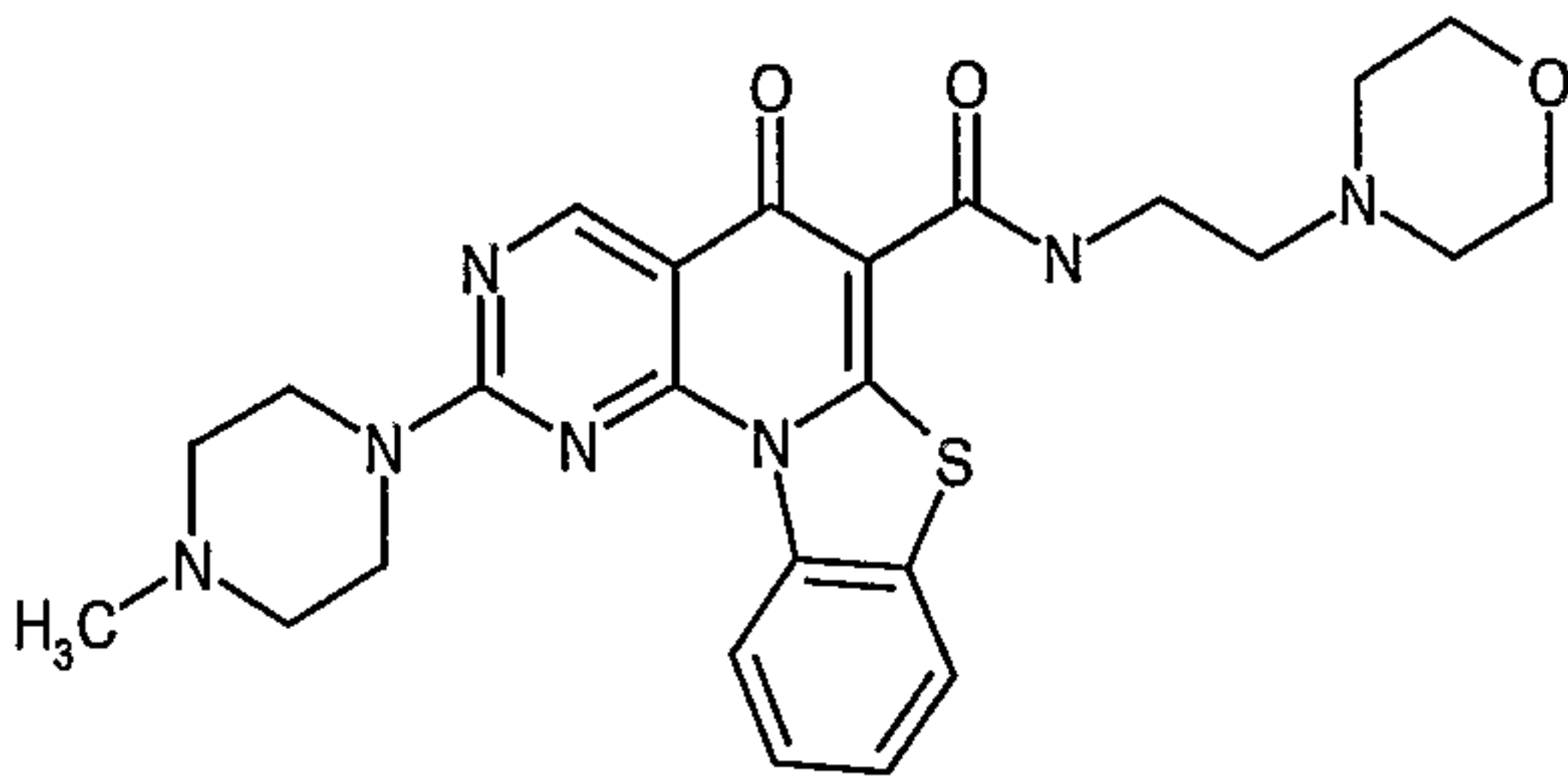
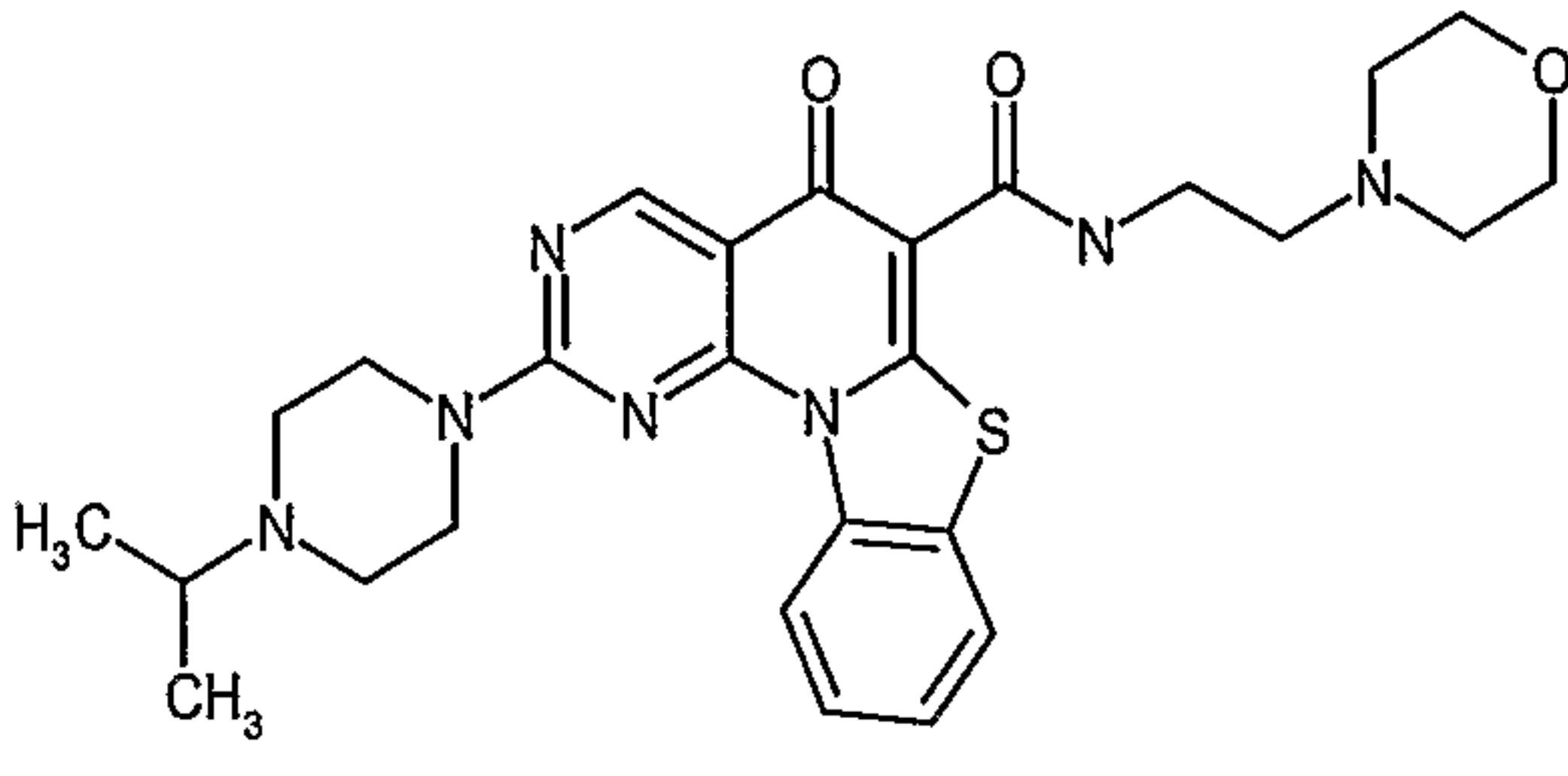
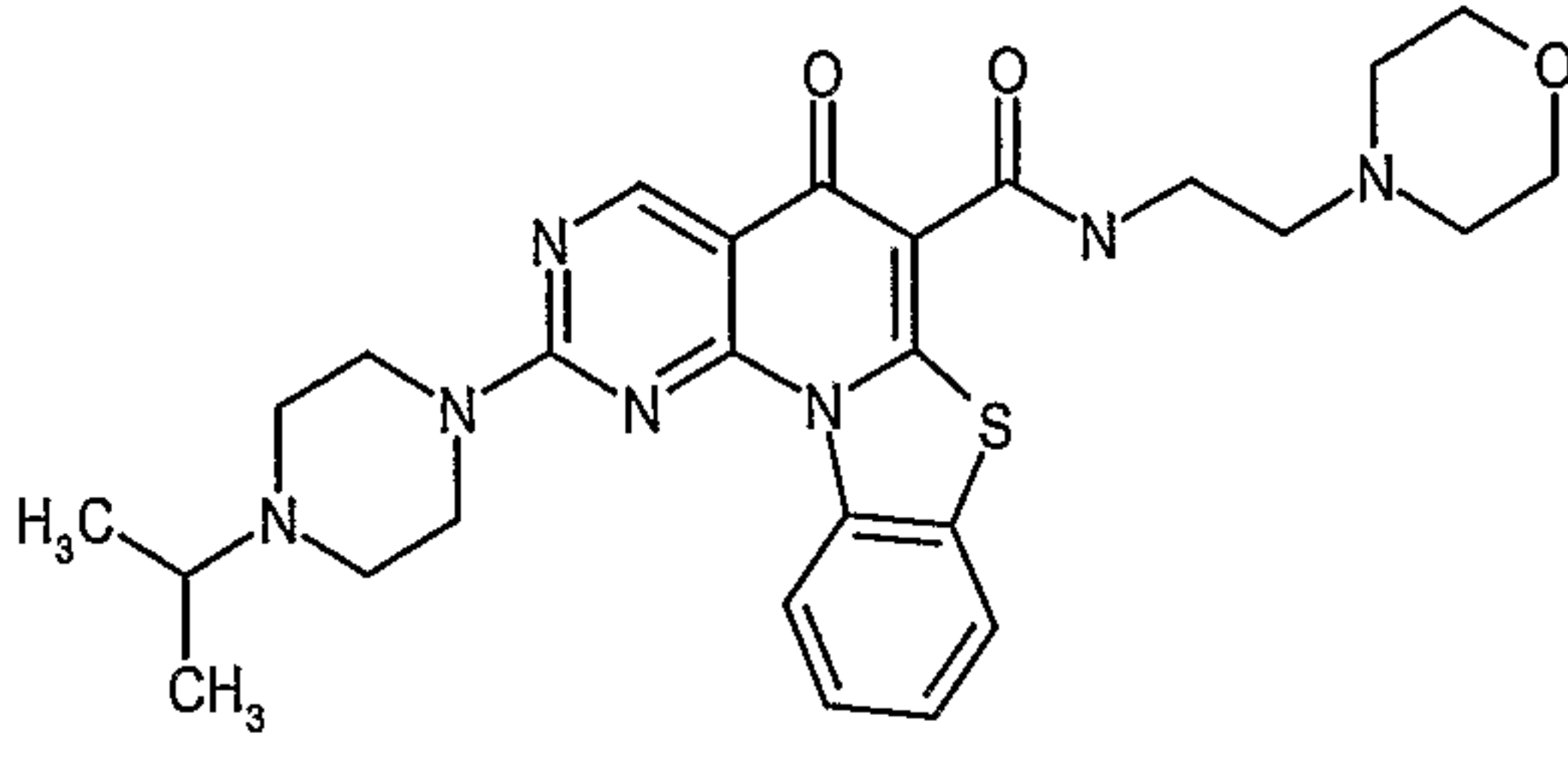
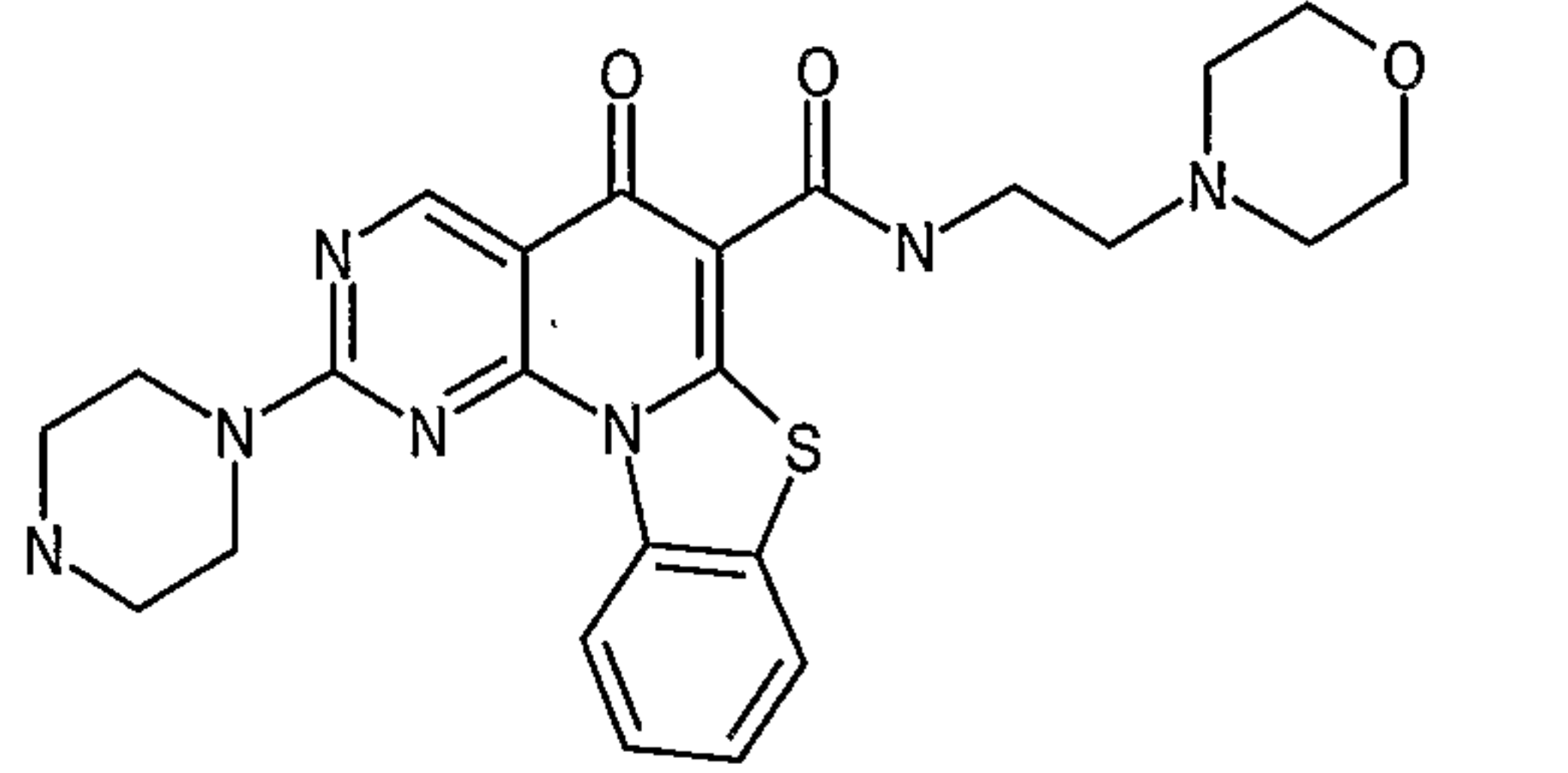
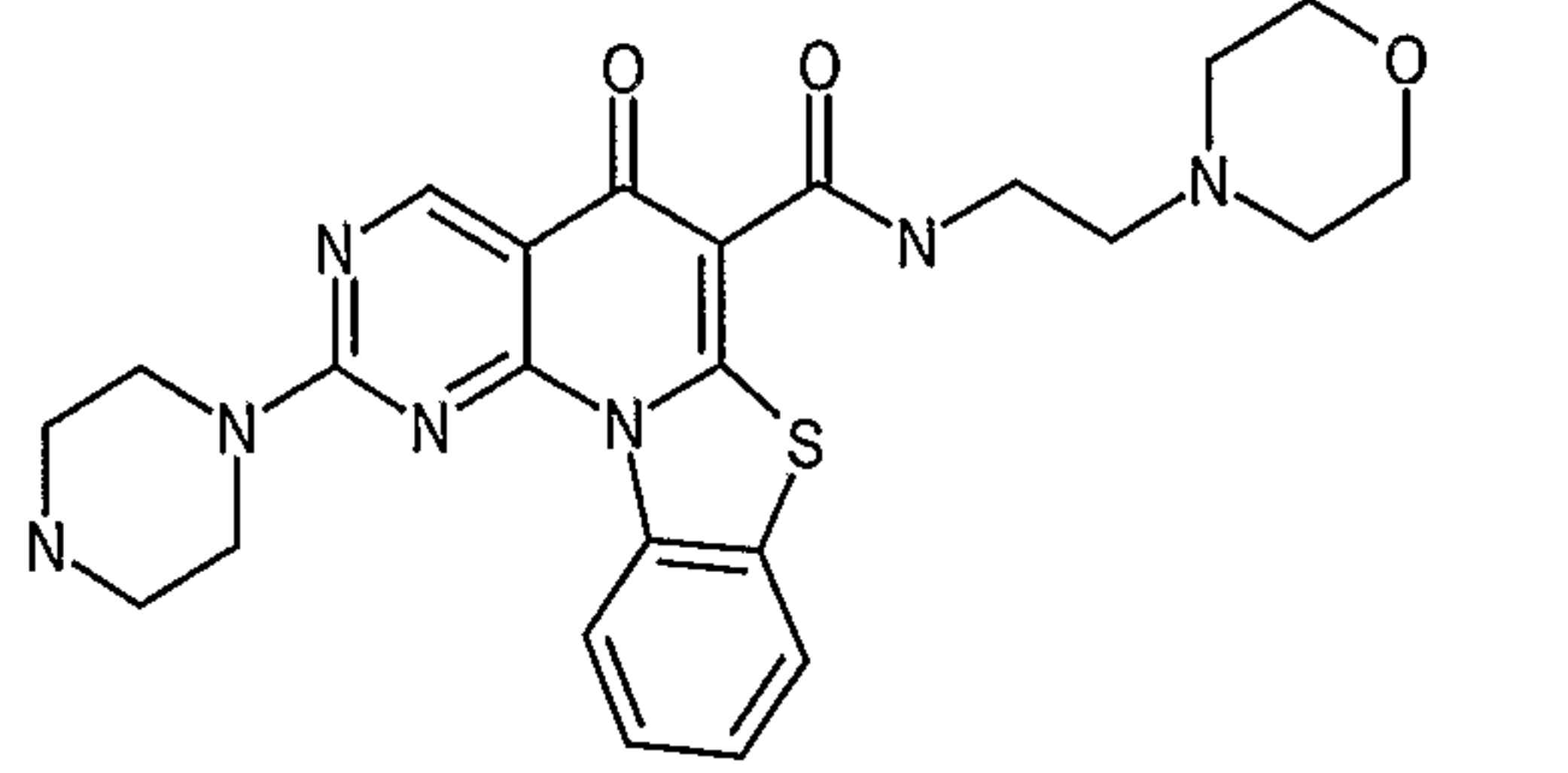
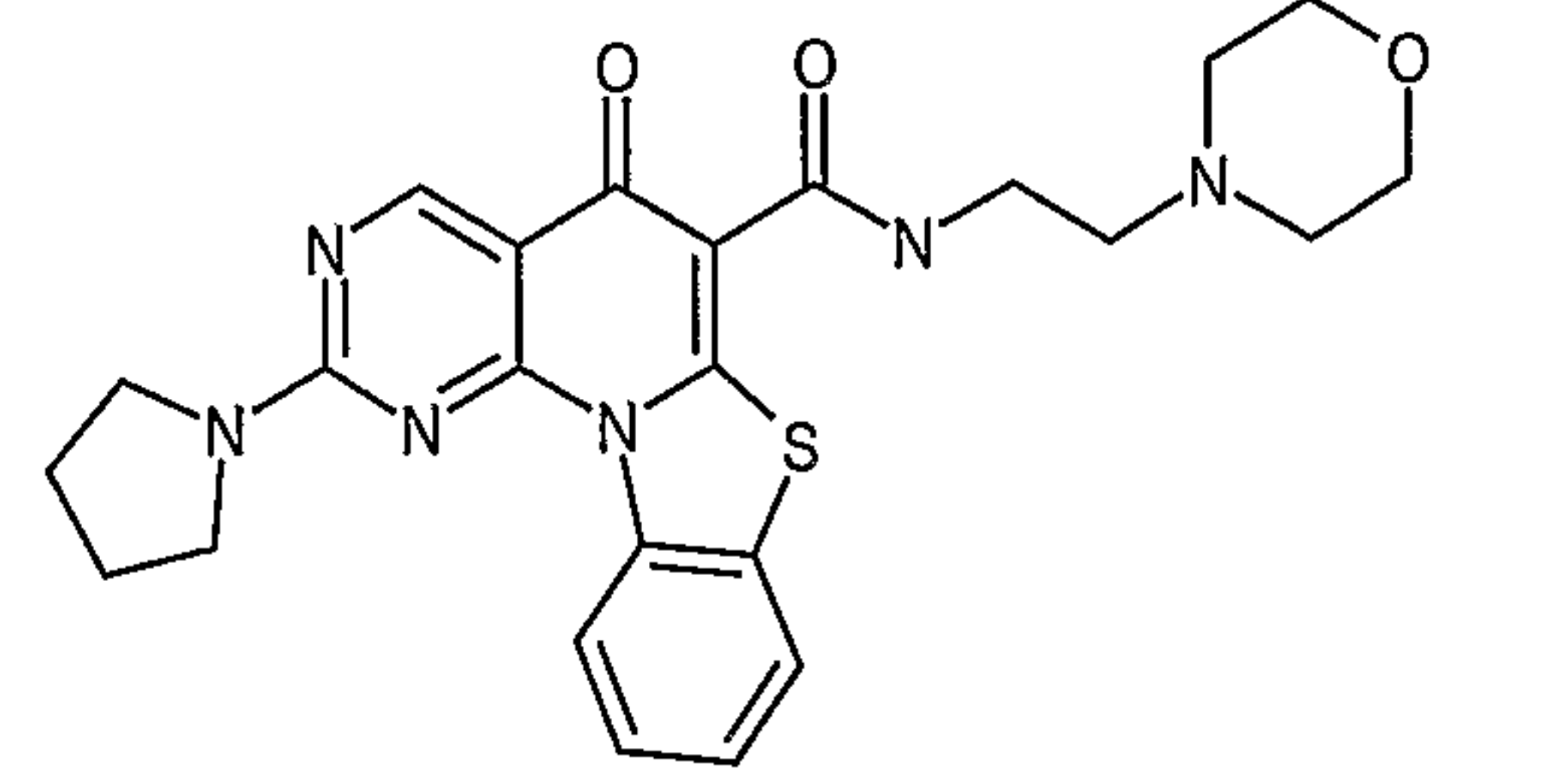
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 0.26   |
|   | Miapaca | 0.23   |
|  | HCT-116 | 3.3    |
|  | MiaPaca | 0.3    |
|  | HCT-116 | 3.7    |
|  | MiaPaca | 0.2    |

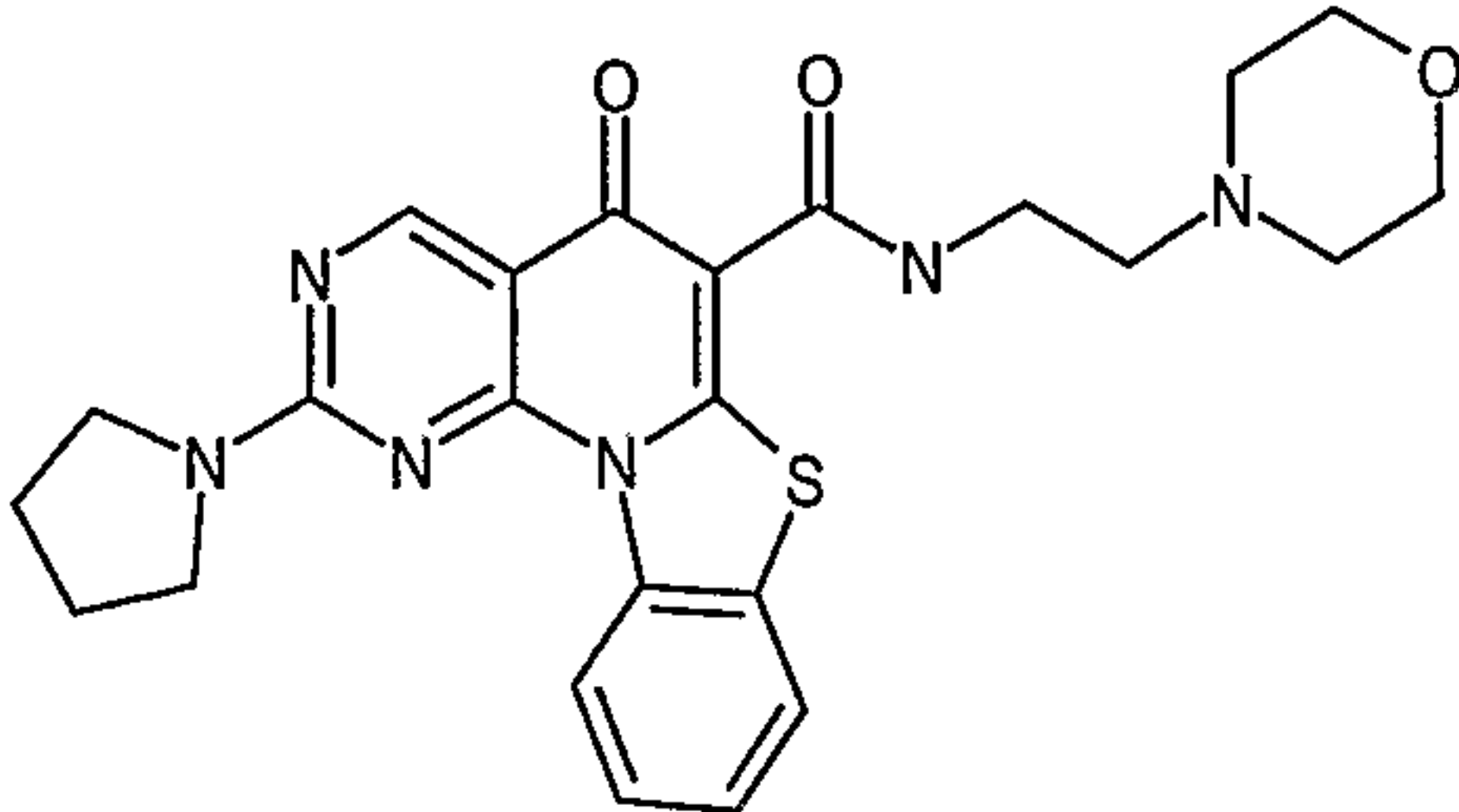
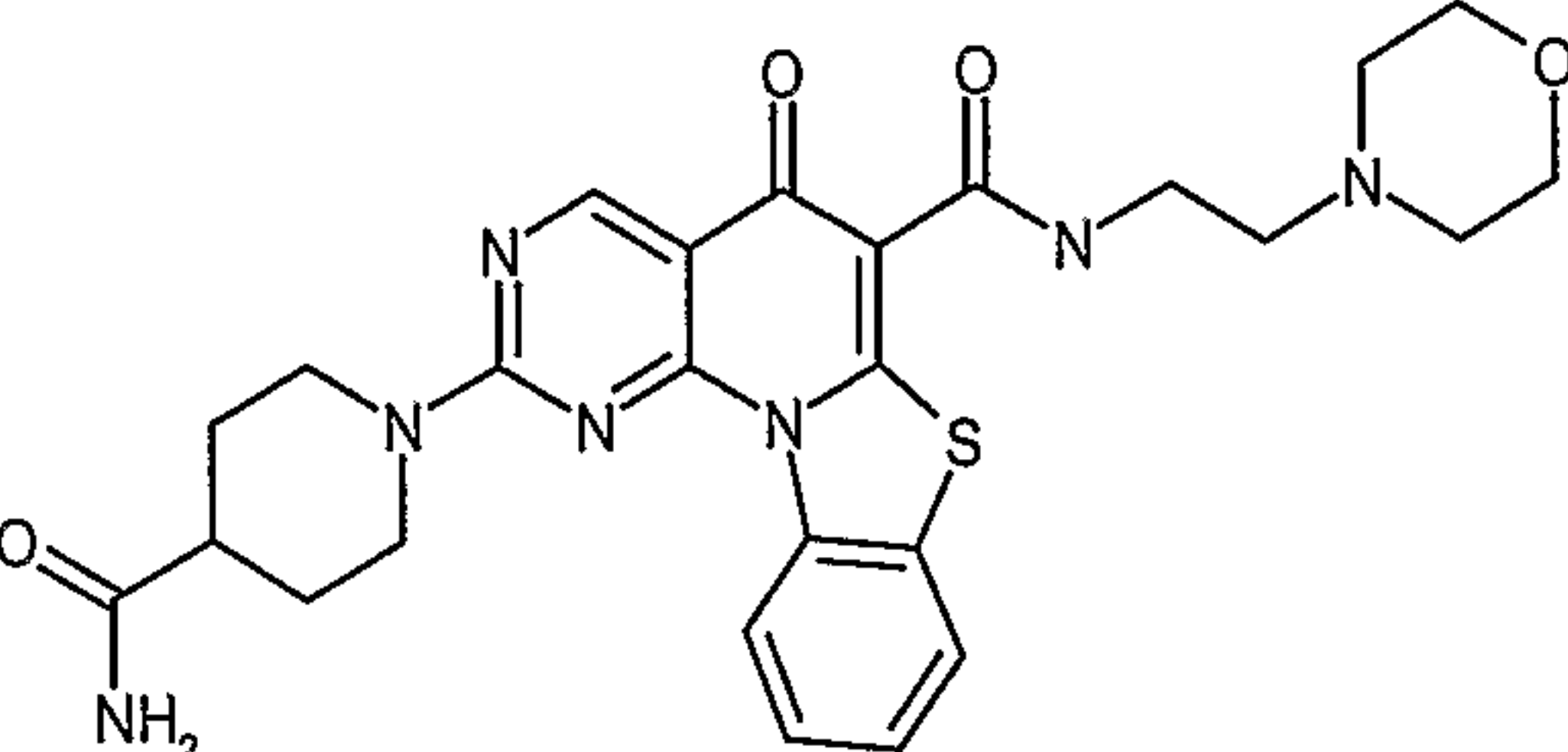
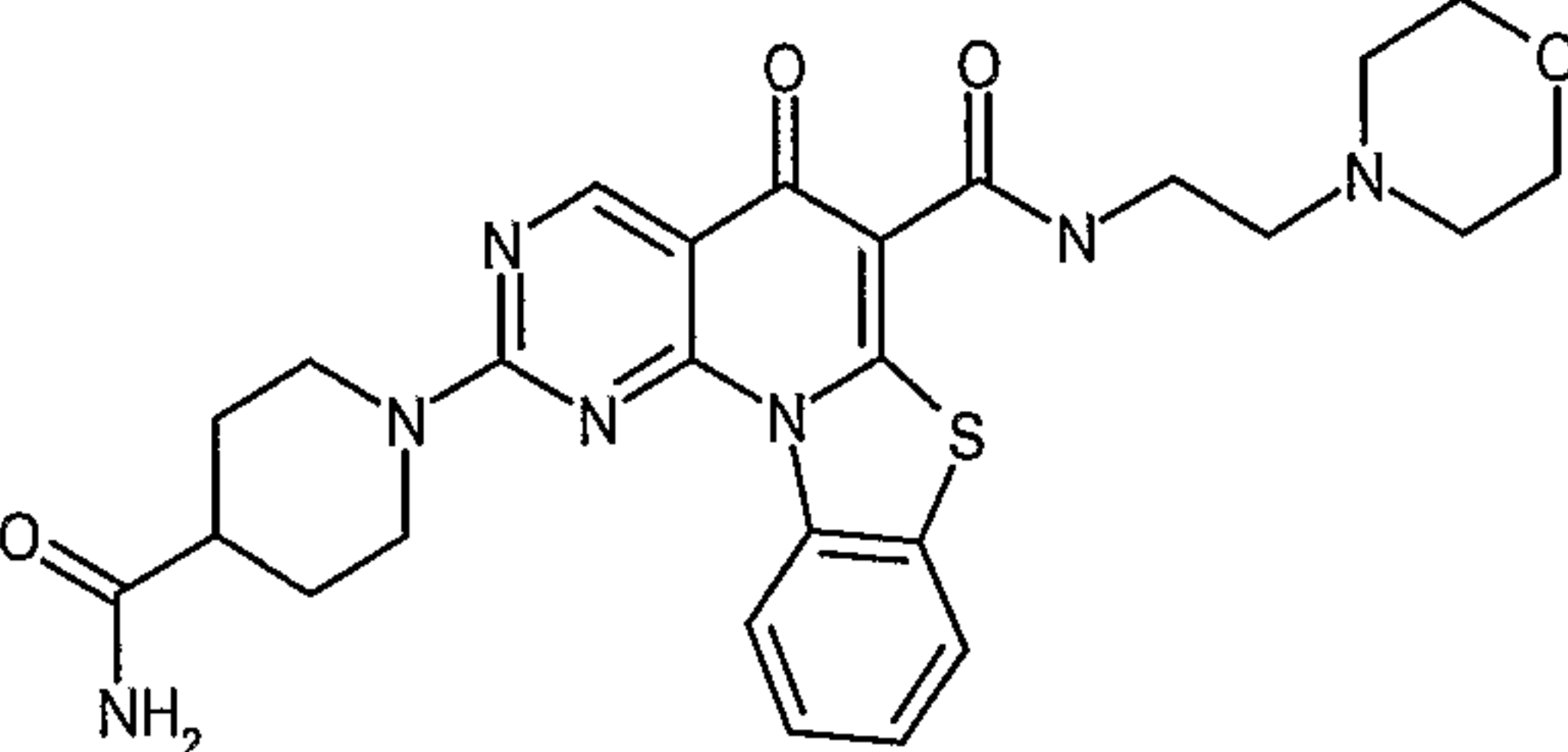
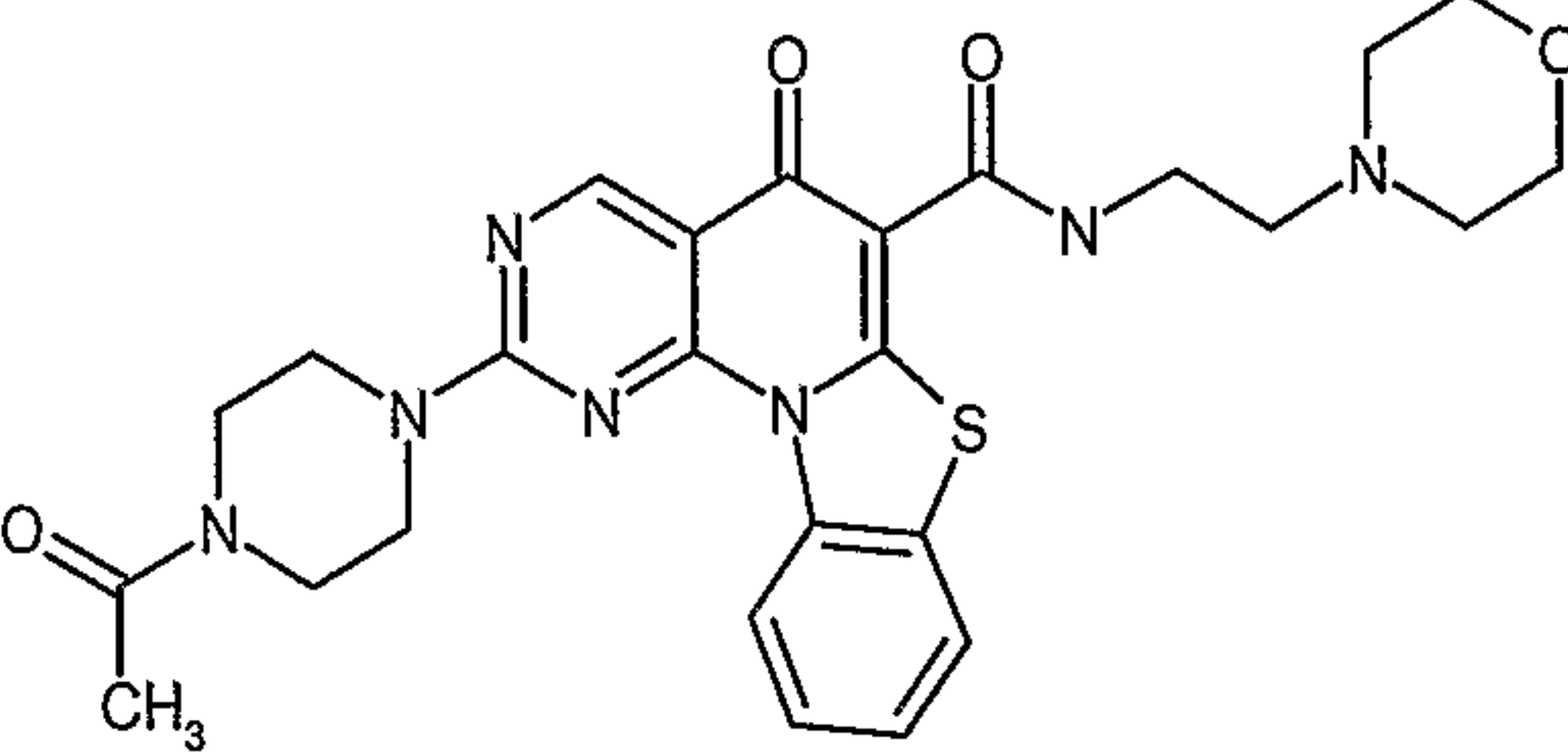
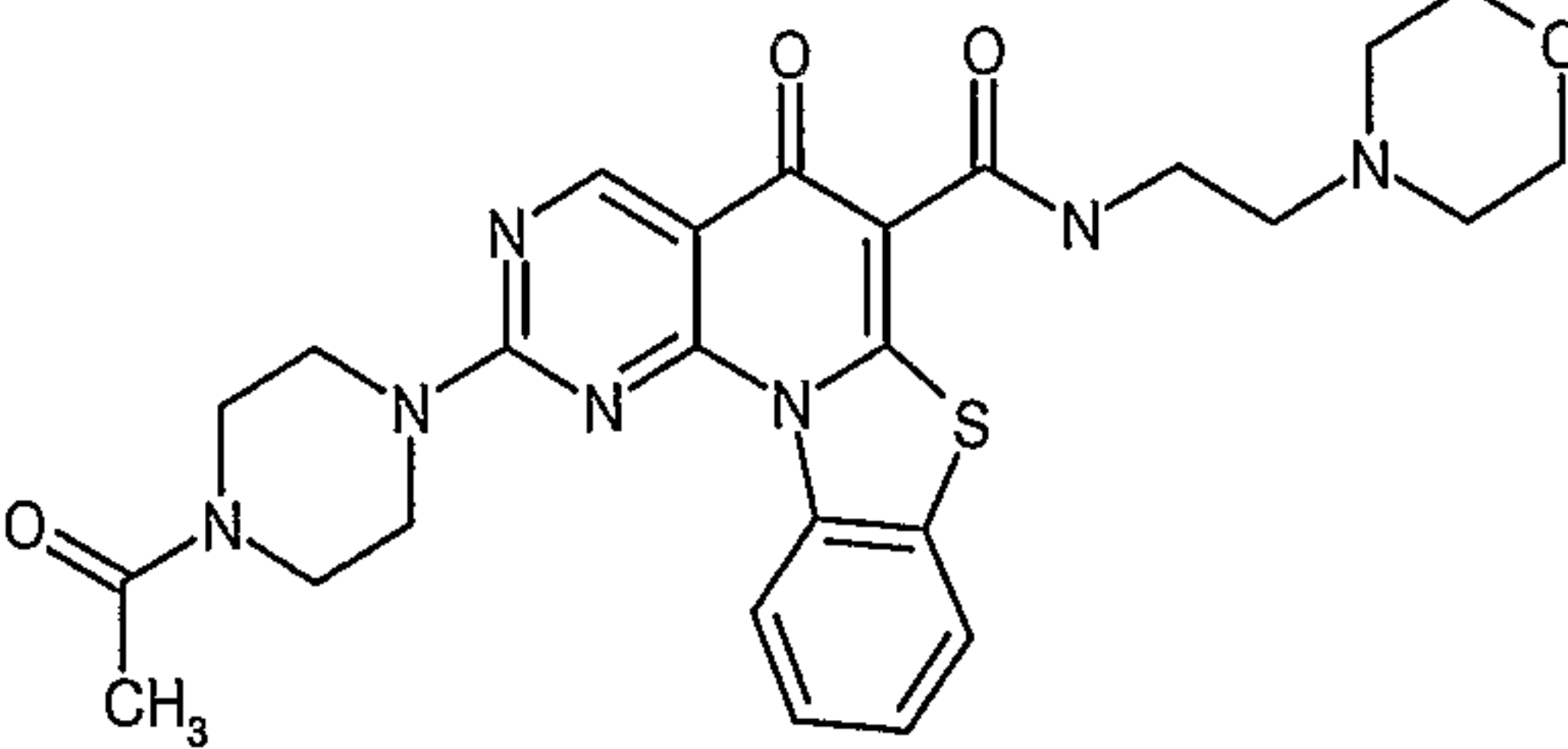
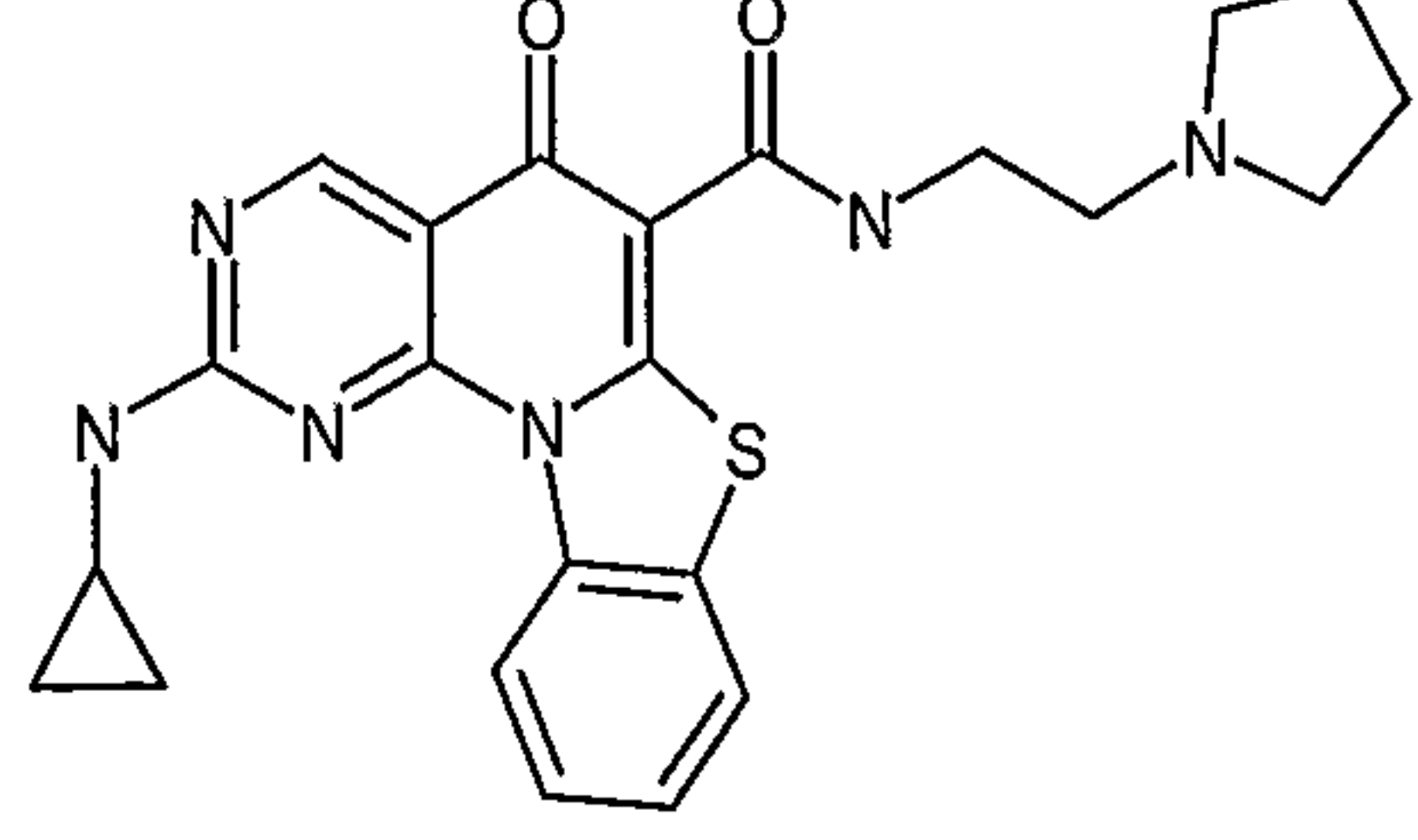
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 4.1    |
|   | MiaPaca | 0.3    |
|  | HCT-116 | 3.4    |
|  | MiaPaca | 0.4    |
|  | HCT-116 | 3.5    |
|  | MiaPaca | 2.3    |

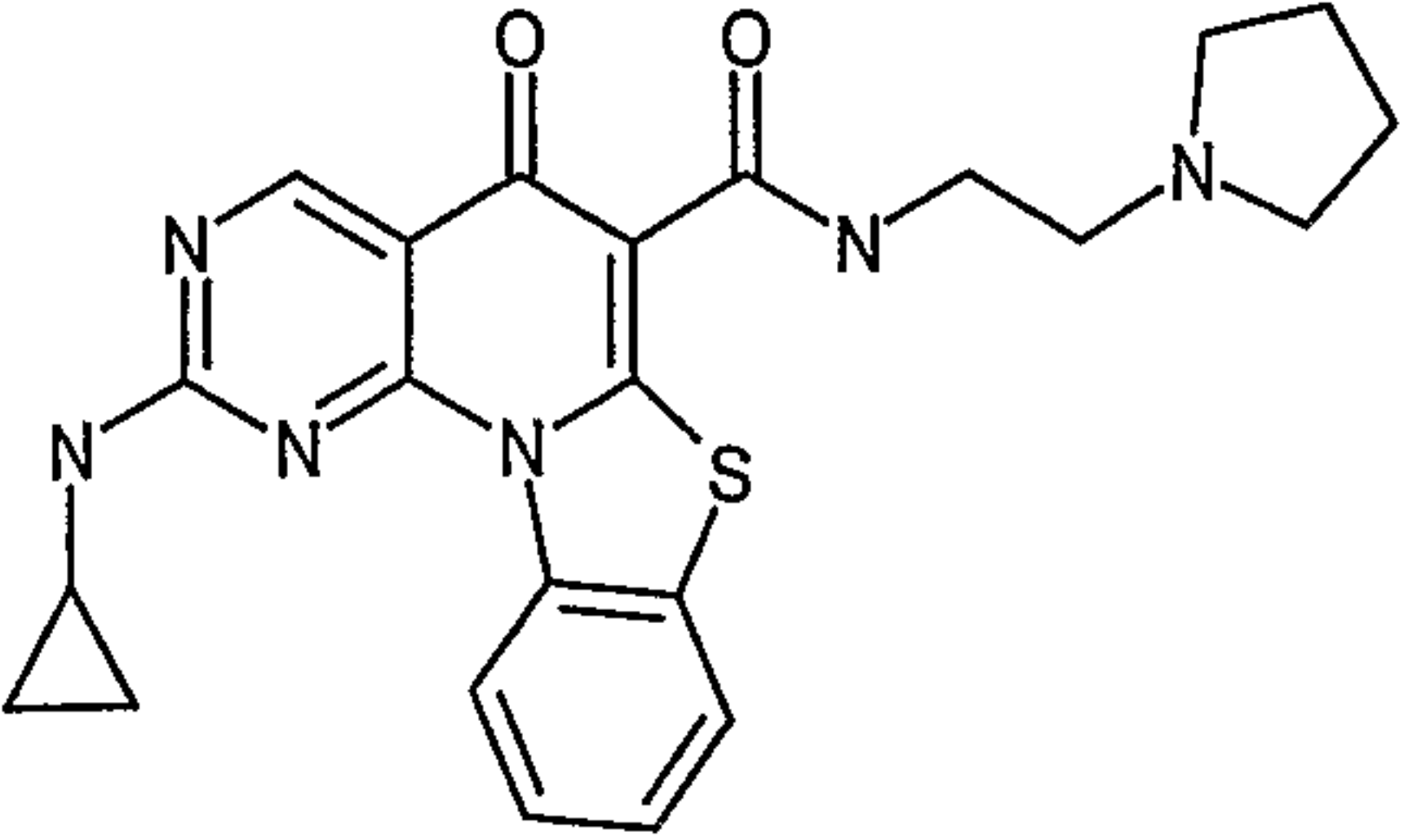
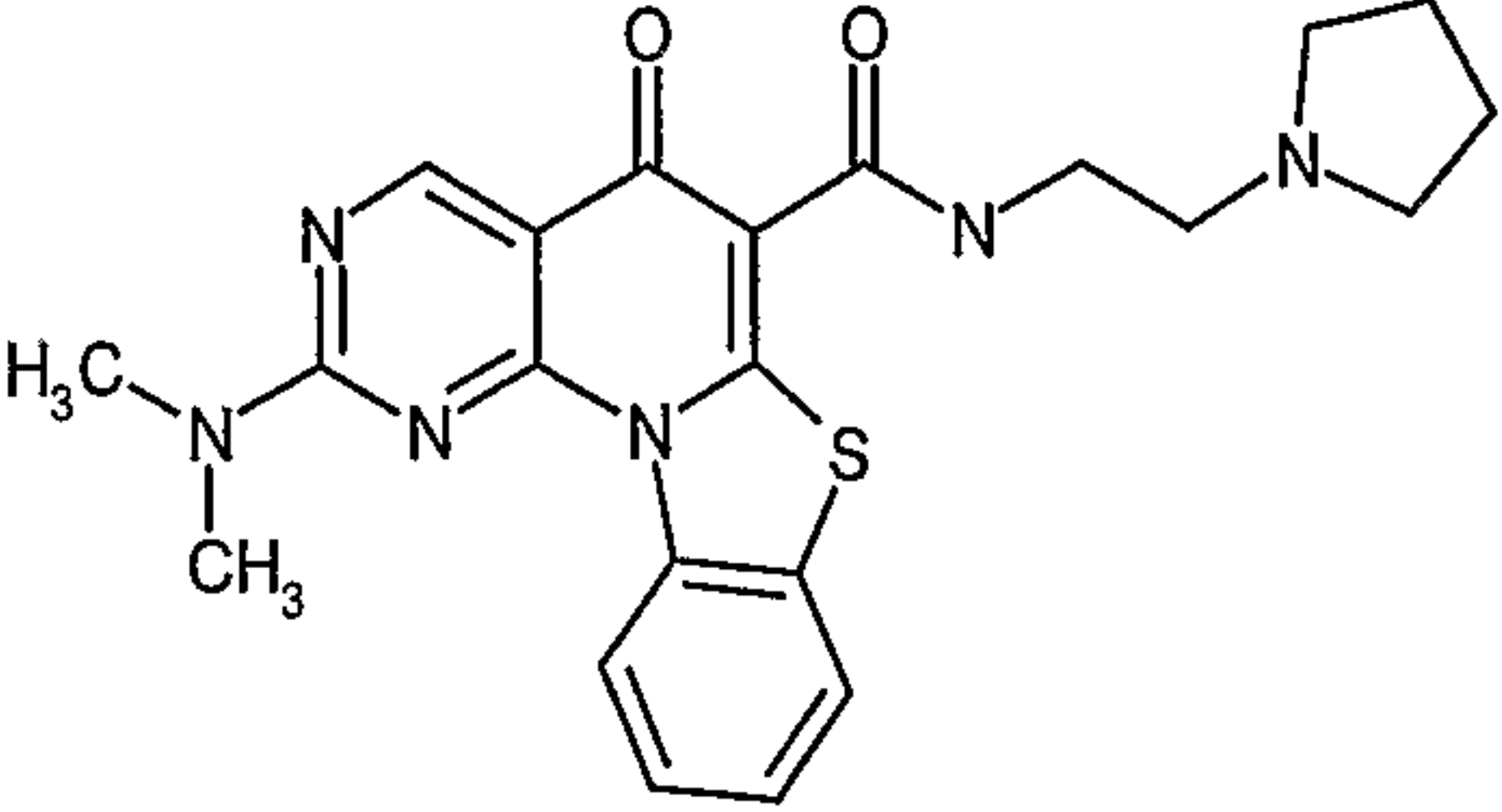
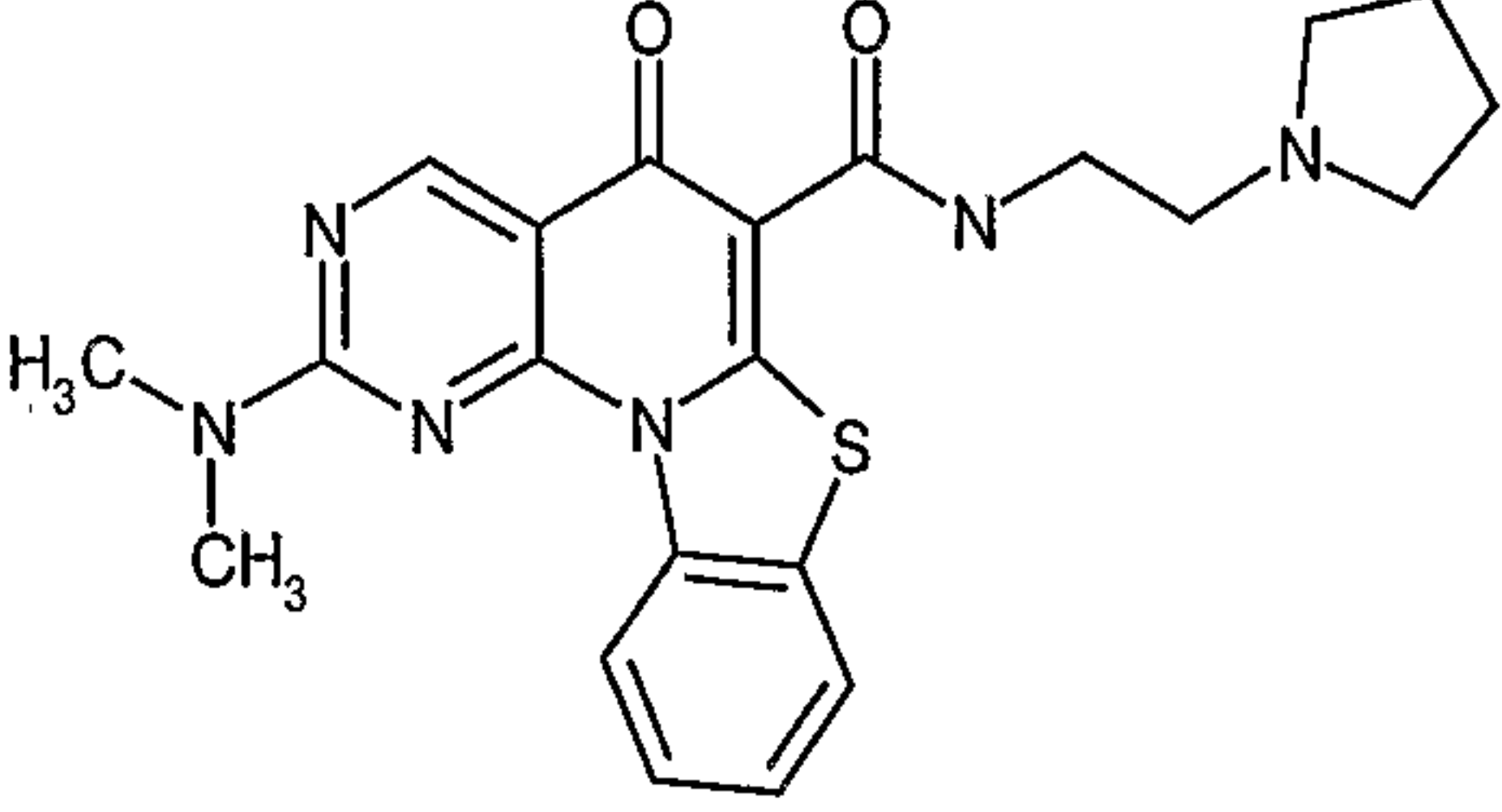
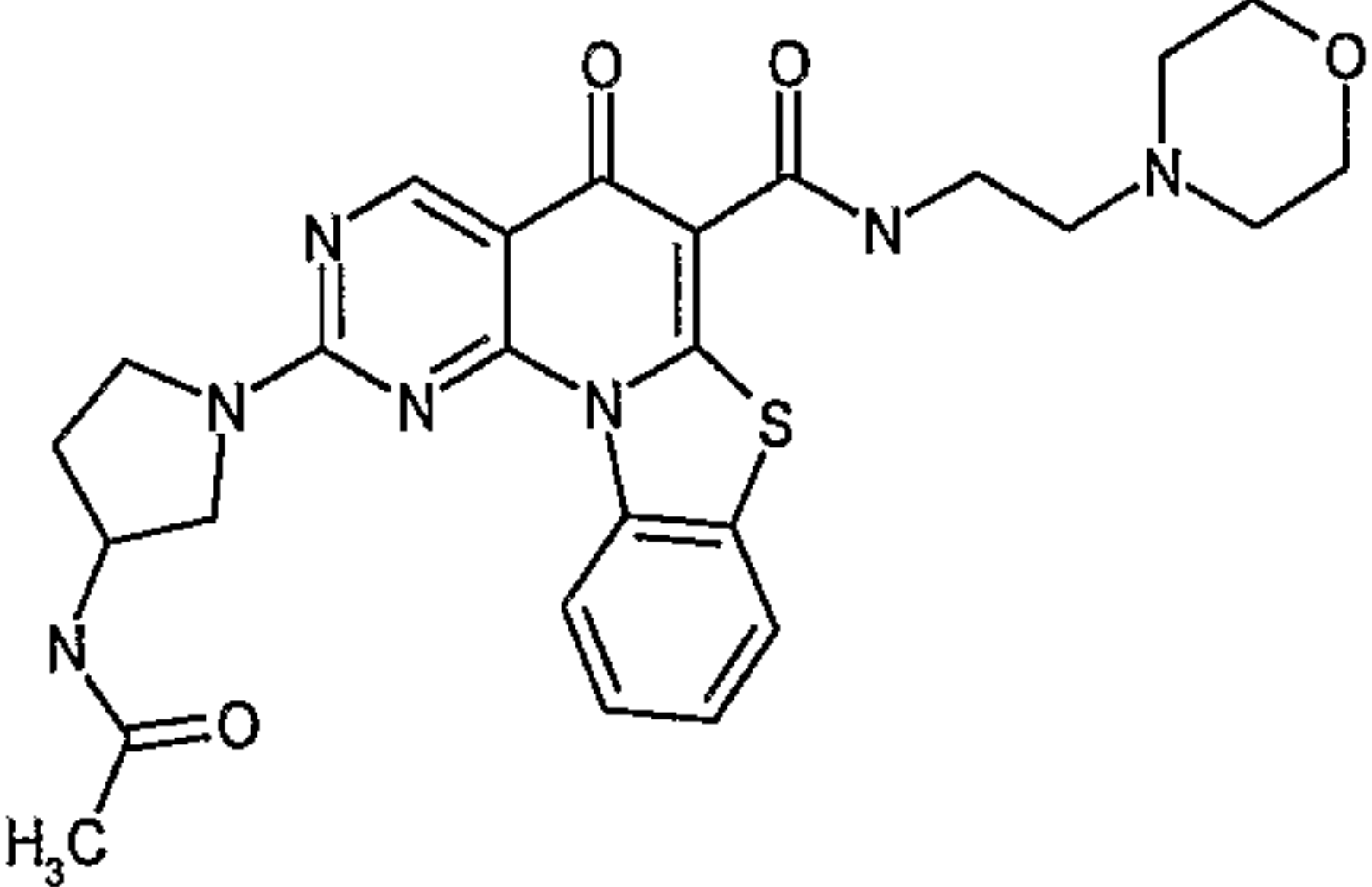
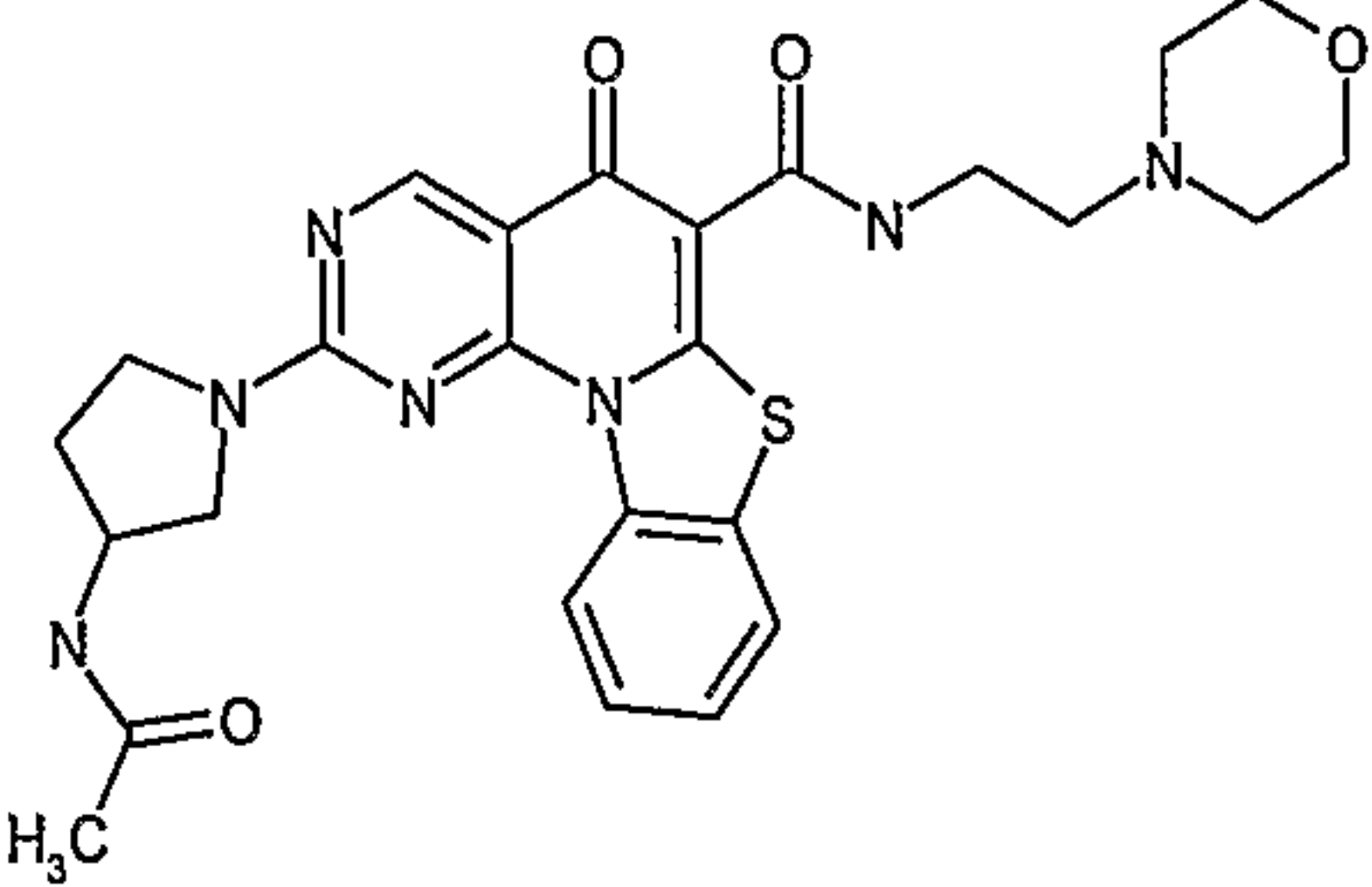
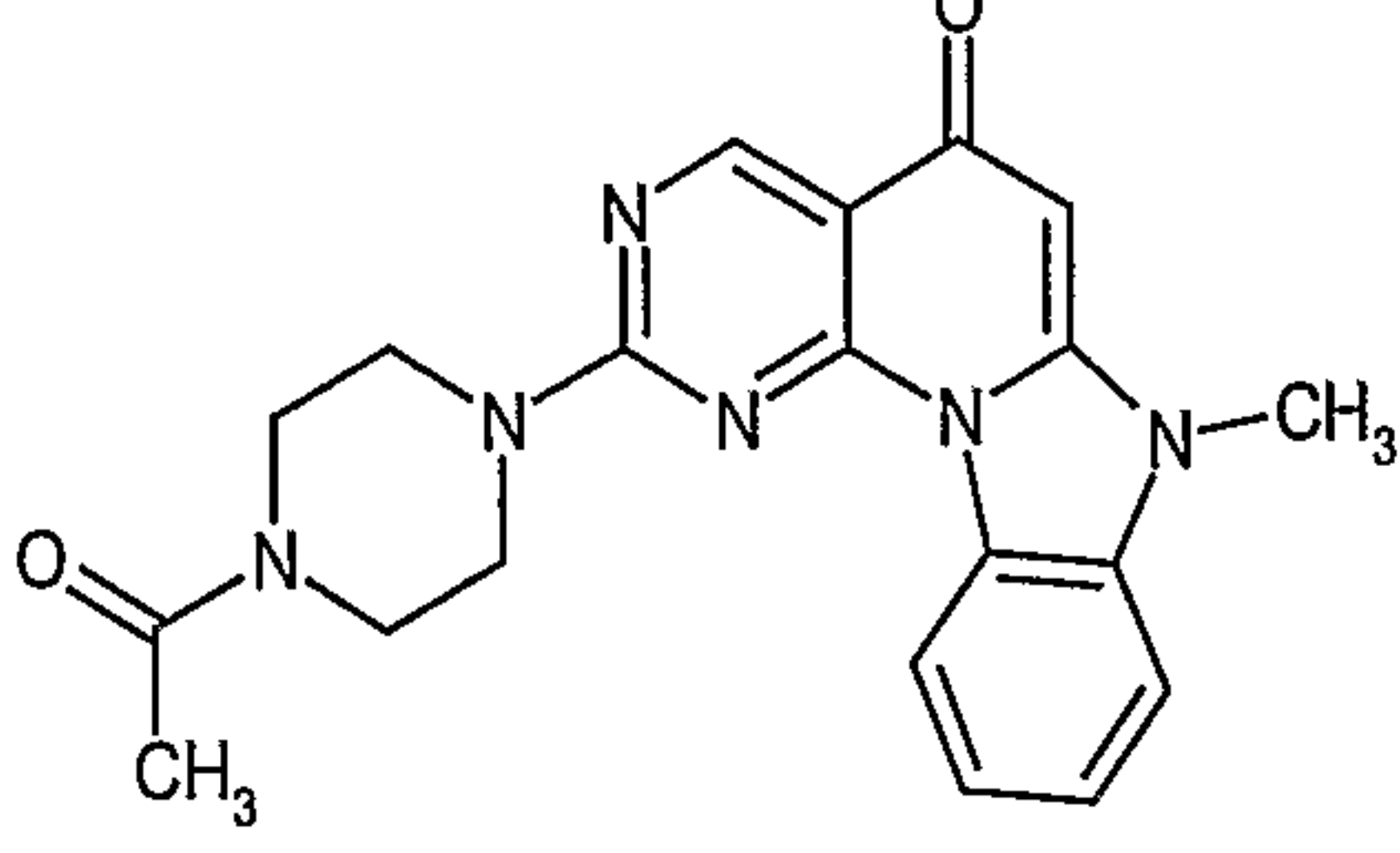
| MOLSTRUCTURE                                                                                                                                                                     | M CELL  | M DATA |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------|--------|
|  <chem>CC(=O)N[C@H]1CCN1c2ccc3c(c2)c(=O)n(c3c4ccccc4S5C(=O)N(CCCN5C6CCCC6)C6=O)c7ccncc7</chem>  | HCT-116 | 3.4    |
|  <chem>CC(=O)N[C@H]1CCN1c2ccc3c(c2)c(=O)n(c3c4ccccc4S5C(=O)N(CCCN5C6CCCC6)C6=O)c7ccncc7</chem> | MiaPaca | 0.7    |
|  <chem>NC(=O)N1CCCC1c2ccc3c(c2)c(=O)n(c3c4ccccc4S5C(=O)N(CCCN5C6CCCC6)C6=O)c7ccncc7</chem>    | HCT-116 | 3.7    |
|  <chem>NC(=O)N1CCCC1c2ccc3c(c2)c(=O)n(c3c4ccccc4S5C(=O)N(CCCN5C6CCCC6)C6=O)c7ccncc7</chem>    | MiaPaca | 0.2    |
|  <chem>C1CCN(C1)CCN(CCCN2C3CCCC3C(=O)N2C4=CC=CC=C4S5C(=O)N(CCCN5C6CCCC6)C6=O)c7ccncc7</chem> | HCT-116 | 0.3    |

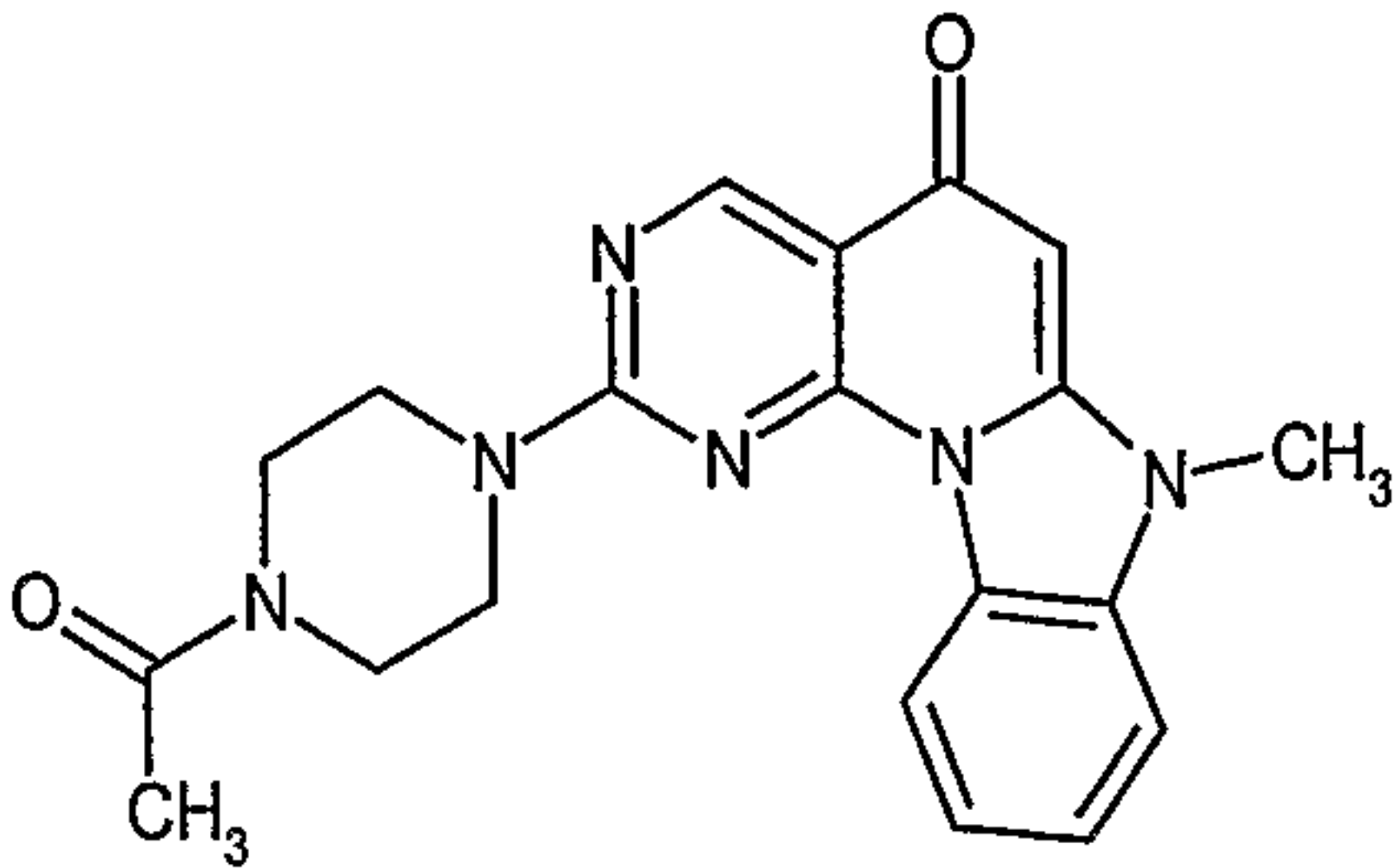
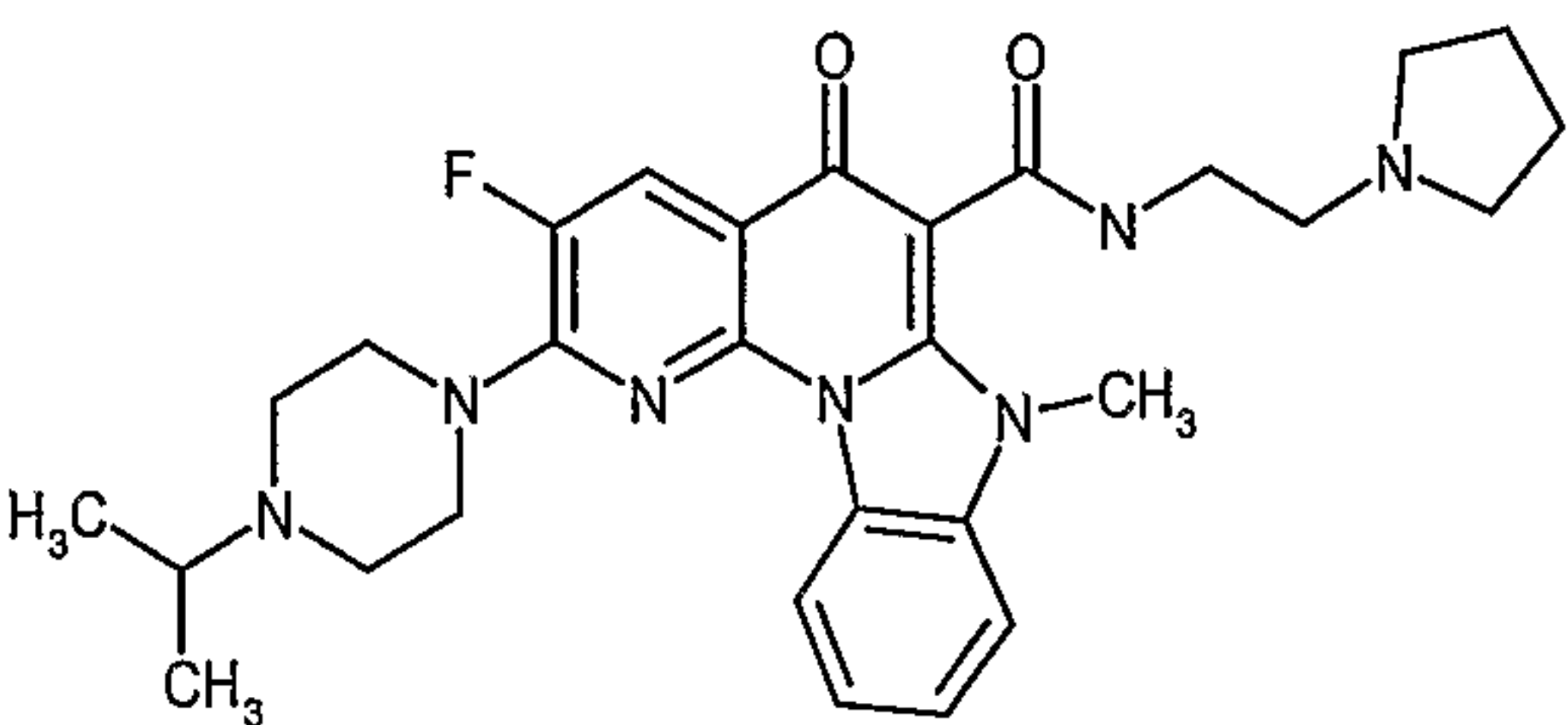
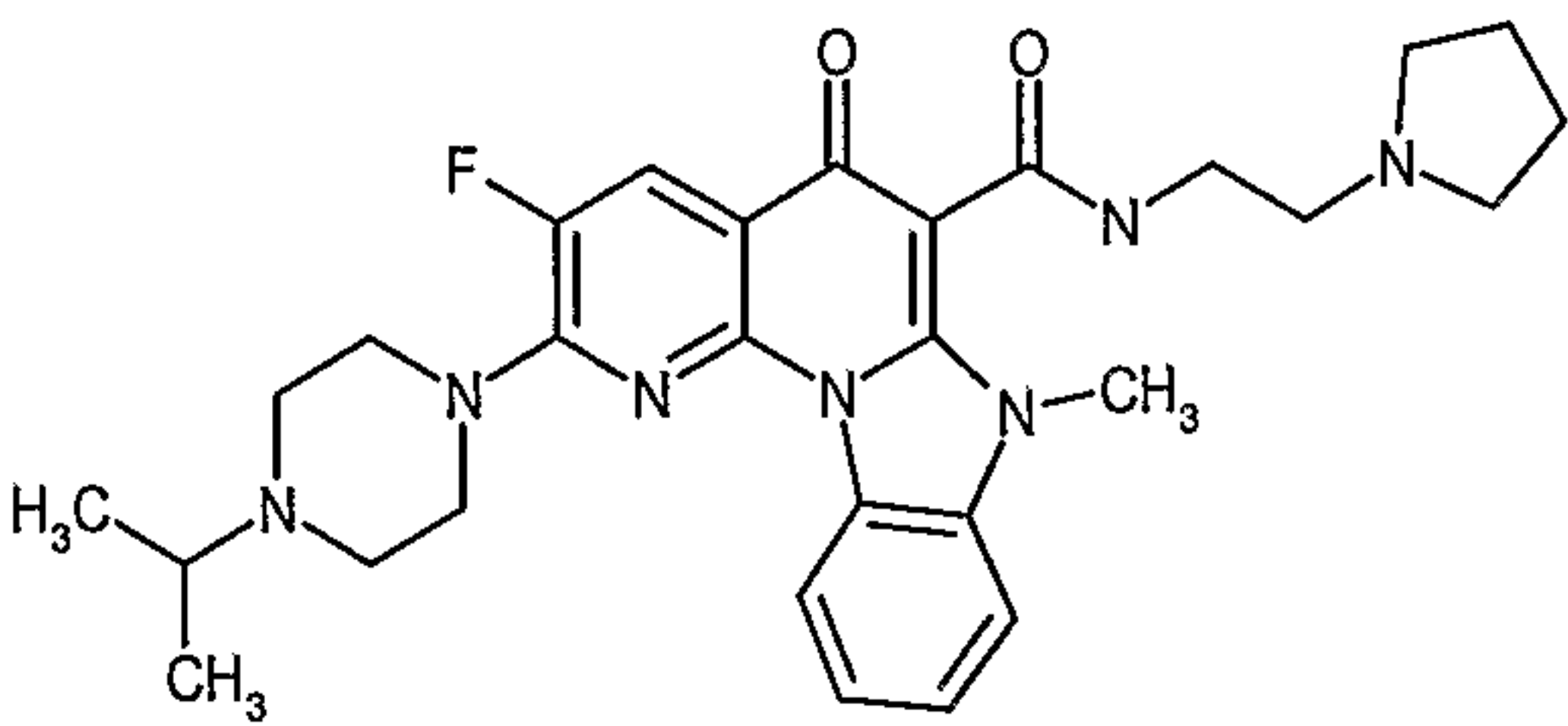
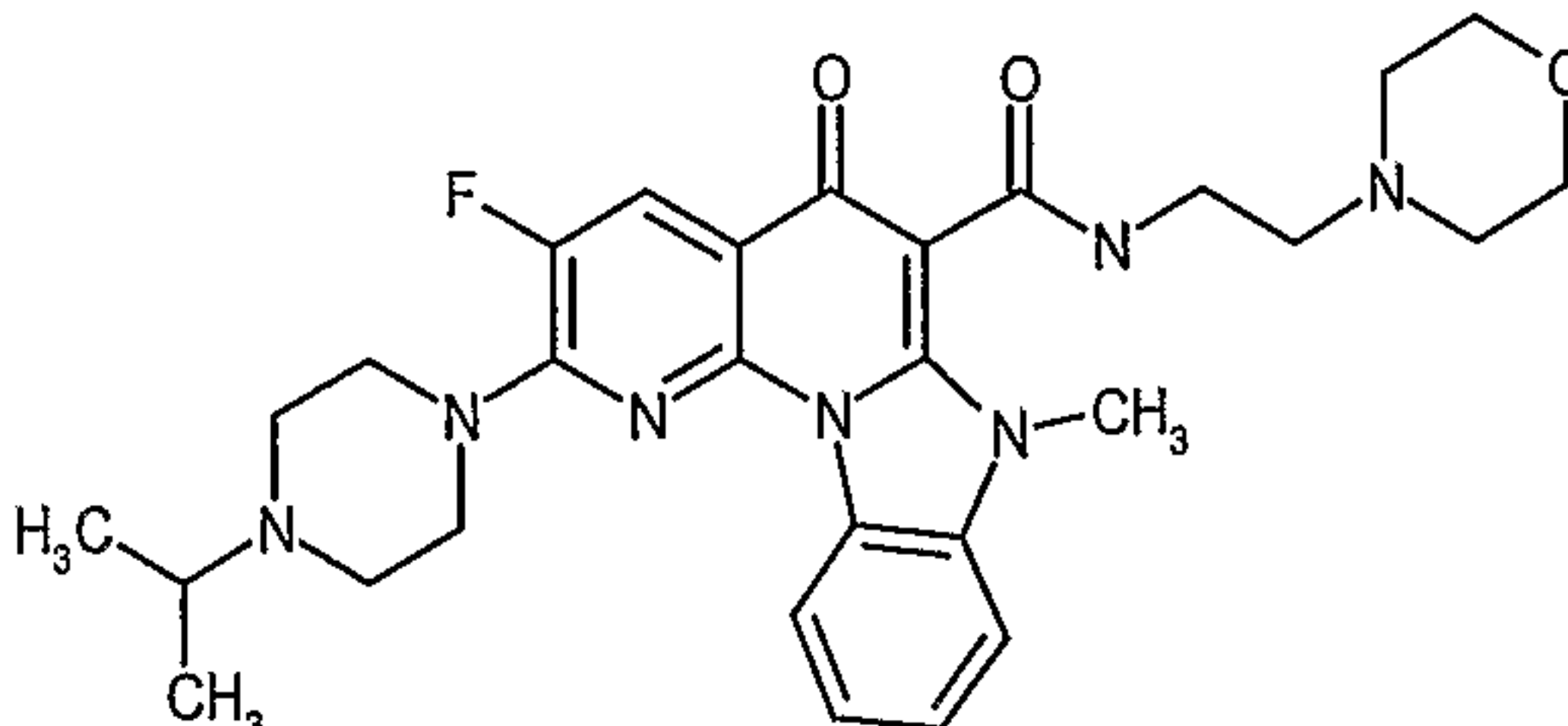
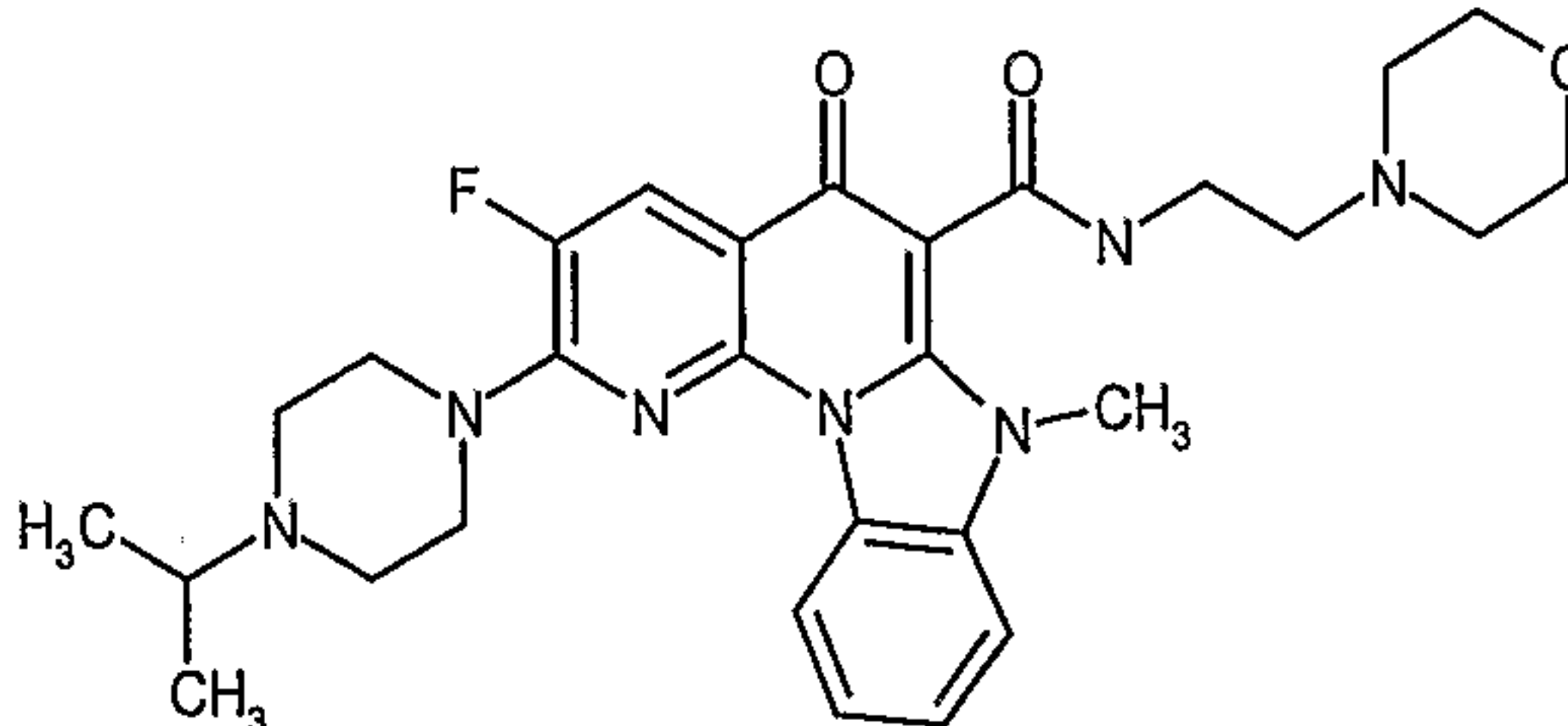
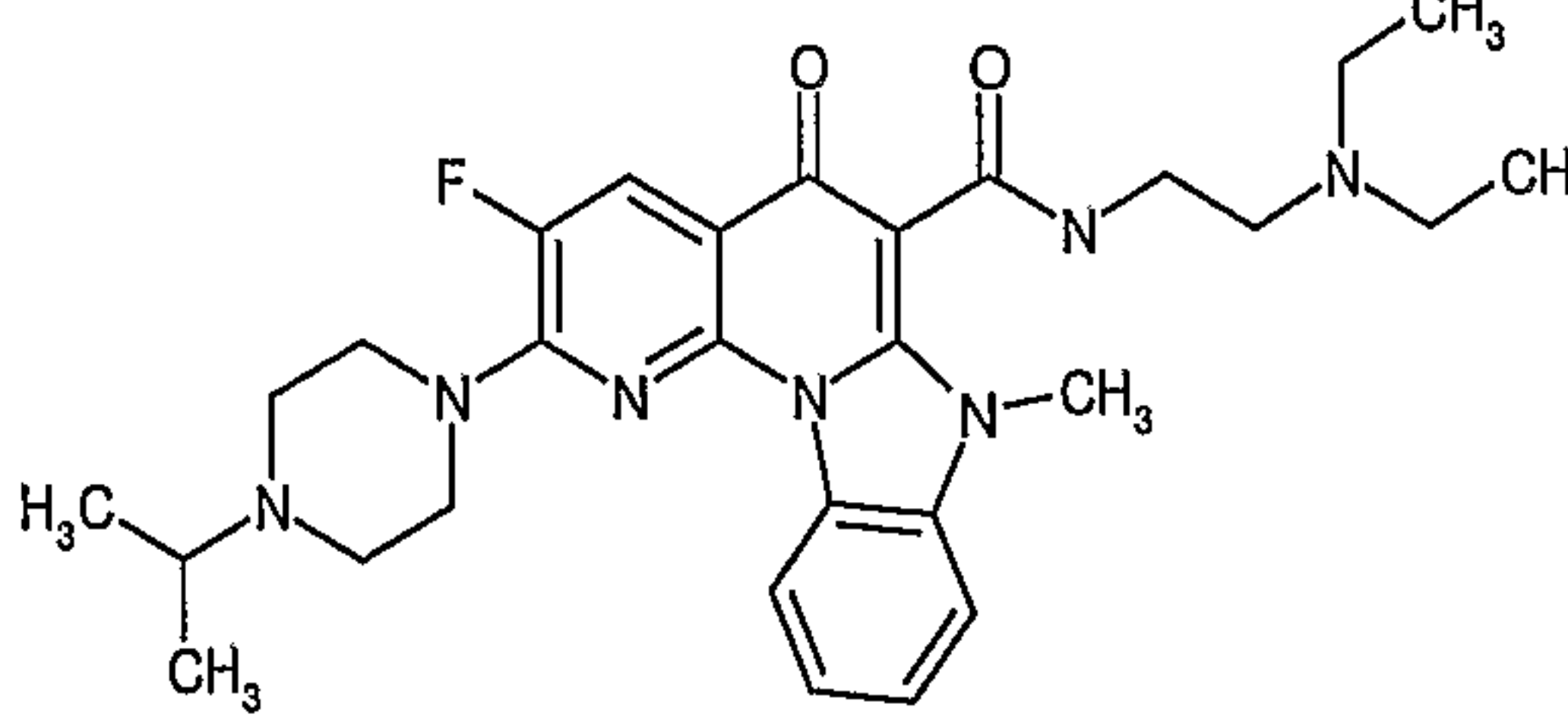
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | 0.2    |
|   | HCT-116 | 0.3    |
|  | MiaPaca | 0.1    |
|  | HCT-116 | 0.2    |
|  | MiaPaca | 0.0    |
|  | HCT-116 | 3.5    |

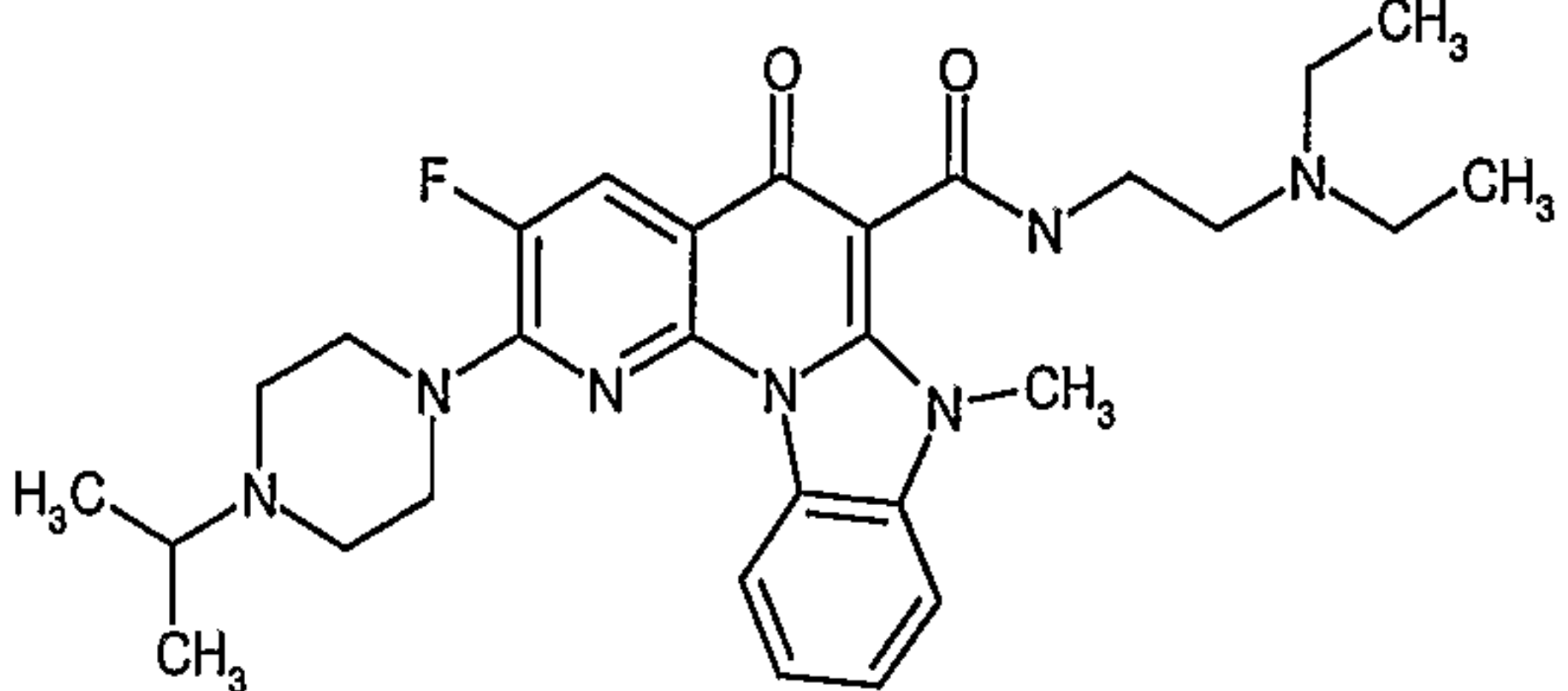
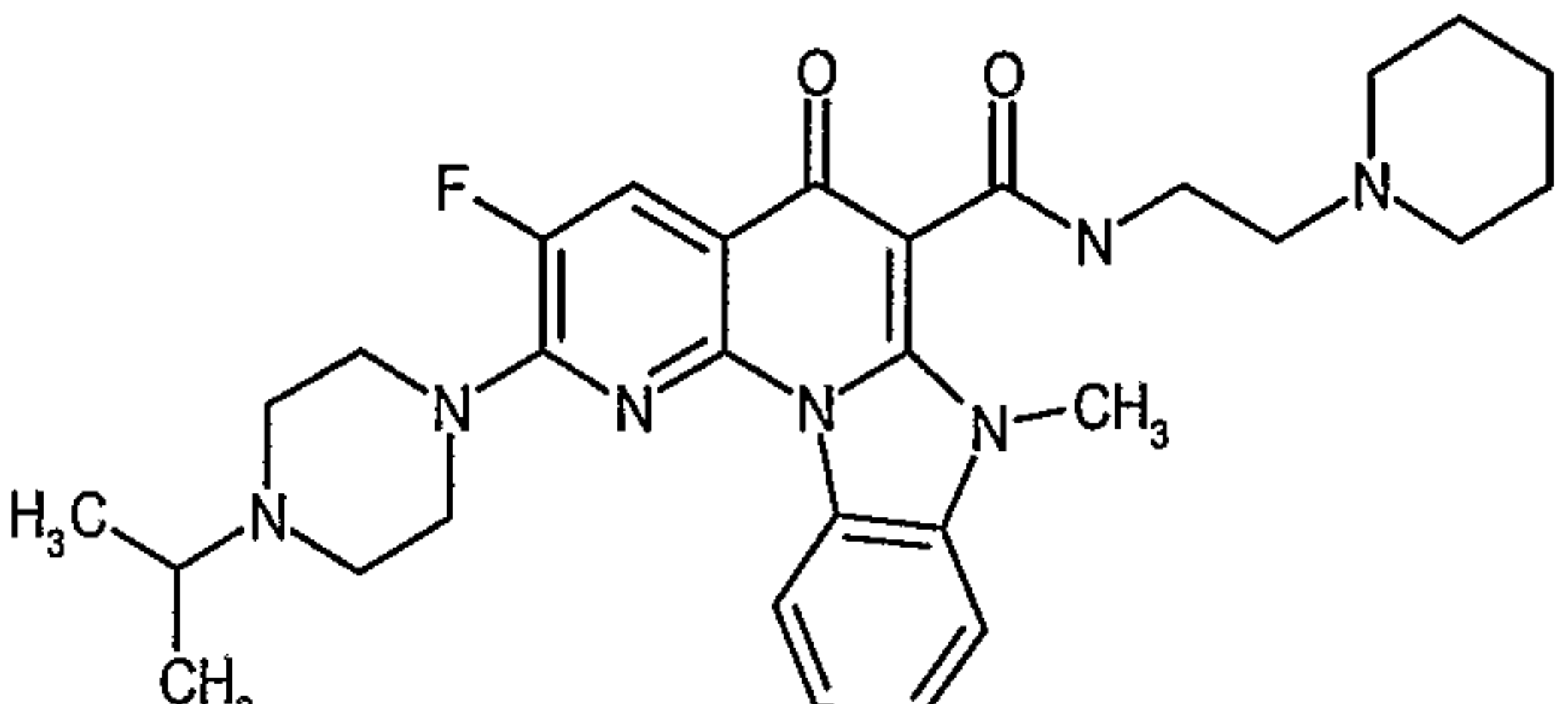
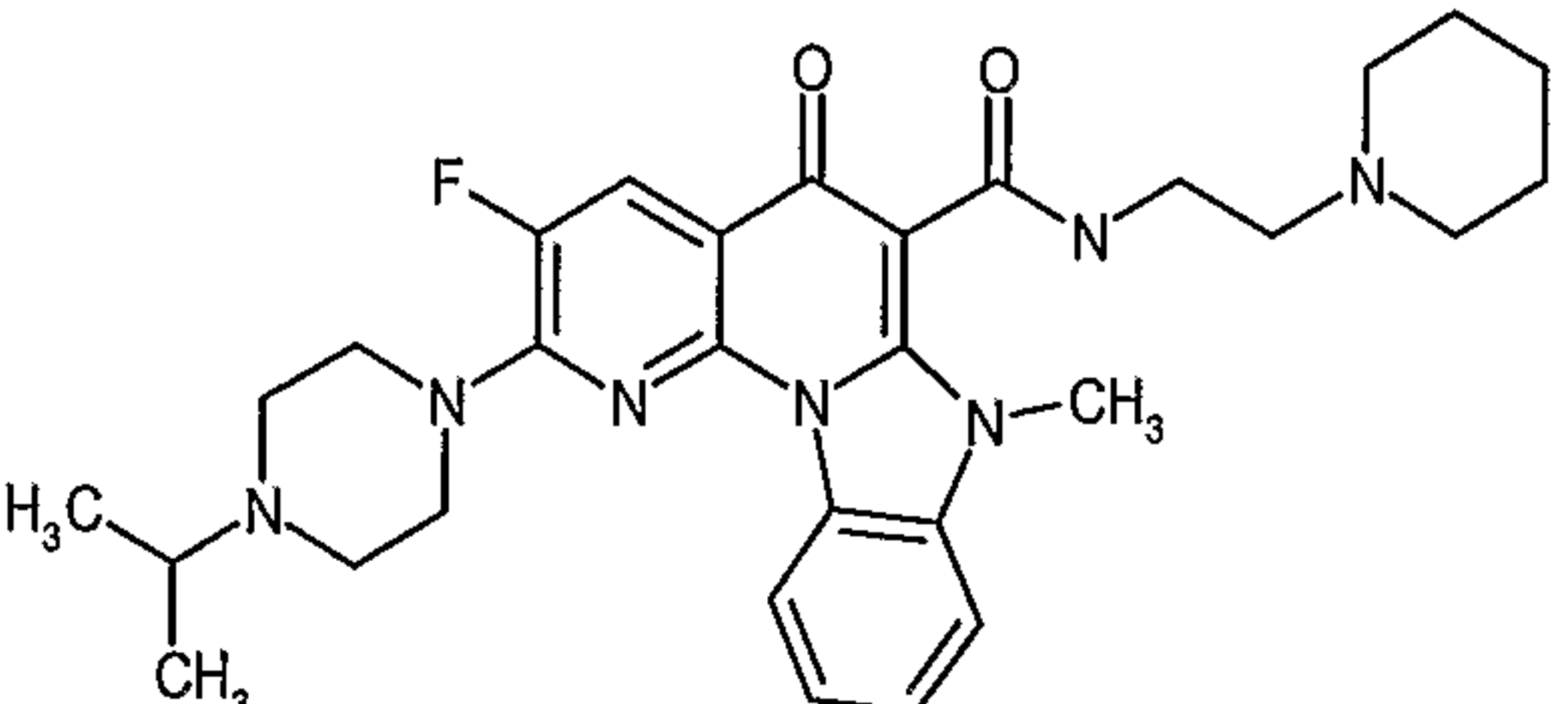
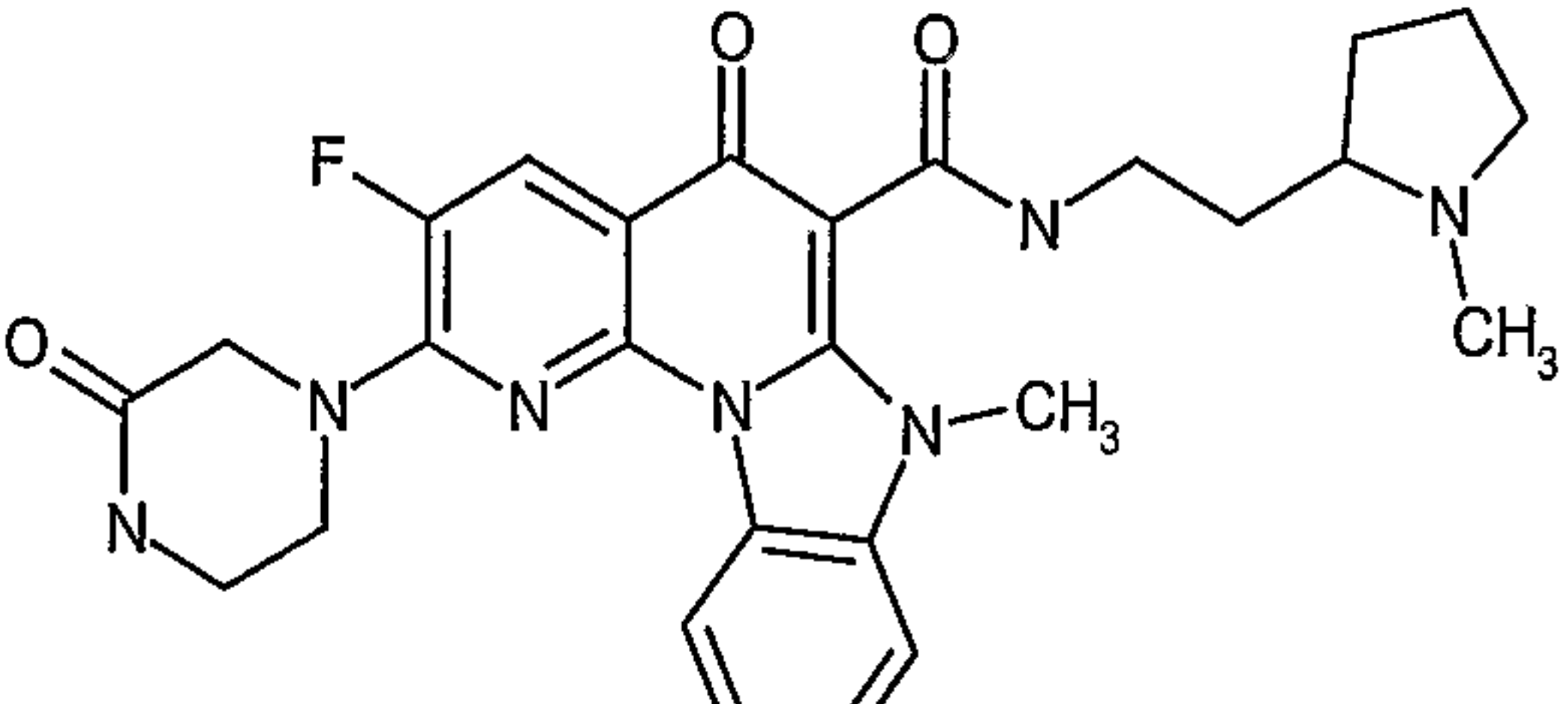
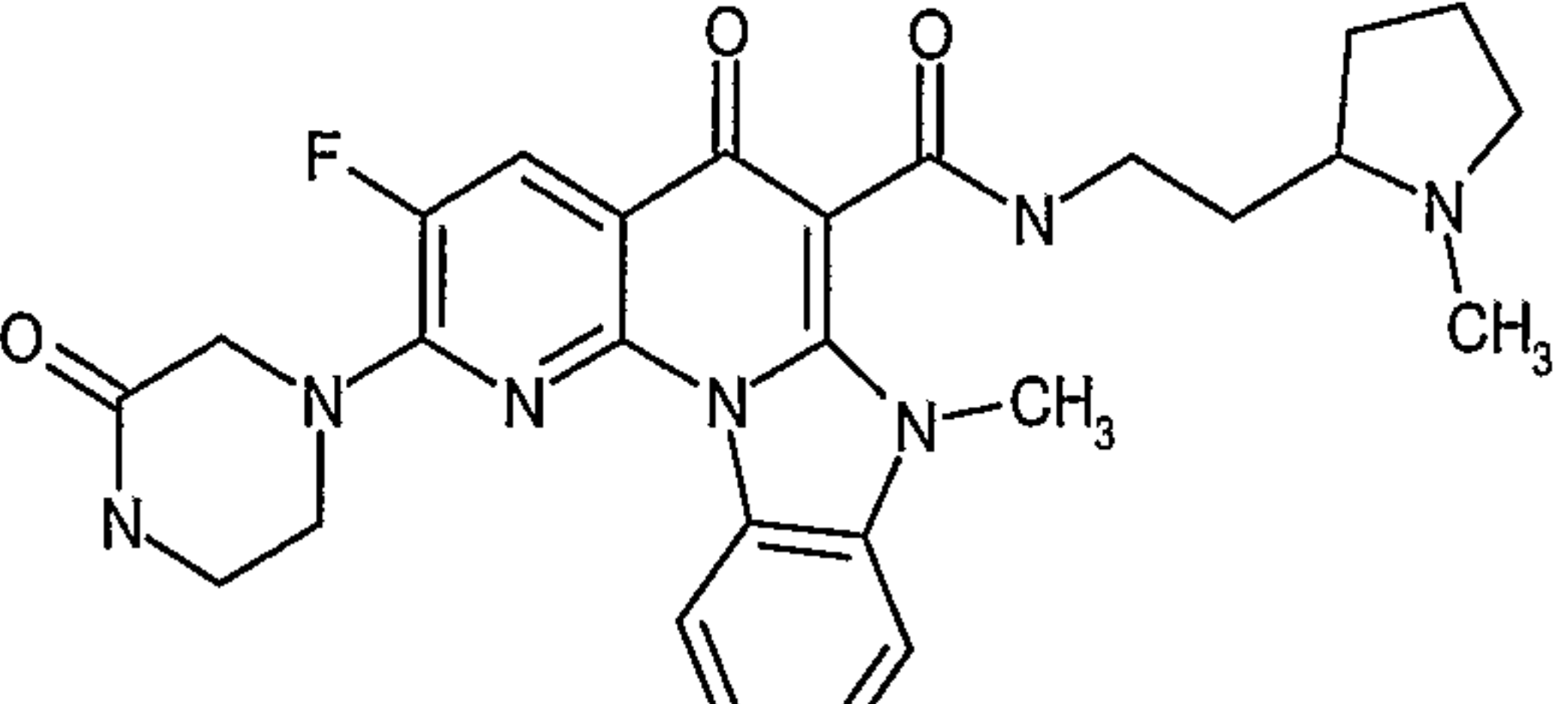
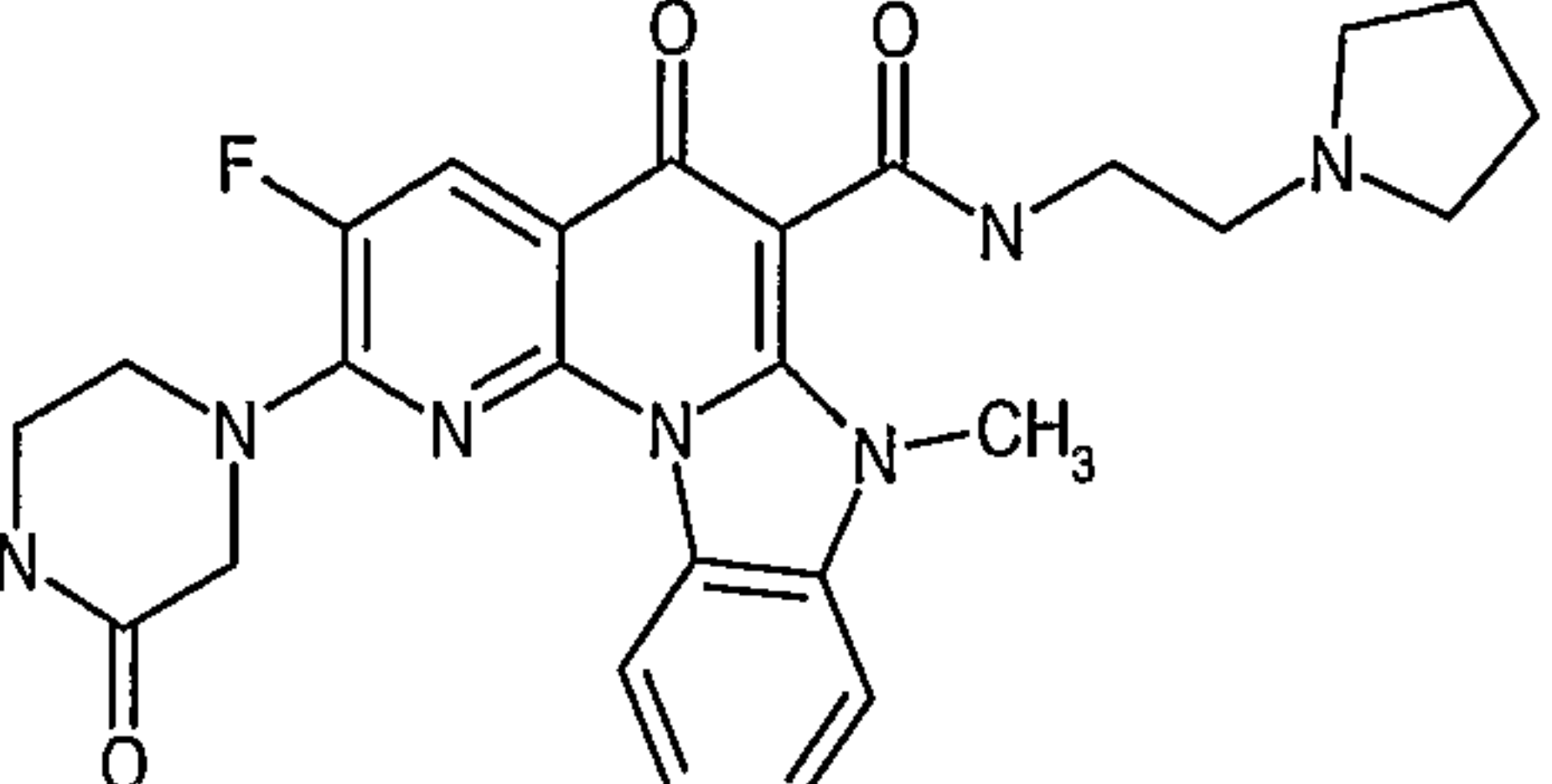
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | MiaPaca | 3.2    |
|    | HCT-116 | 0.3    |
|   | MiaPaca | 0.2    |
|  | HCT-116 | 0.04   |
|  | MiaPaca | 0.02   |
|  | HCT-116 | 0.04   |

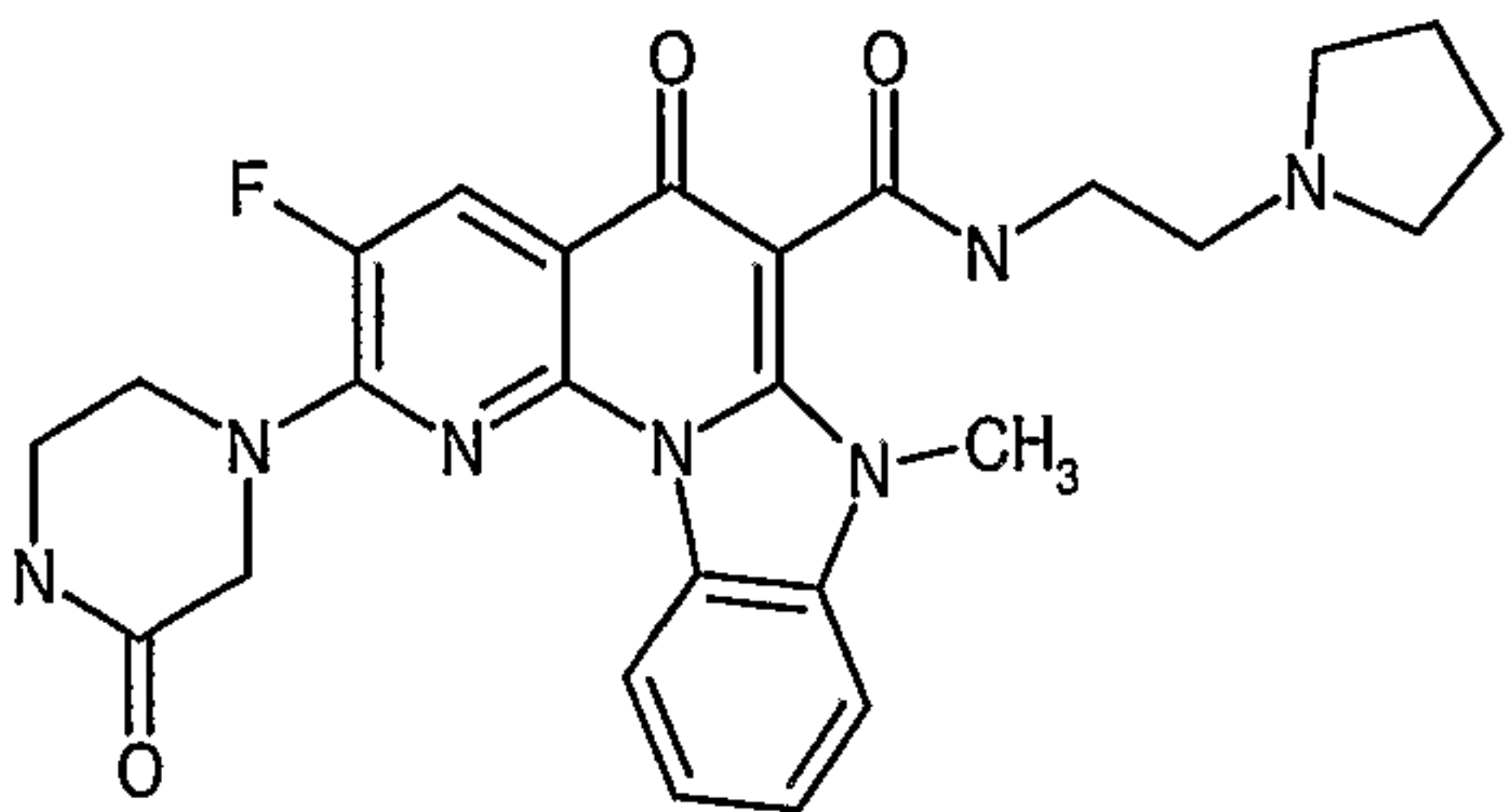
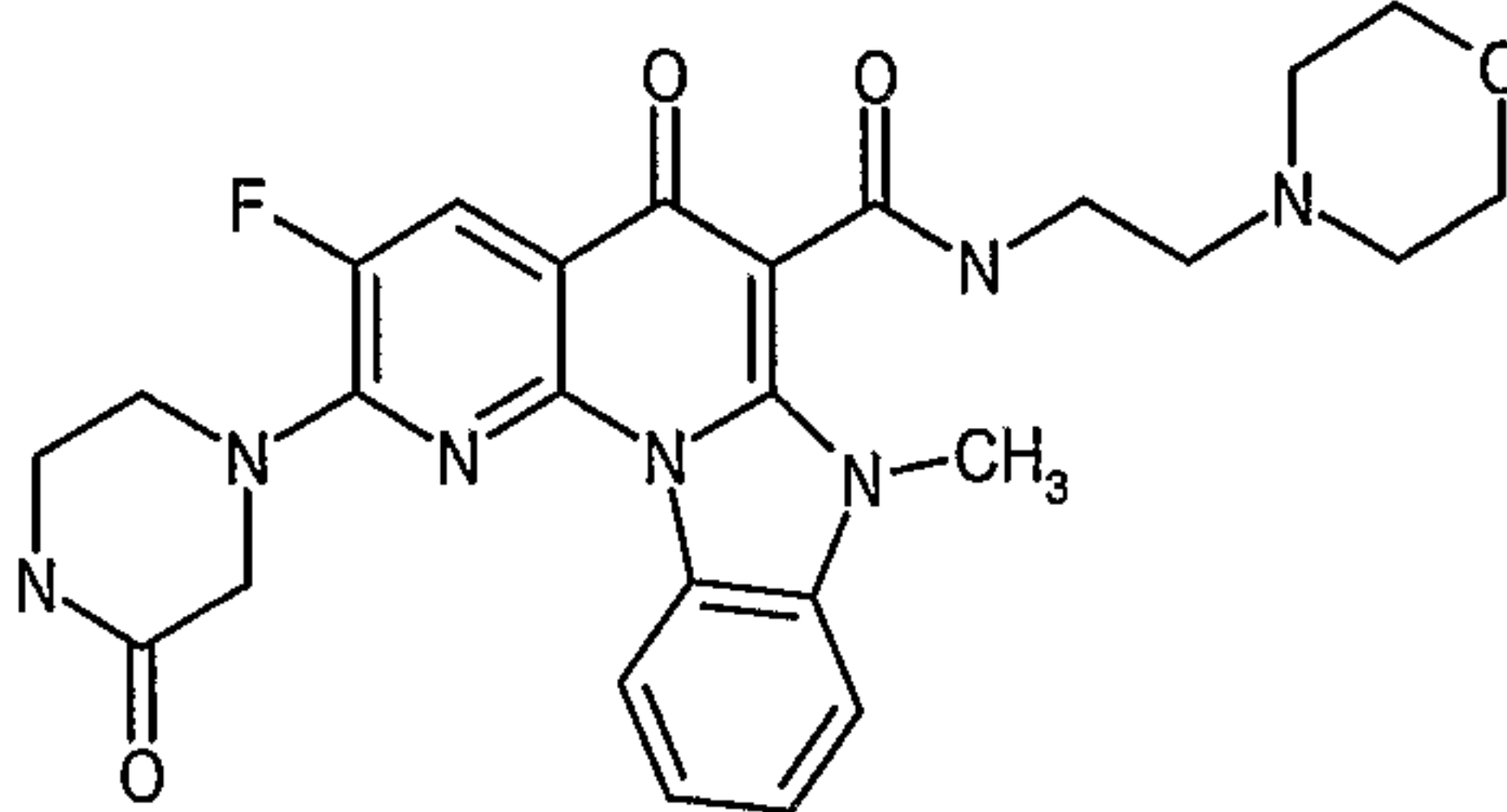
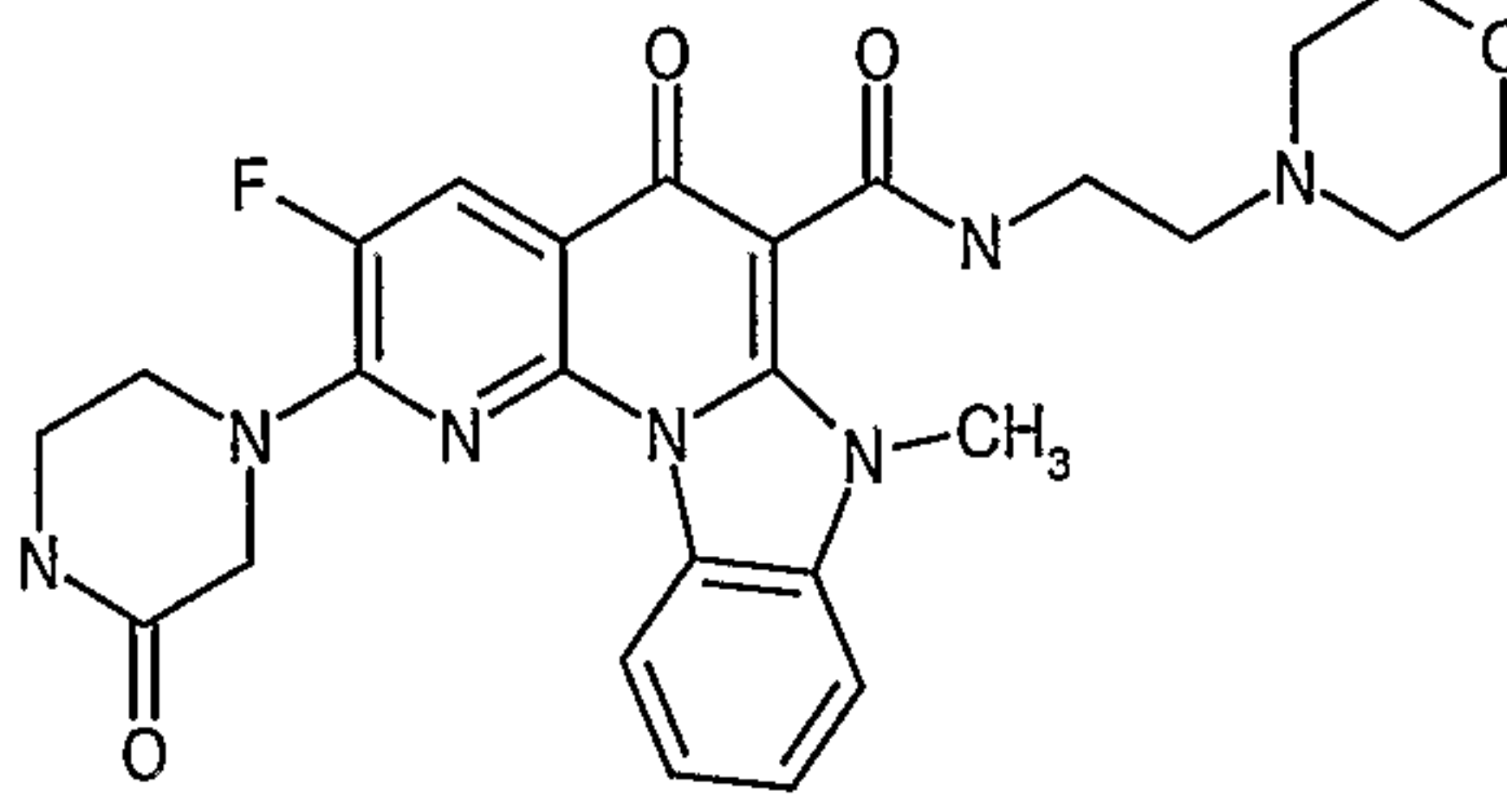
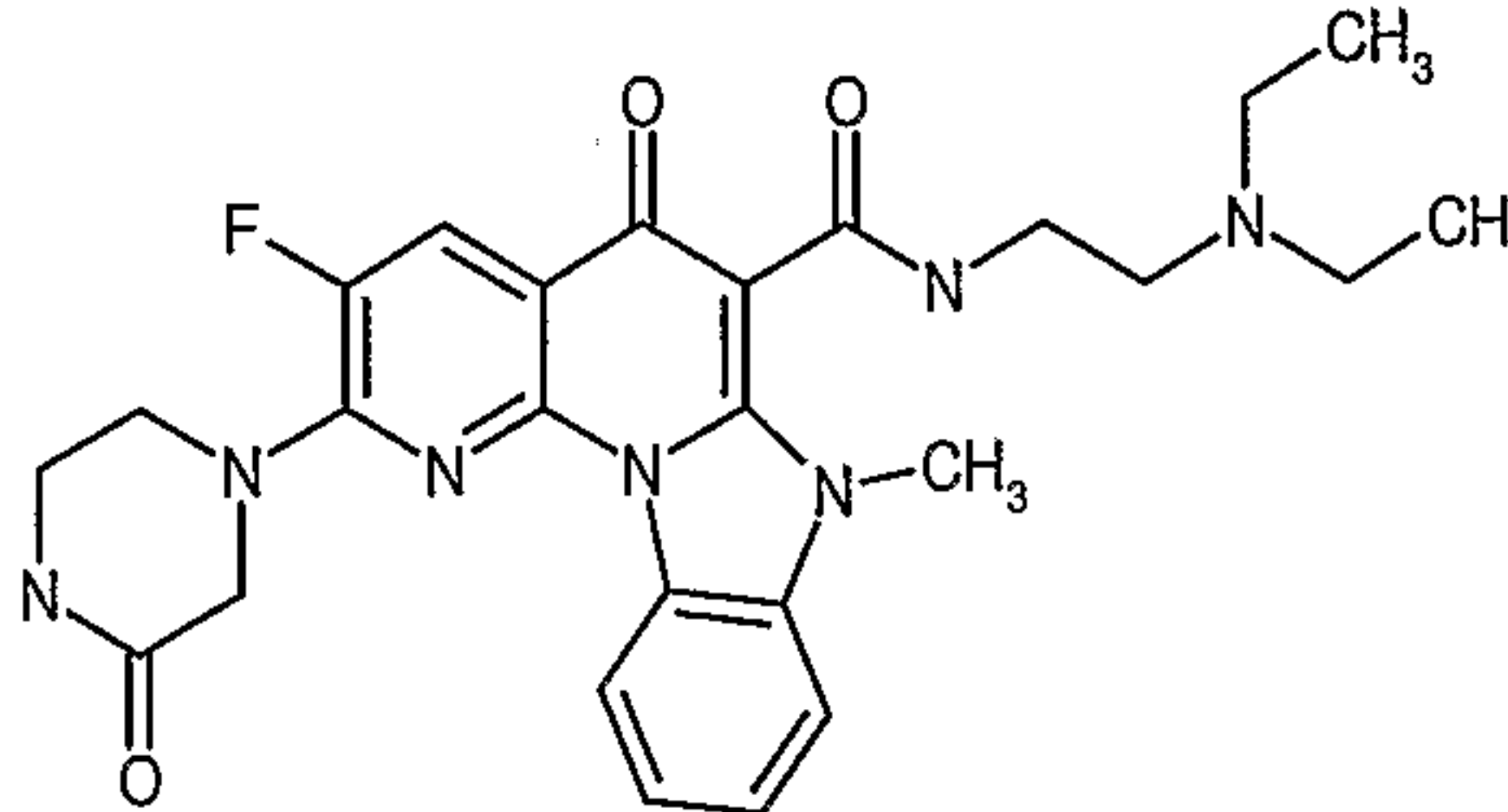
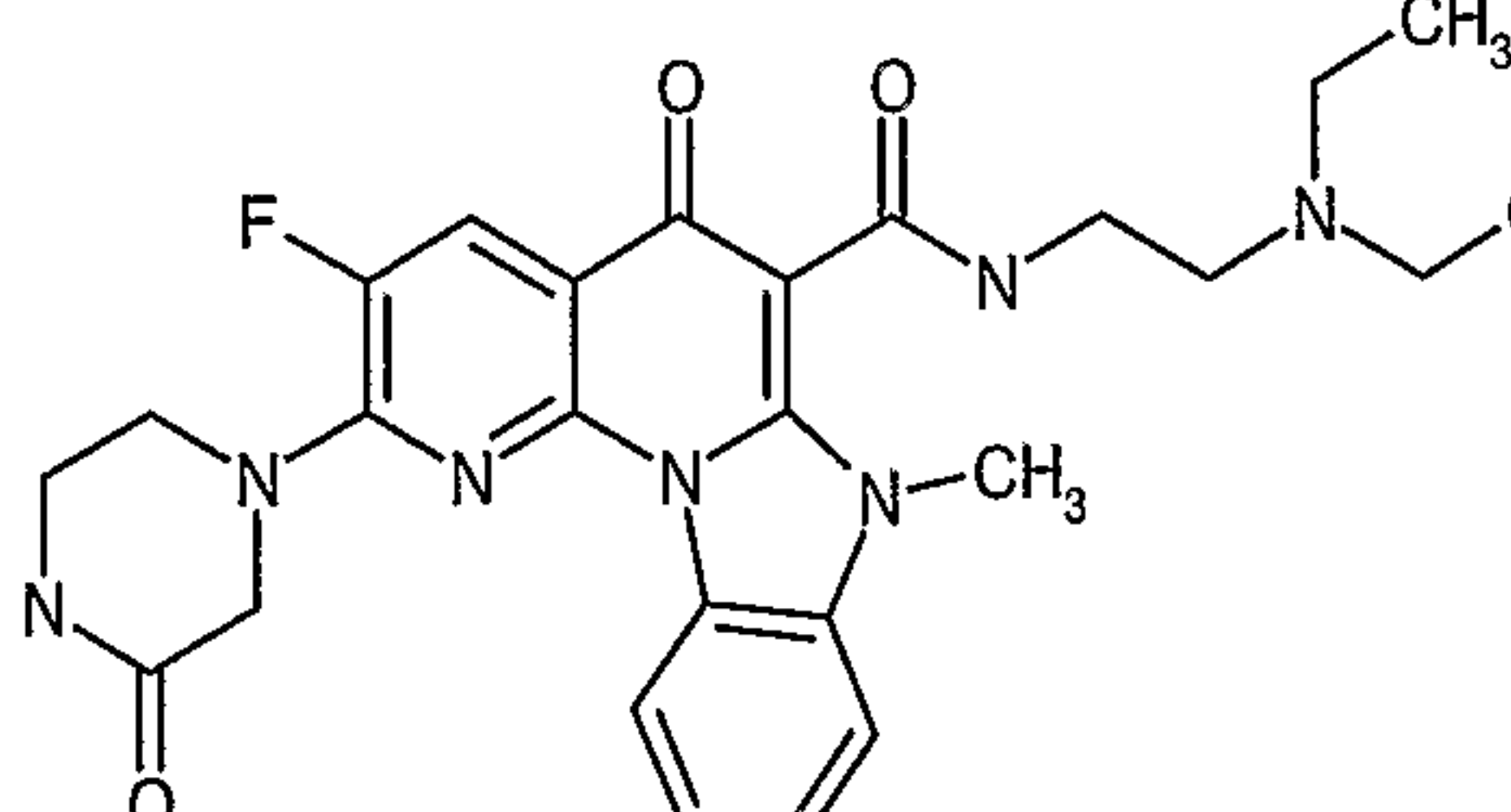
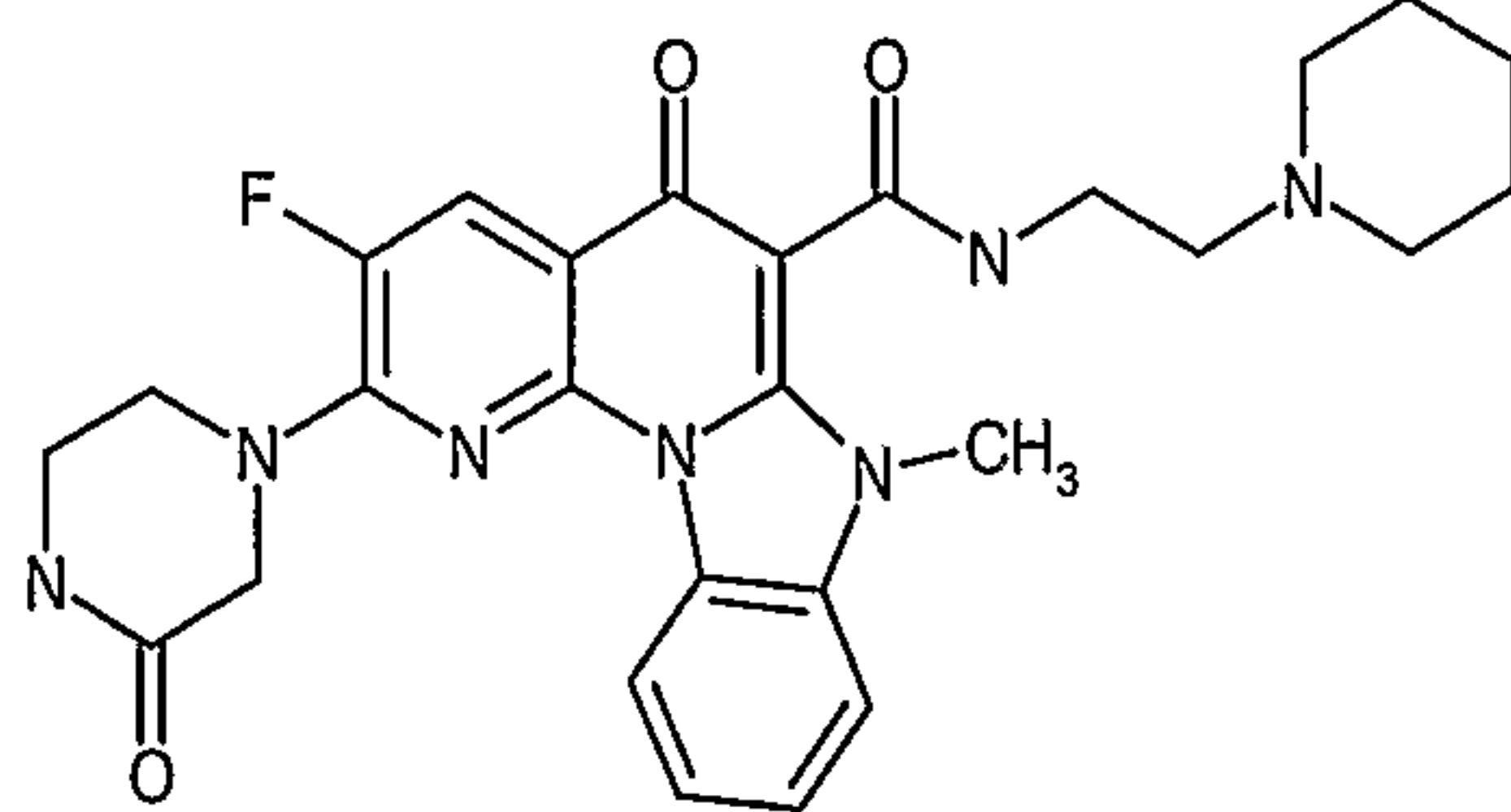
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | 0.02   |
|   | HCT-116 | 0.04   |
|  | MiaPaca | 0.03   |
|  | MiaPaca | <0.01  |
|  | HCT-116 | <0.01  |
|  | HCT-116 | 3.4    |

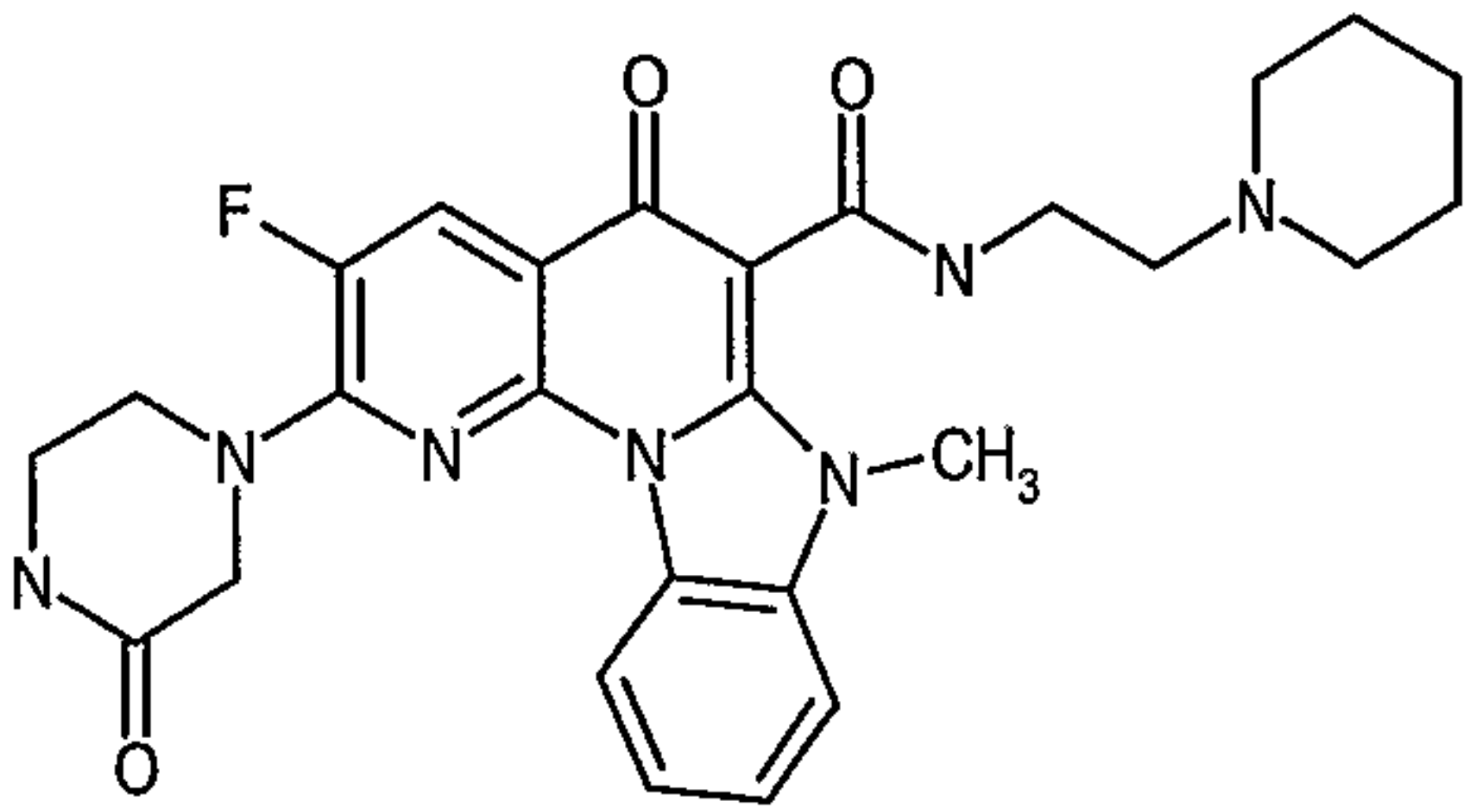
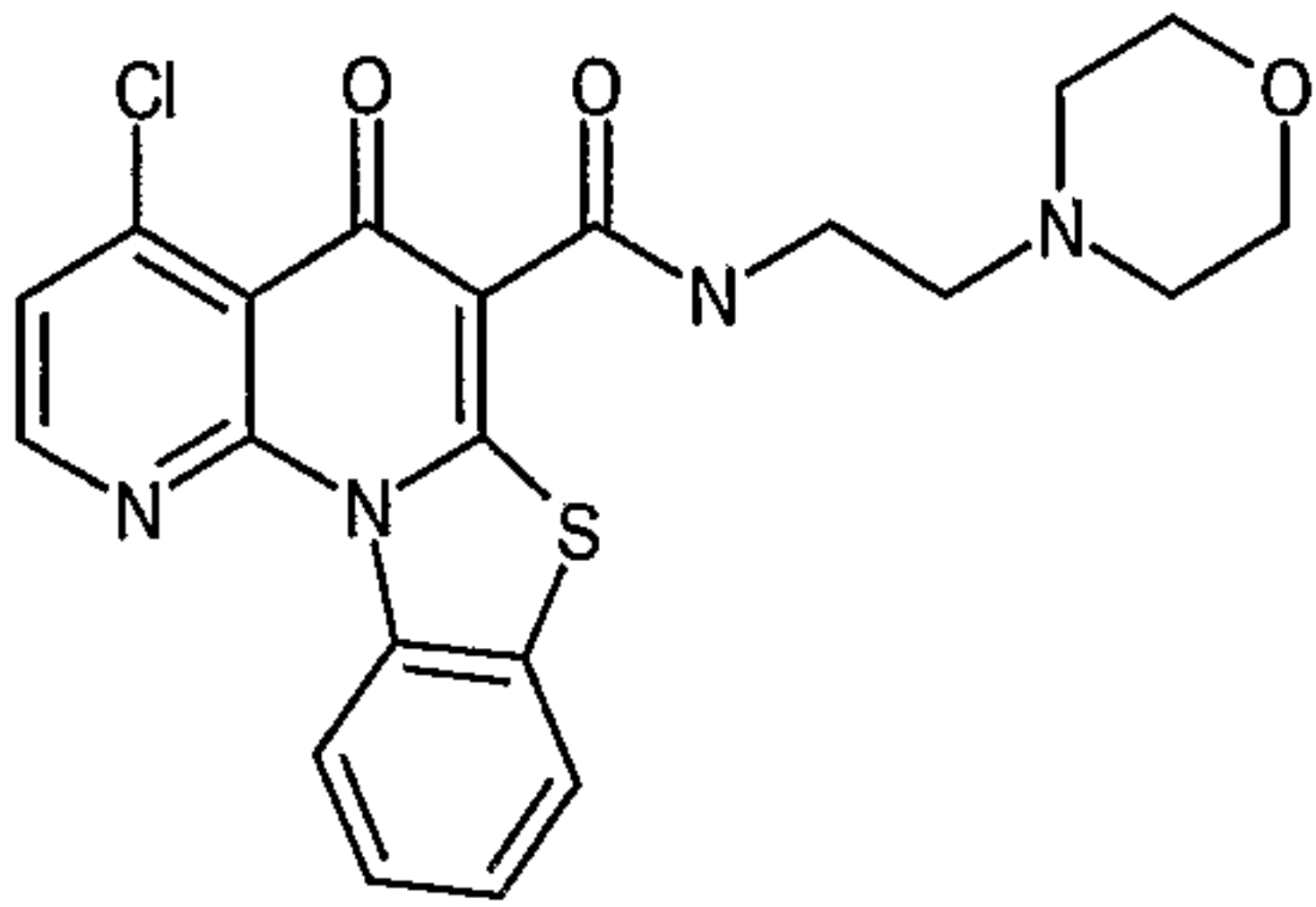
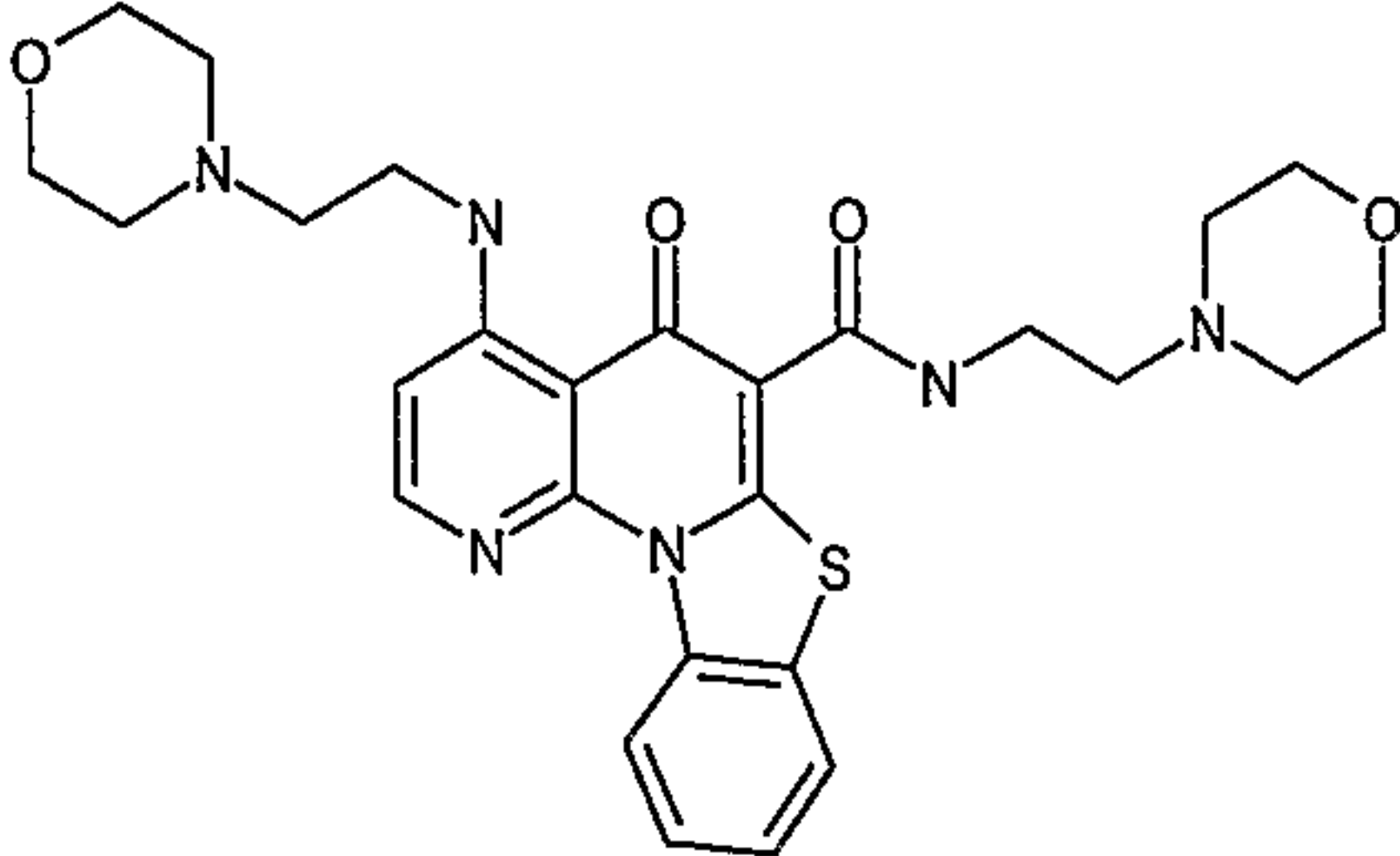
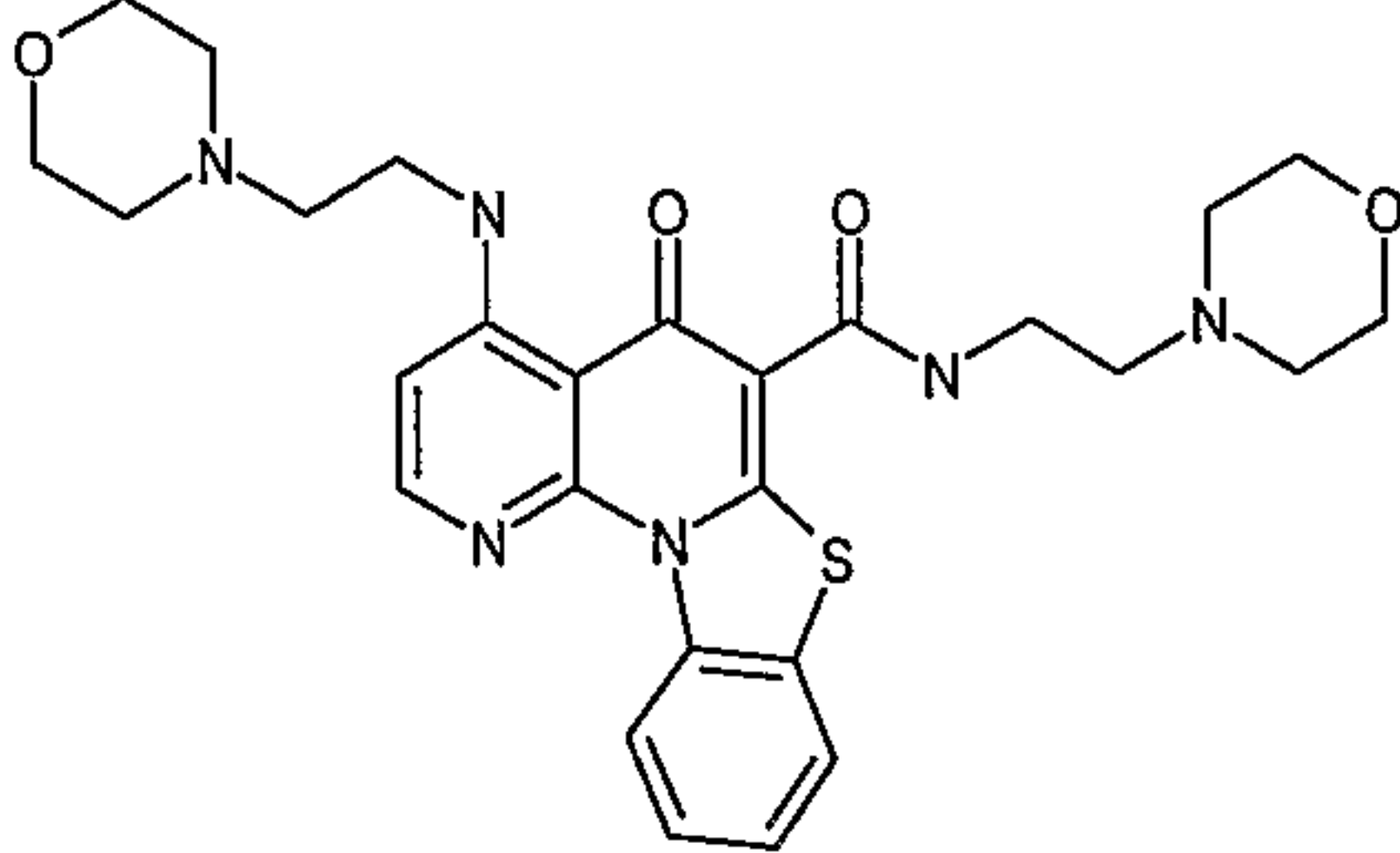
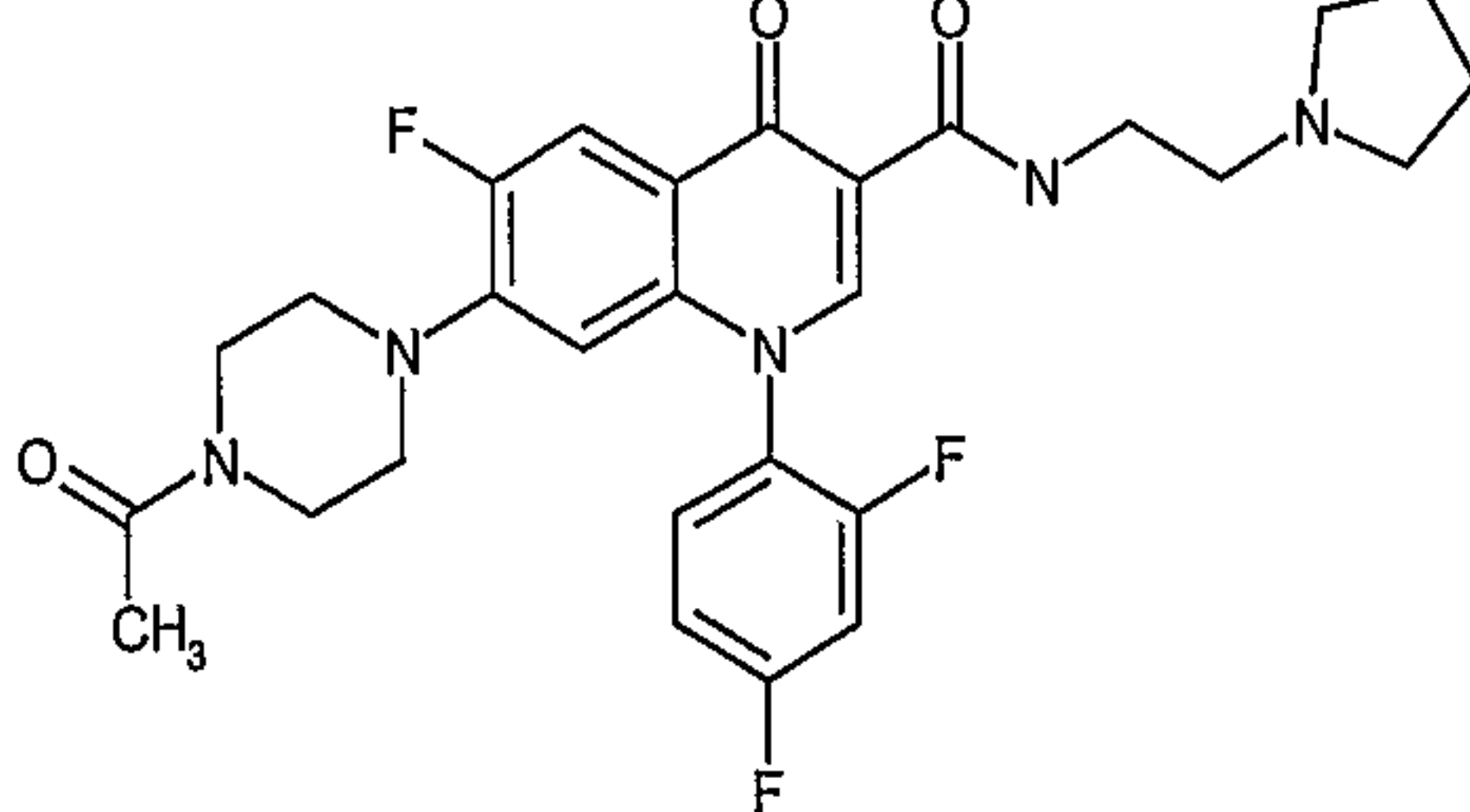
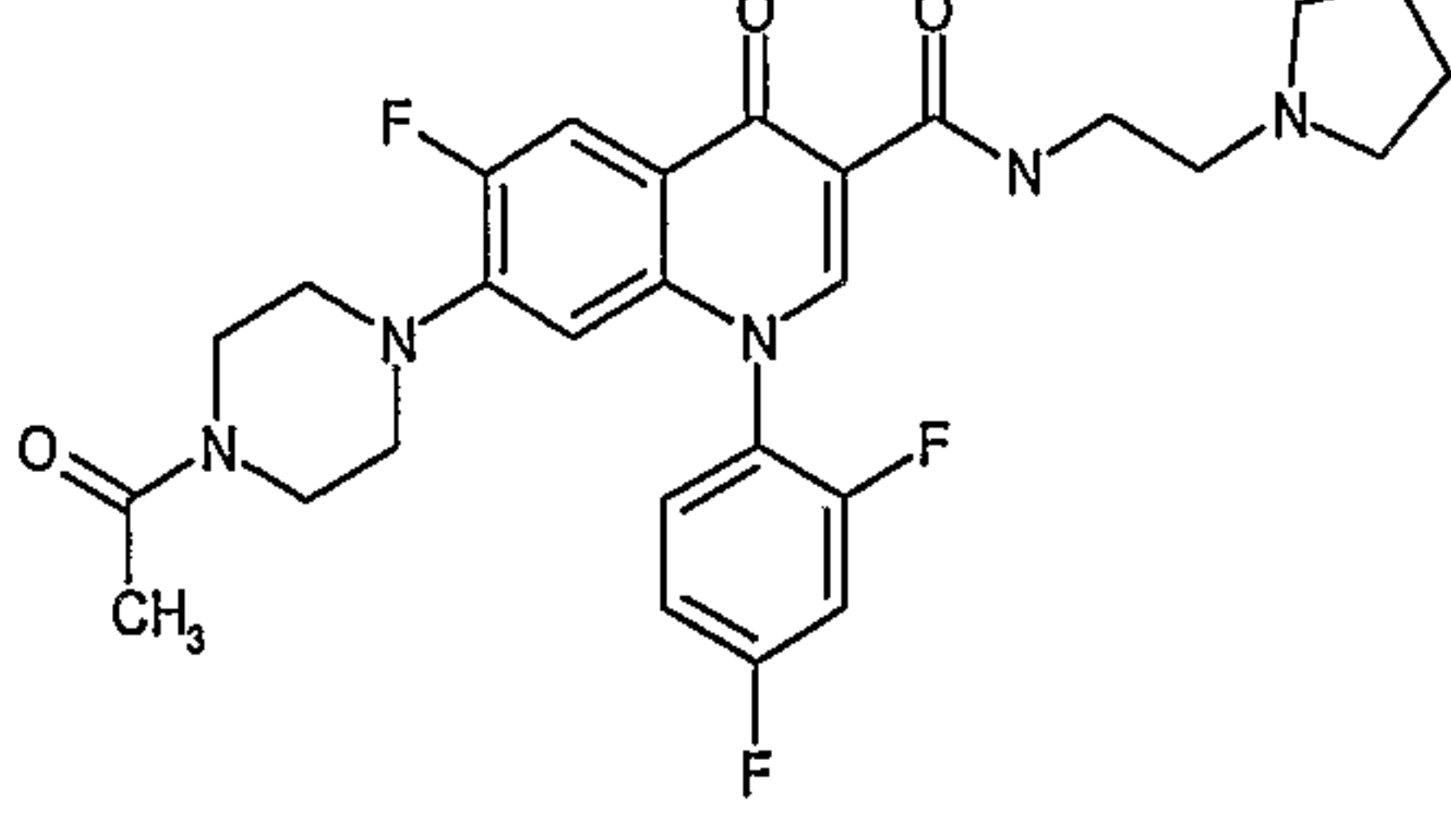
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | MiaPaca | 2.3    |
|   | HCT-116 | 0.7    |
|  | MiaPaca | 0.5    |
|  | HCT-116 | 0.2    |
|  | MiaPaca | 0.2    |
|   | HCT-116 | 0.2    |

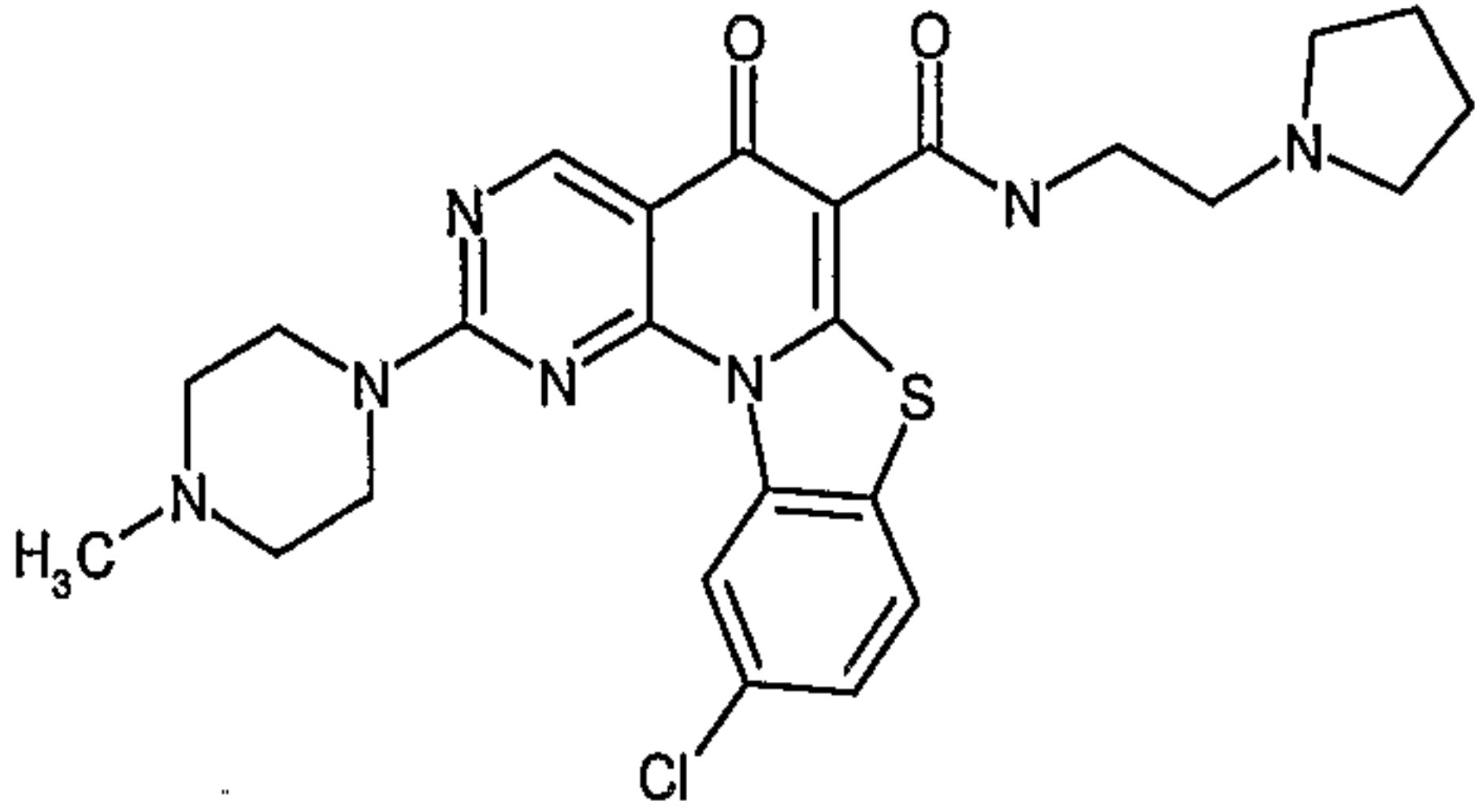
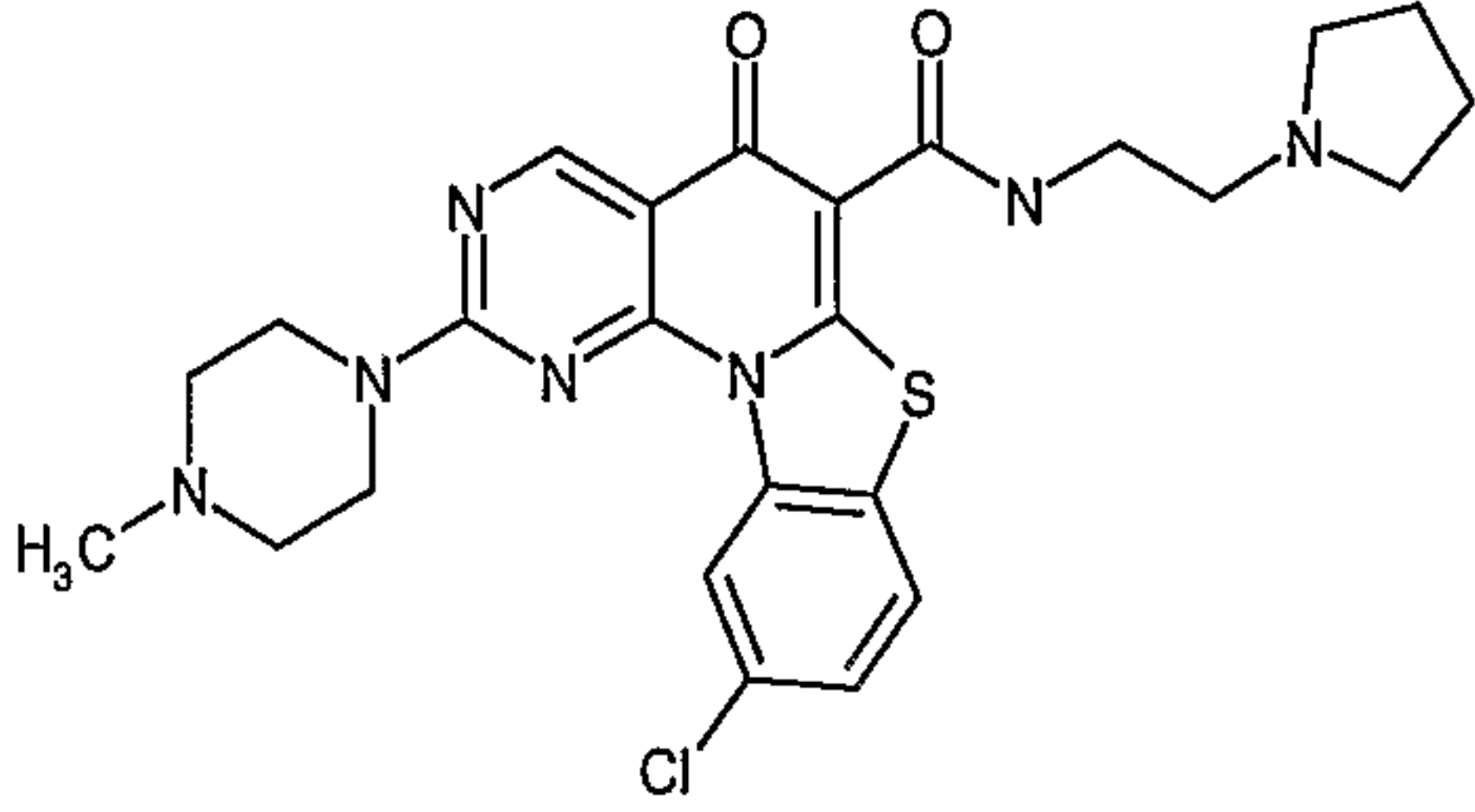
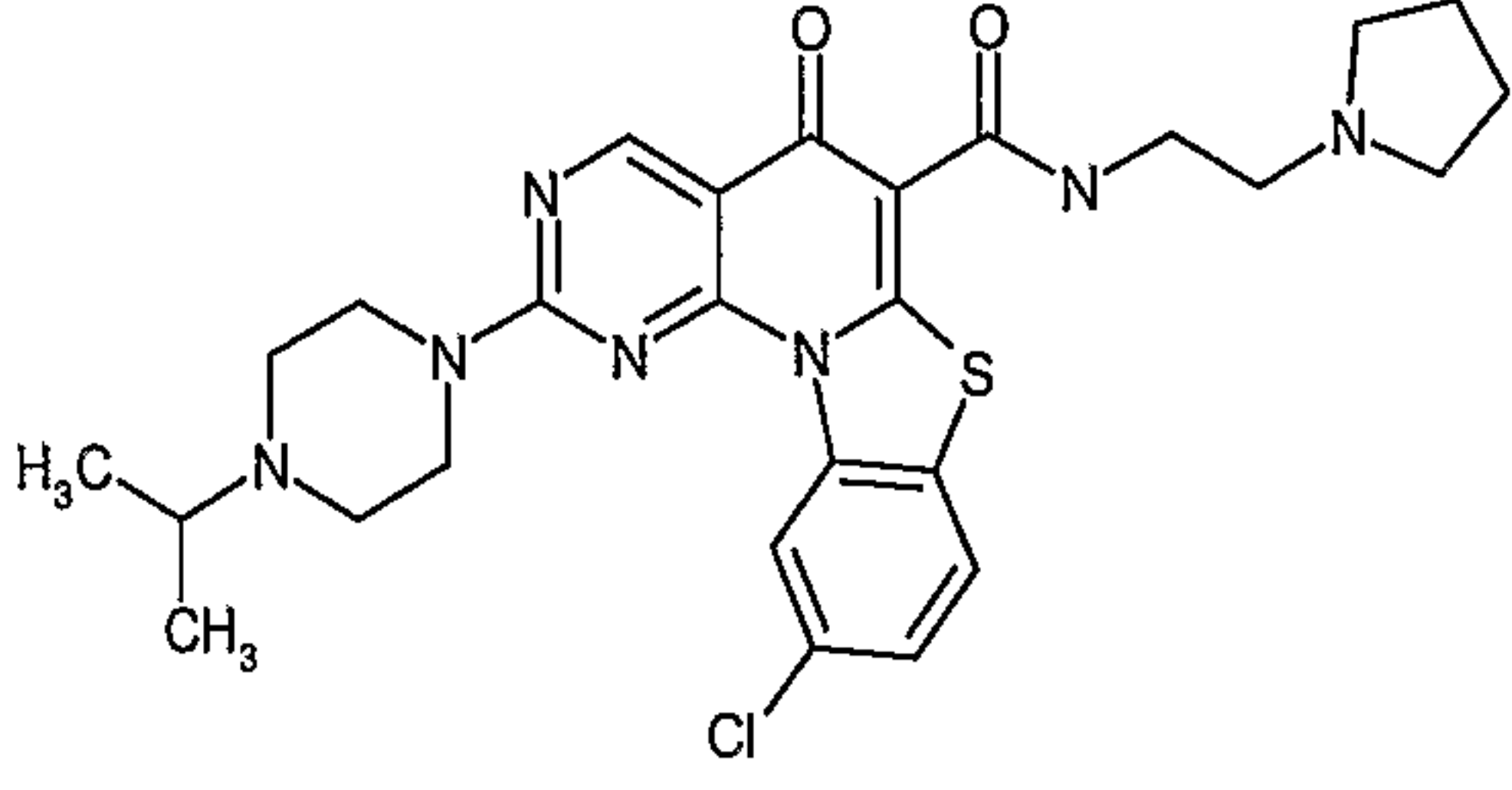
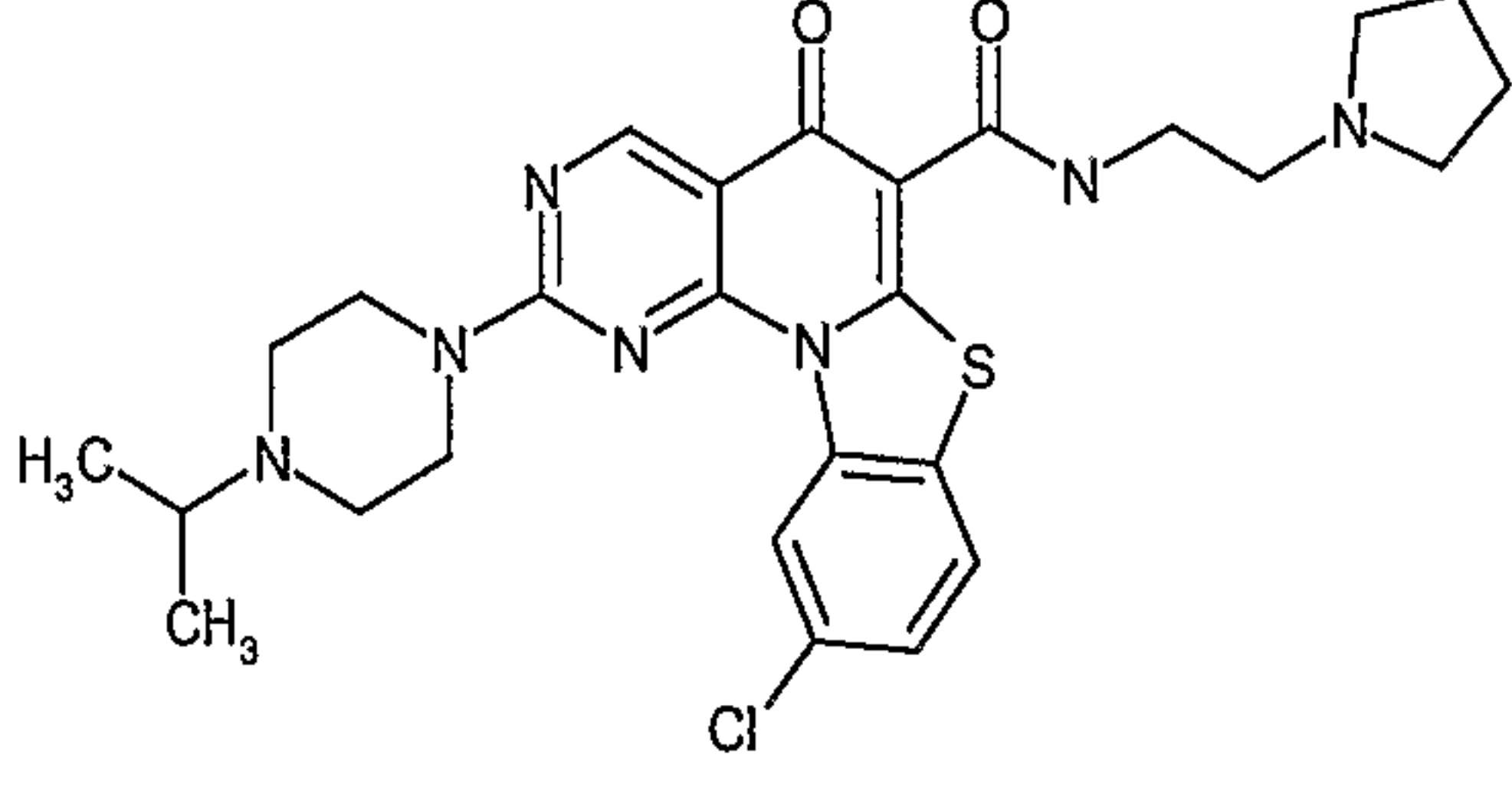
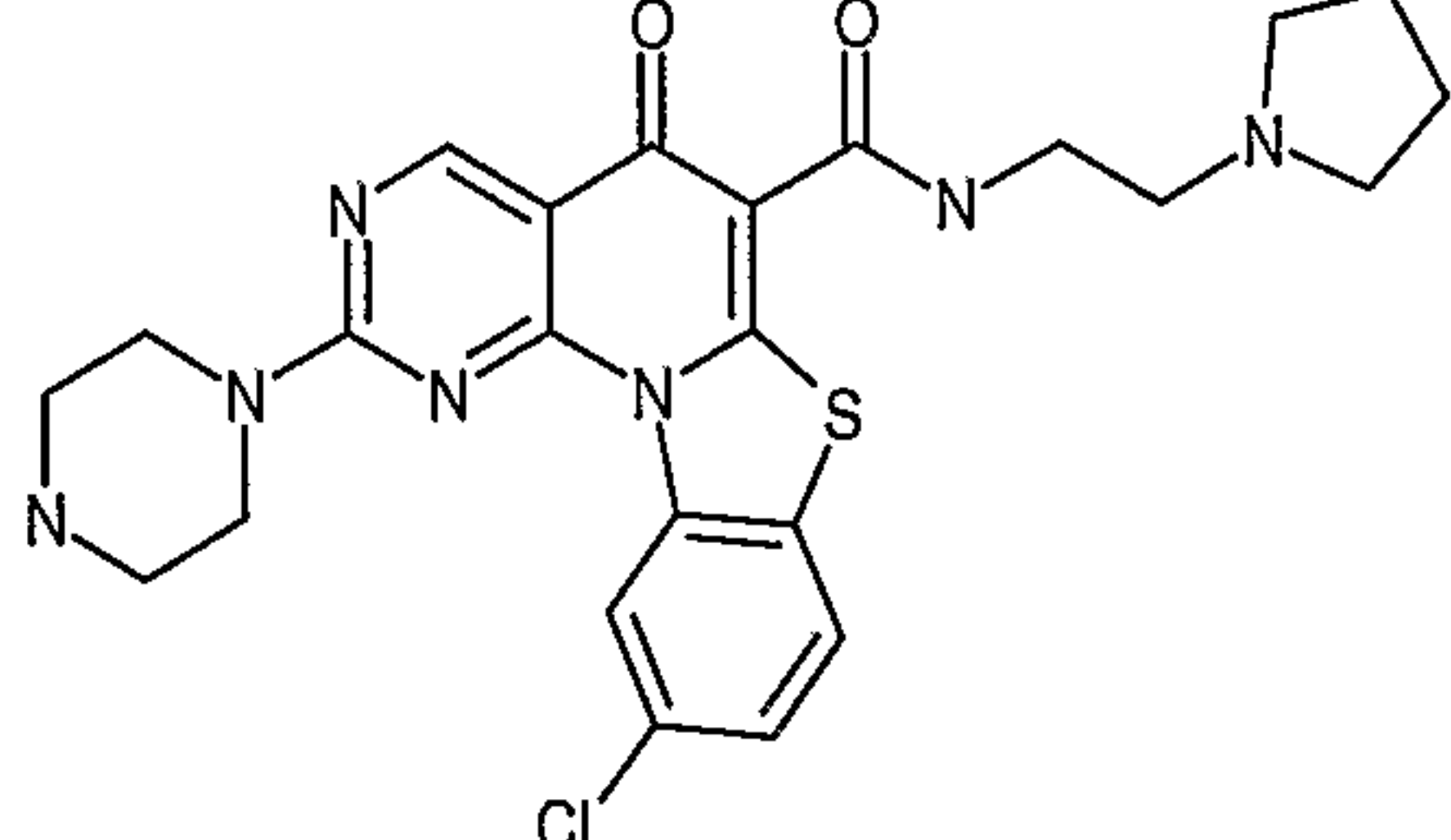
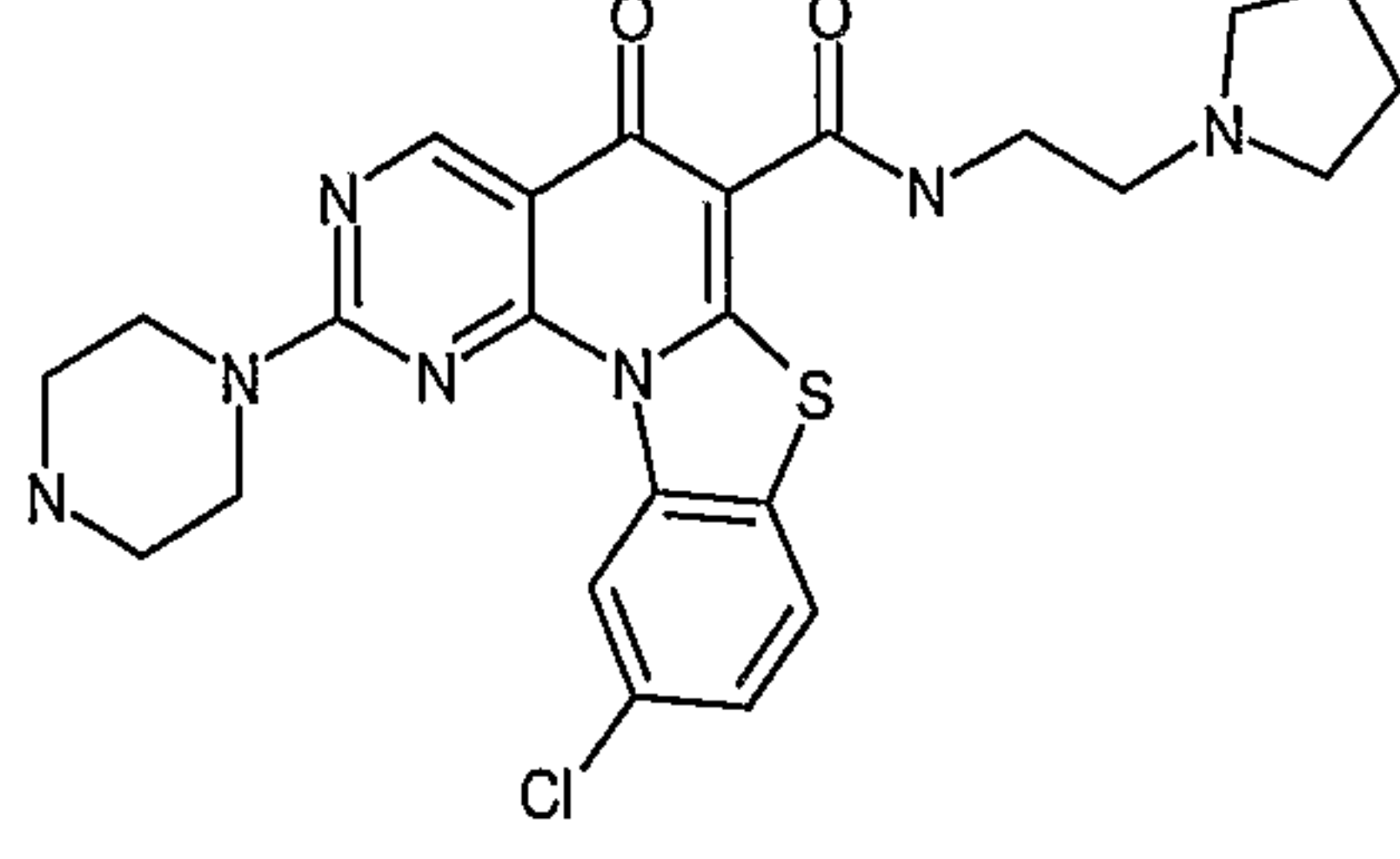
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | 0.03   |
|   | HCT-116 | 0.3    |
|  | MiaPaca | 0.02   |
|  | HCT-116 | 0.2    |
|  | MiaPaca | 0.2    |
|  | HCT-116 | 2.8    |

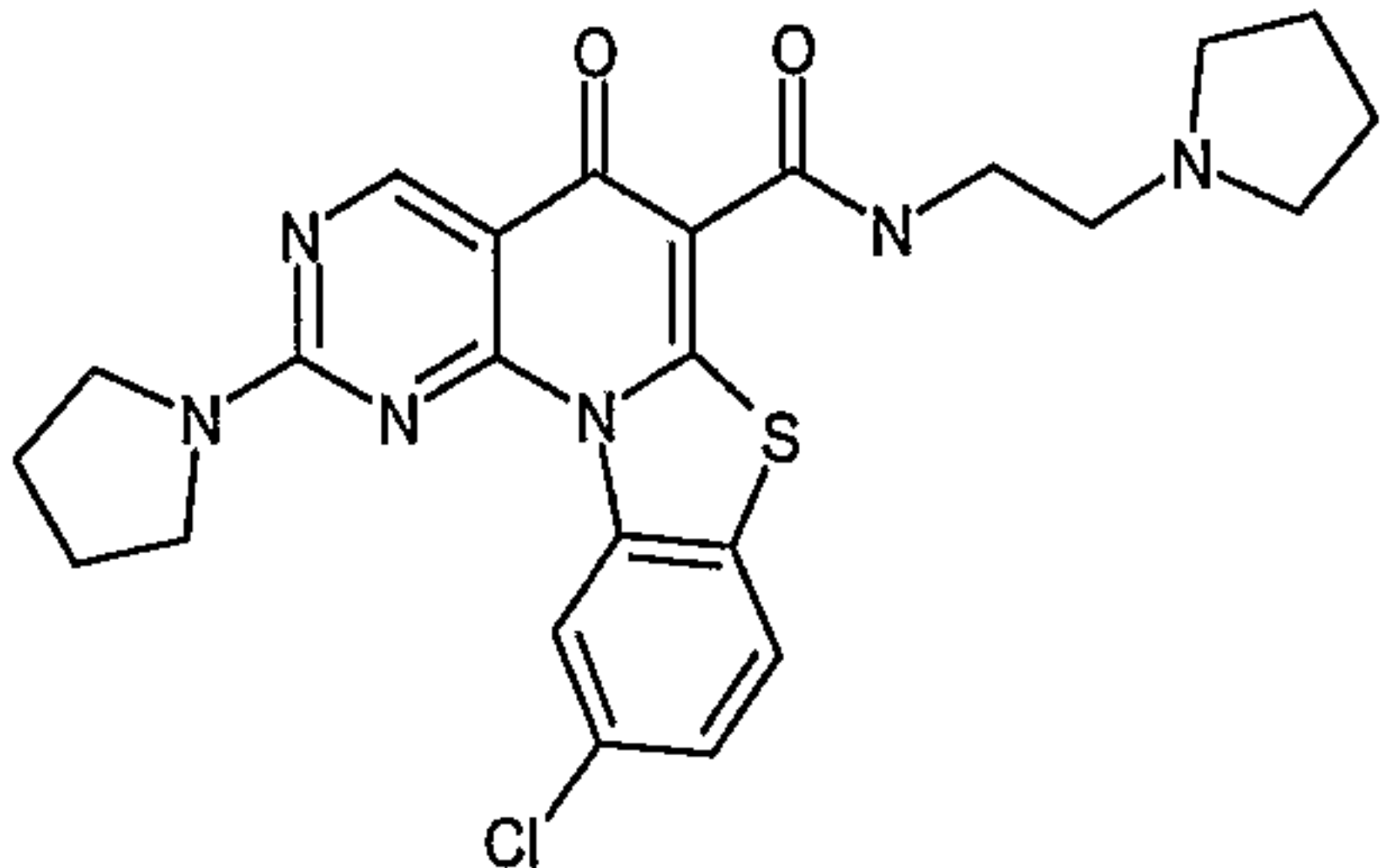
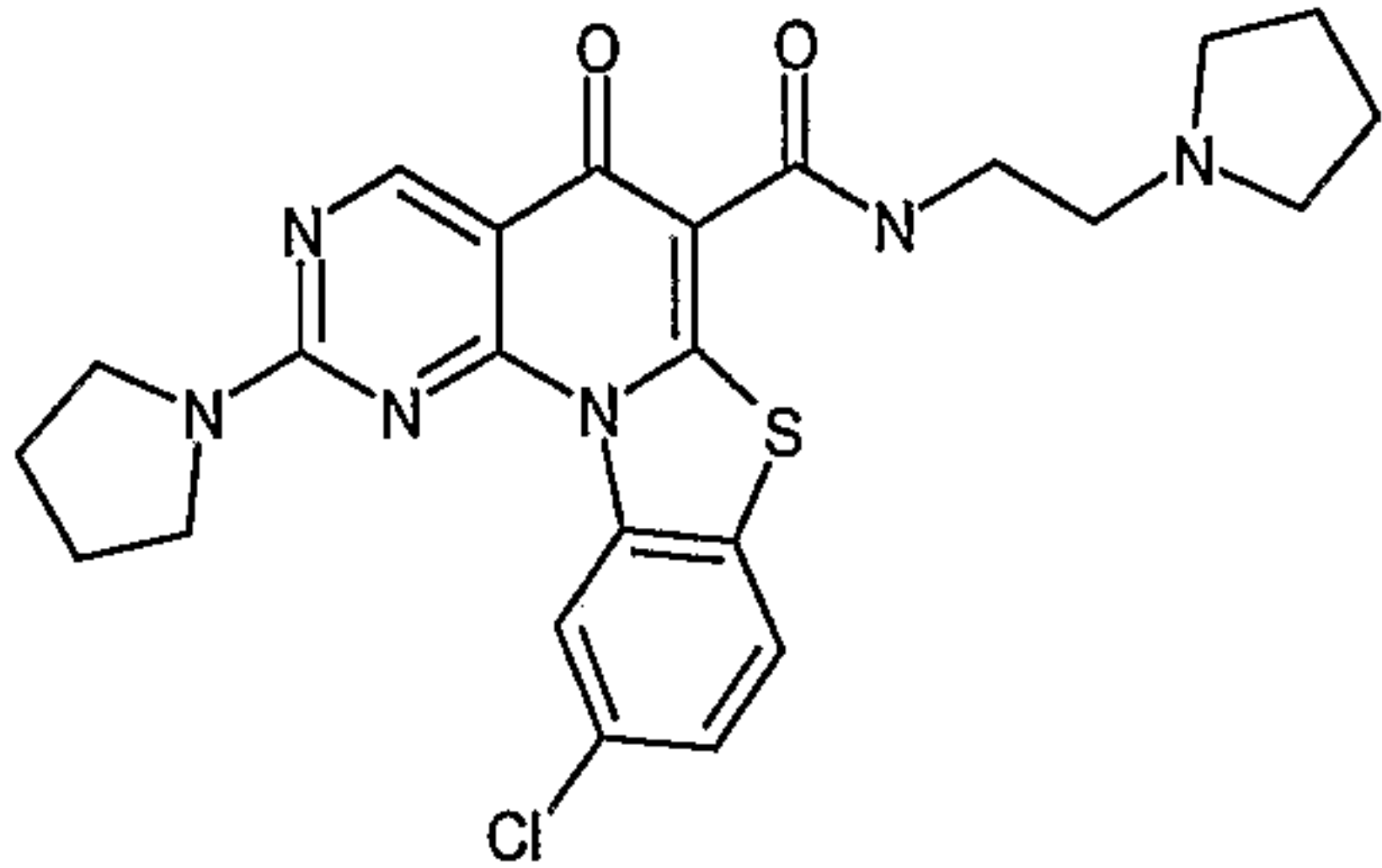
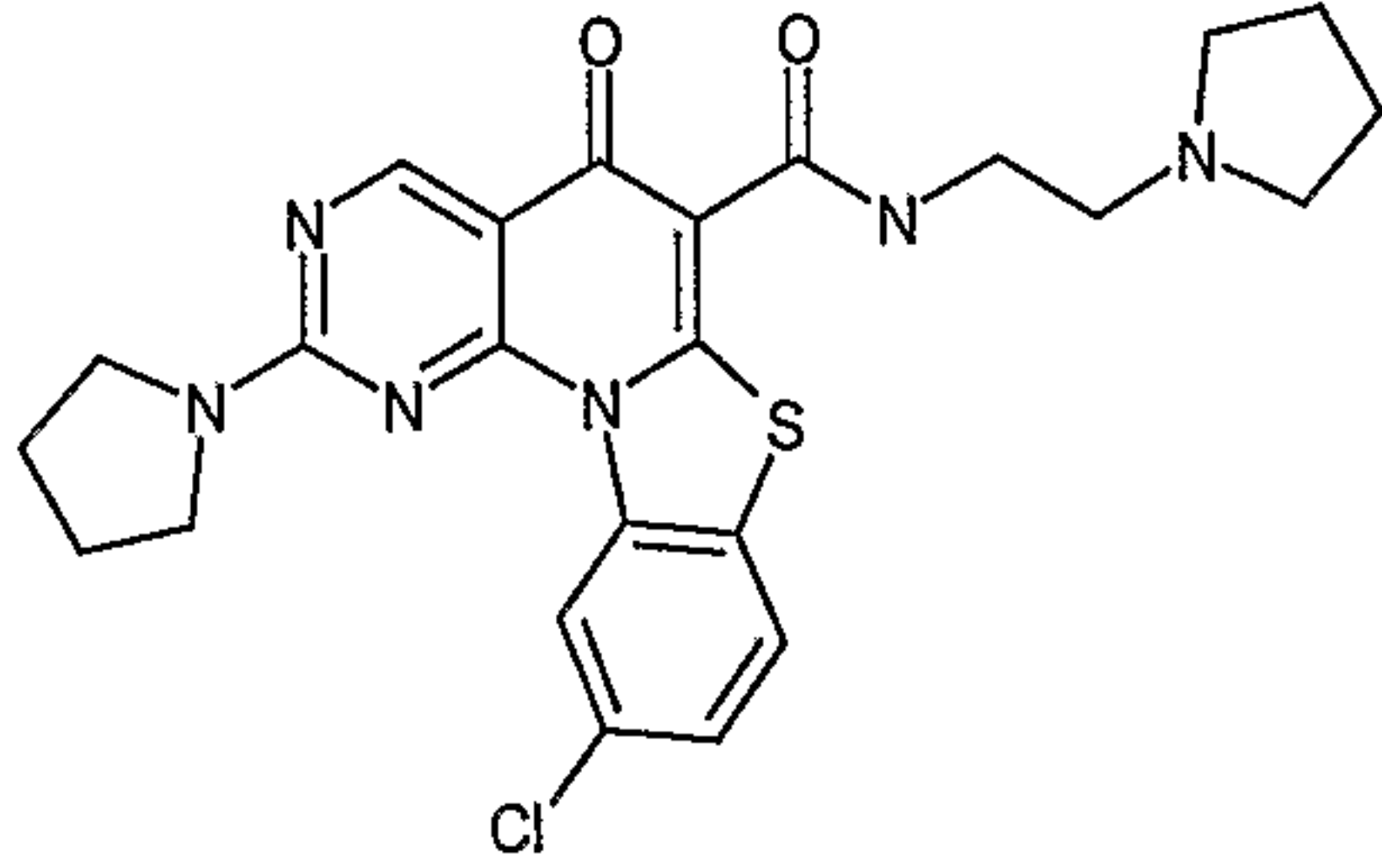
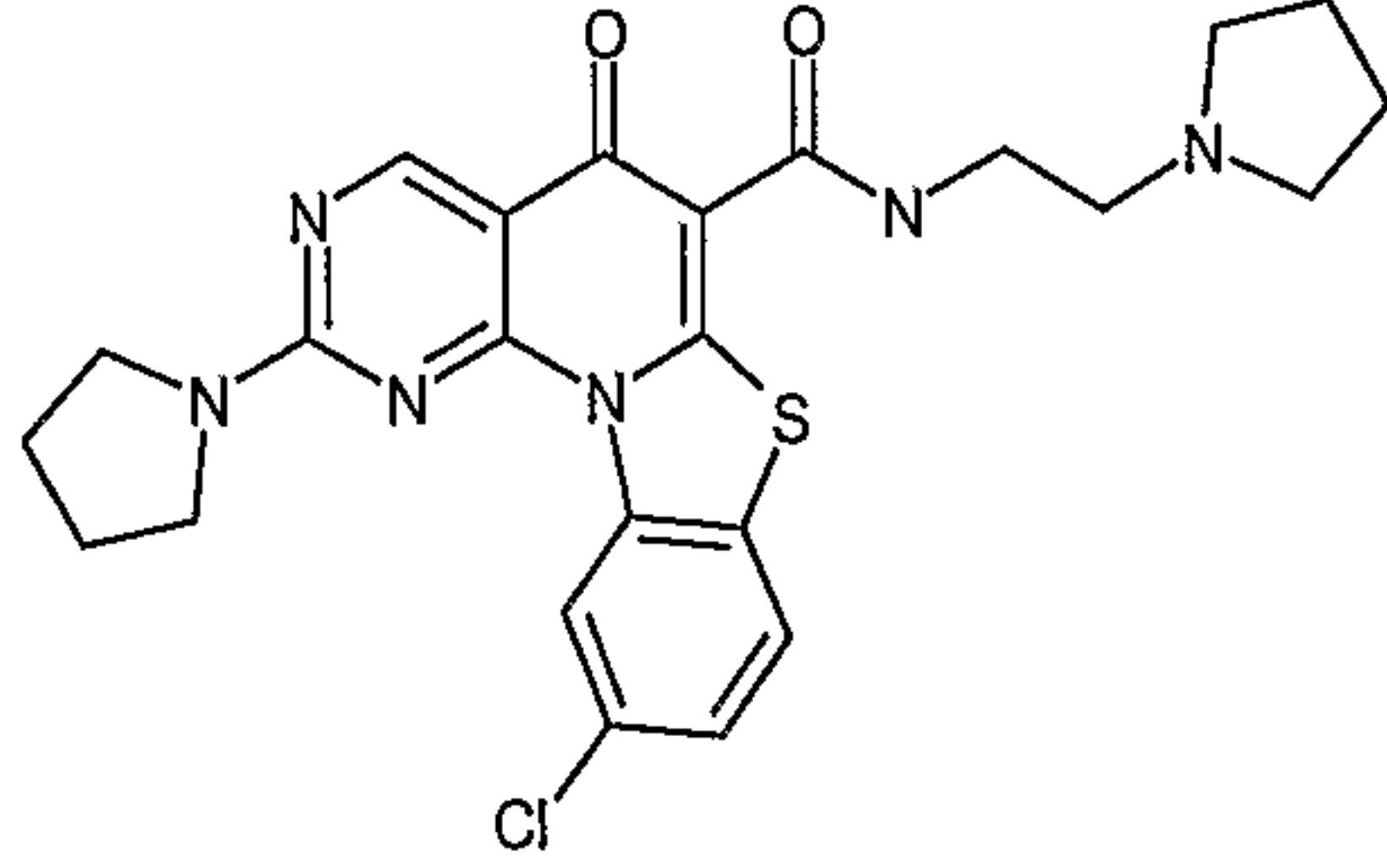
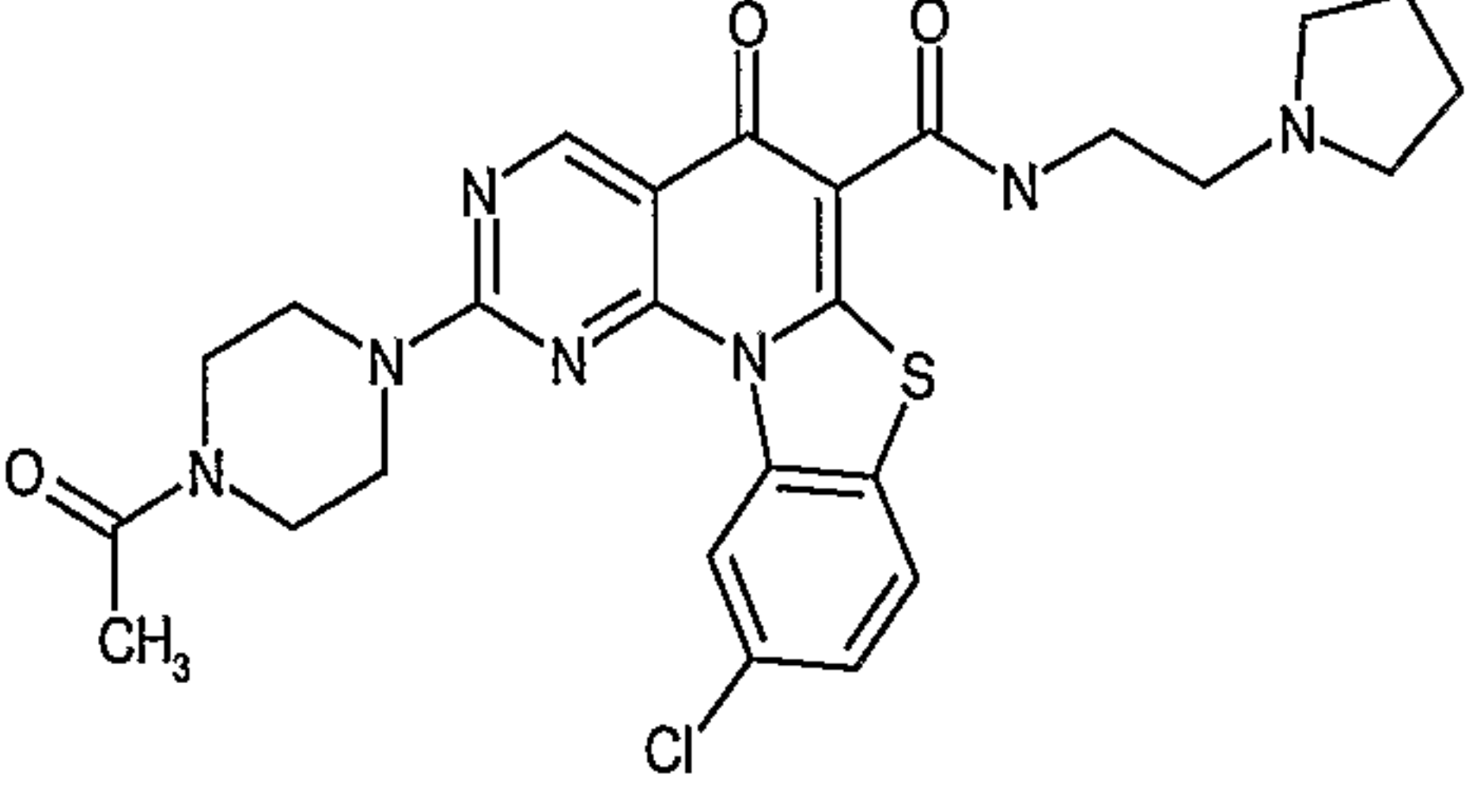
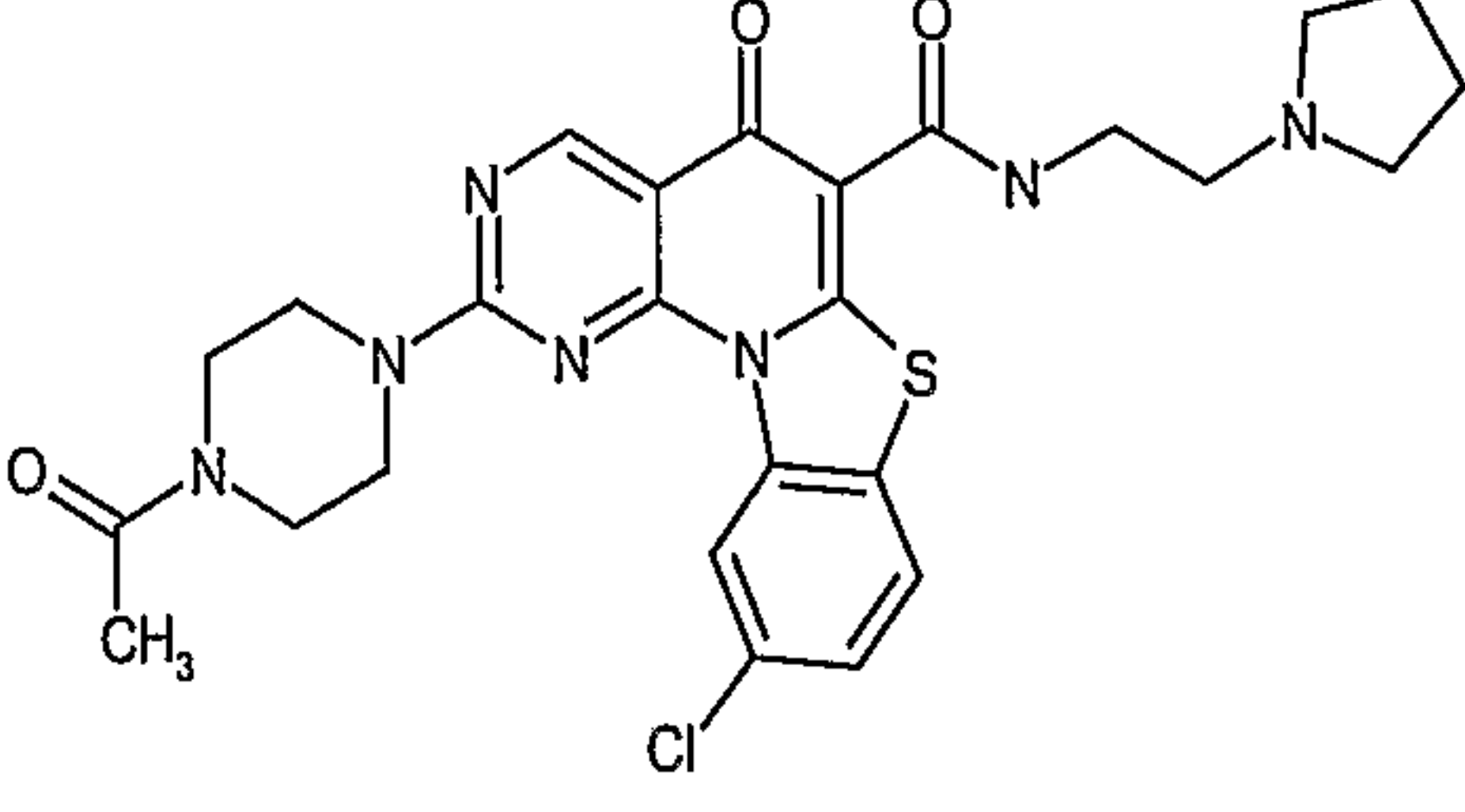
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | MiaPaca | 2.1    |
|   | HCT-116 | 0.04   |
|  | MiaPaca | 0.02   |
|  | HCT-116 | 0.2    |
|  | MiaPaca | 0.05   |
|  | HCT-116 | 0.04   |

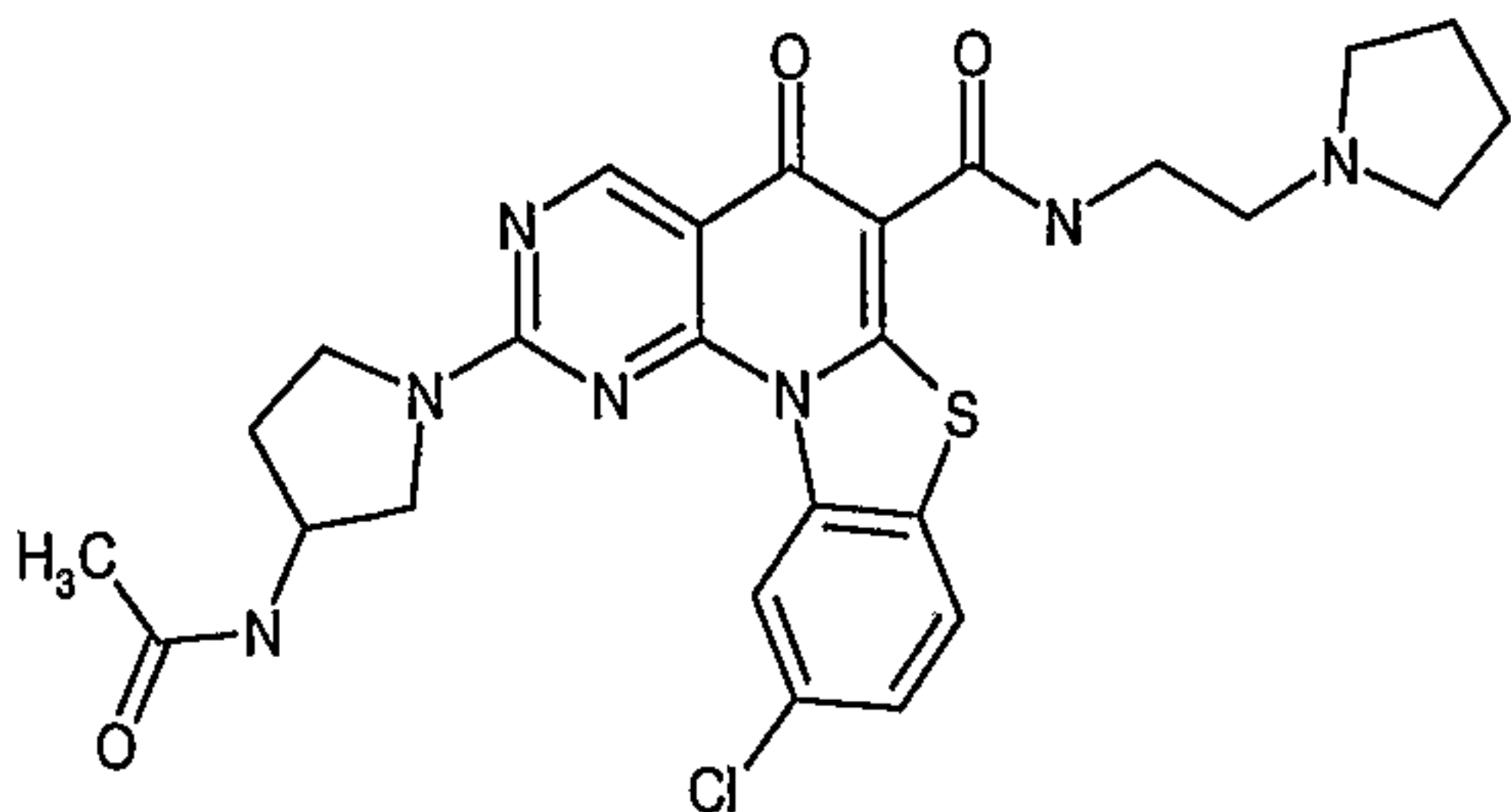
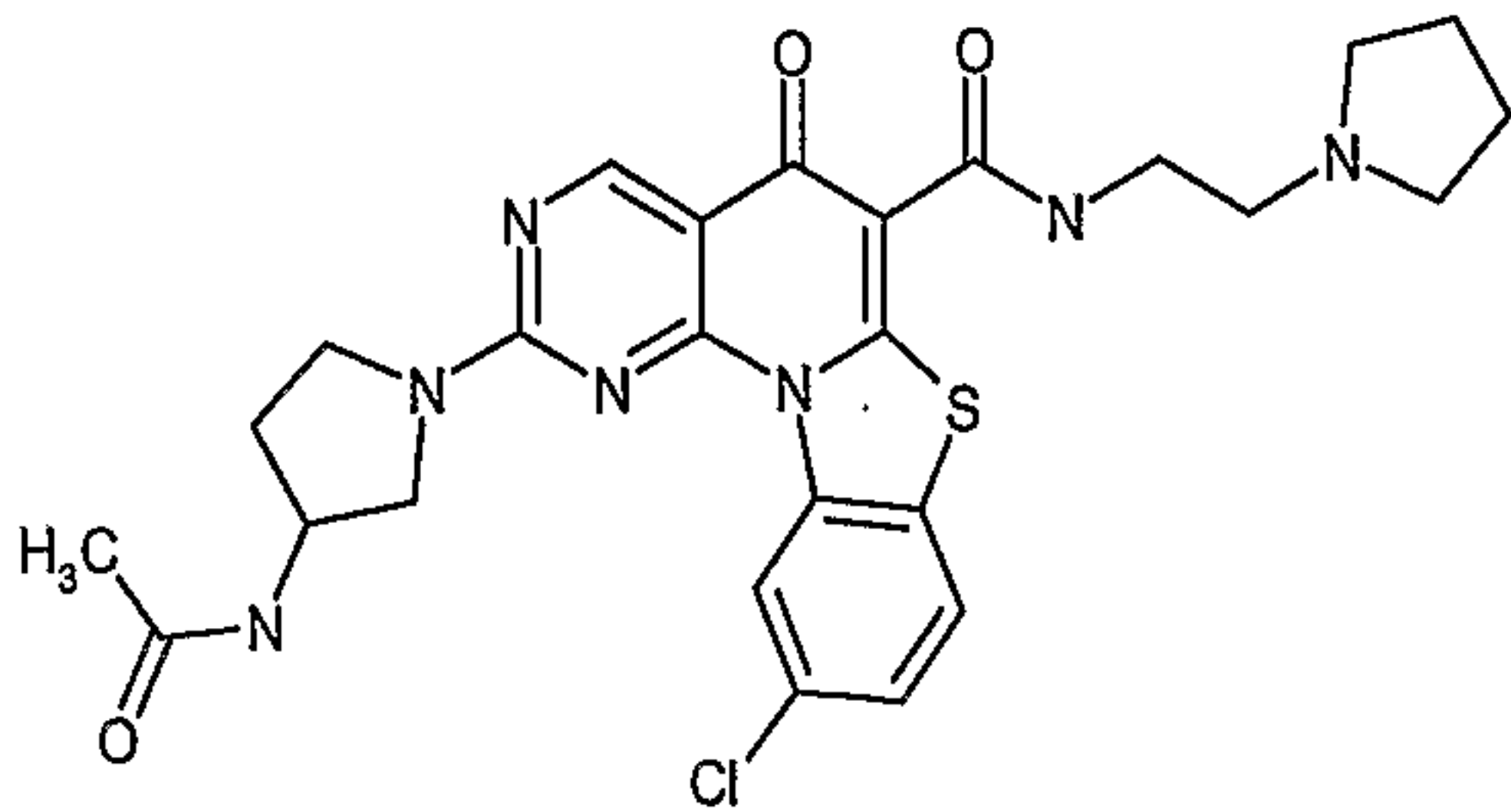
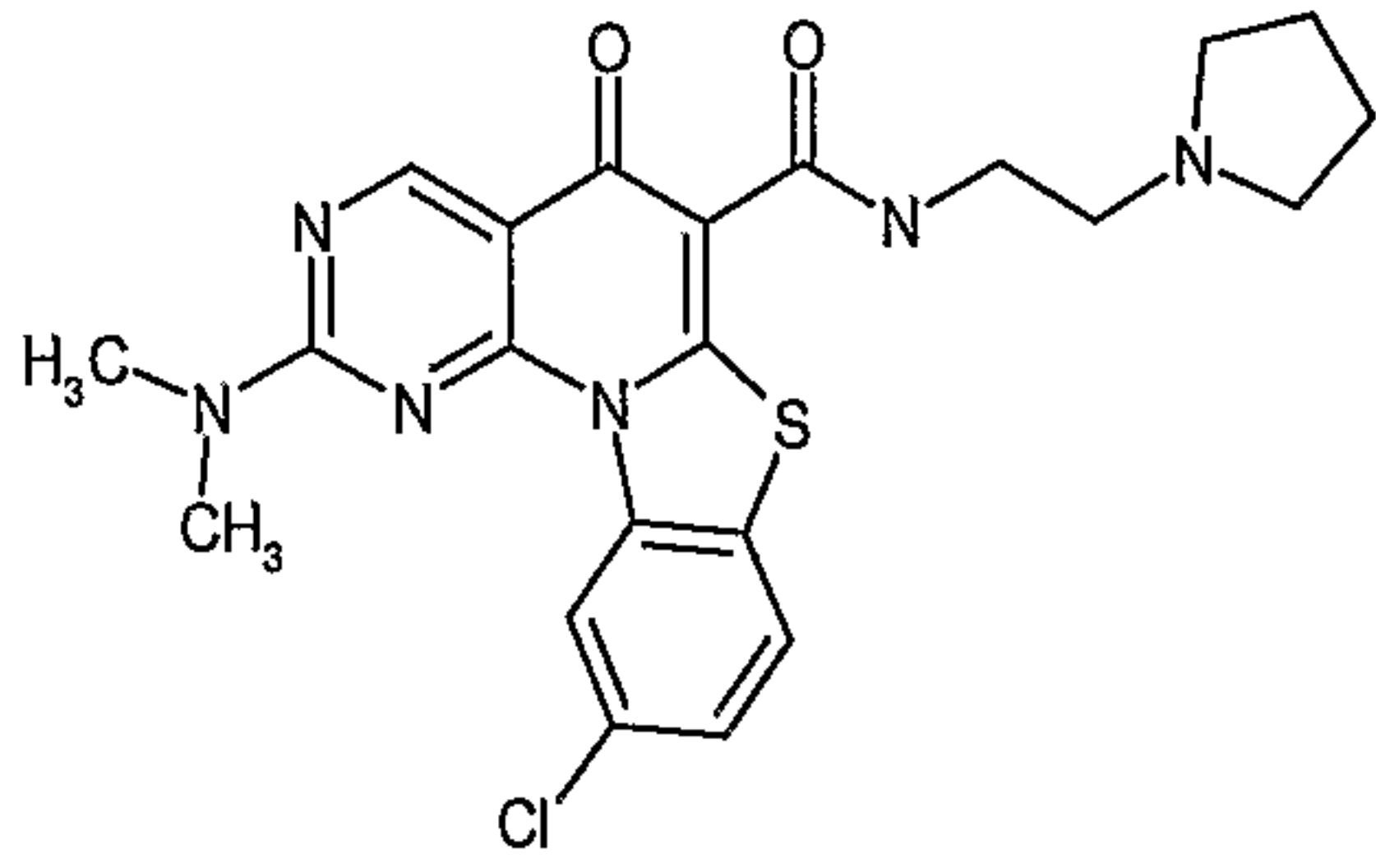
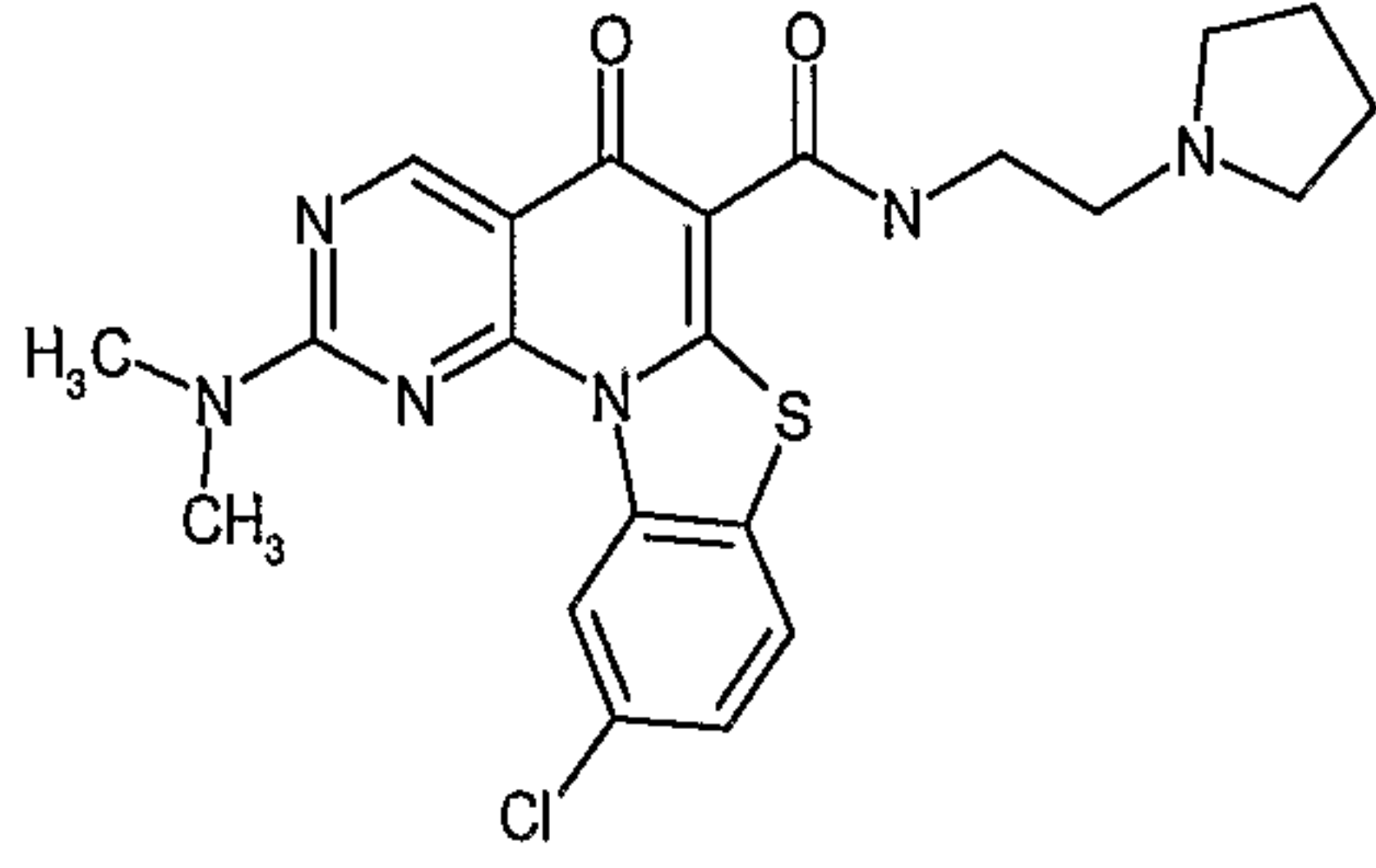
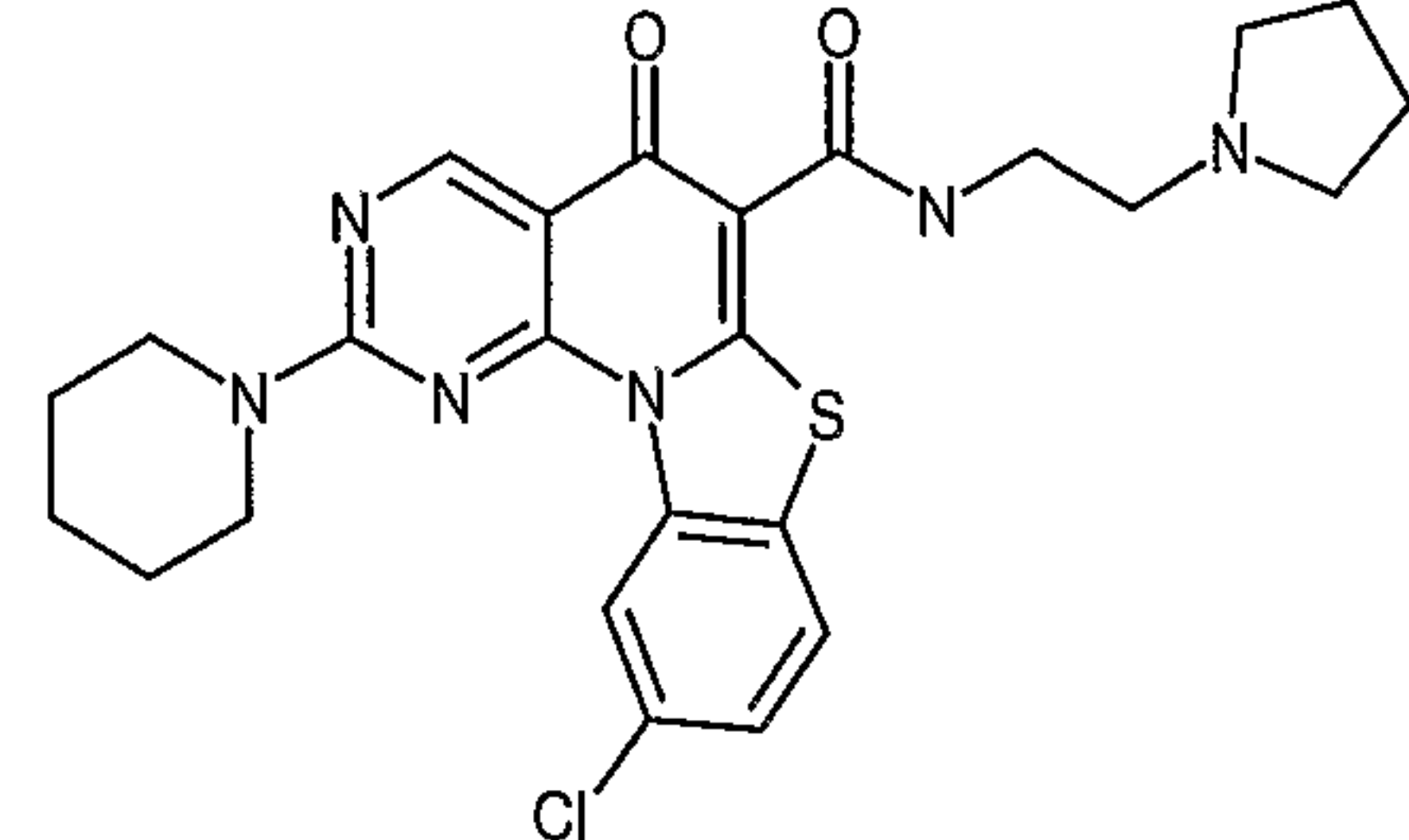
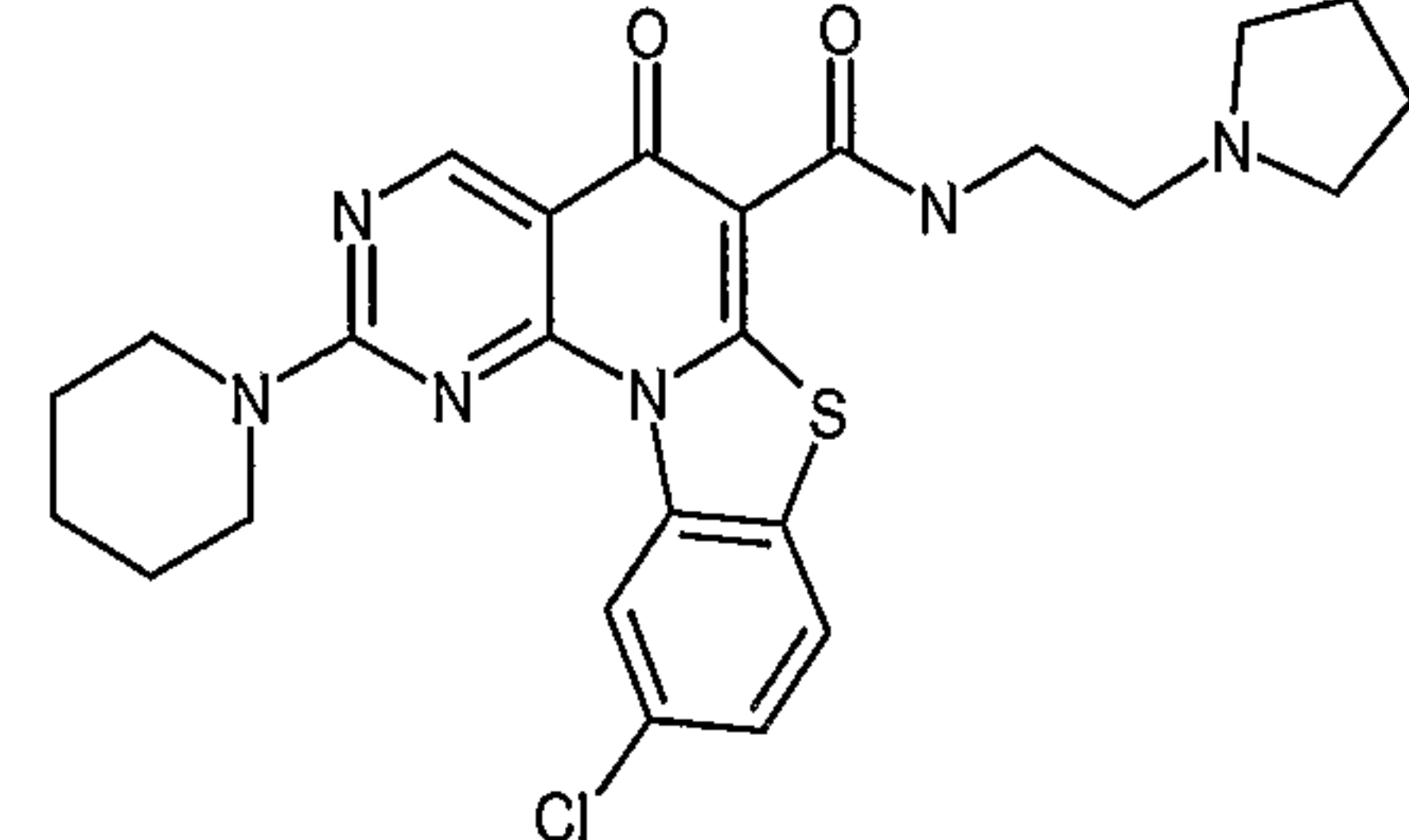
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | 0.01   |
|   | HCT-116 | 0.04   |
|  | MiaPaca | 0.02   |
|  | HCT-116 | >10    |
|  | MiaPaca | 3.9    |
|   | HCT-116 | 1.2    |

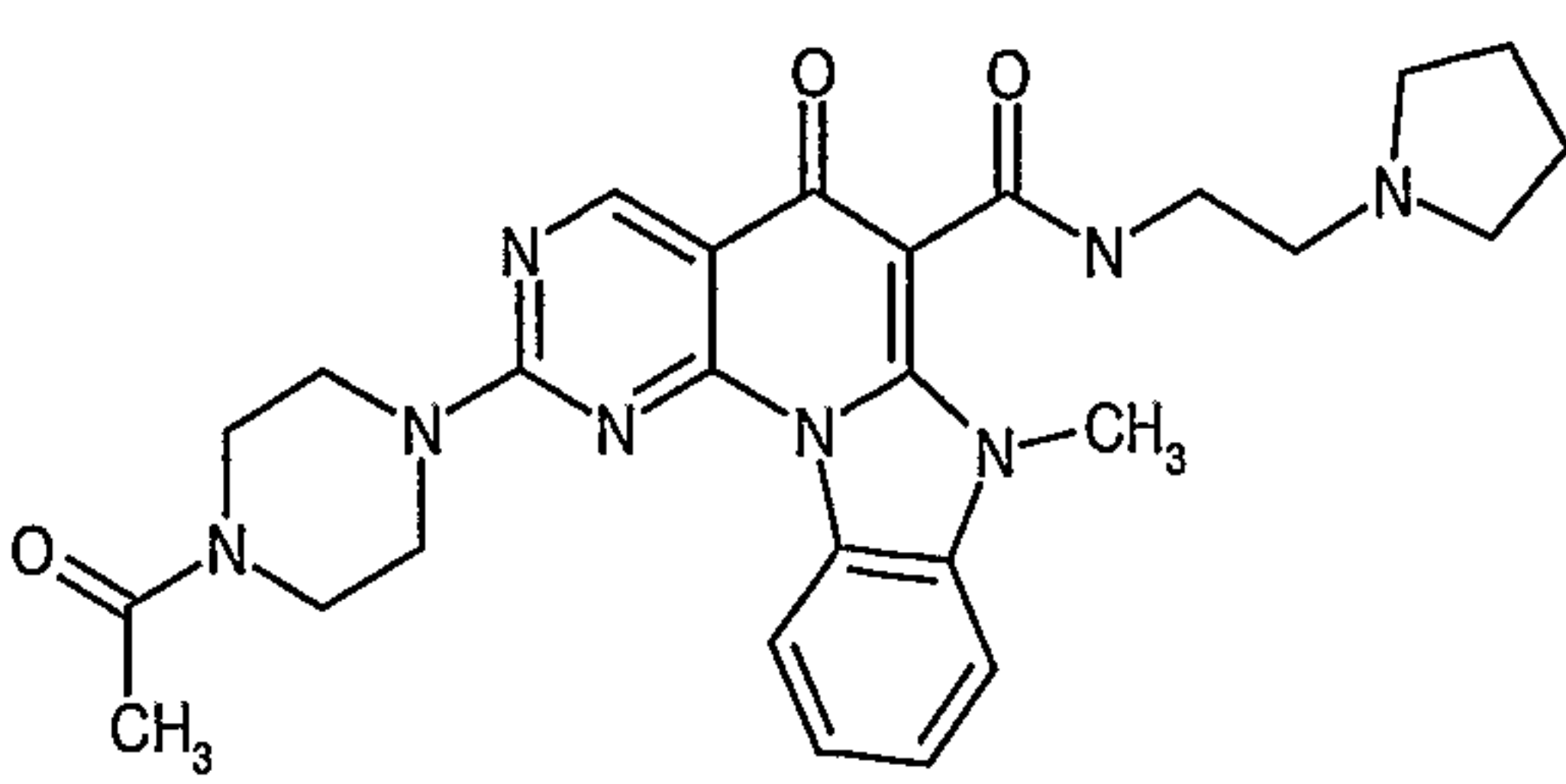
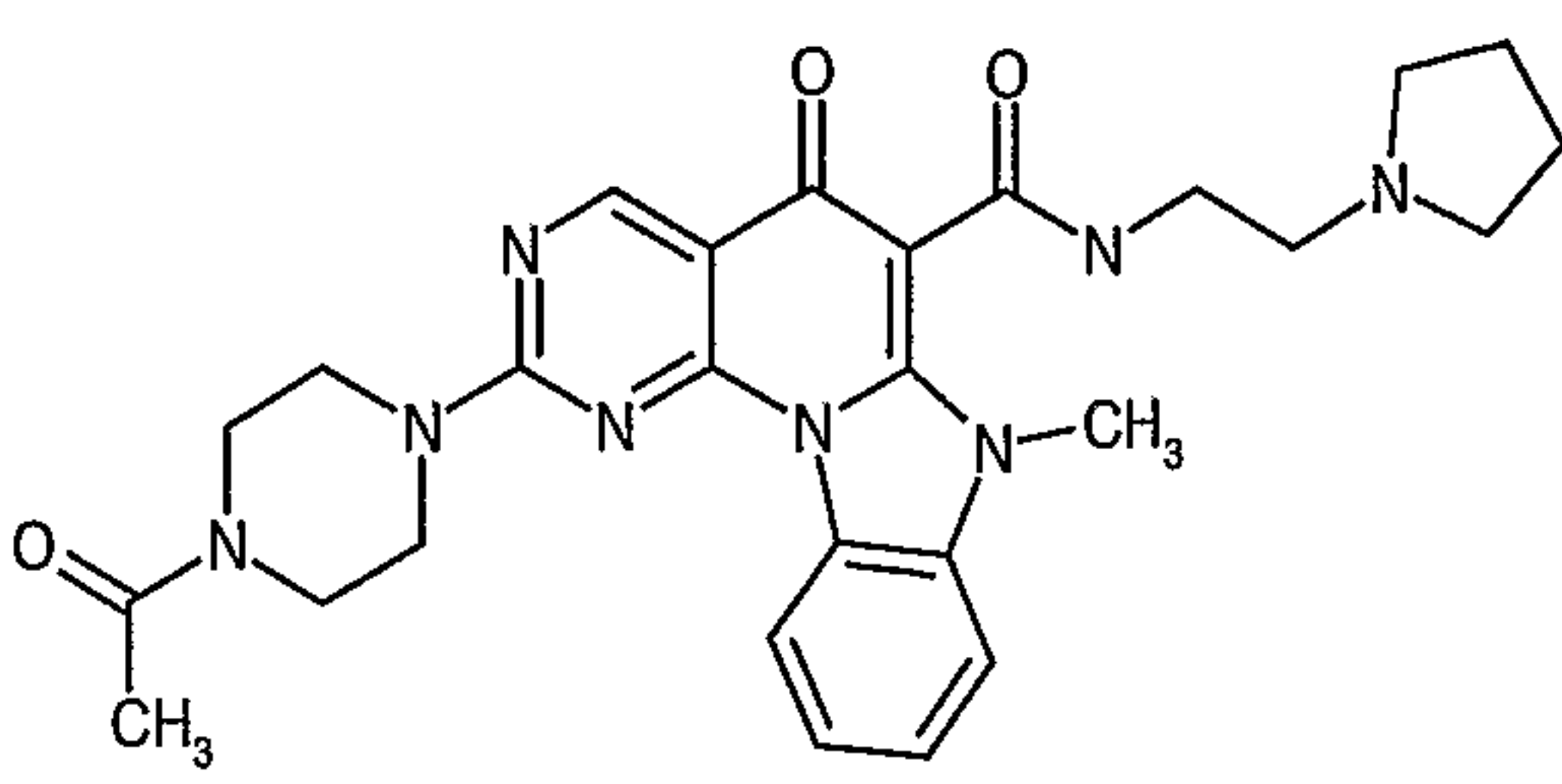
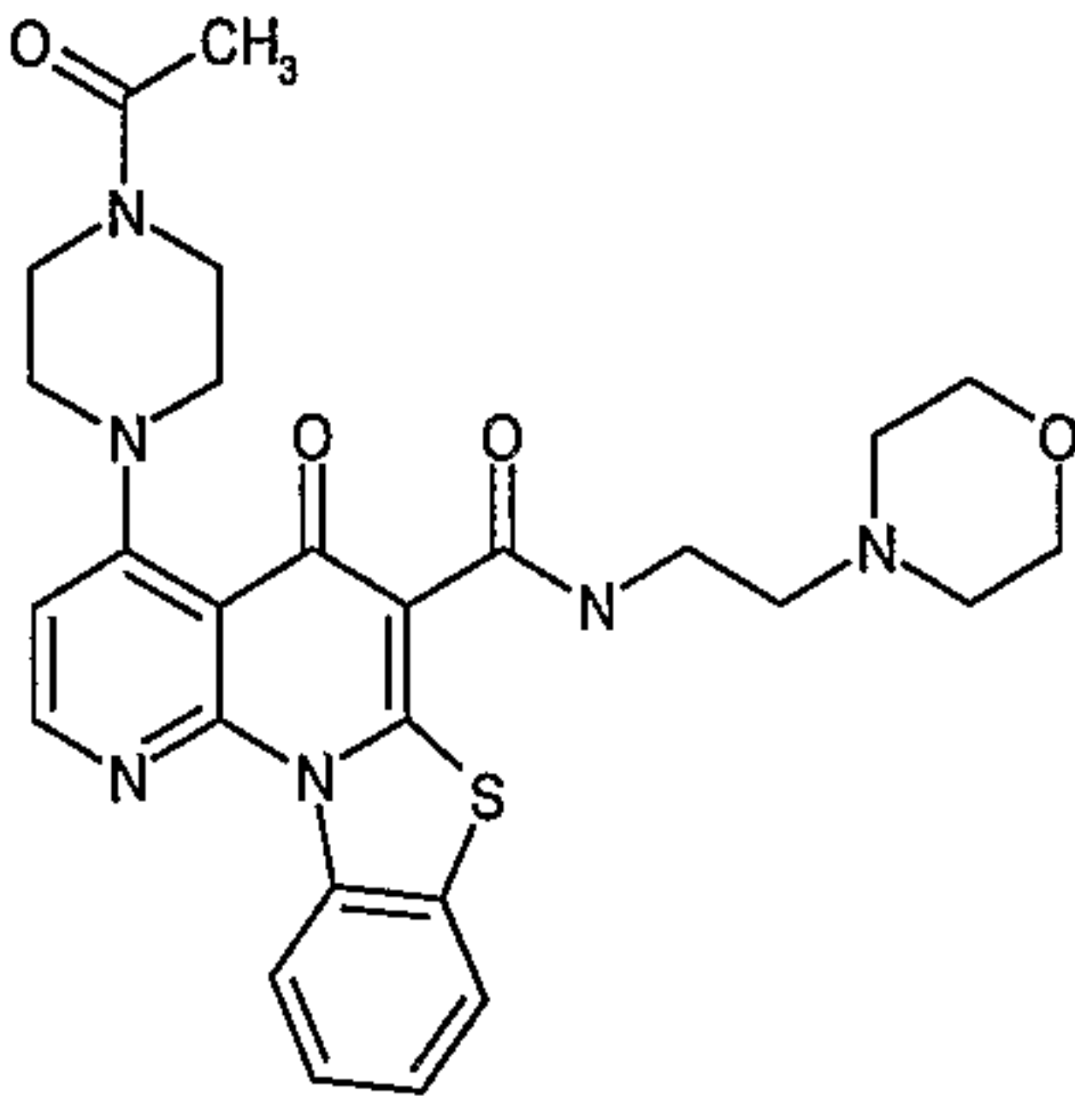
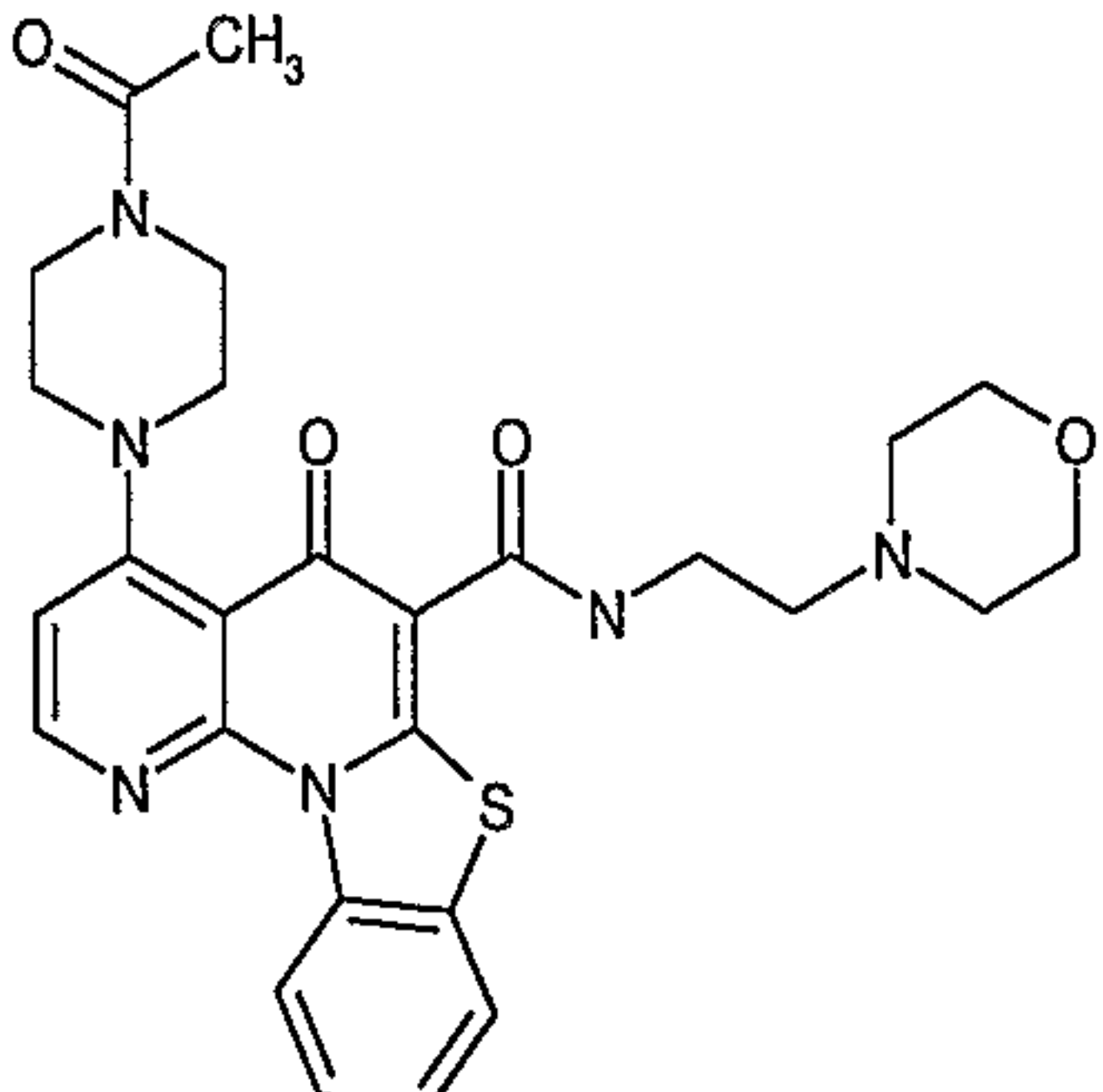
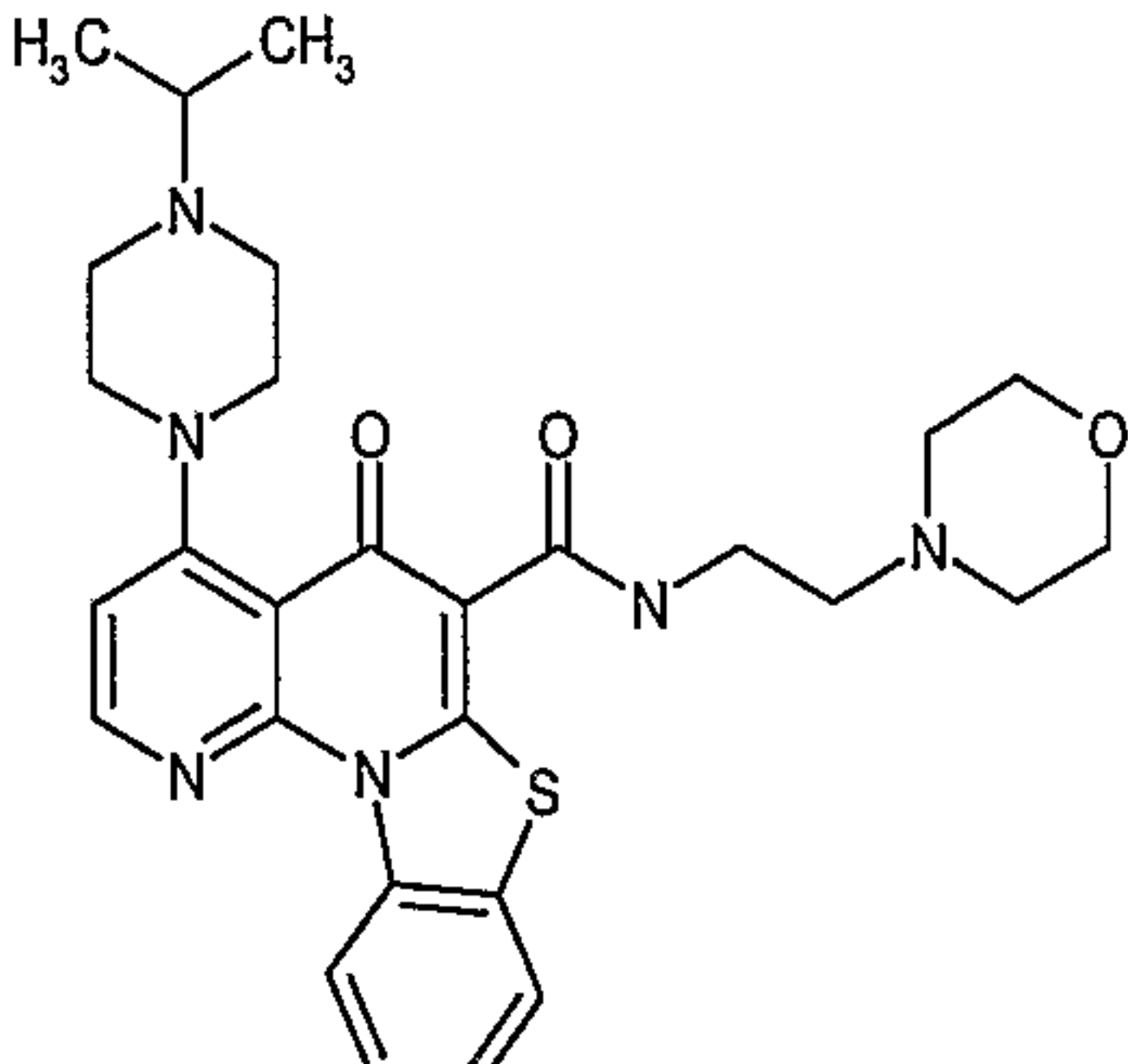
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | 2.3    |
|   | HCT-116 | >10    |
|  | MiaPaca | 2.5    |
|  | HCT-116 | 2.8    |
|  | MiaPaca | 1.8    |
|  | HCT-116 | 4.2    |

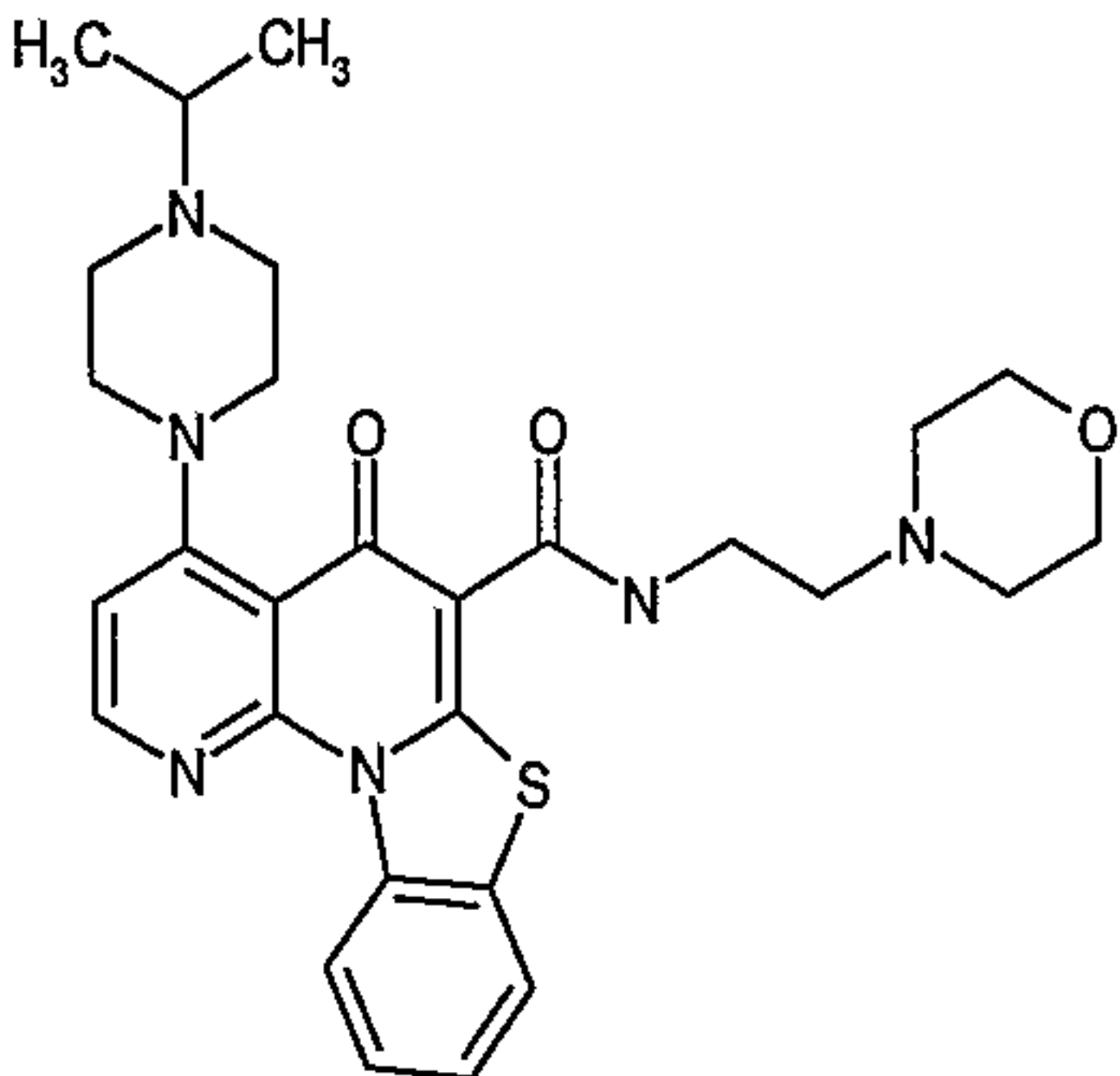
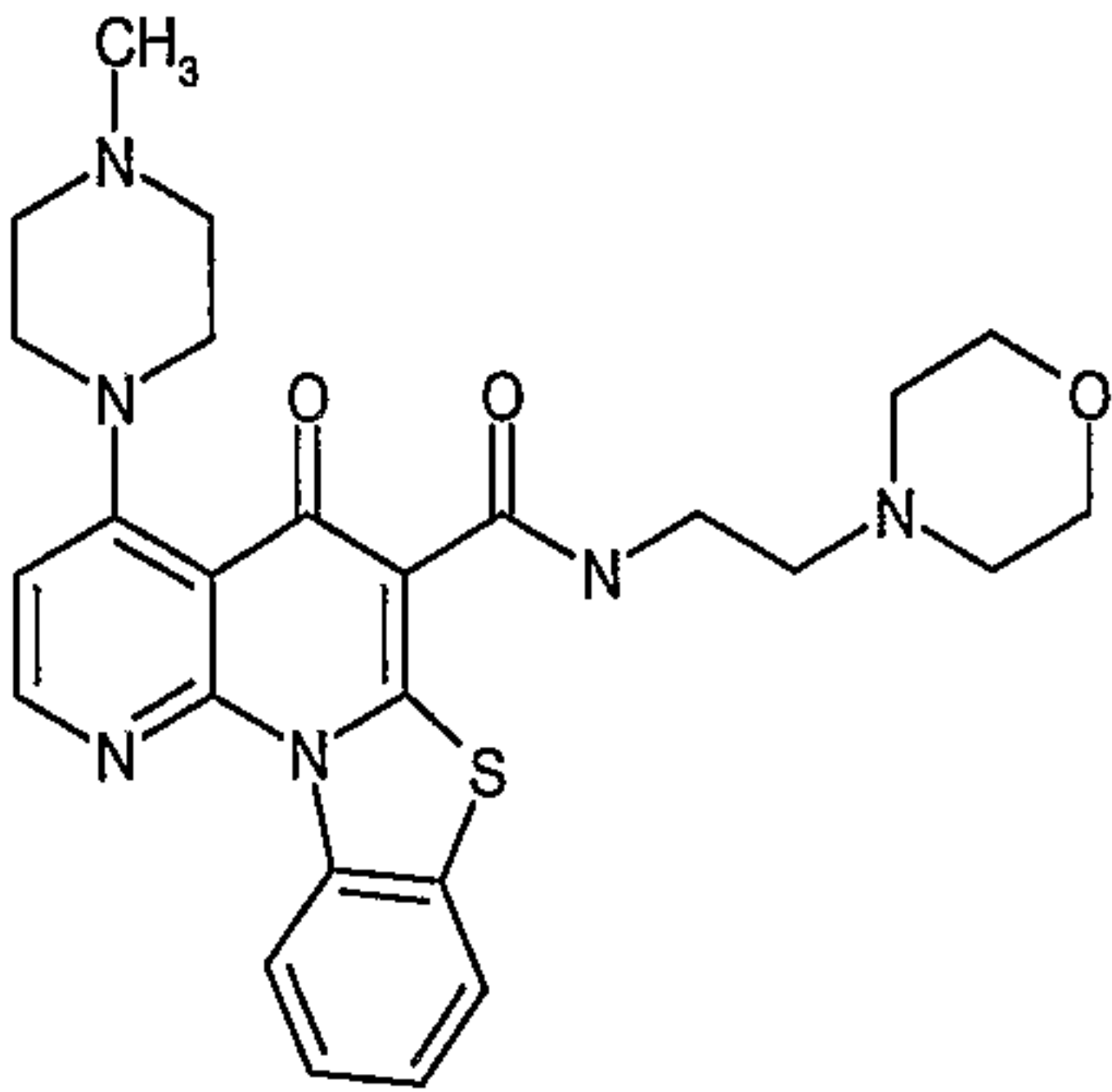
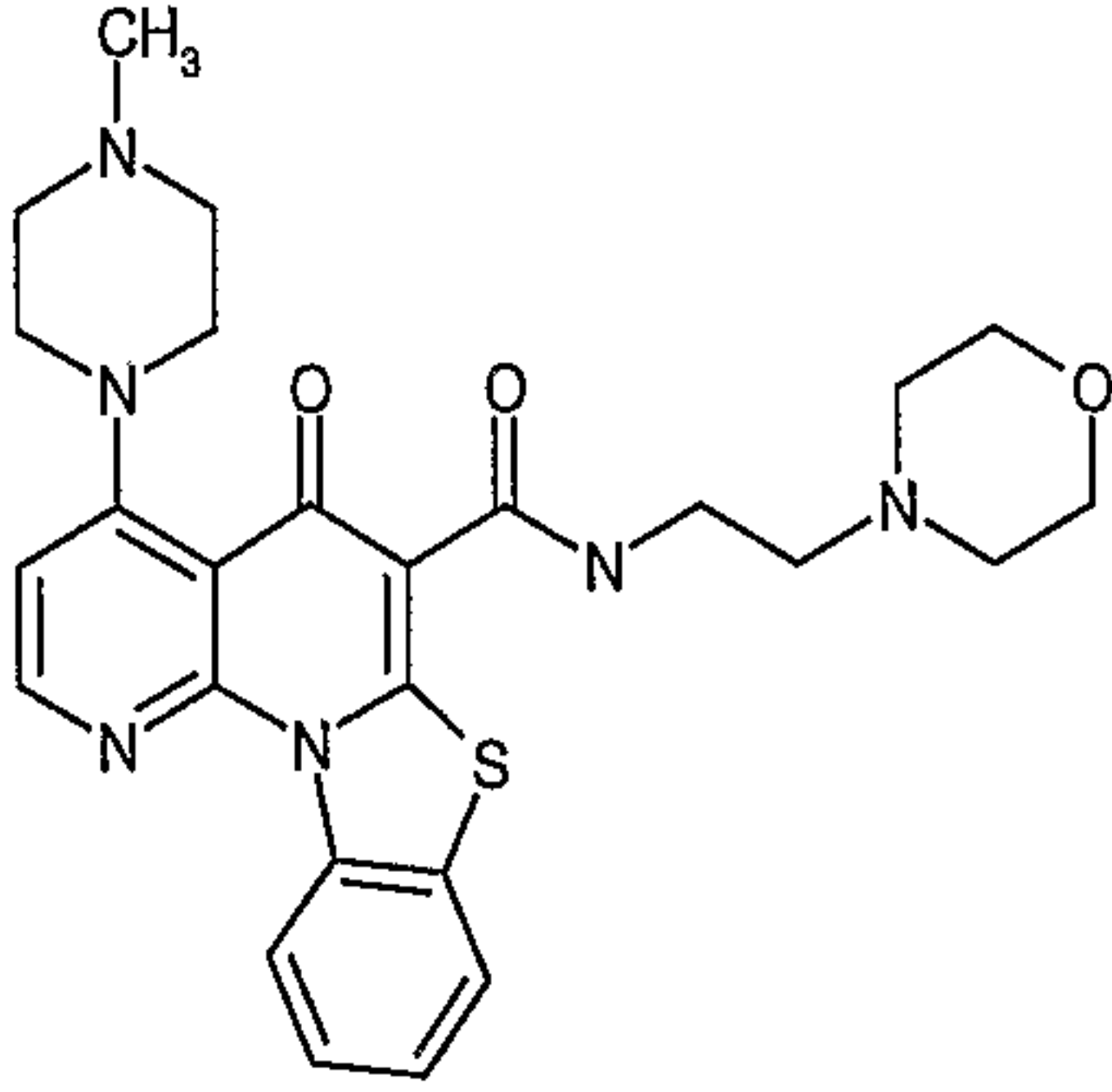
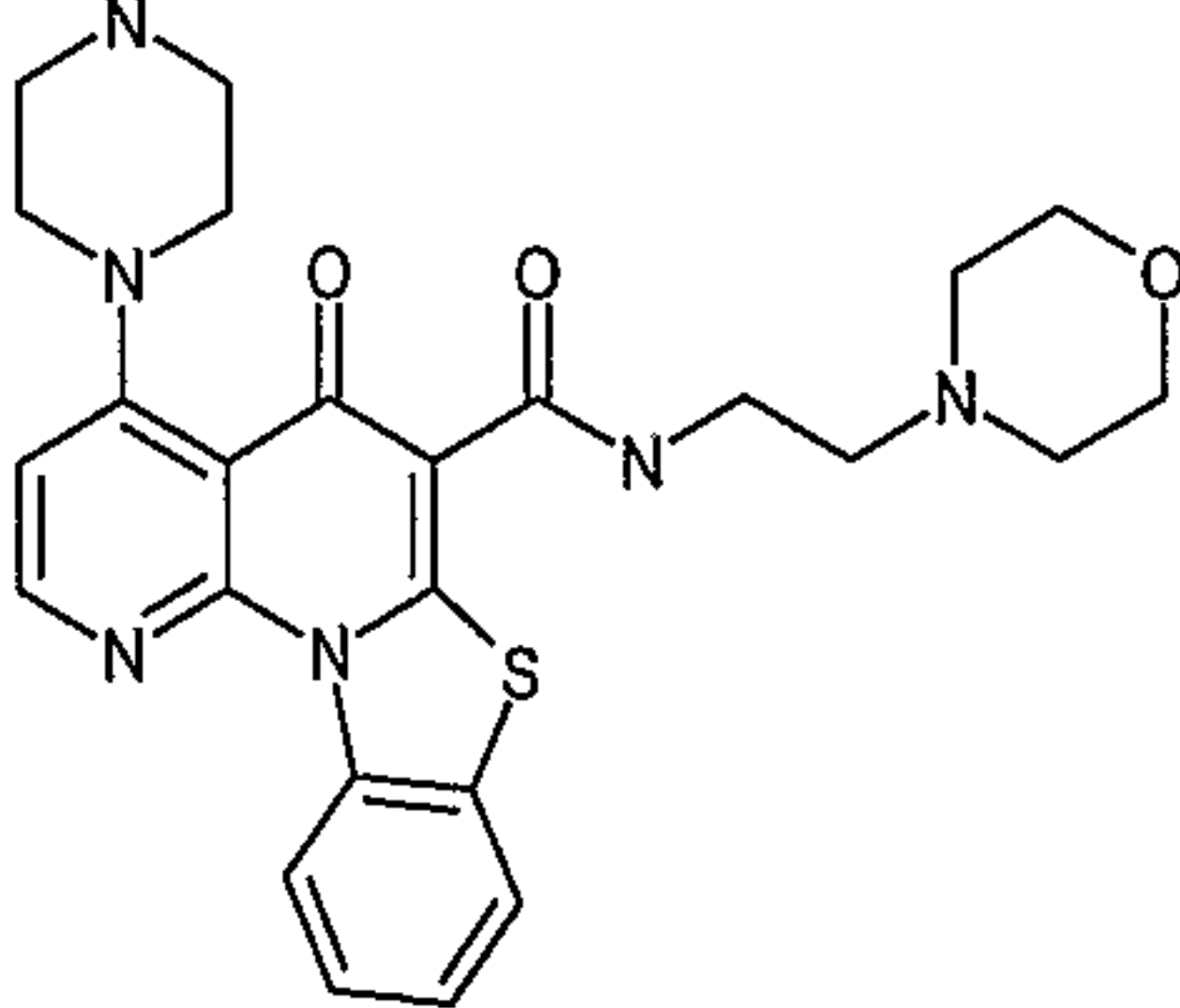
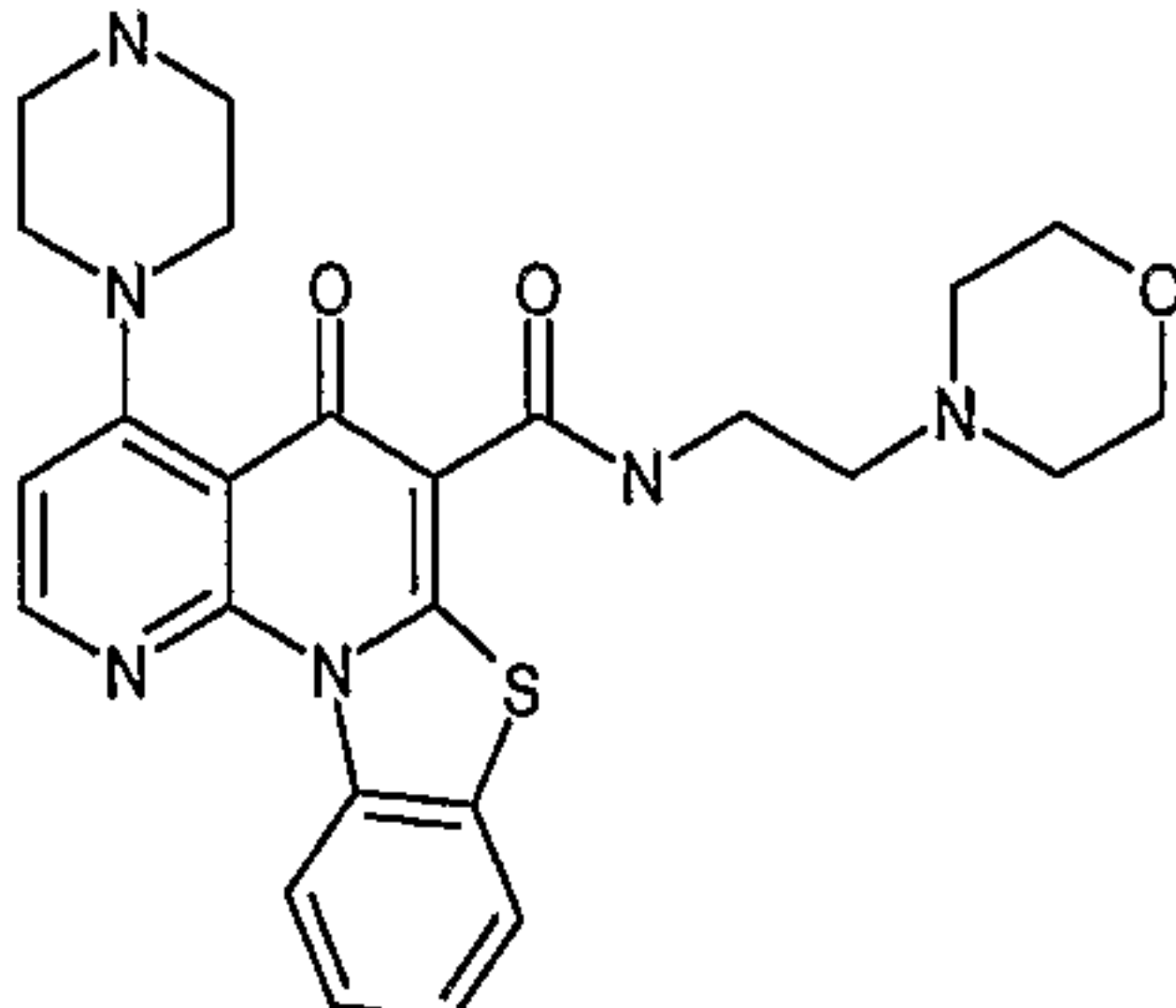
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | 1.4    |
|   | HCT-116 | 3.5    |
|  | HCT-116 | 3.4    |
|  | MiaPaca | 4      |
|  | HCT-116 | >10    |
|  | MiaPaca | 10     |

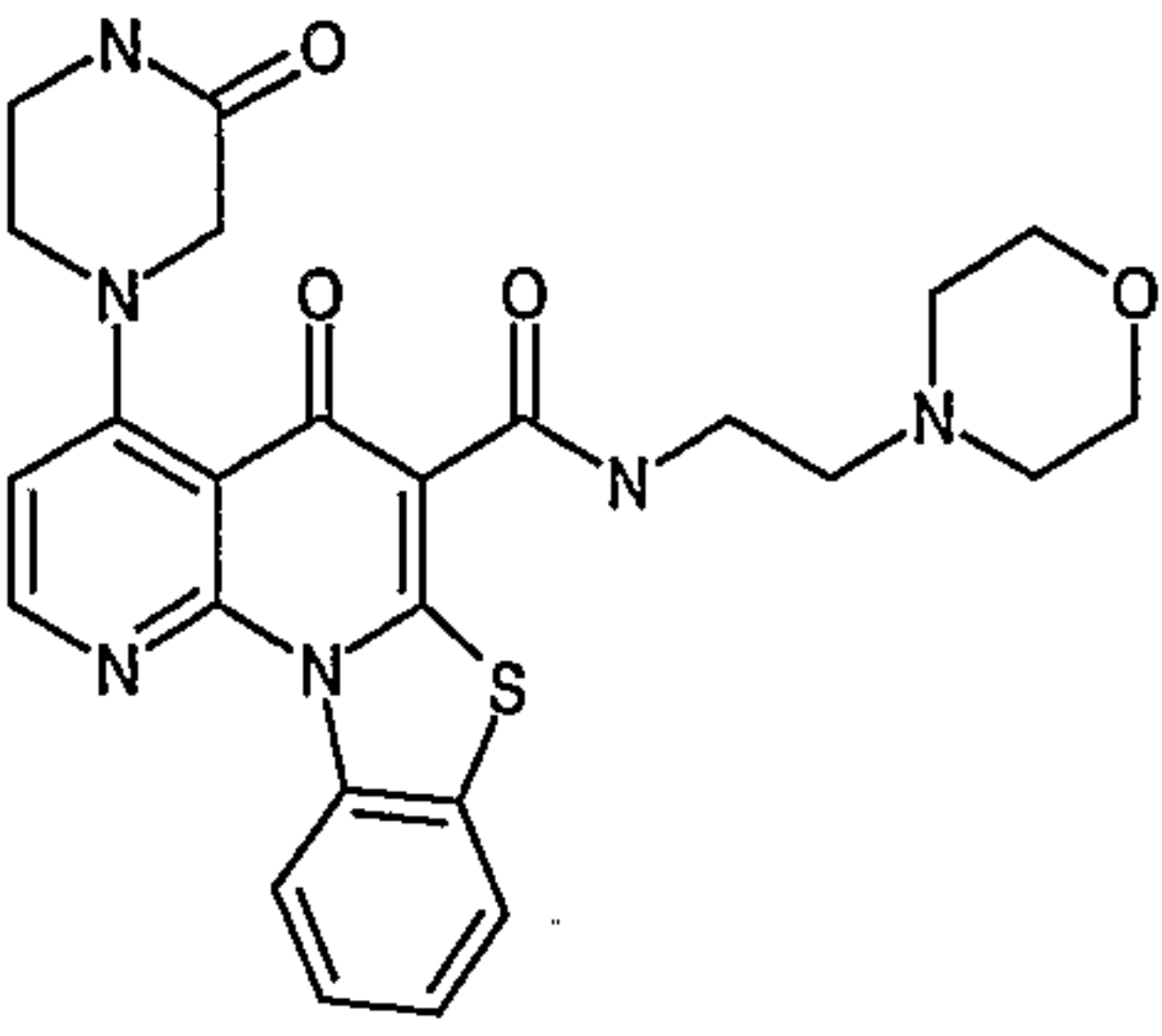
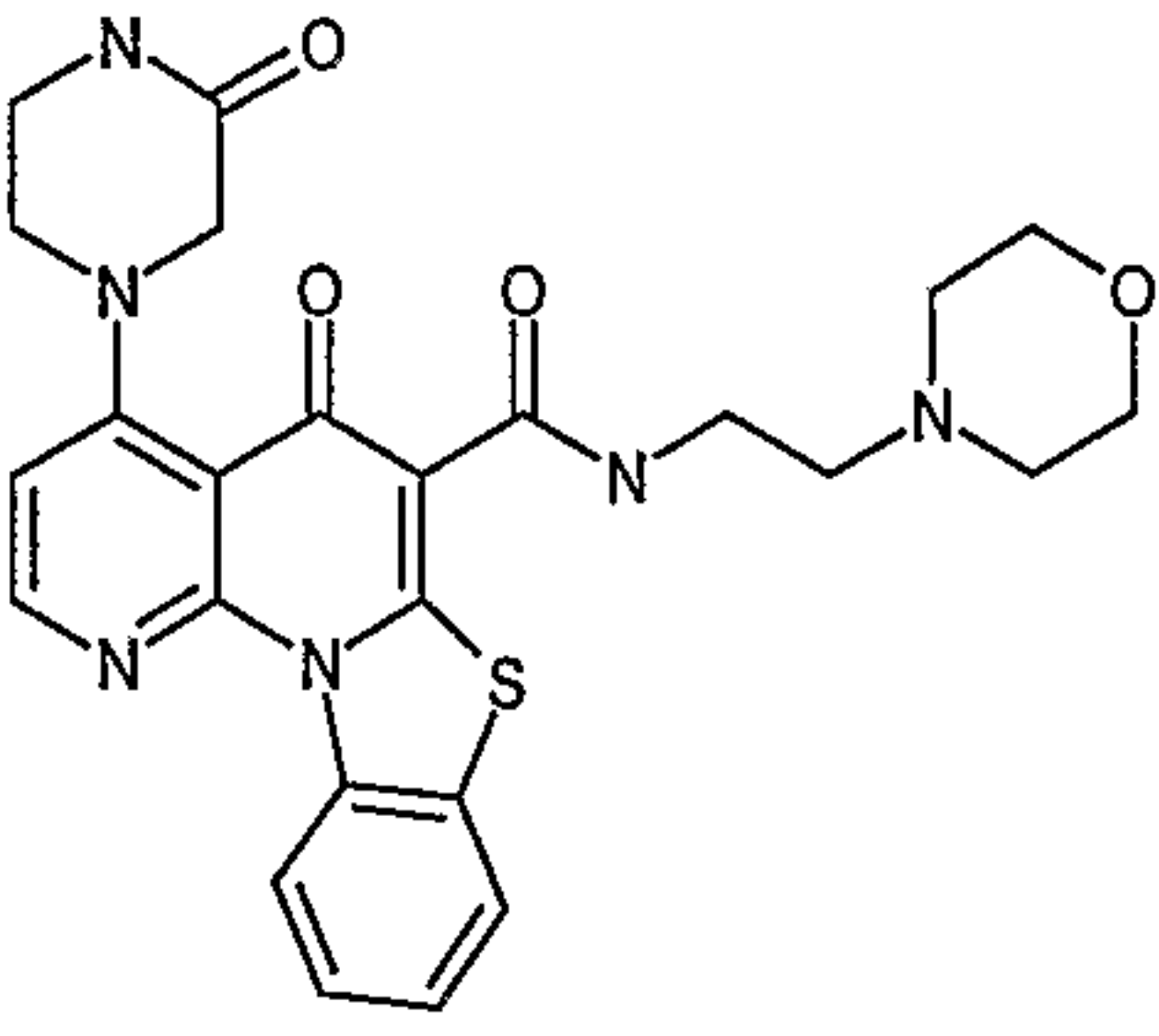
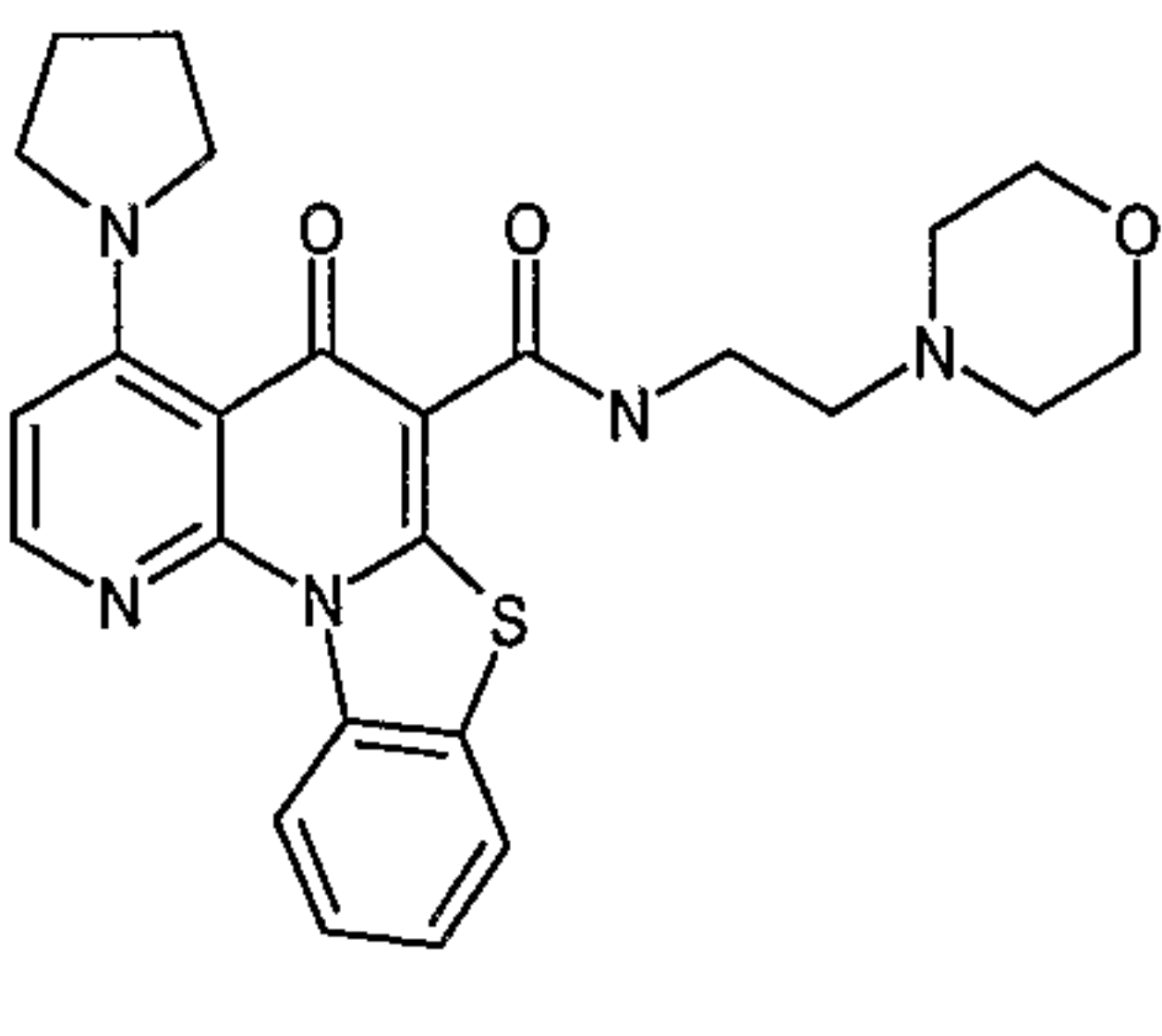
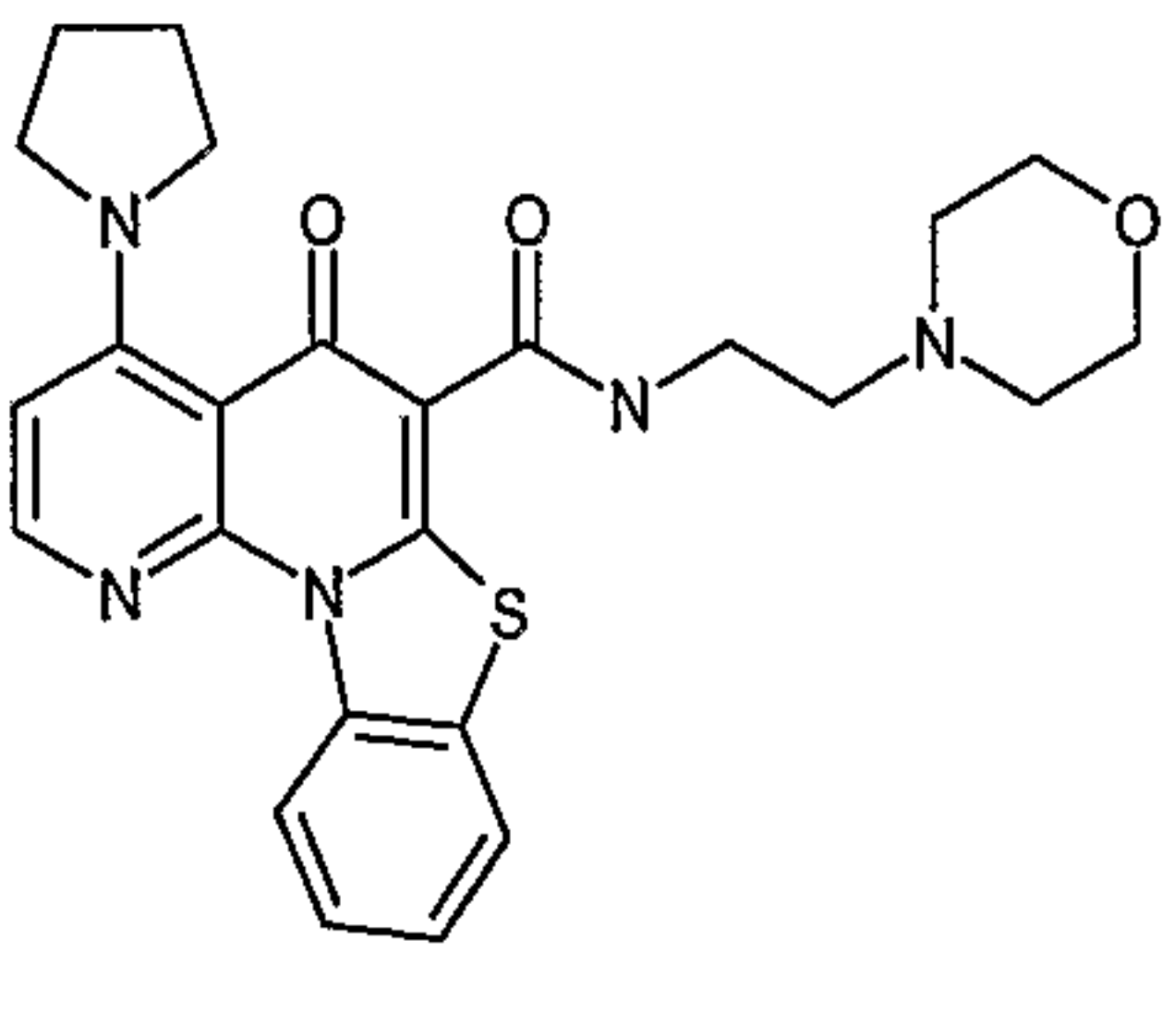
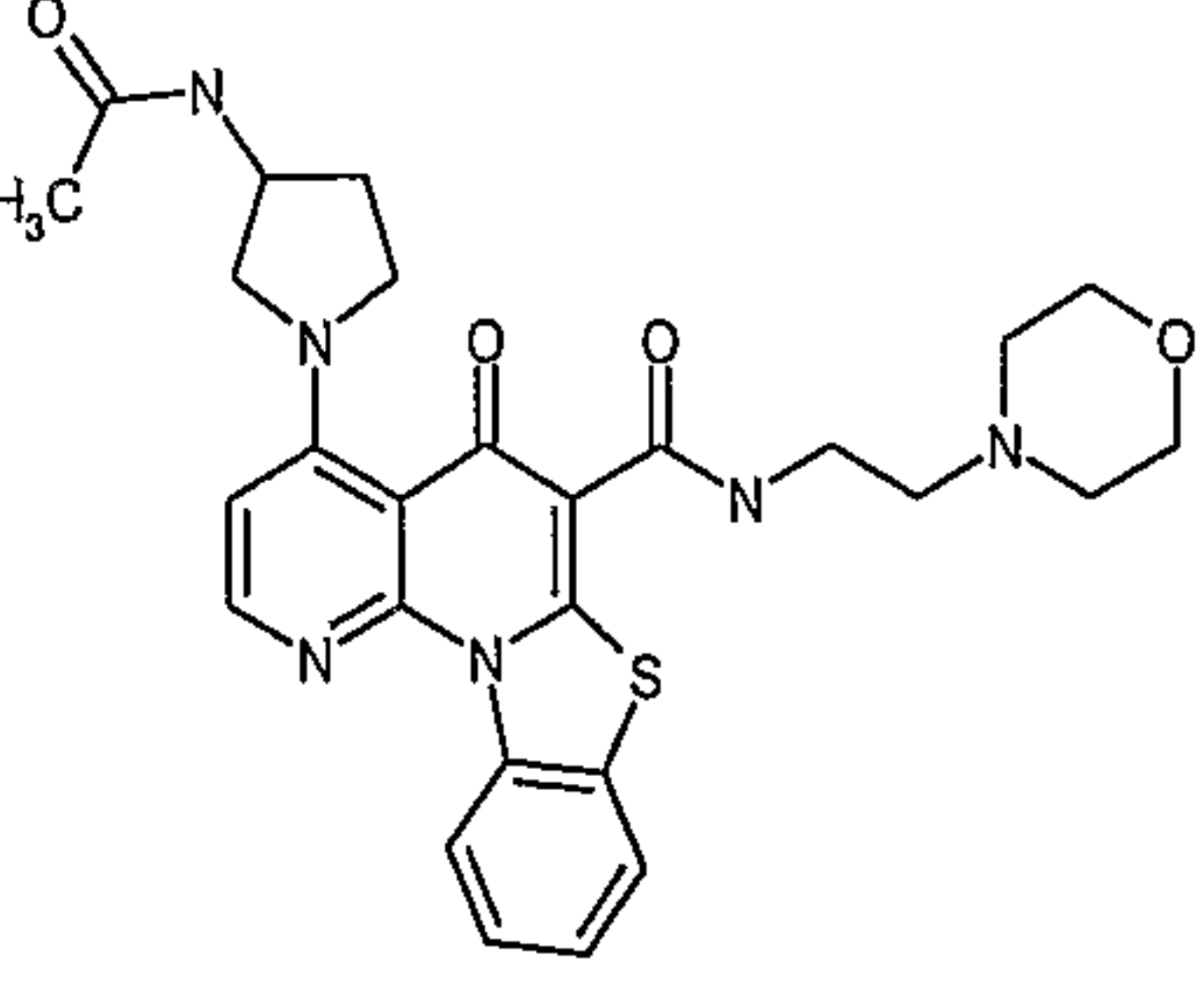
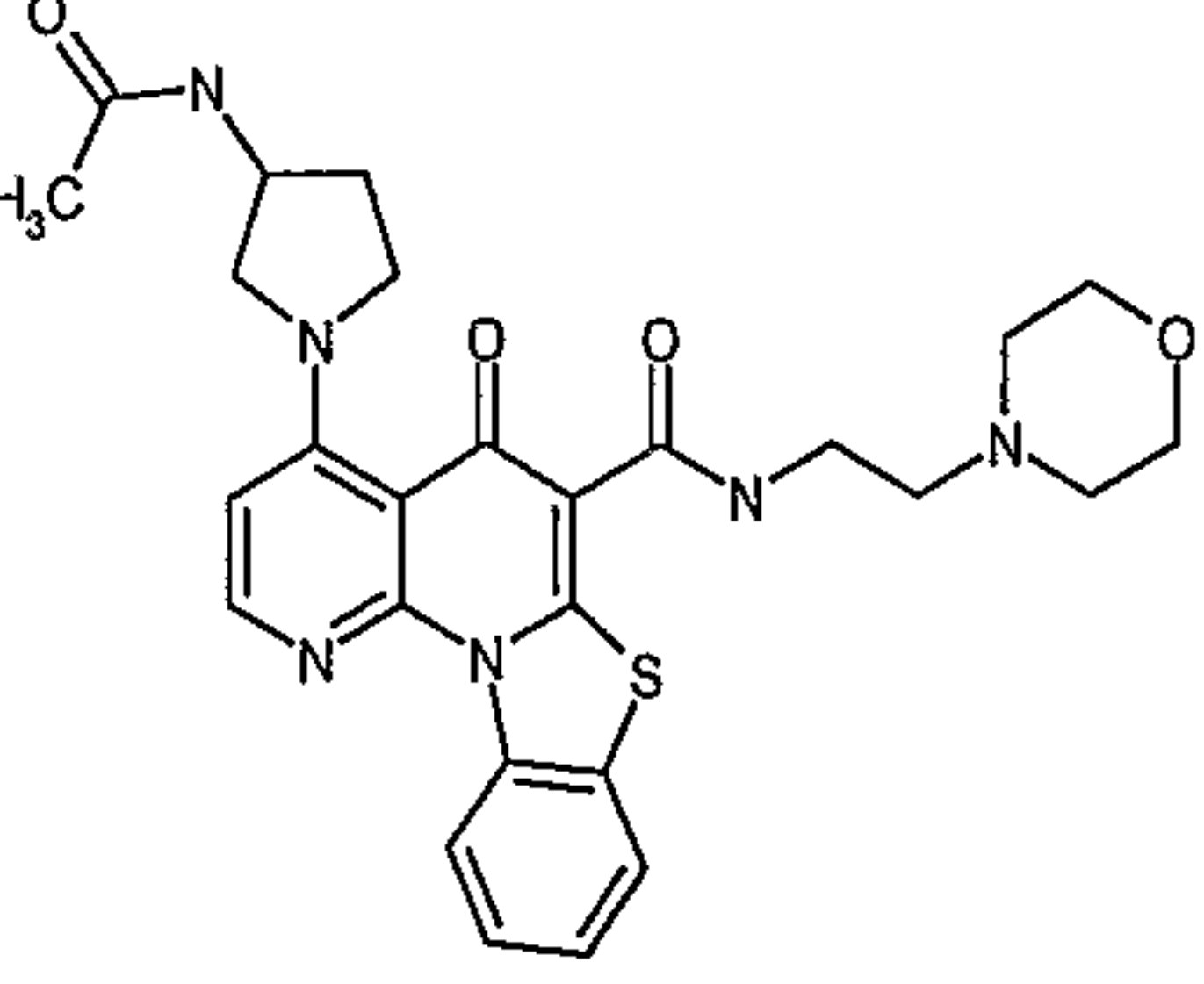
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | MiaPaca | <0.01  |
|    | HCT-116 | 0.024  |
|  | HCT-116 | 0.03   |
|  | MiaPaca | 0.03   |
|   | MiaPaca | 0.03   |
|   | HCT-116 | 0.02   |

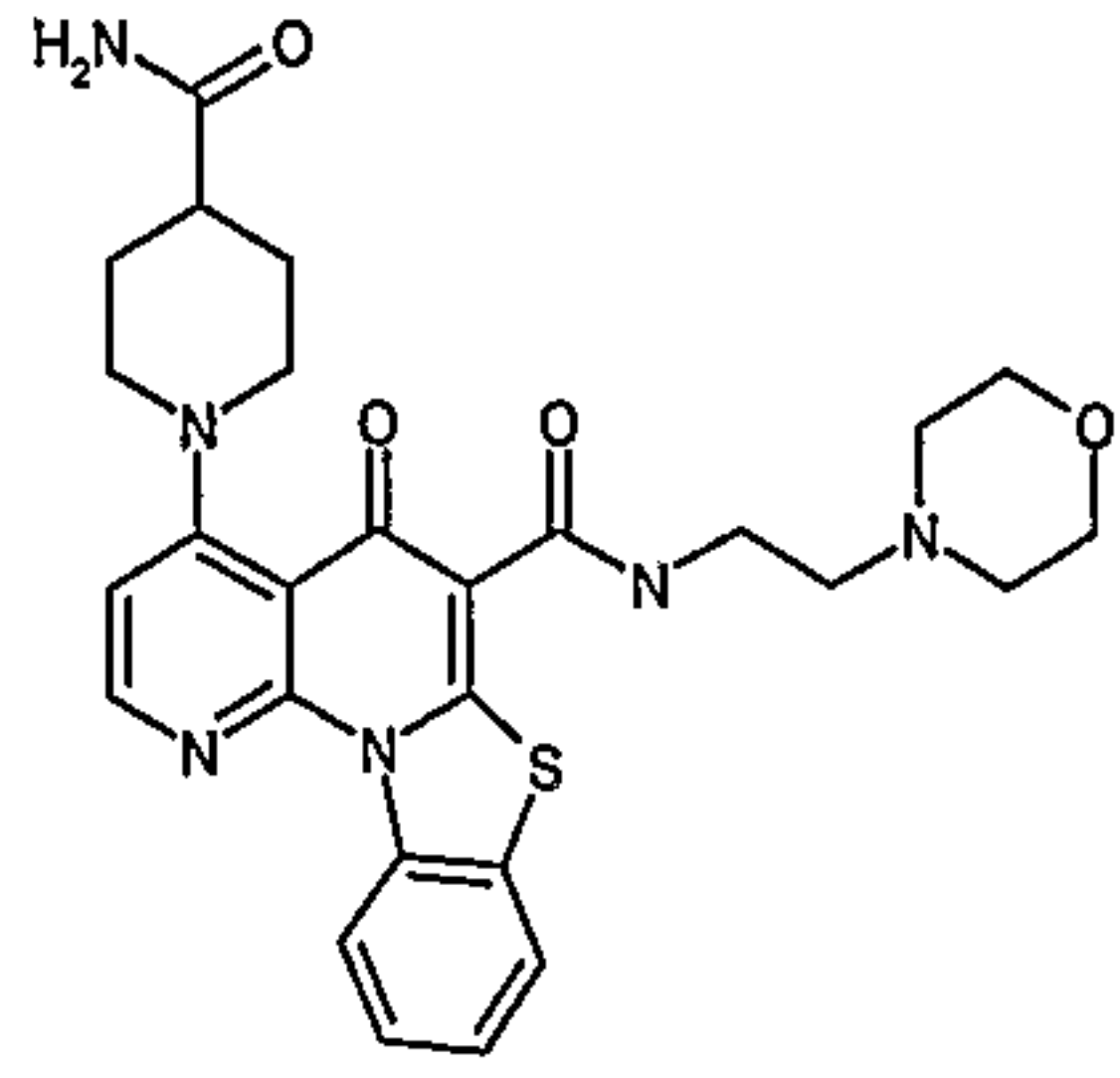
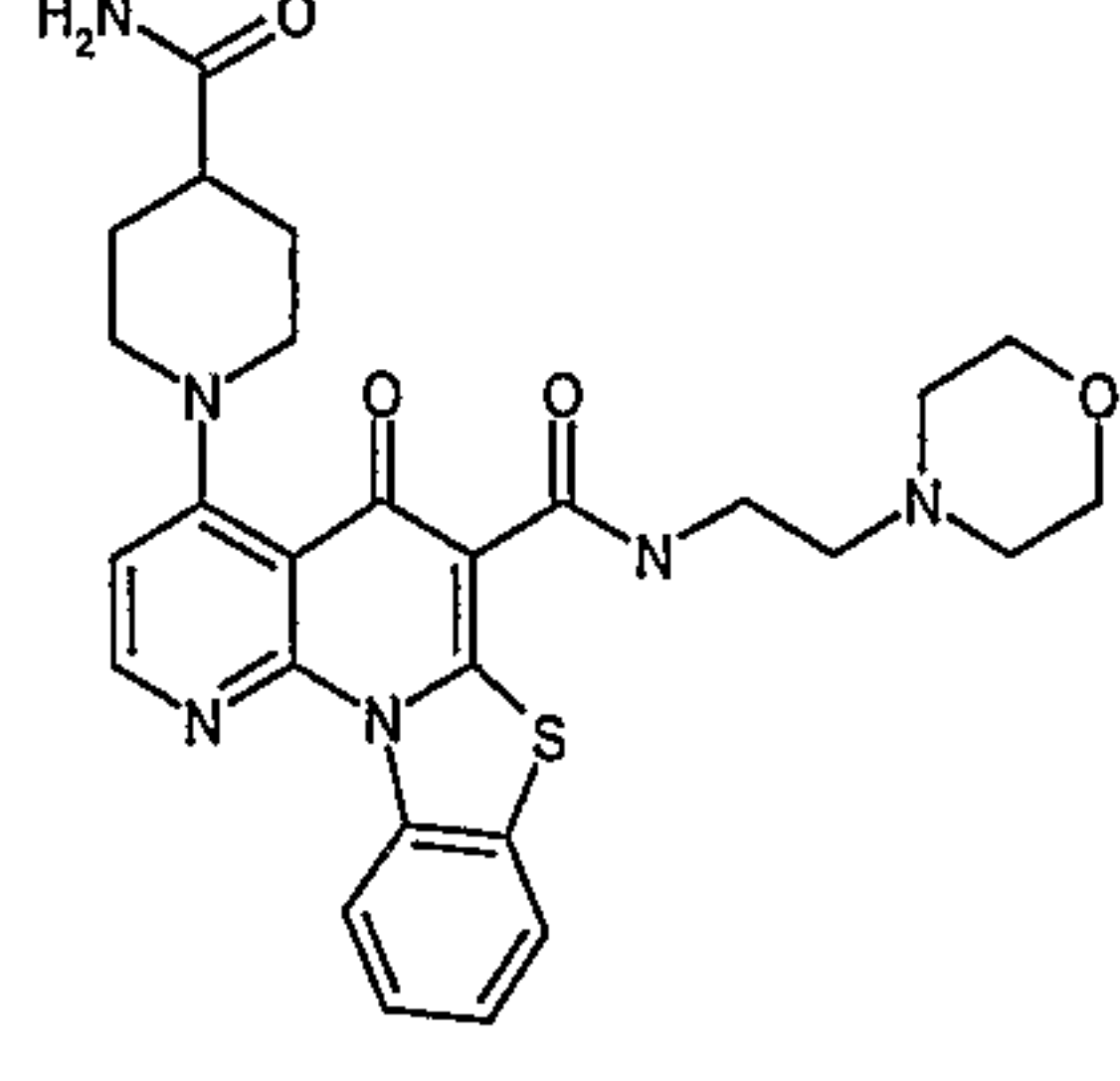
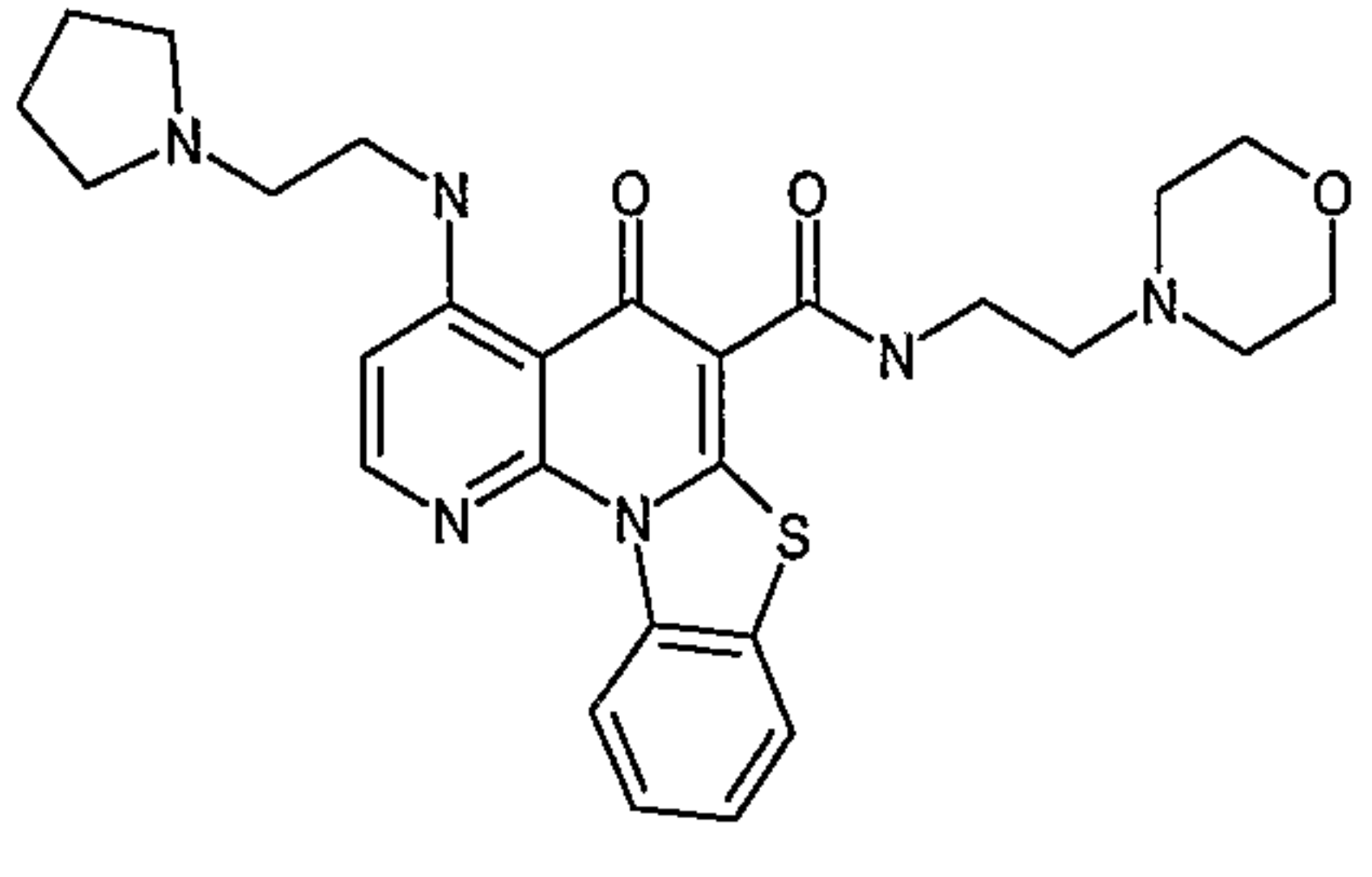
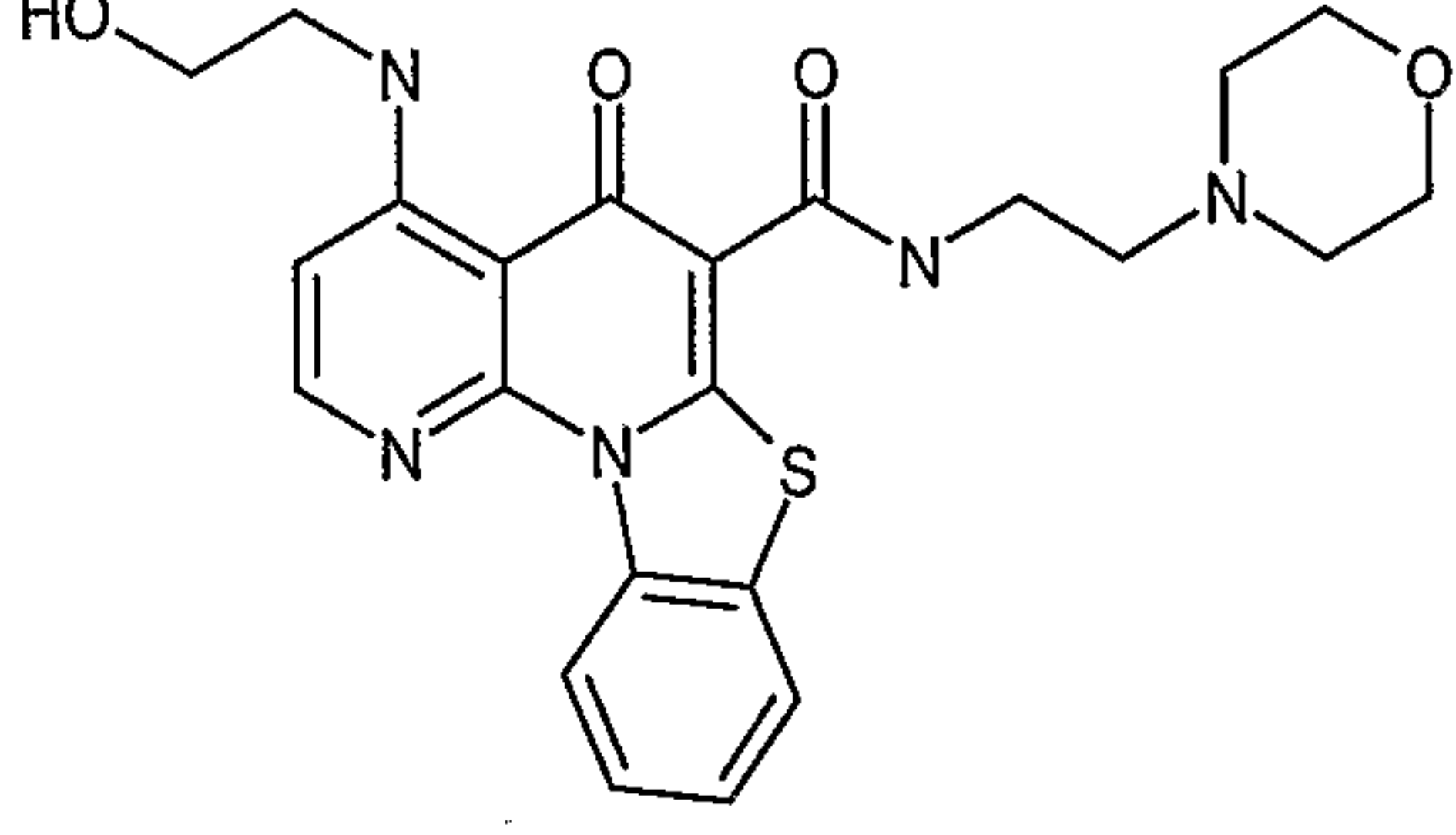
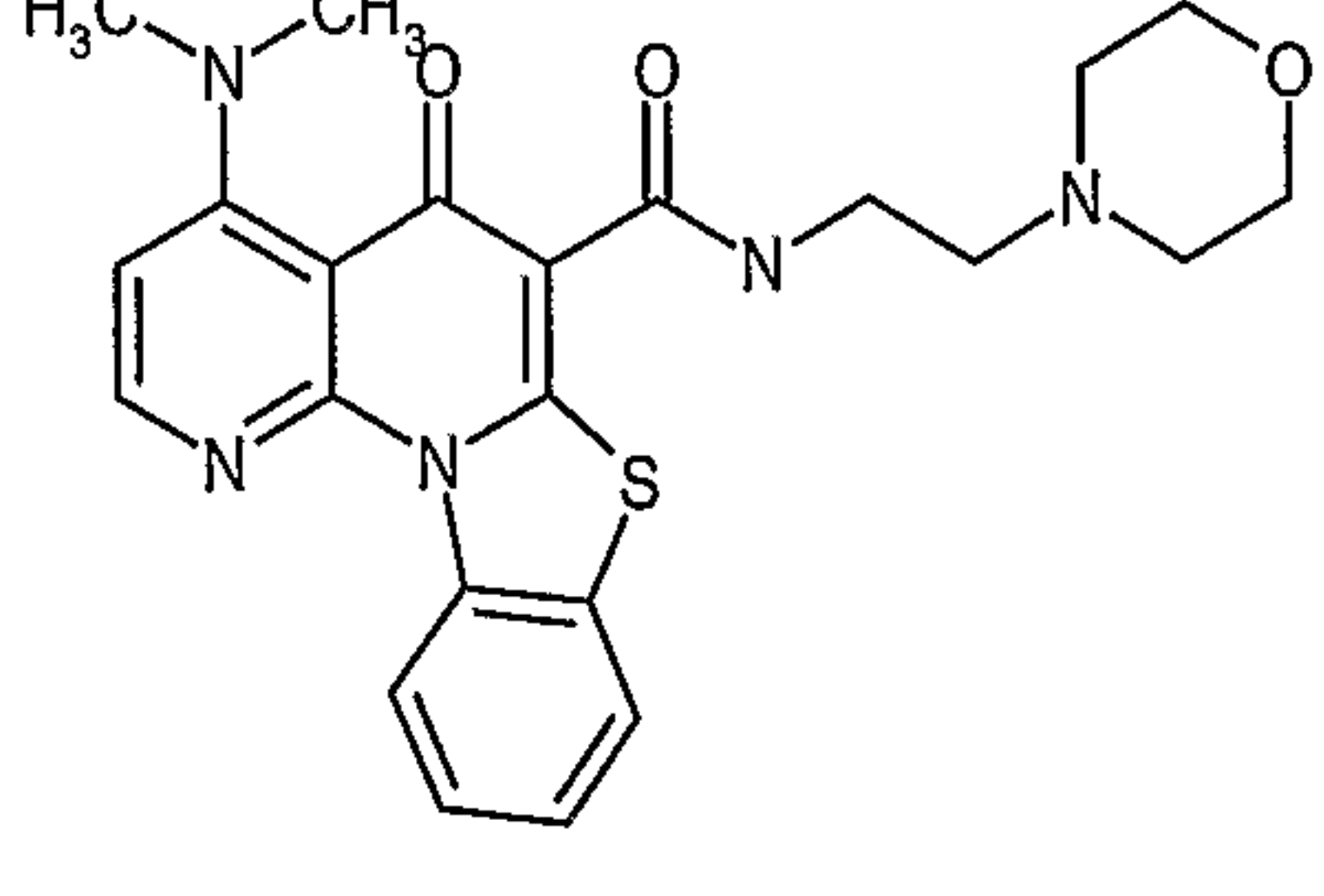
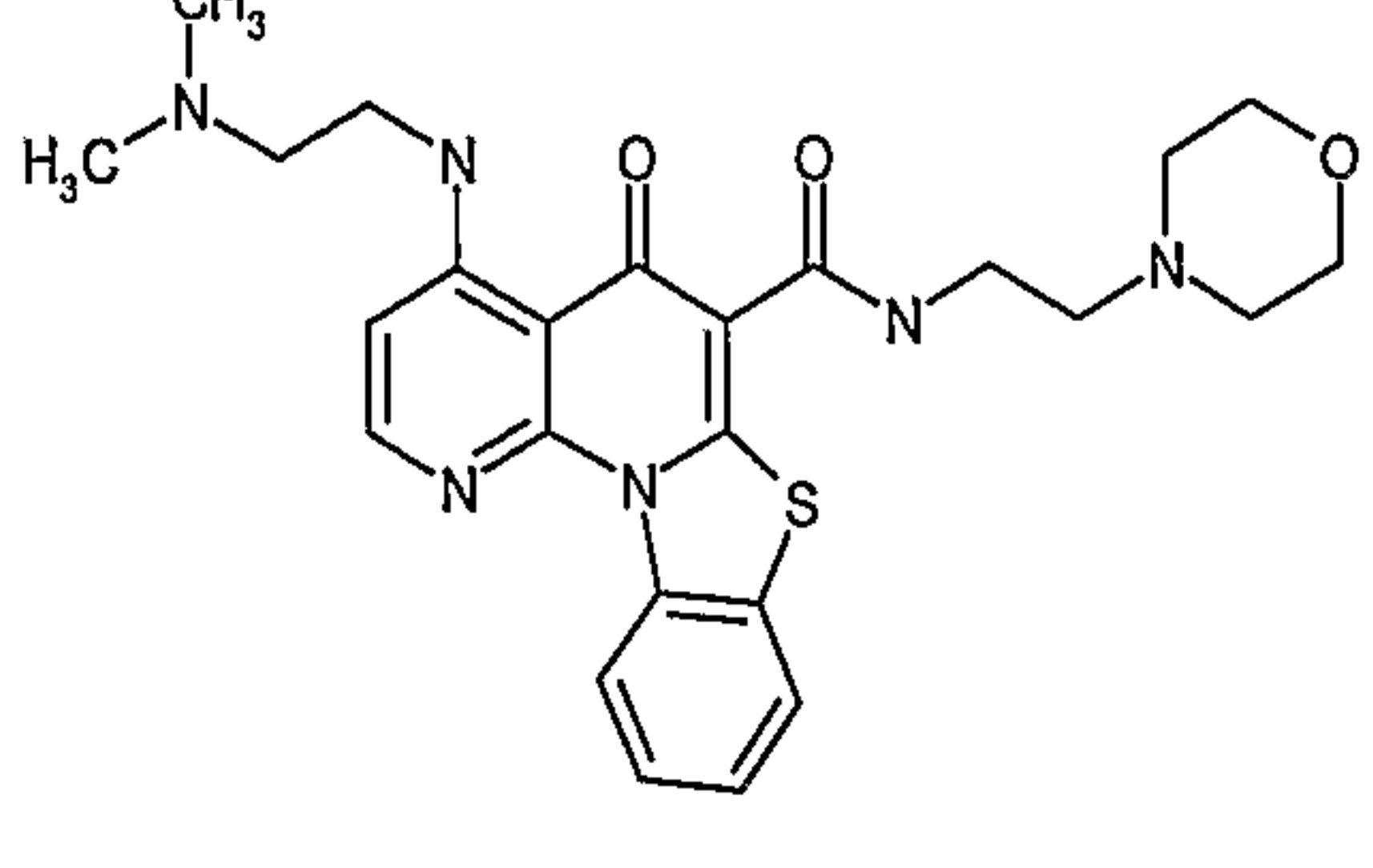
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | 0.05   |
|   | HCT-116 | 0.21   |
|  | MiaPaca | 0.3    |
|  | HCT-116 | 2.40   |
|  | MiaPaca | 0.02   |
|  | HCT-116 | 0.02   |

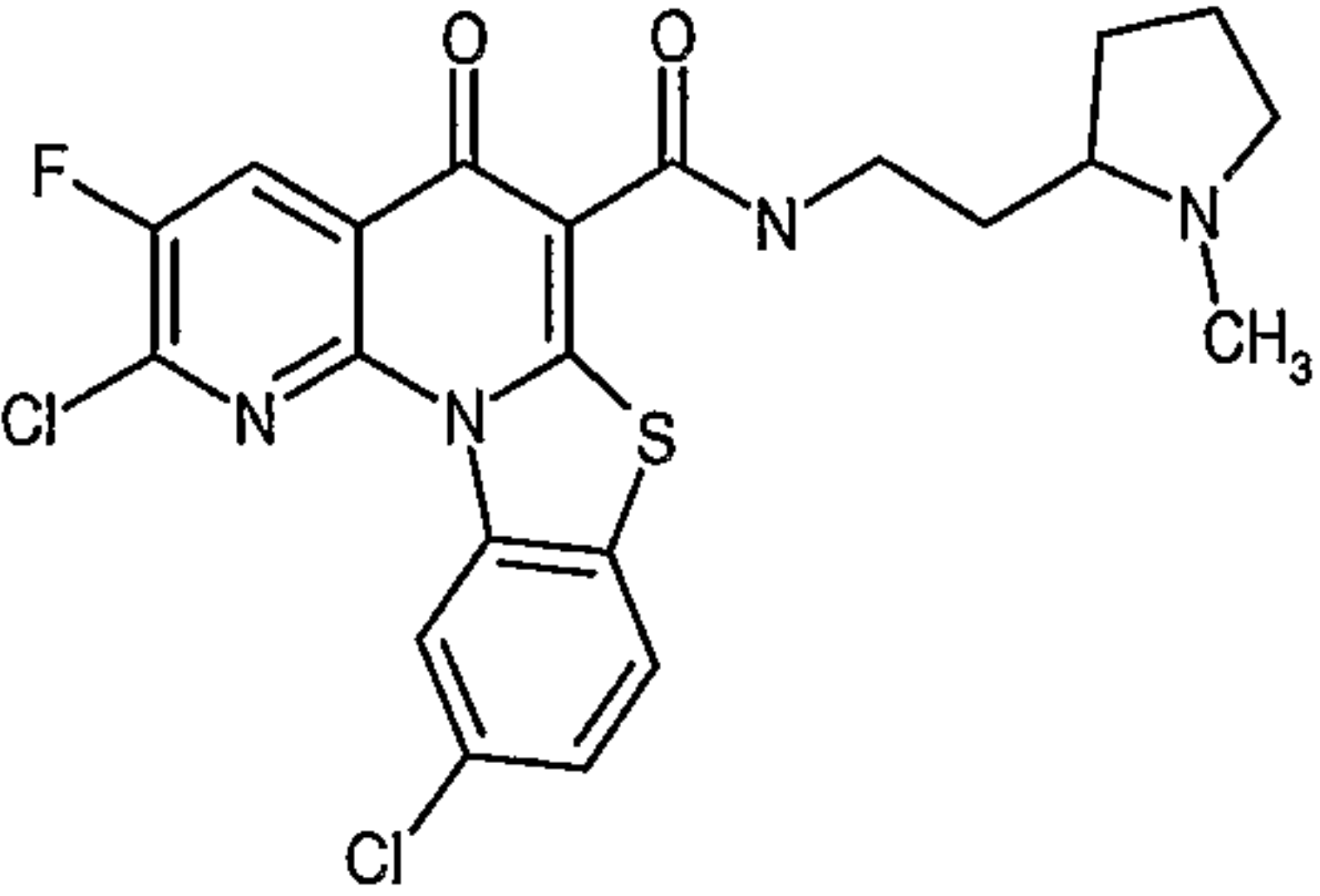
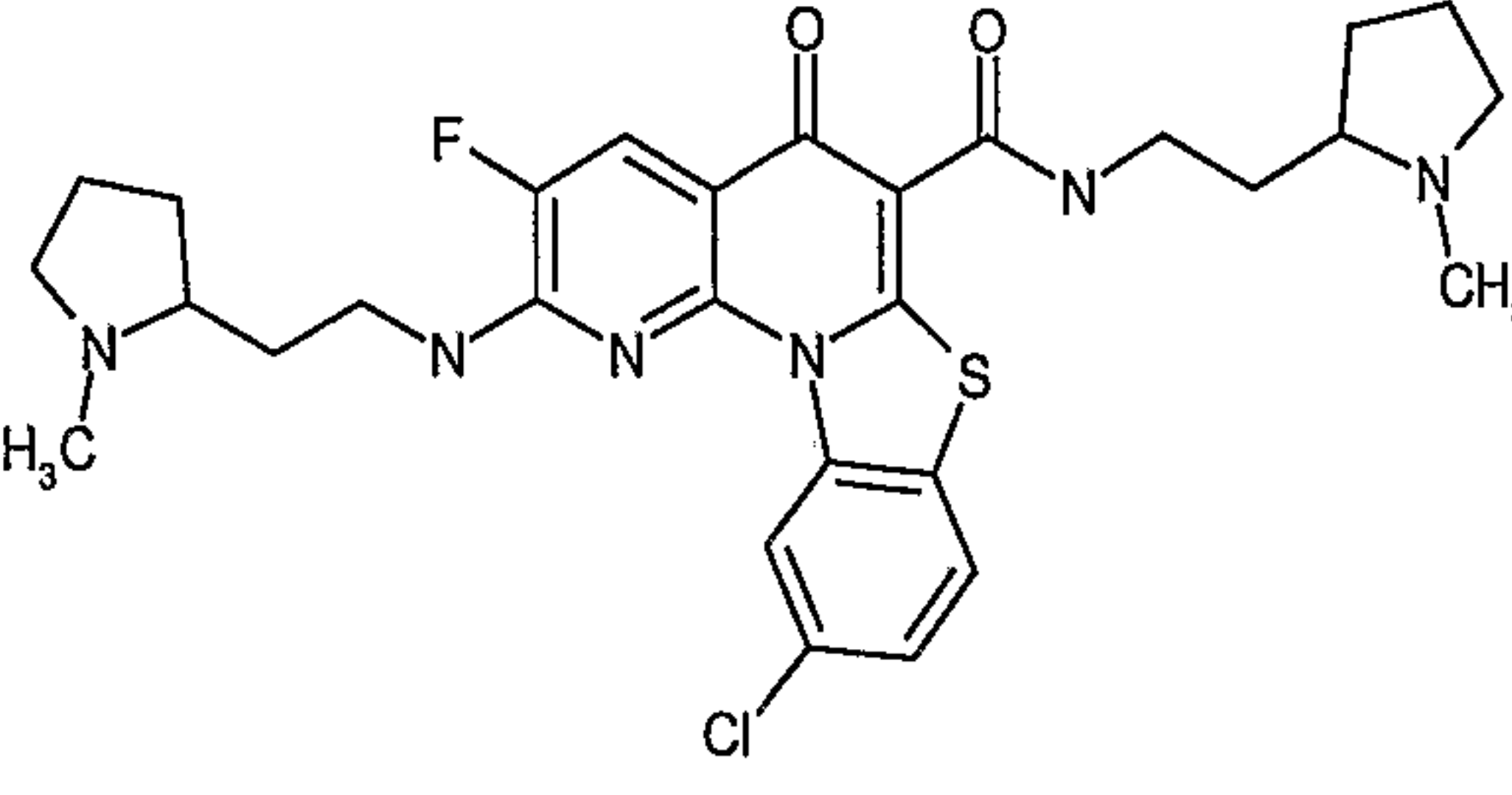
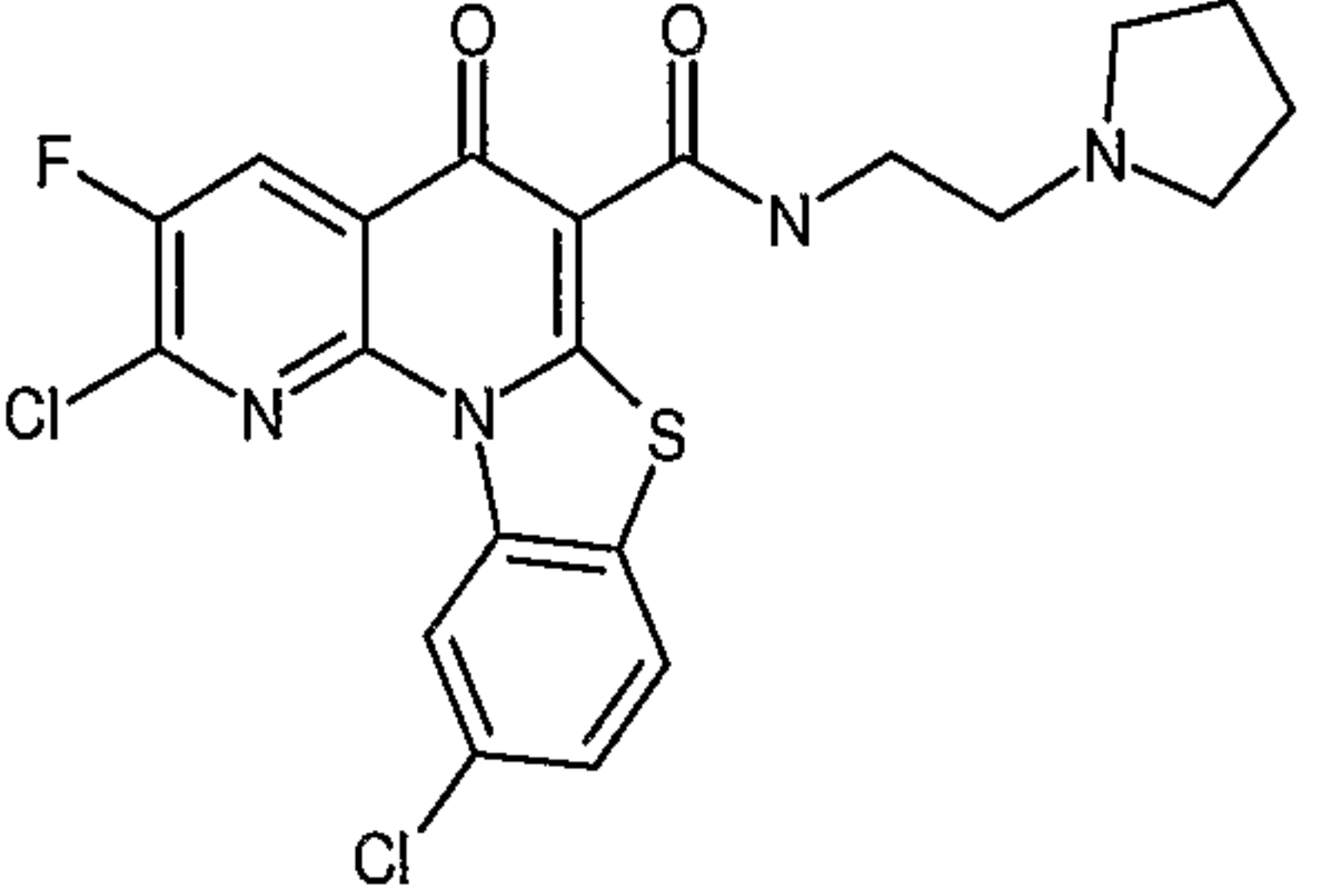
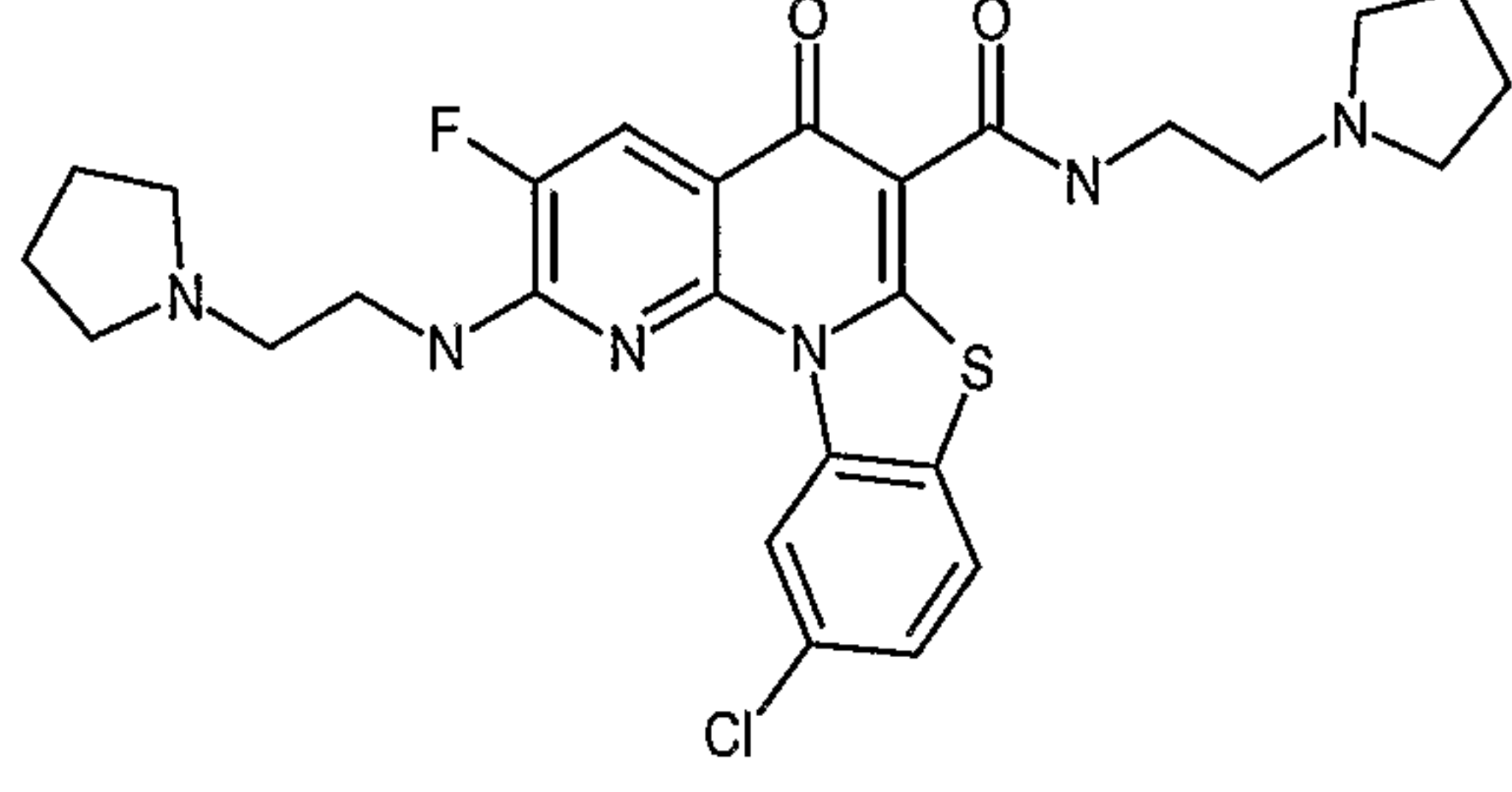
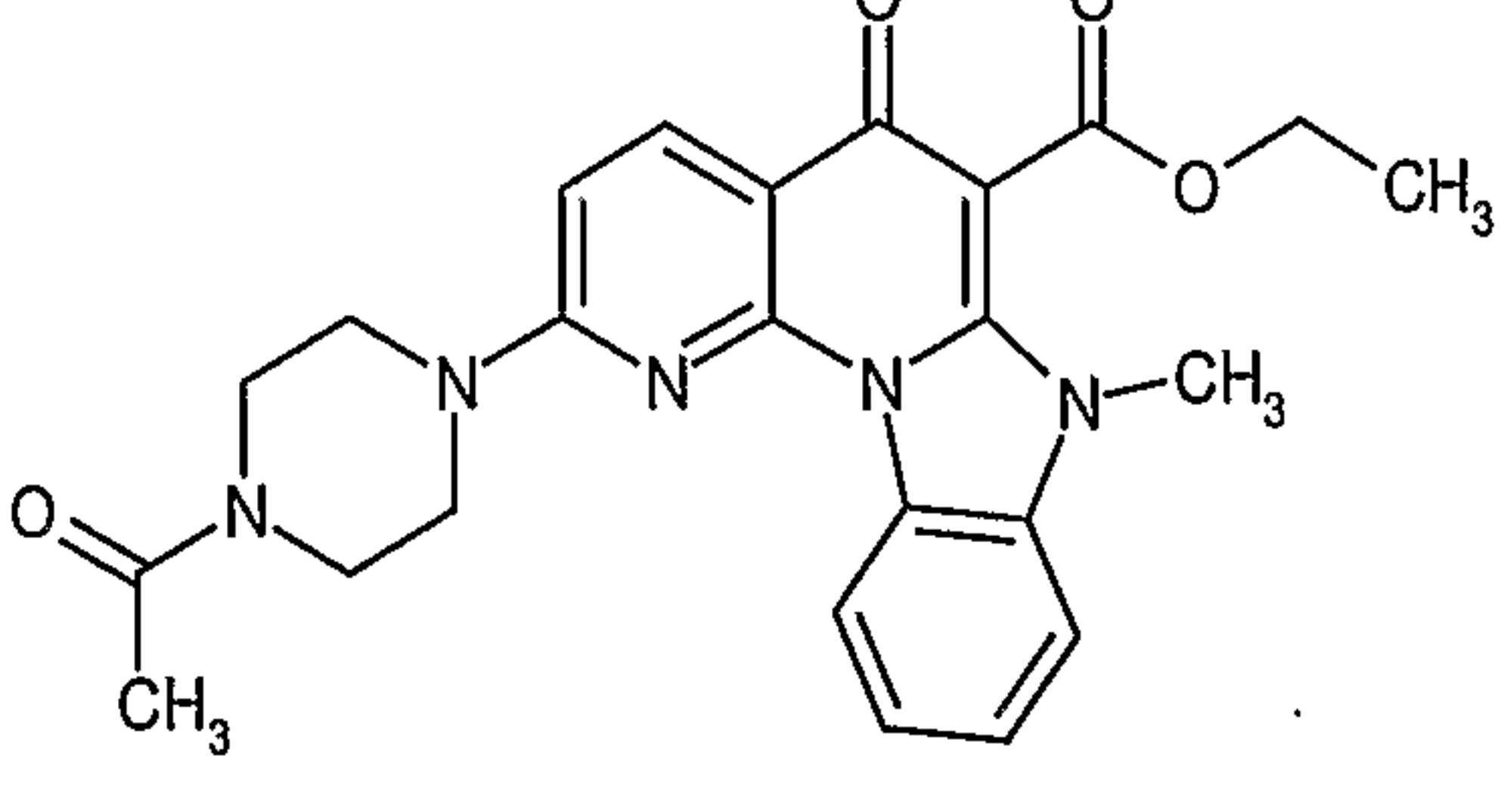
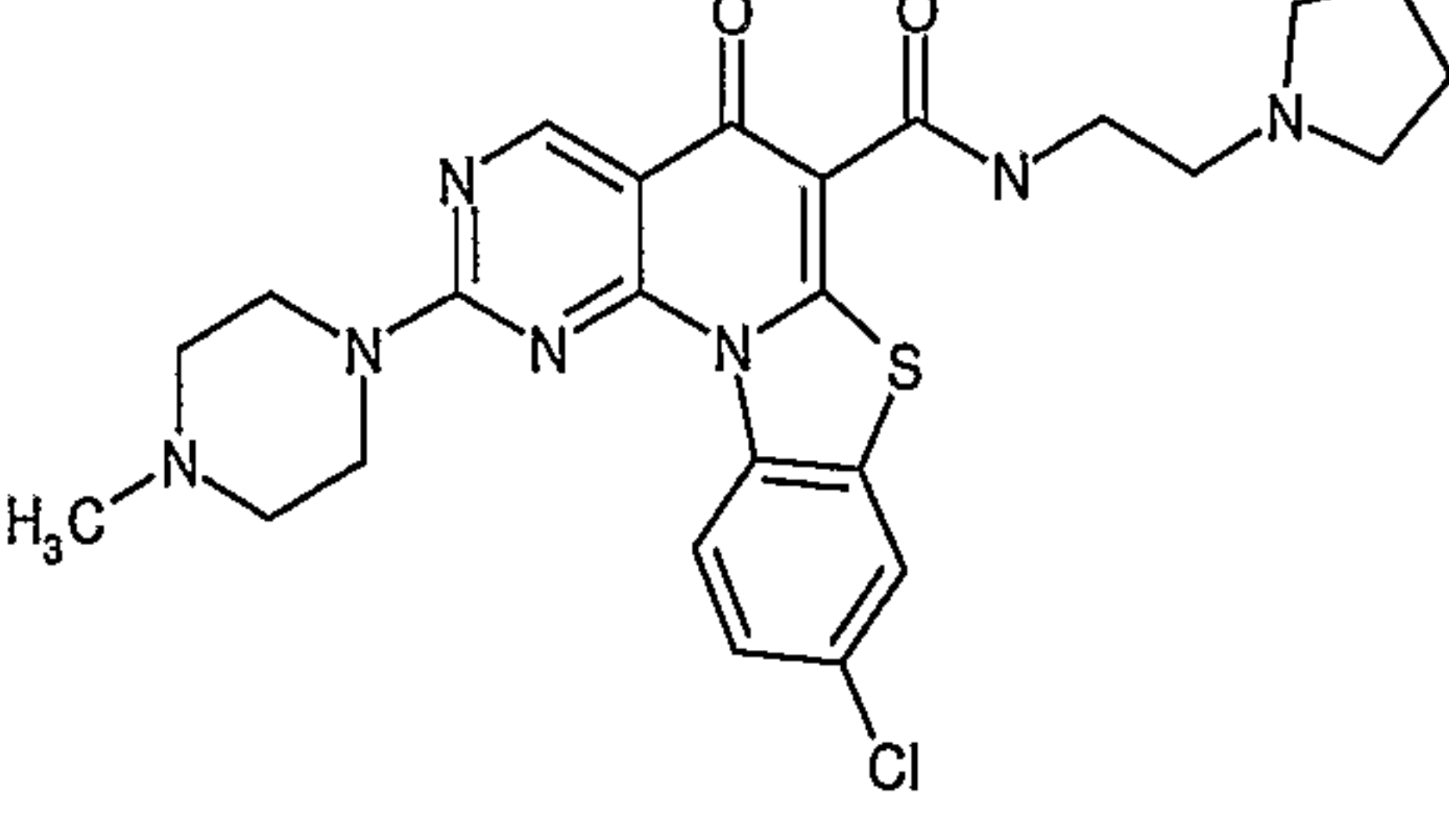
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|   | MiaPaca | 0.03   |
|  | HCT-116 | 0.11   |
|  | MiaPaca | 0.12   |
|  | HCT-116 | 0.16   |
|  | MiaPaca | 0.3    |
|  | HCT-116 | 0.28   |

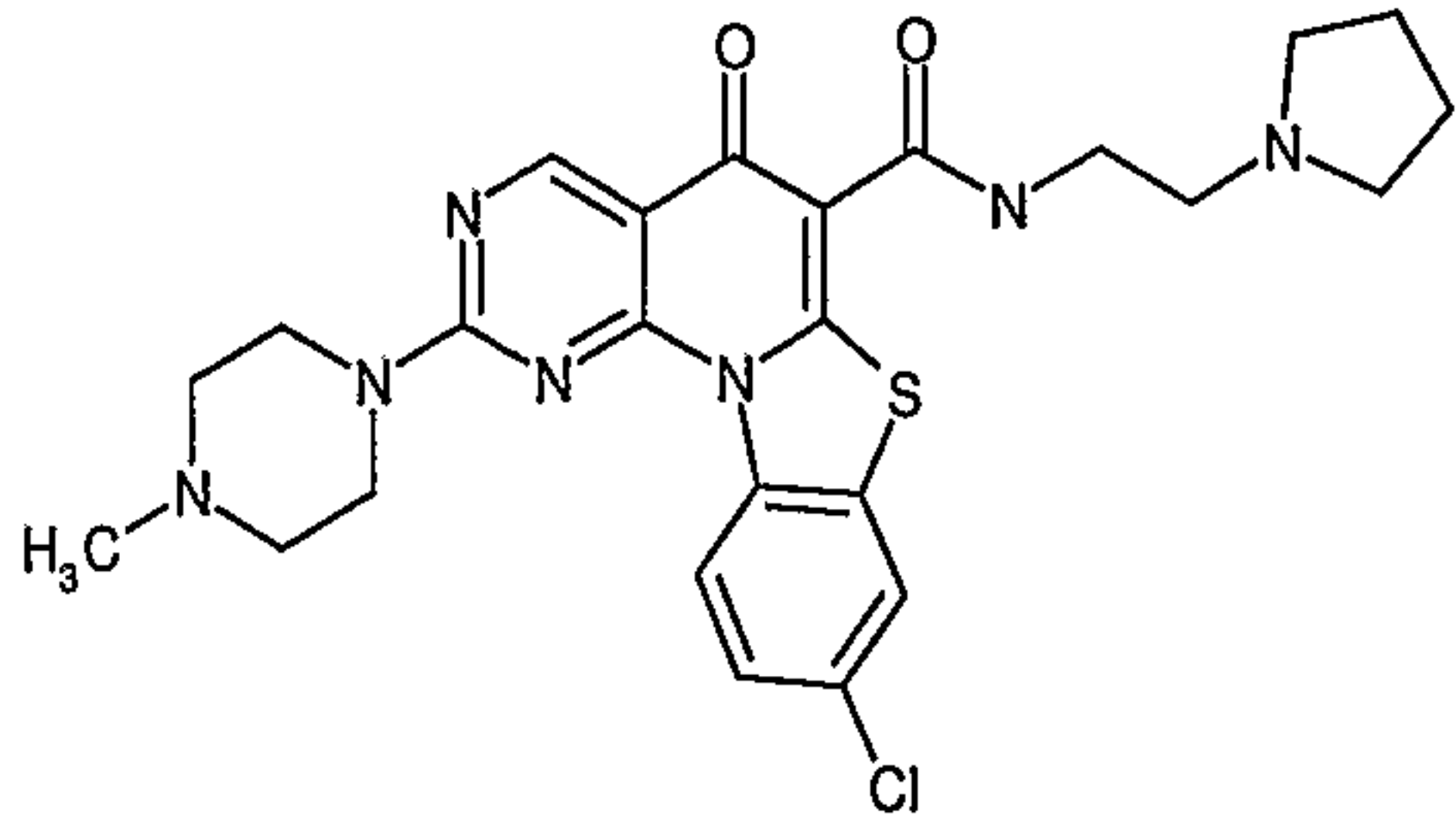
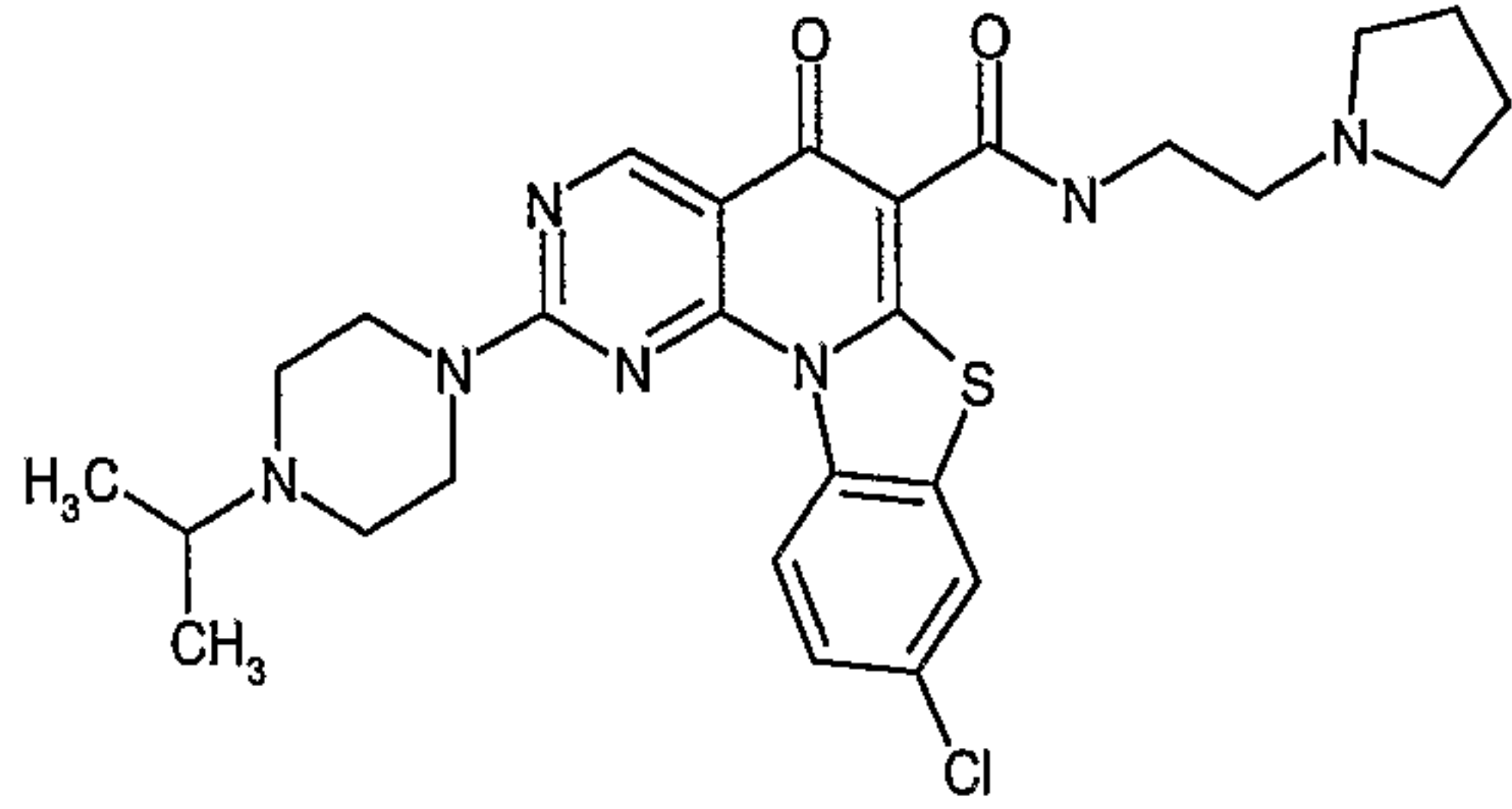
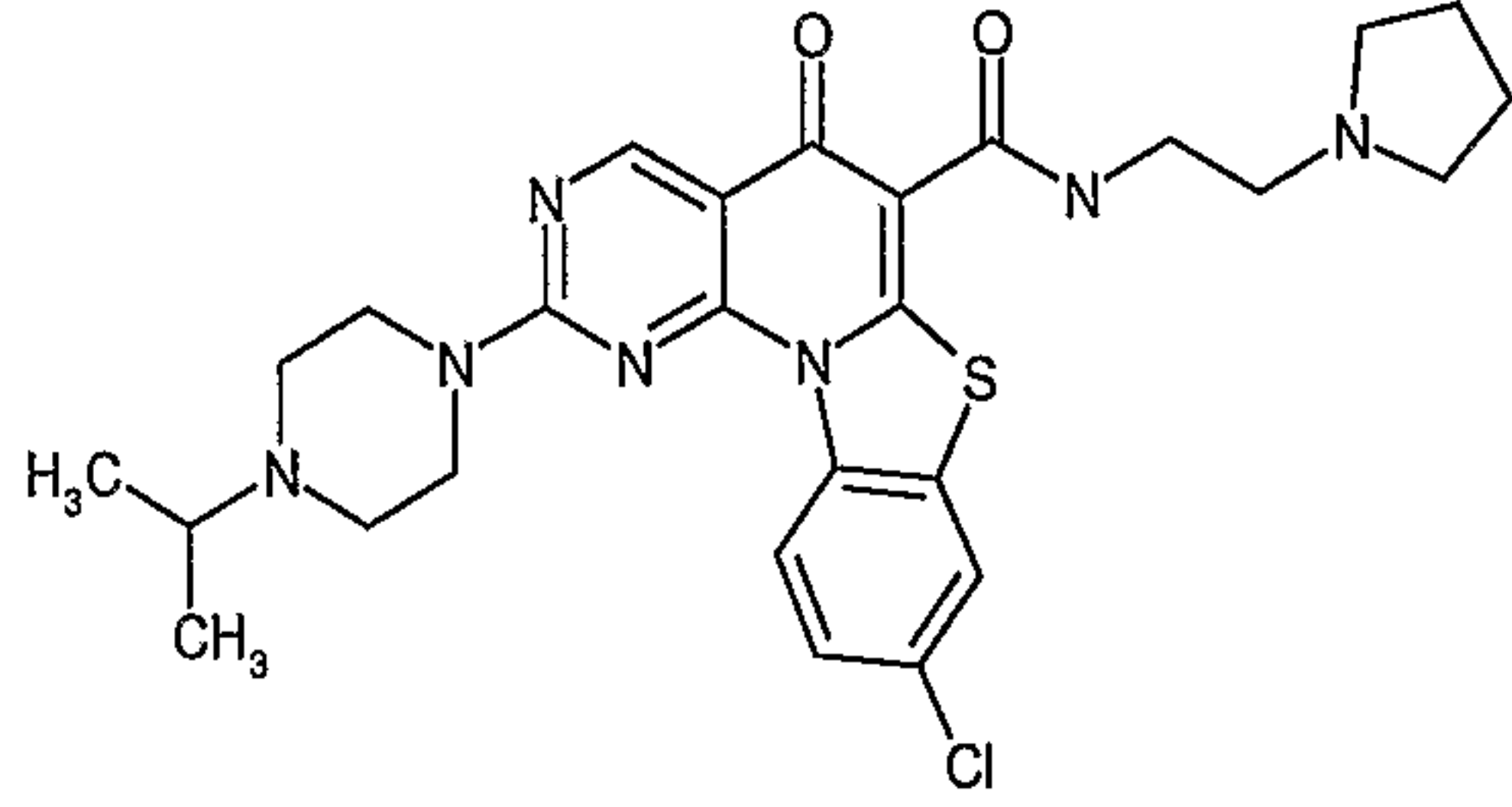
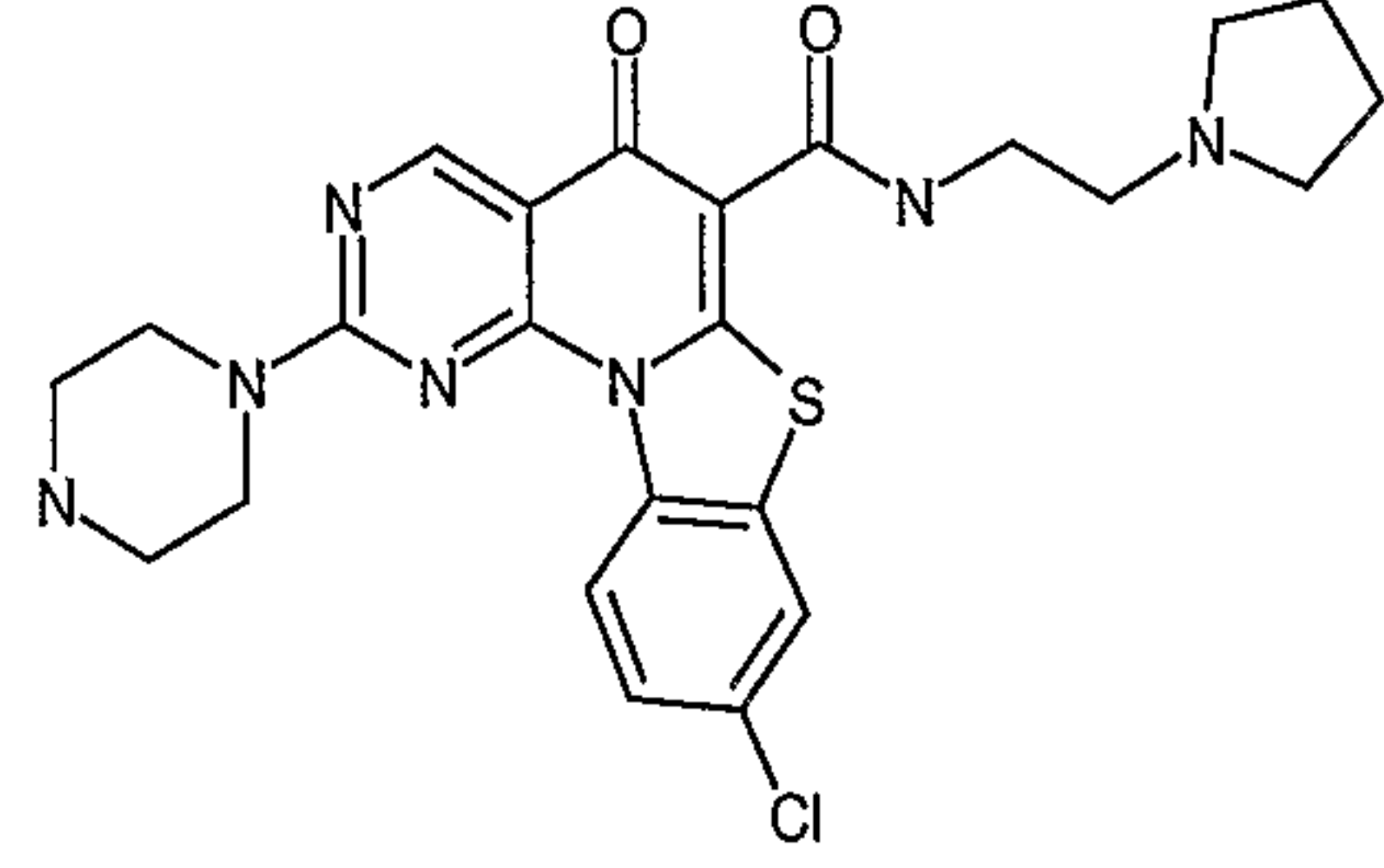
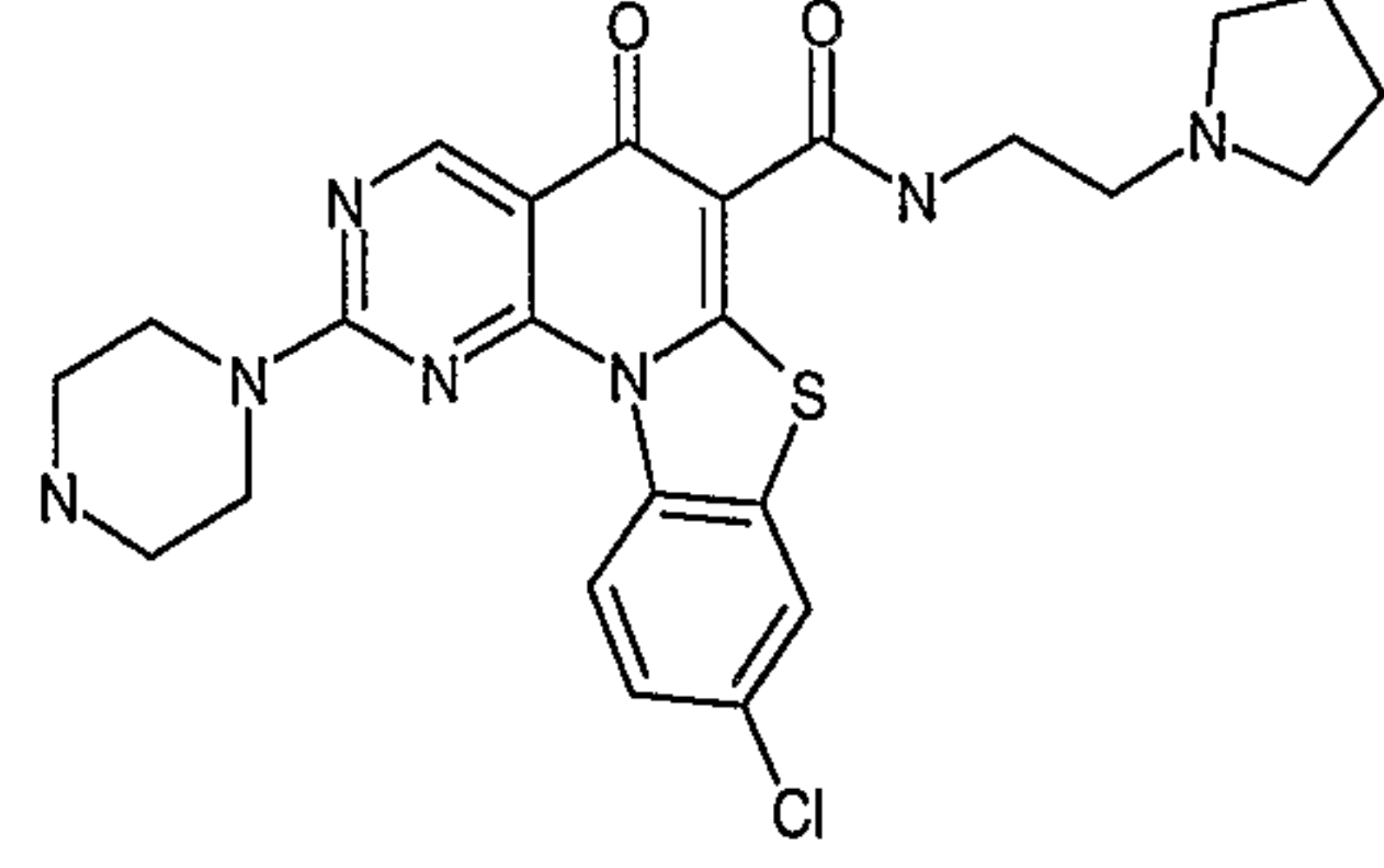
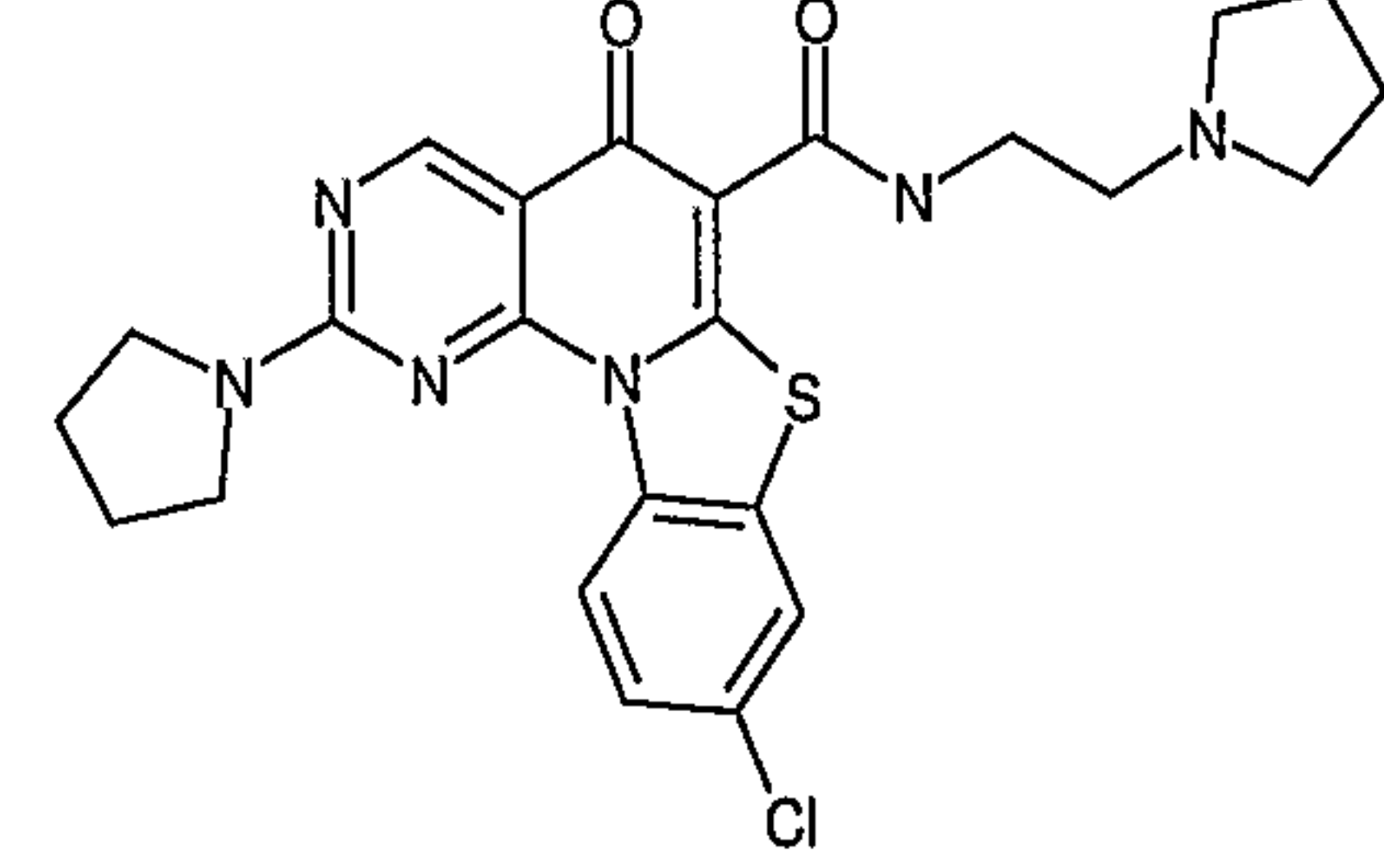
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|   | HCT-116 | 0.22   |
|  | MiaPaca | 0.39   |
|  | MiaPaca | >10    |
|  | HCT-116 | 10     |
|  | HCT-116 | 10     |

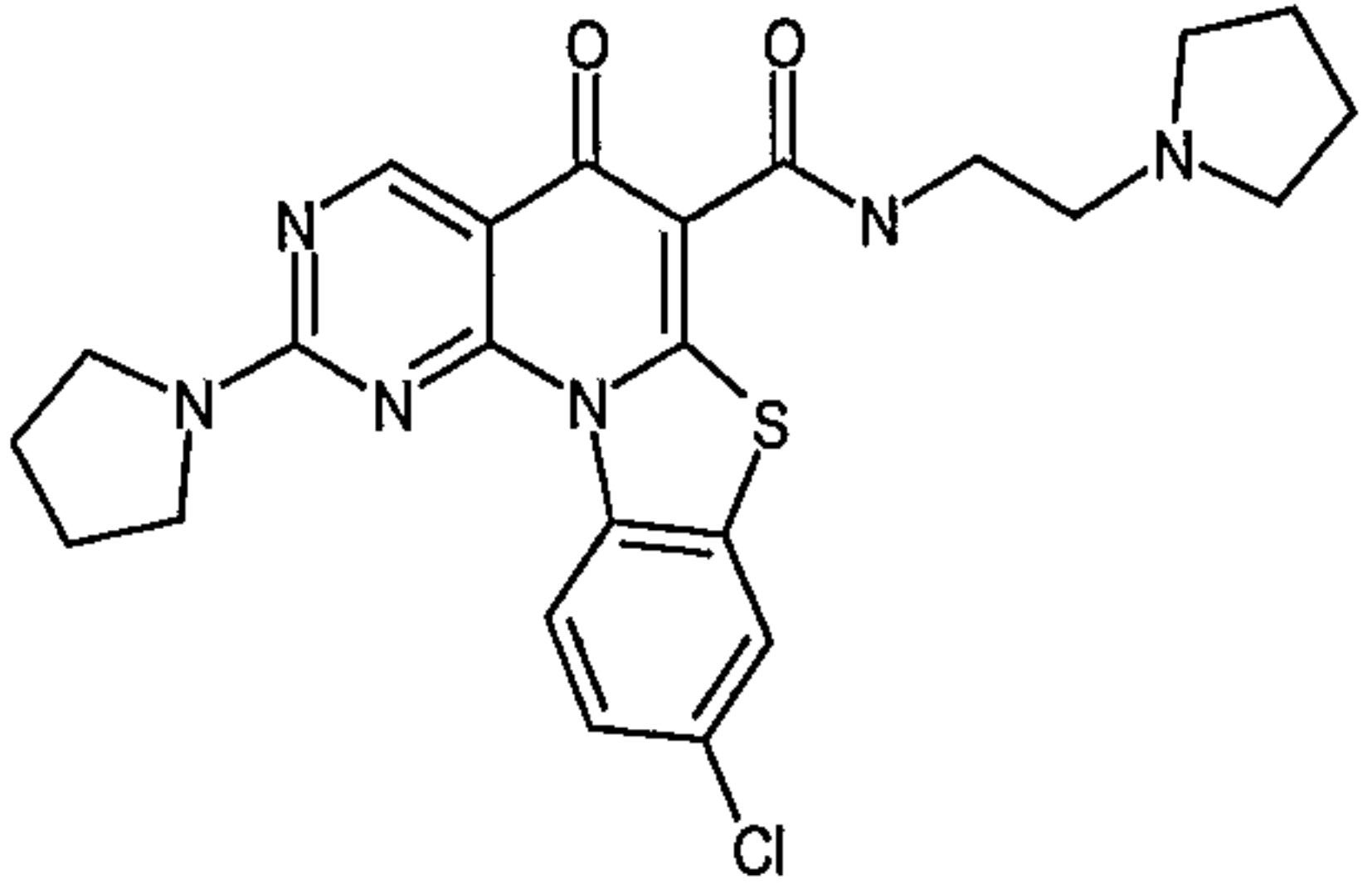
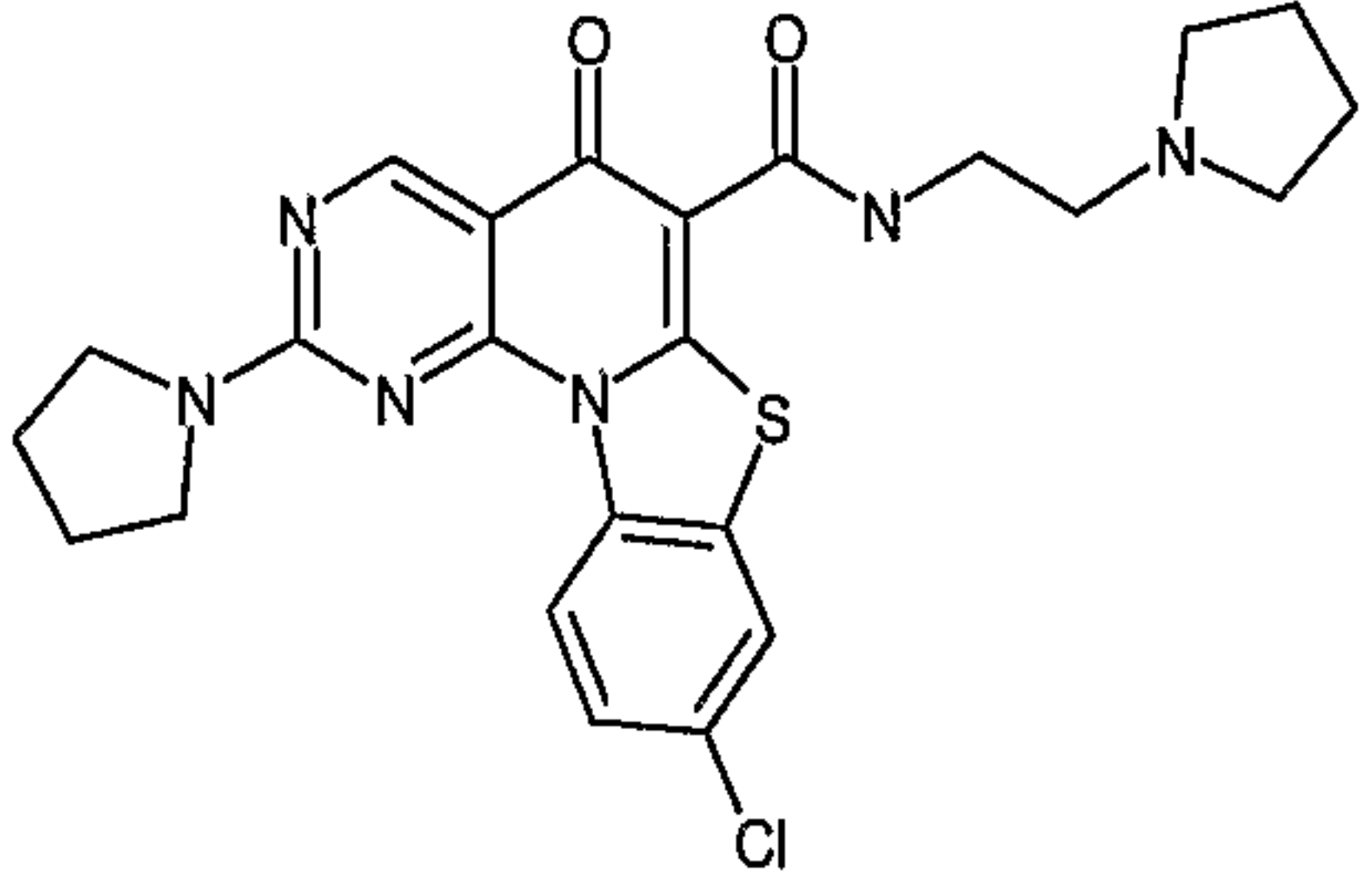
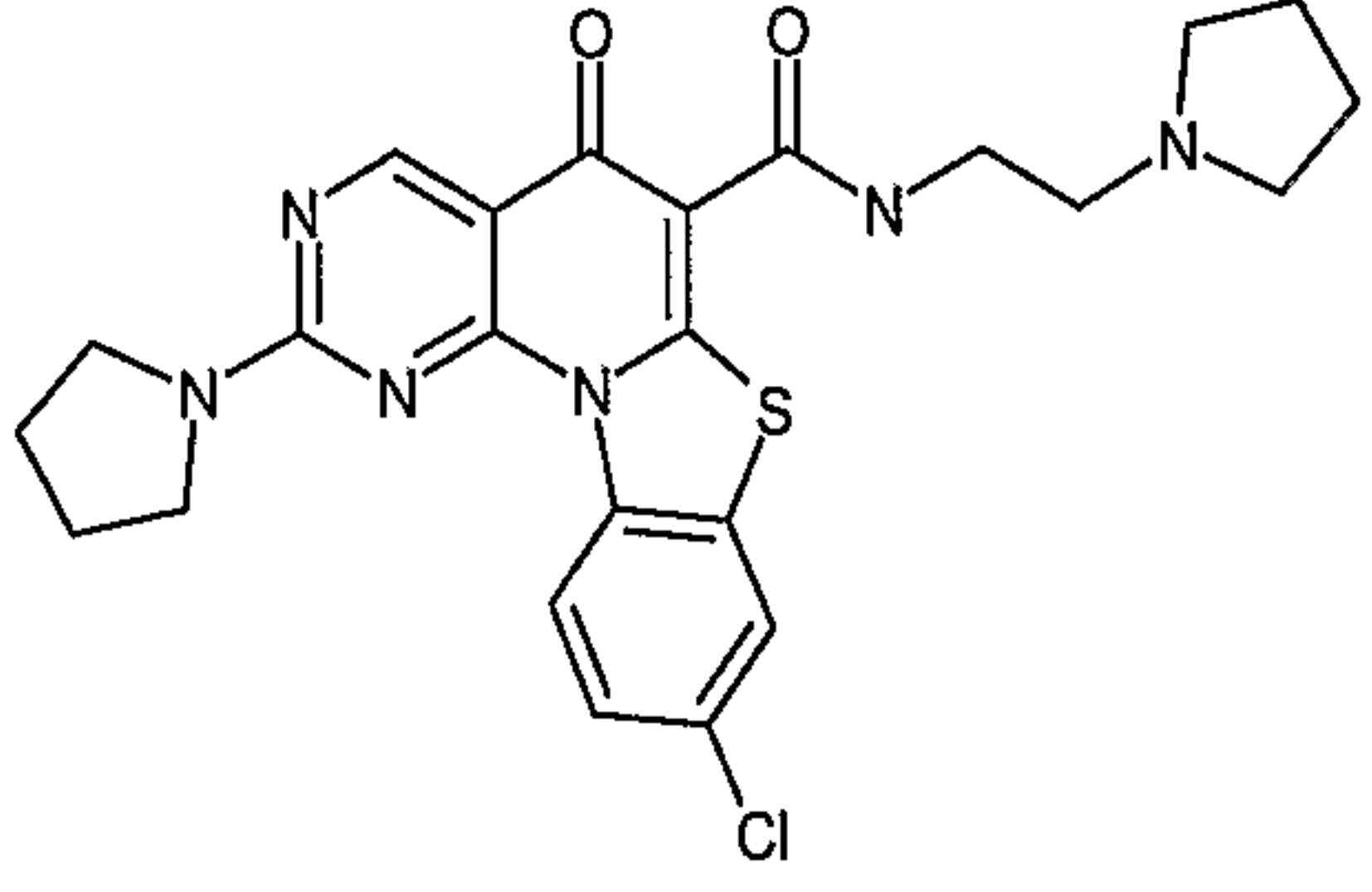
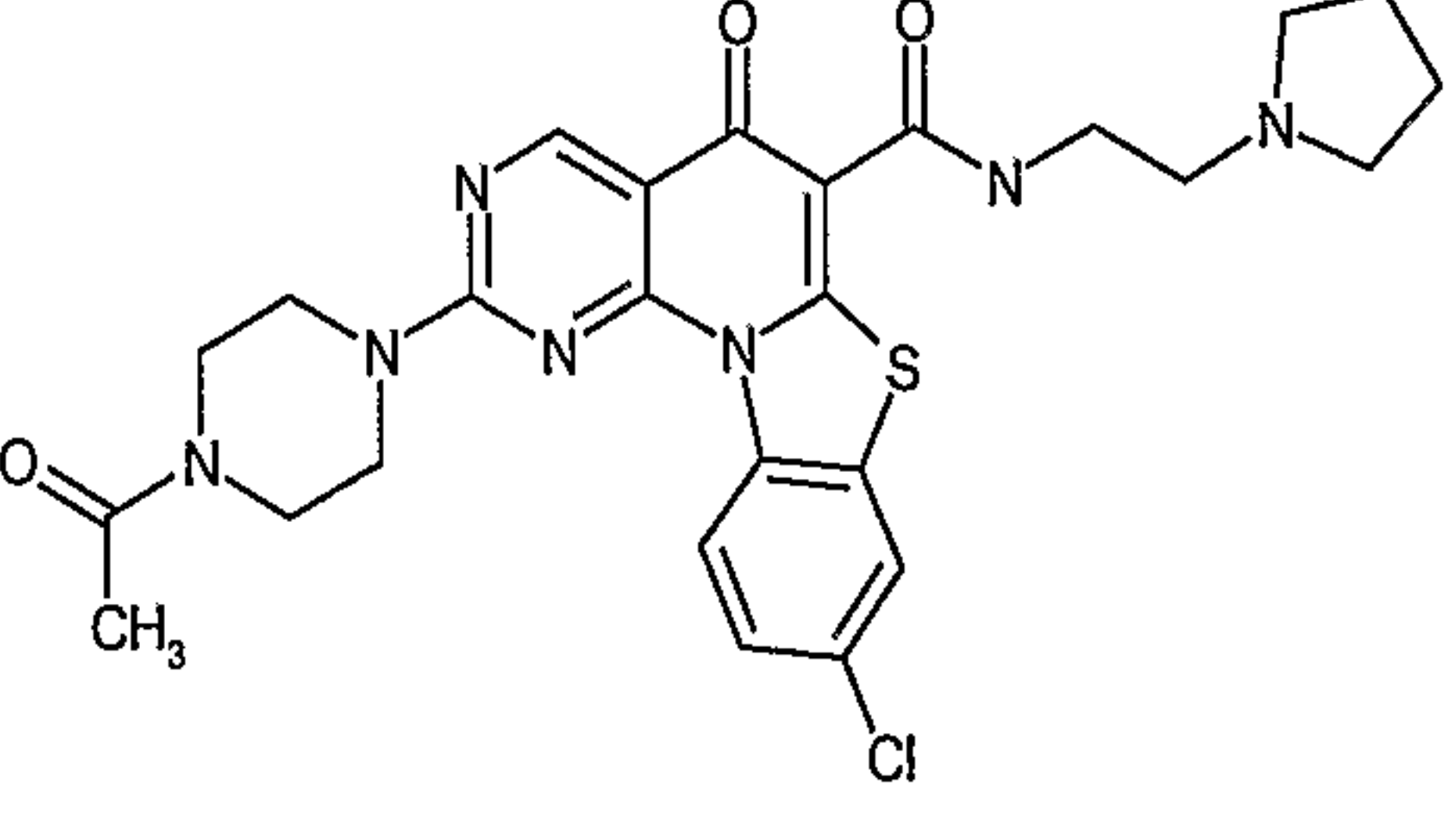
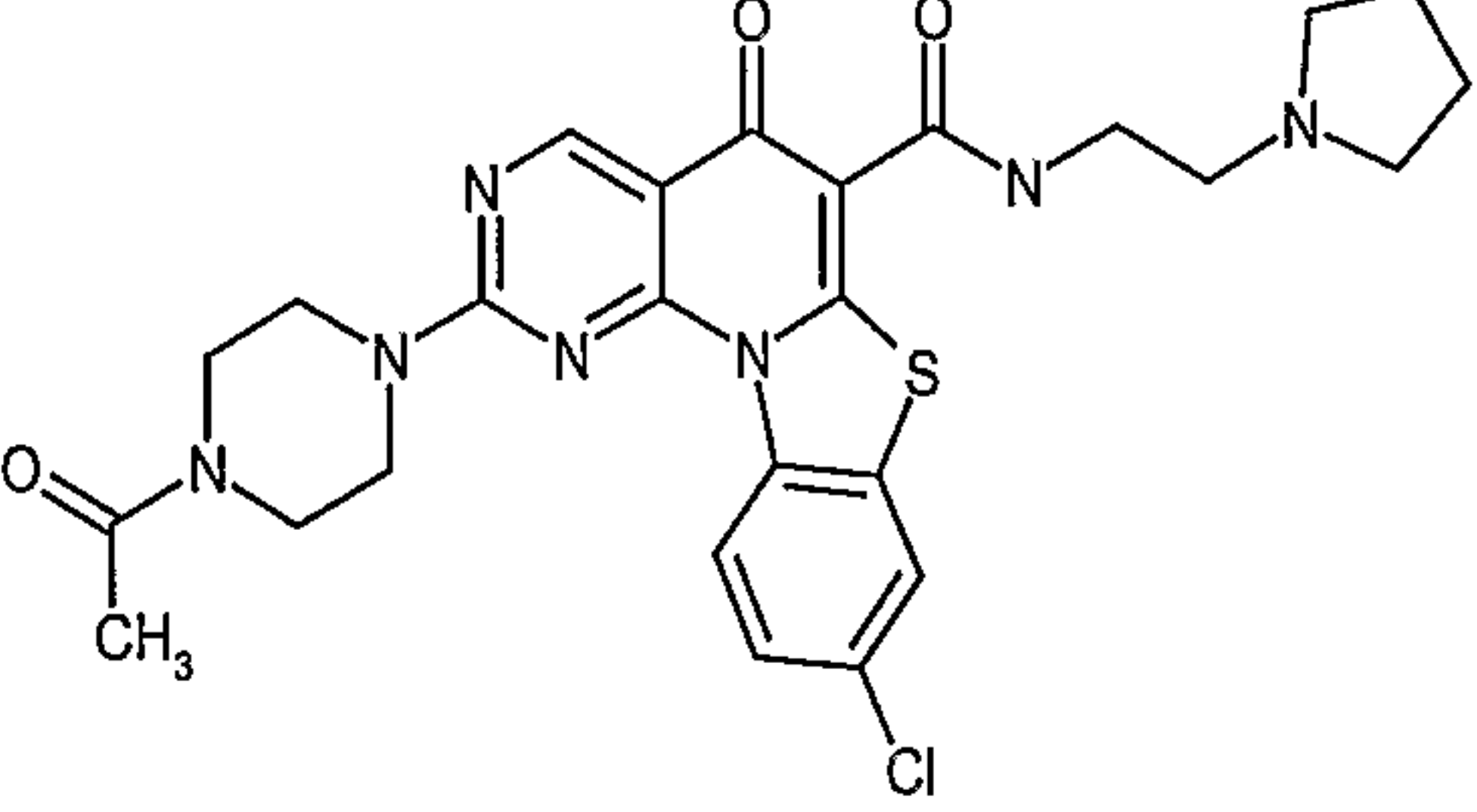
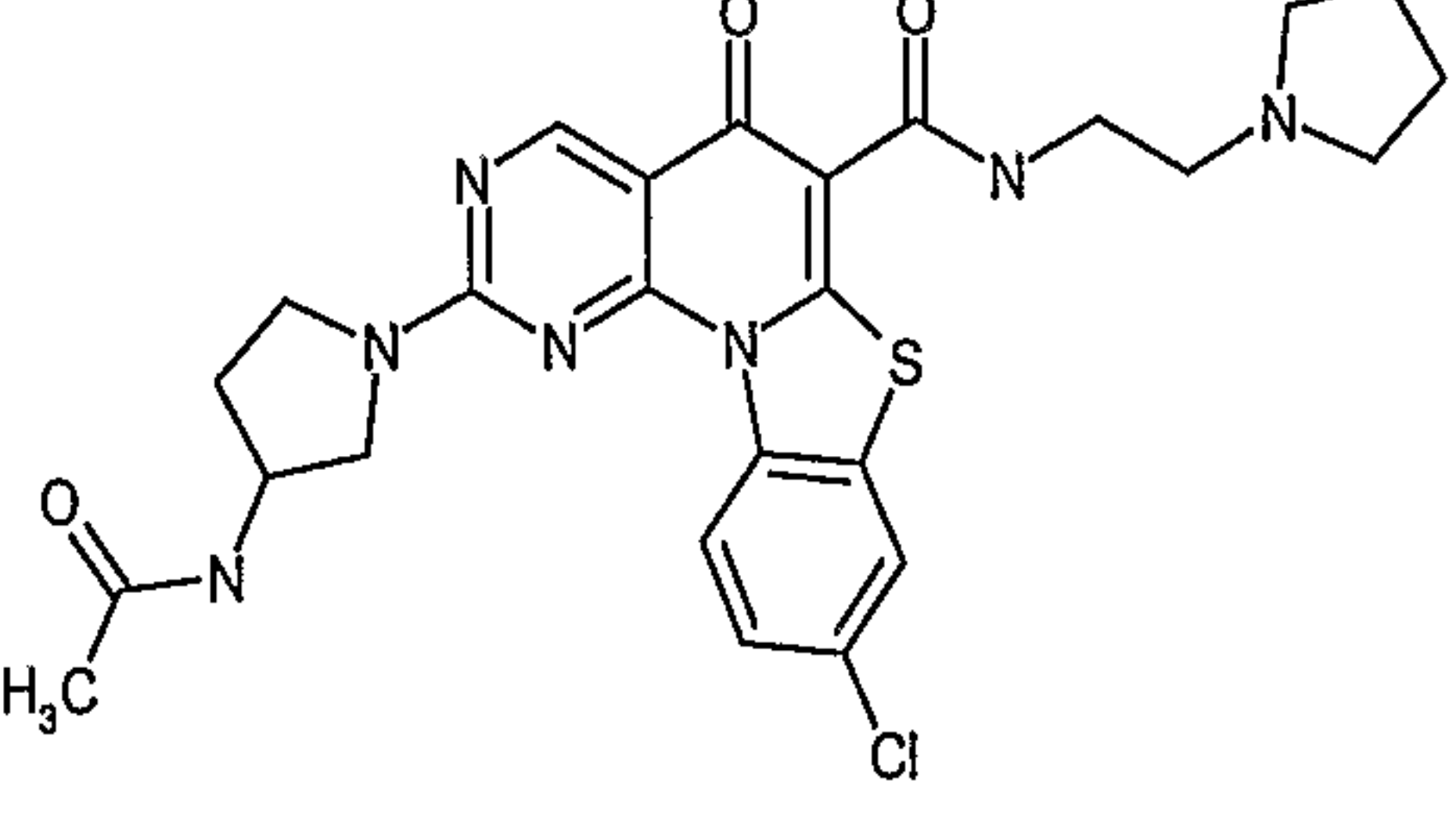
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | 4      |
|   | HCT-116 | 10     |
|  | MiaPaca | 4.2    |
|  | HCT-116 | 3.0    |
|  | MiaPaca | 3.9    |

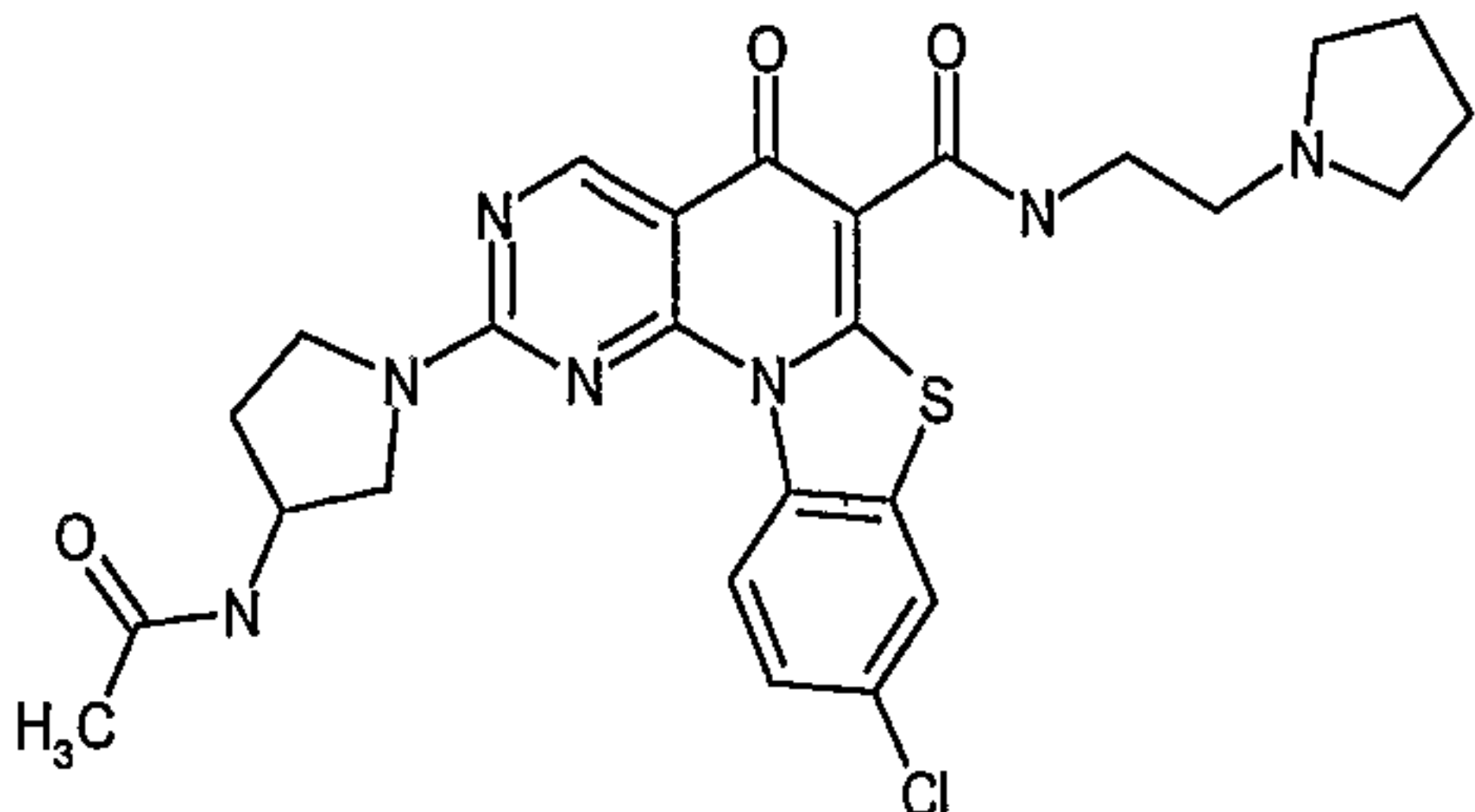
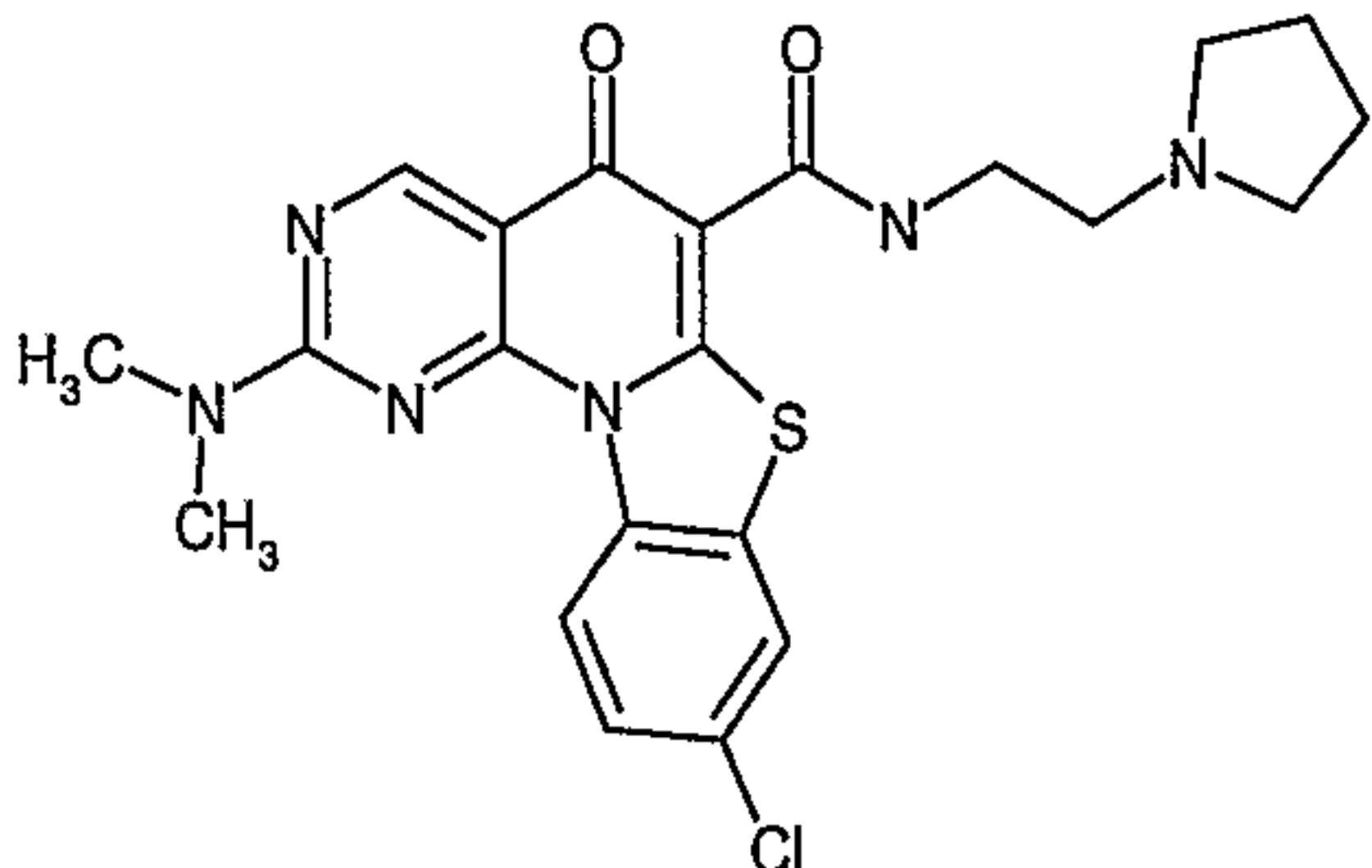
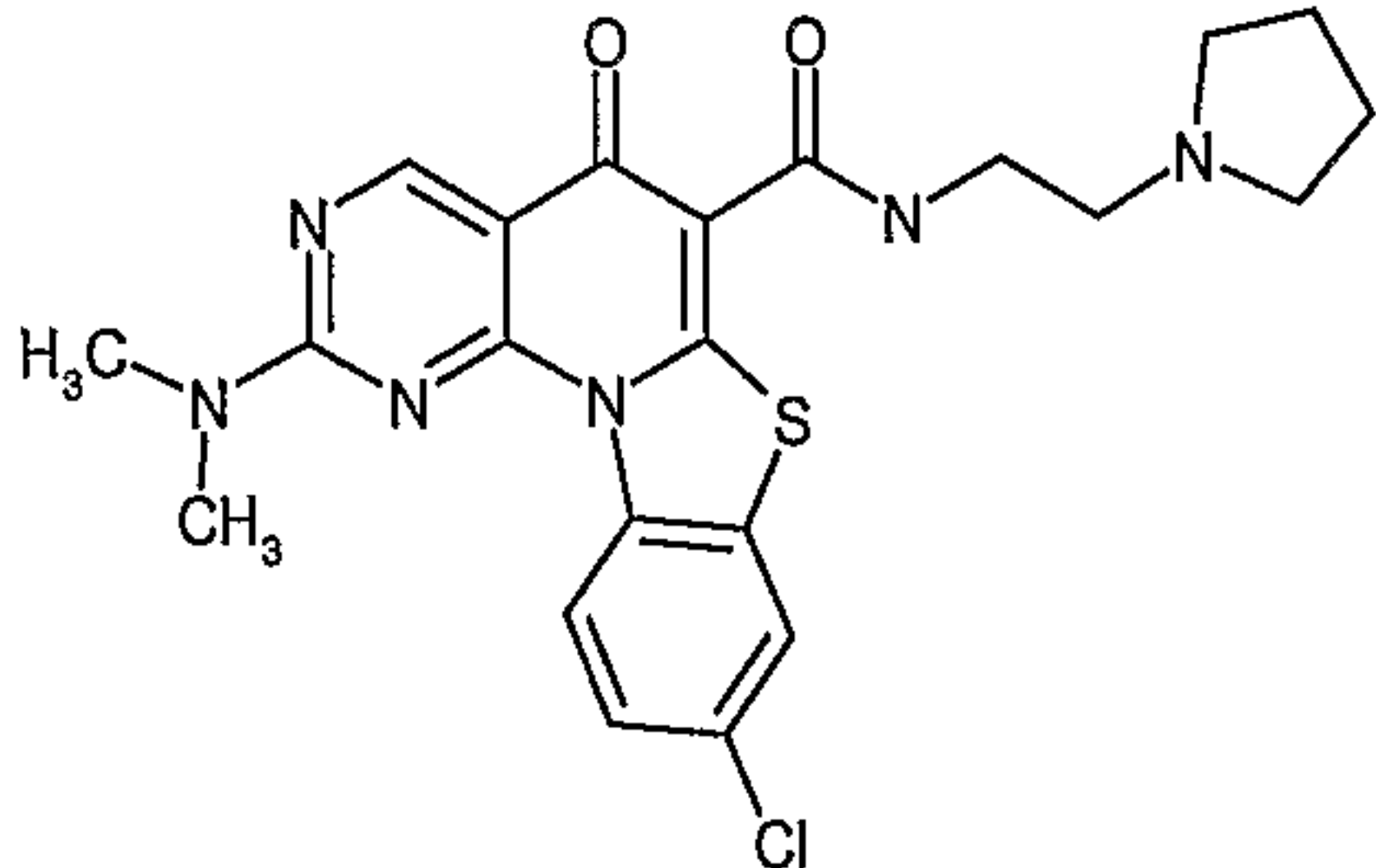
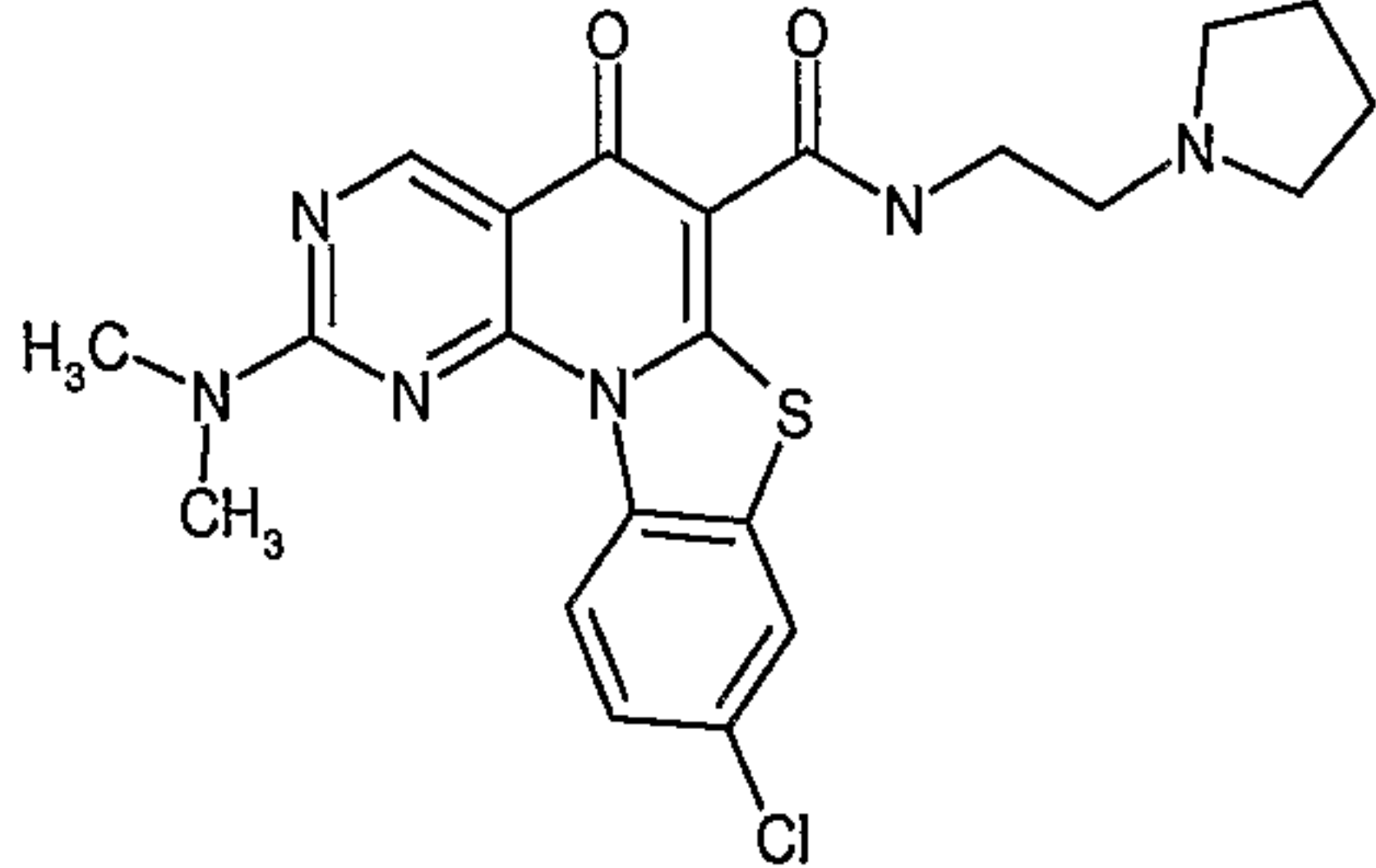
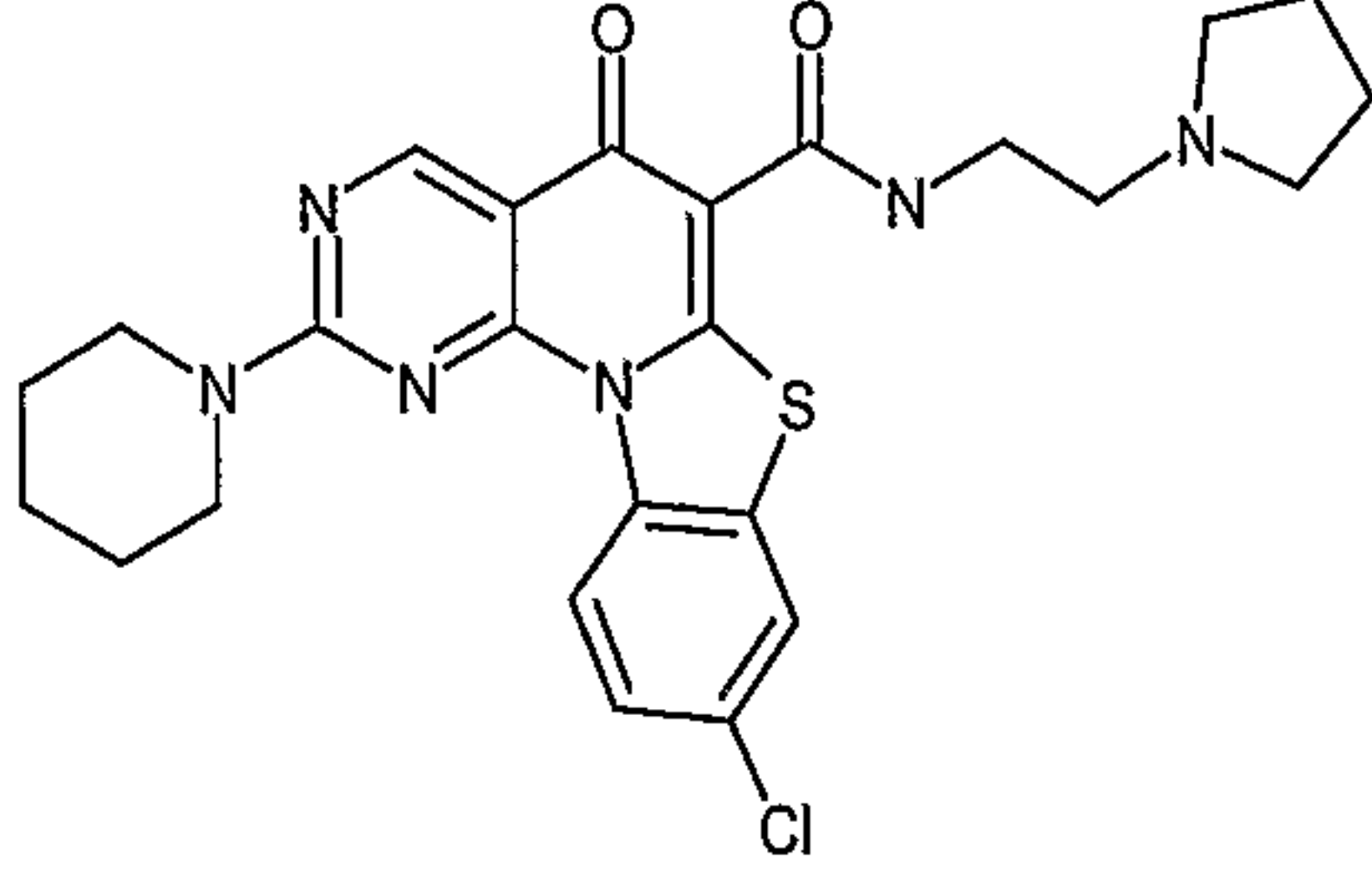
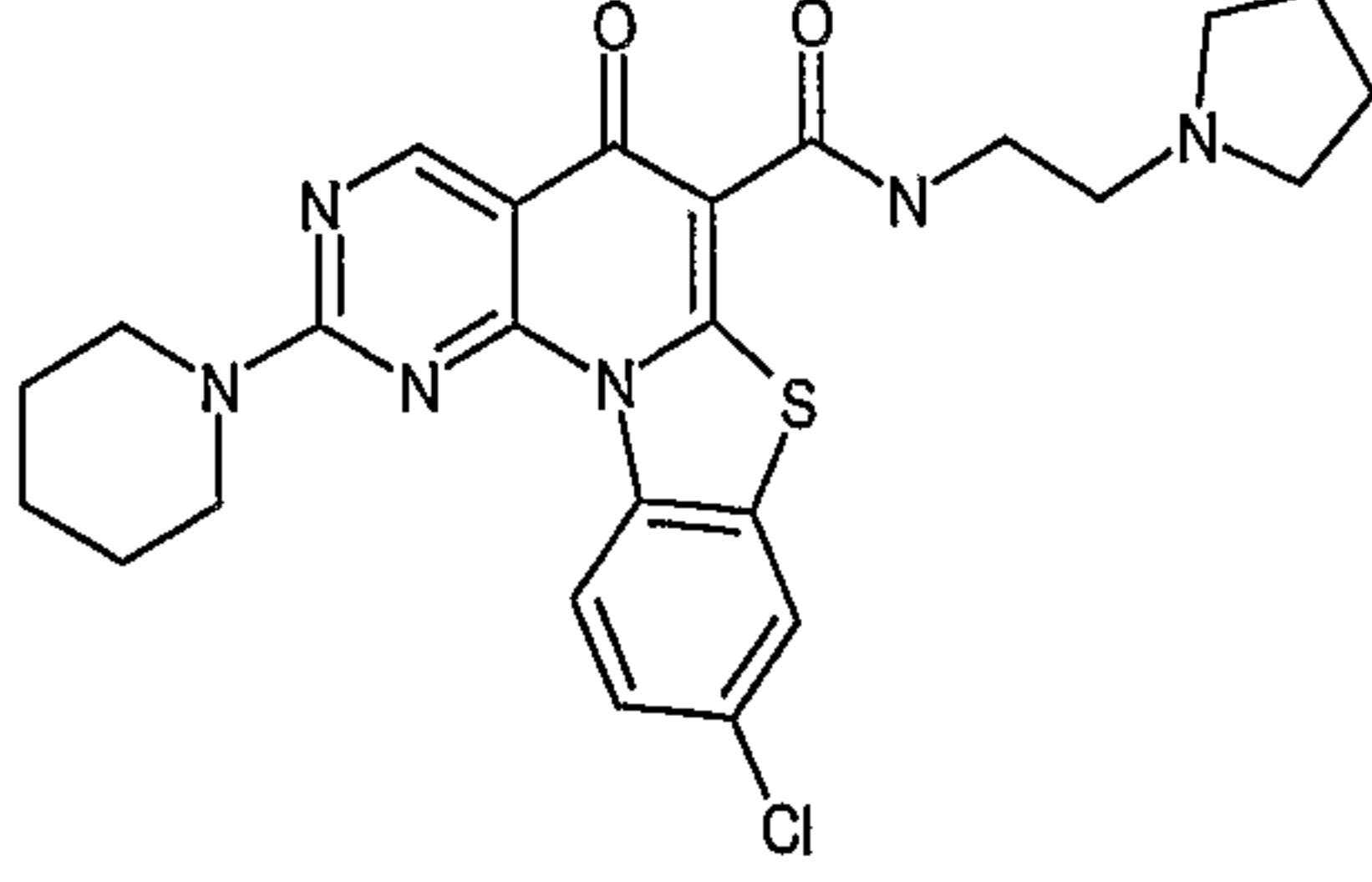
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 4.5    |
|   | MiaPaca | 6.6    |
|  | HCT-116 | 3.5    |
|  | MiaPaca | >10    |
|  | HCT-116 | 10     |
|  | MiaPaca | 4.3    |

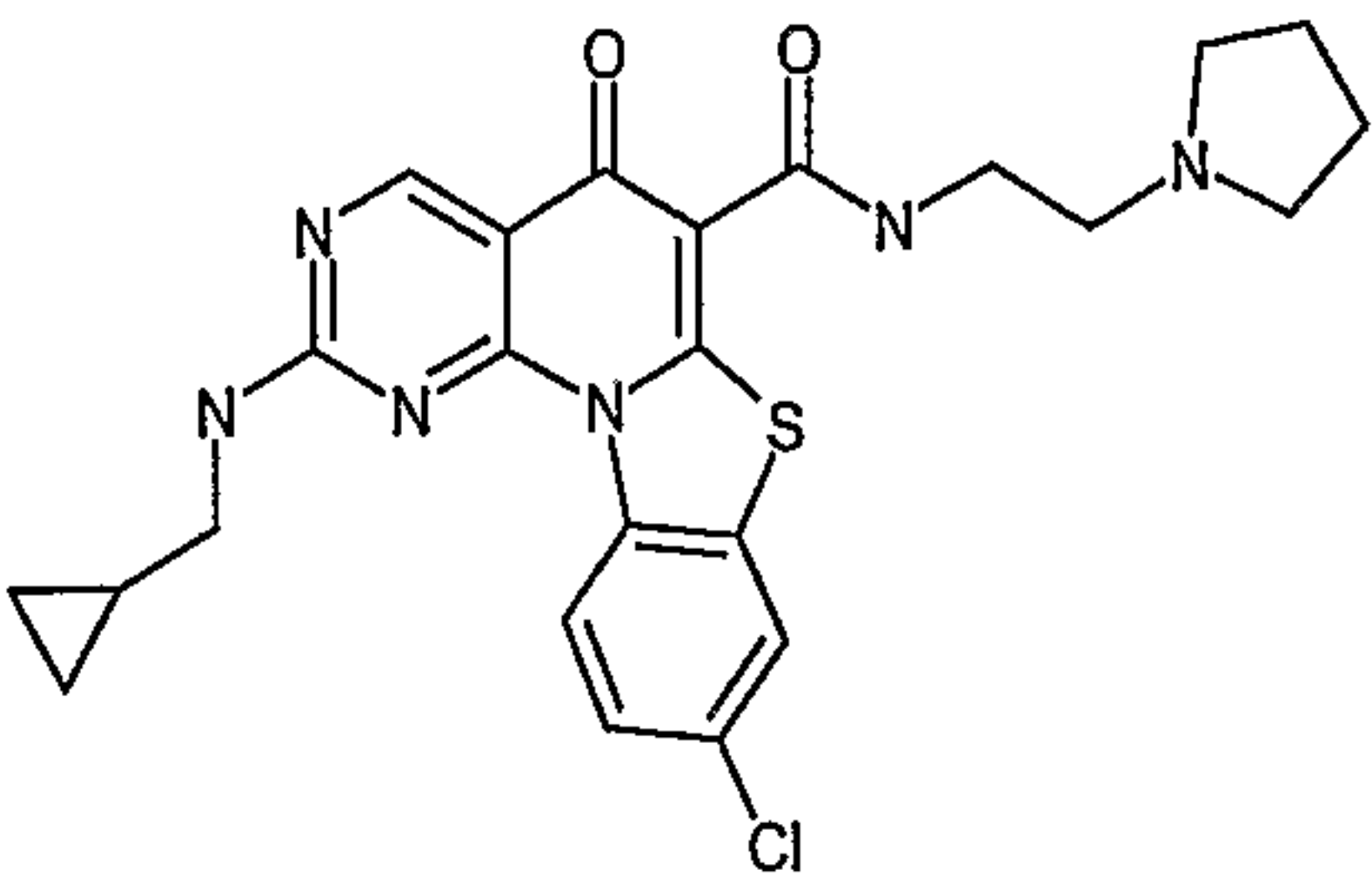
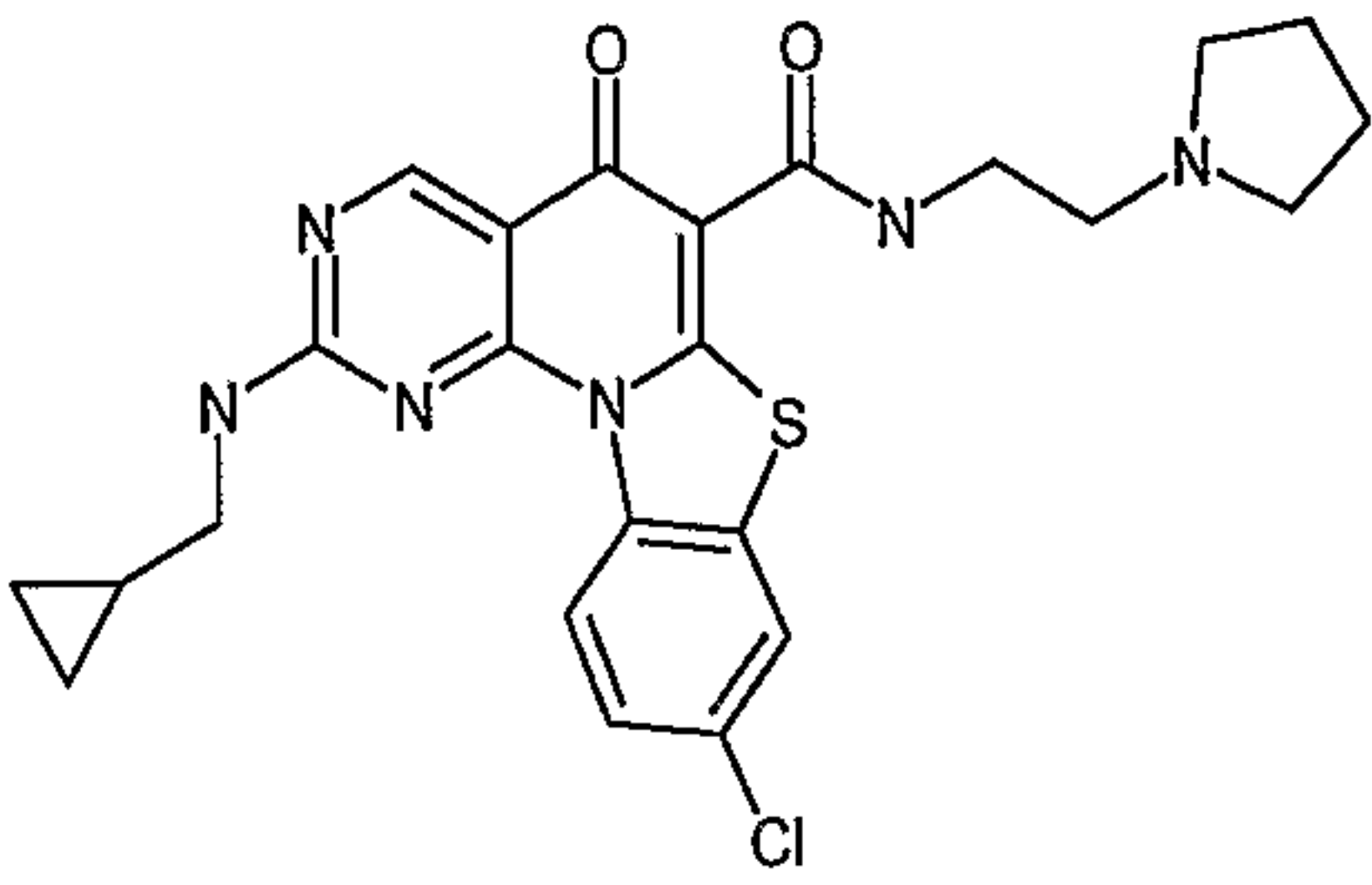
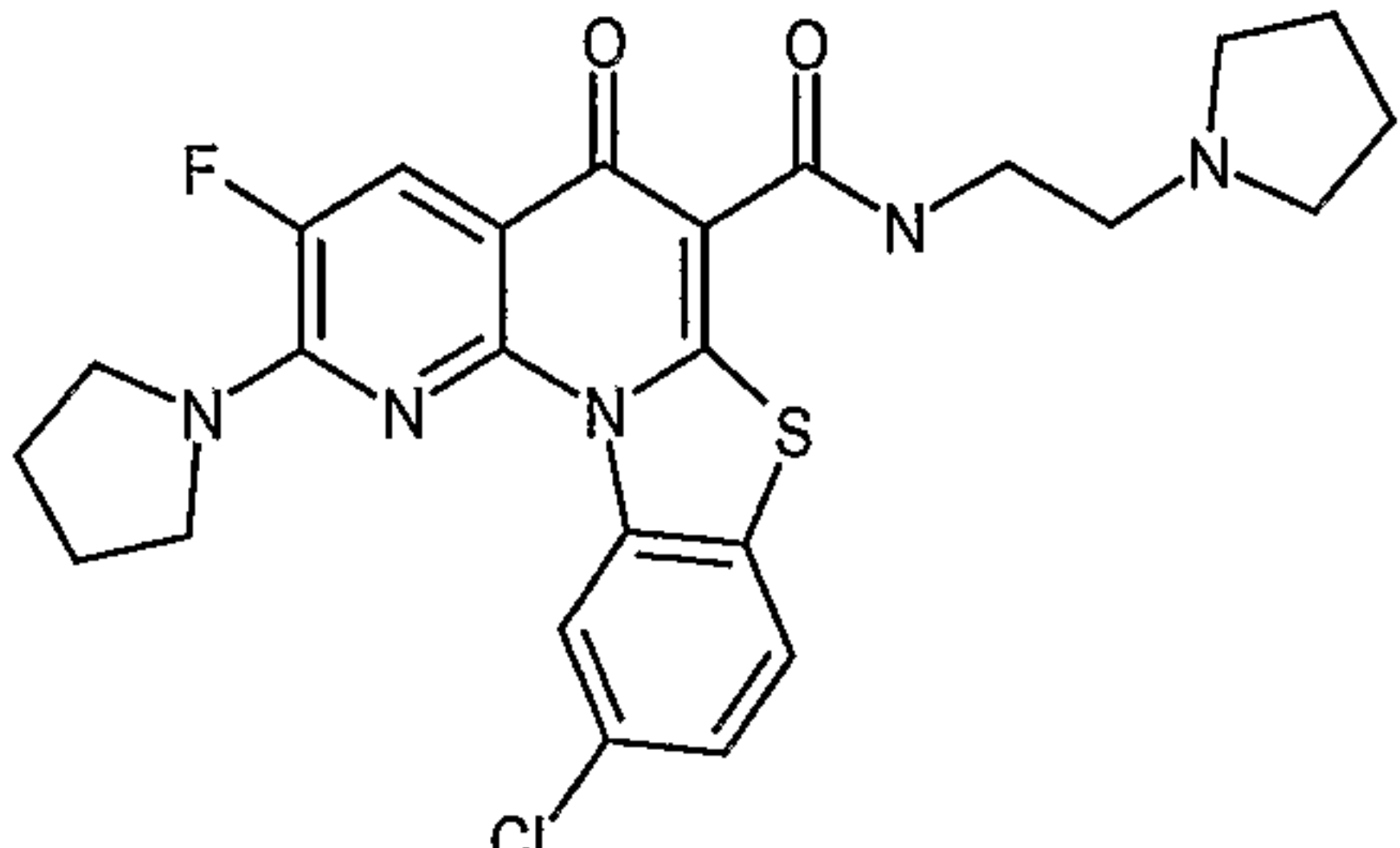
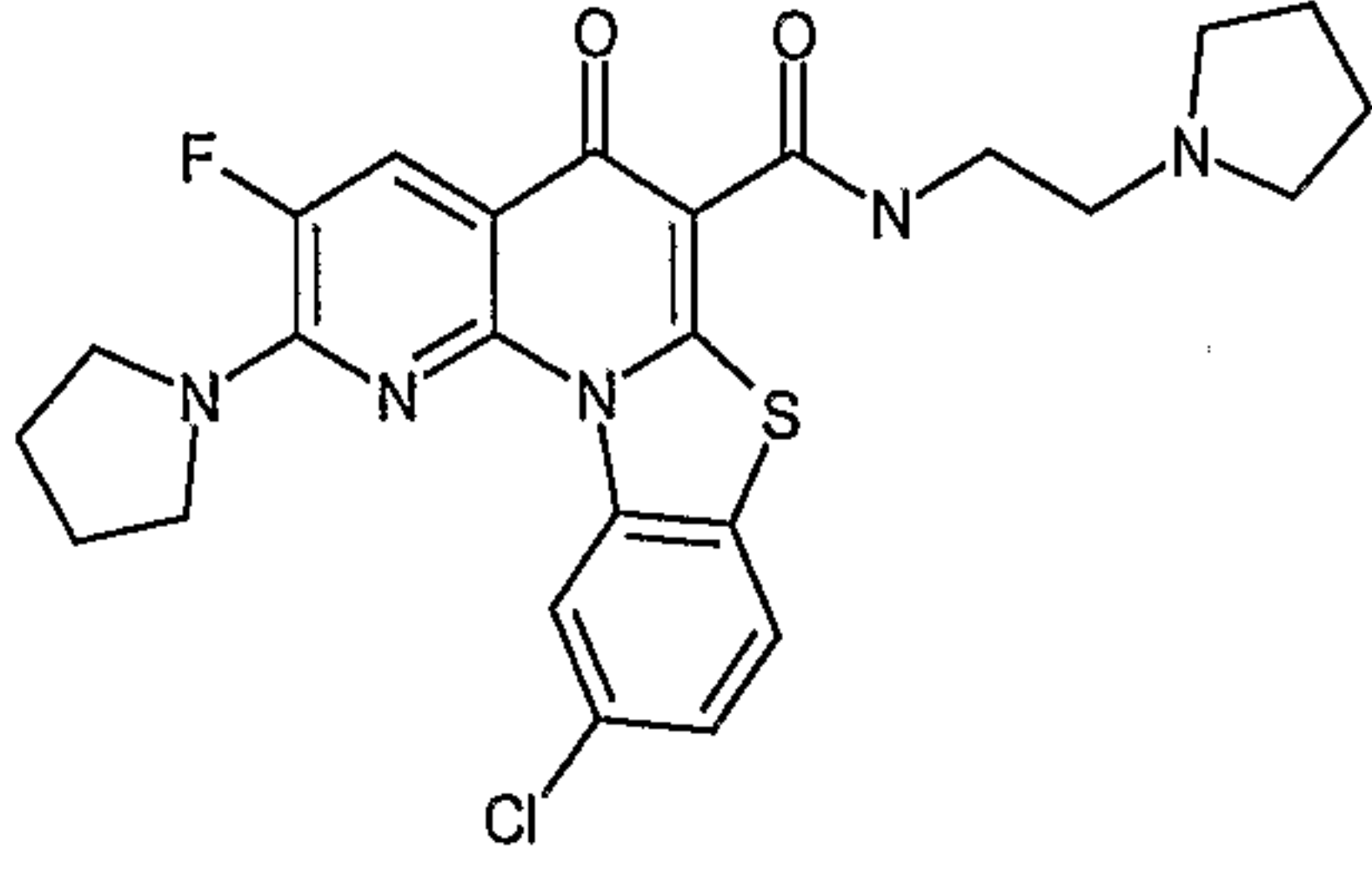
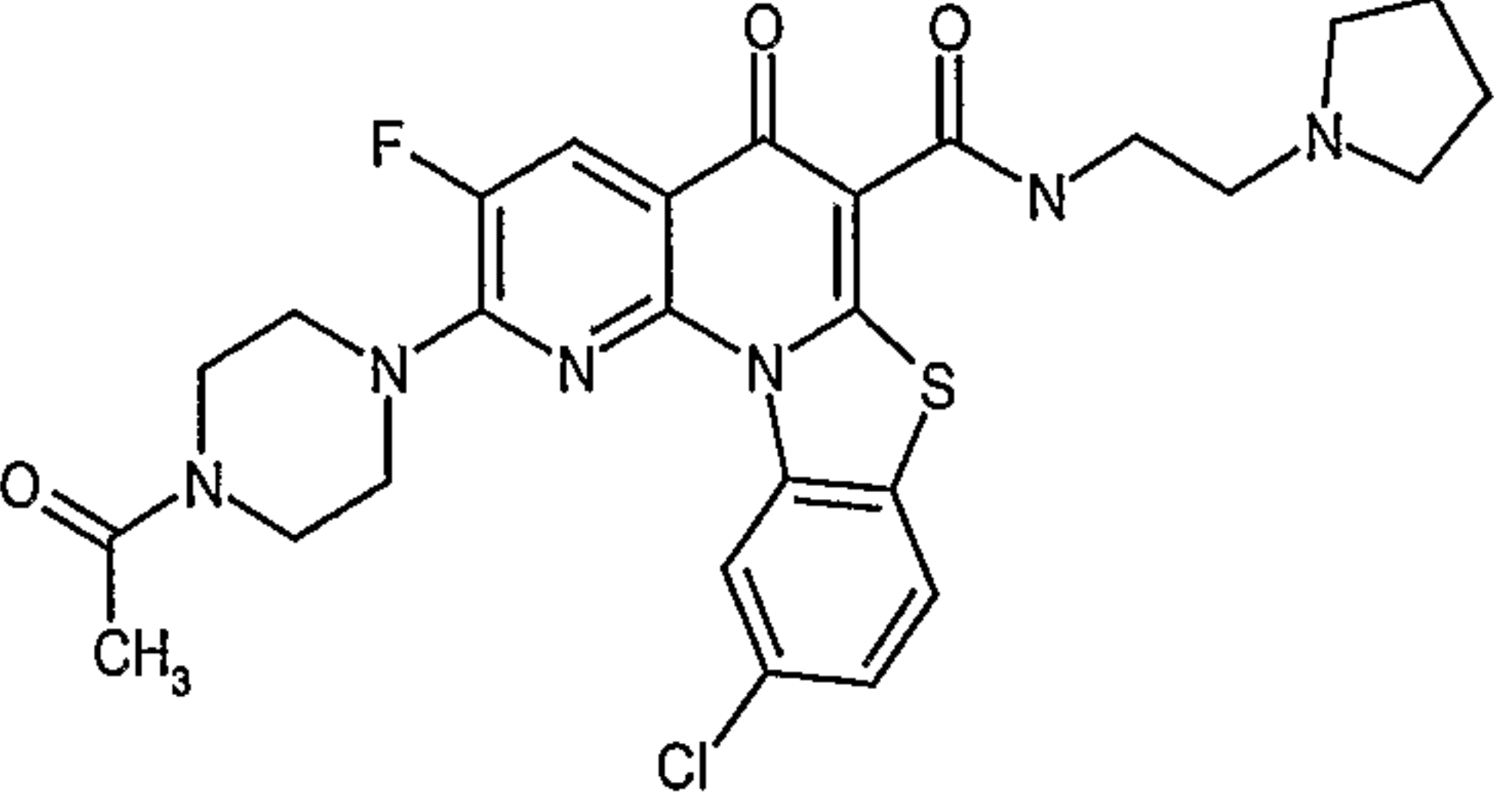
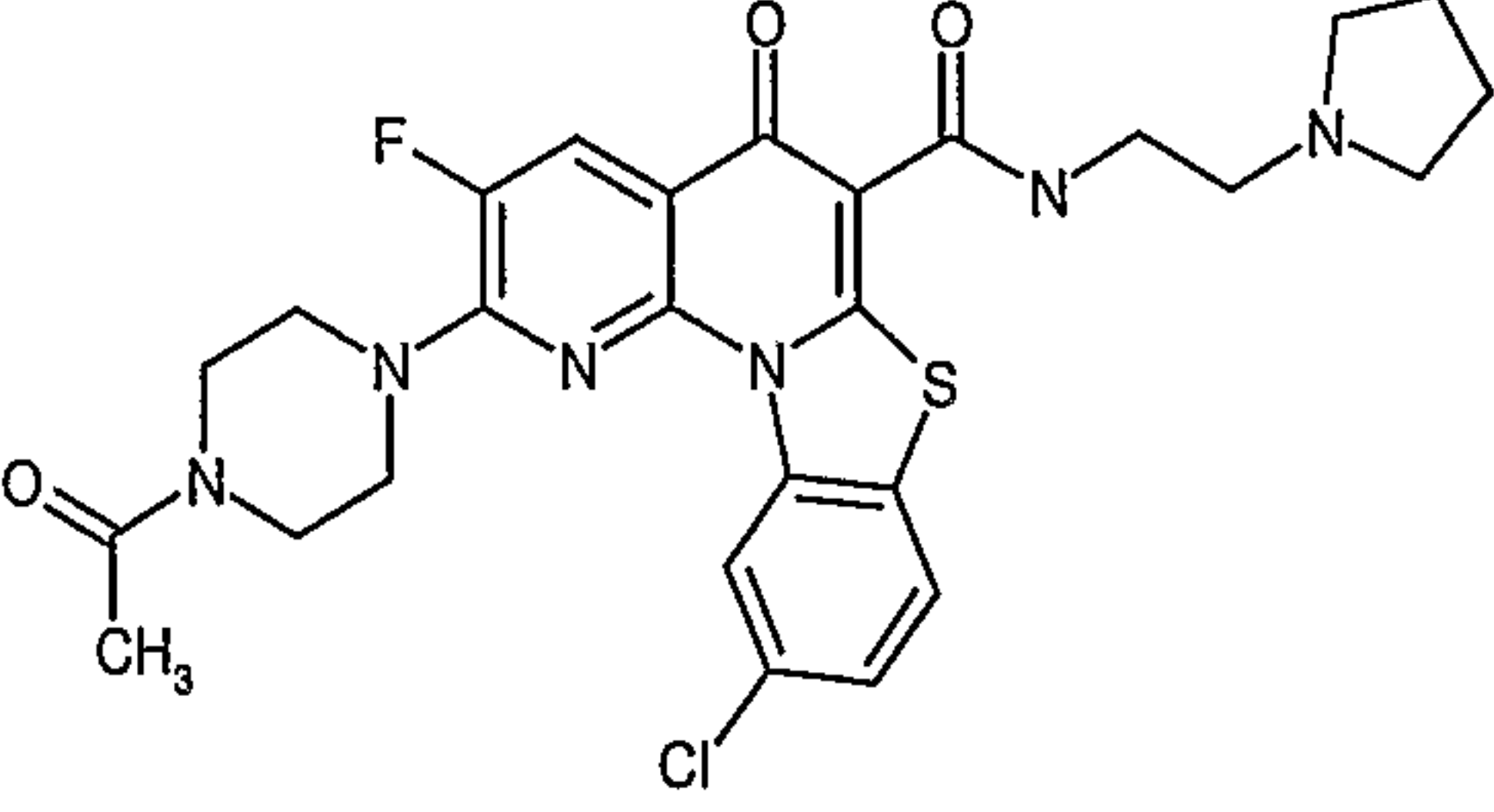
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | >10    |
|   | MiaPaca | >10    |
|  | HCT-116 | 0.25   |
|  | HCT-116 | 10     |
|  | HCT-116 | >10    |
|  | HCT-116 | 0.28   |

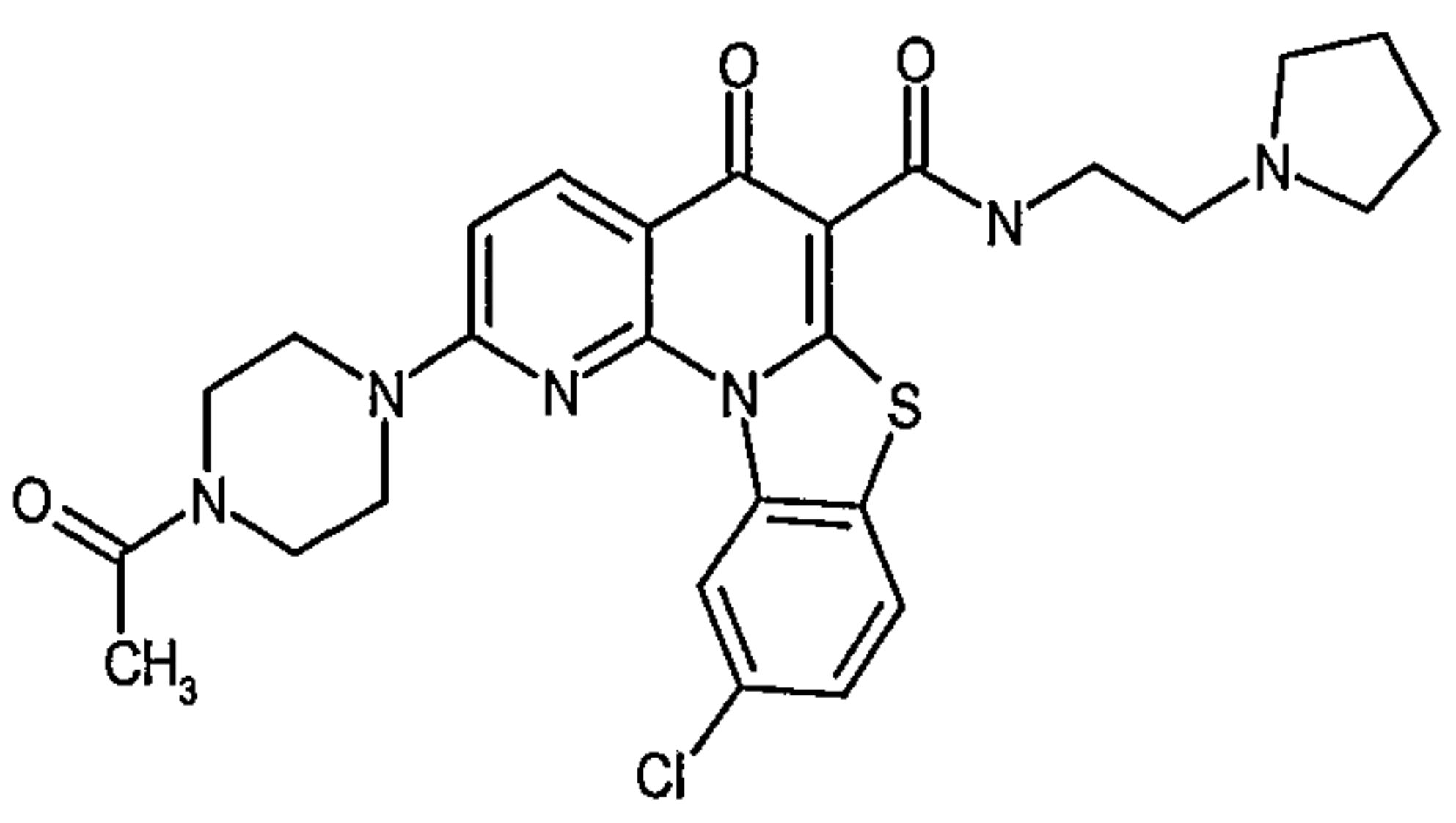
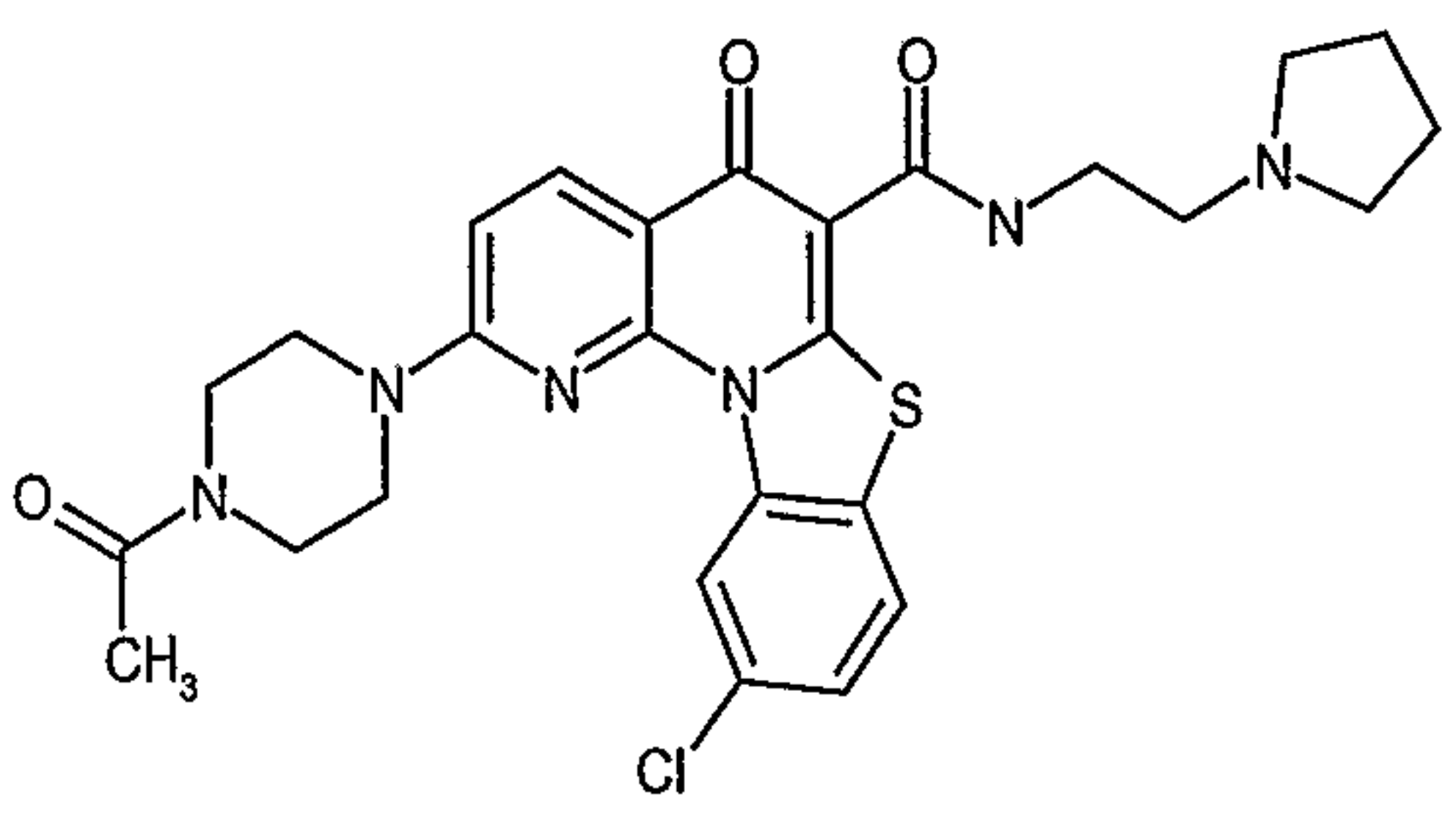
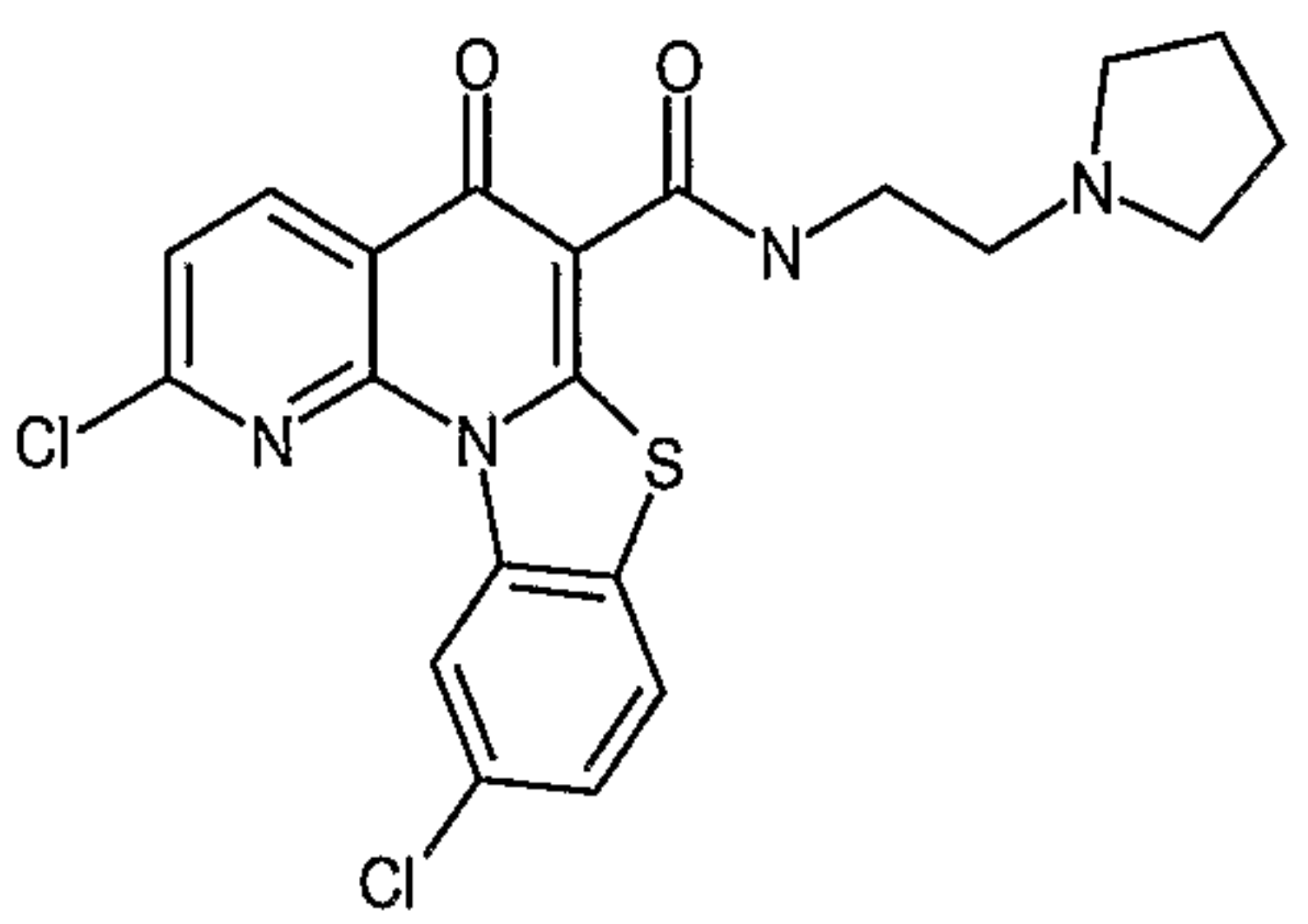
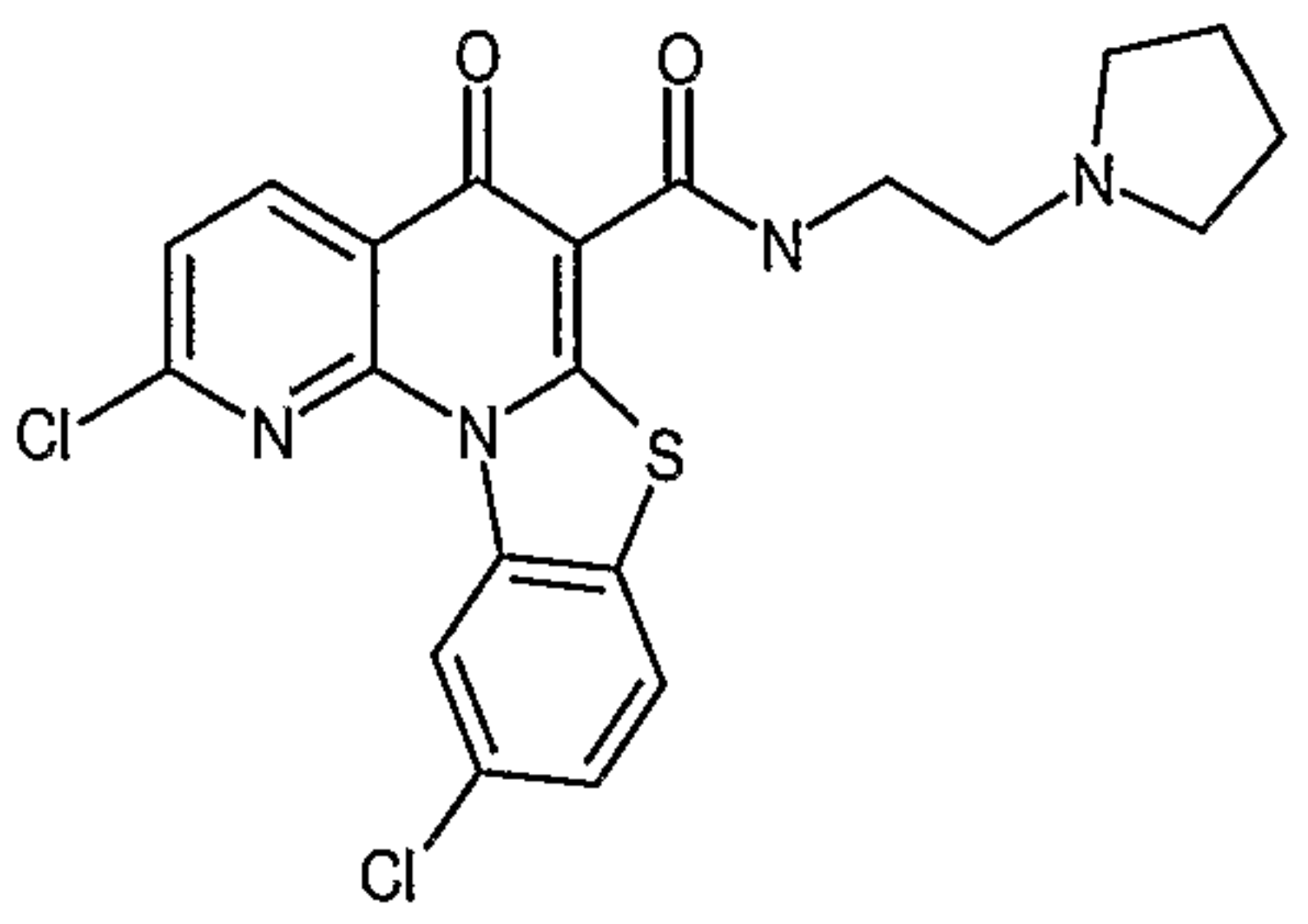
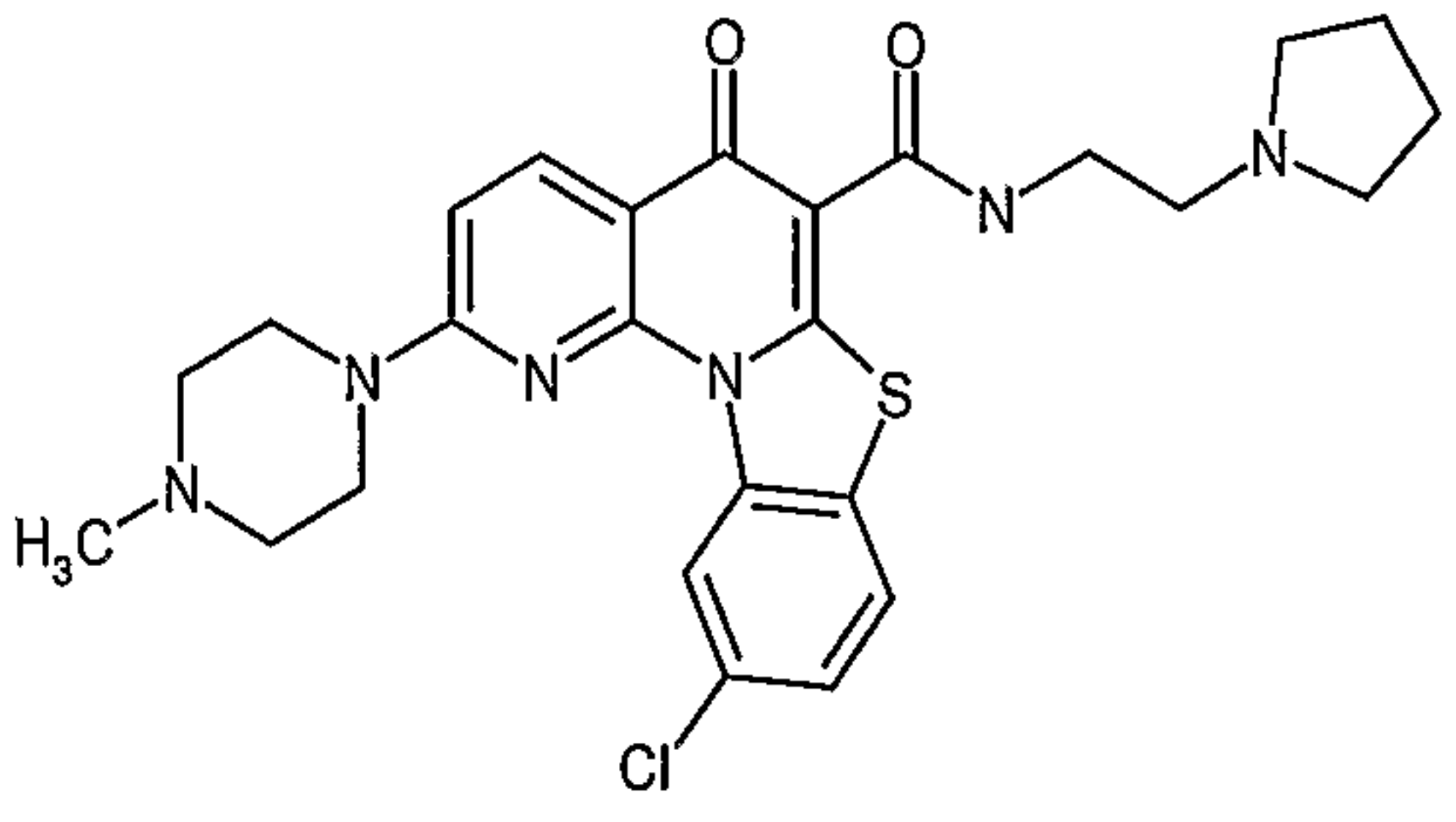
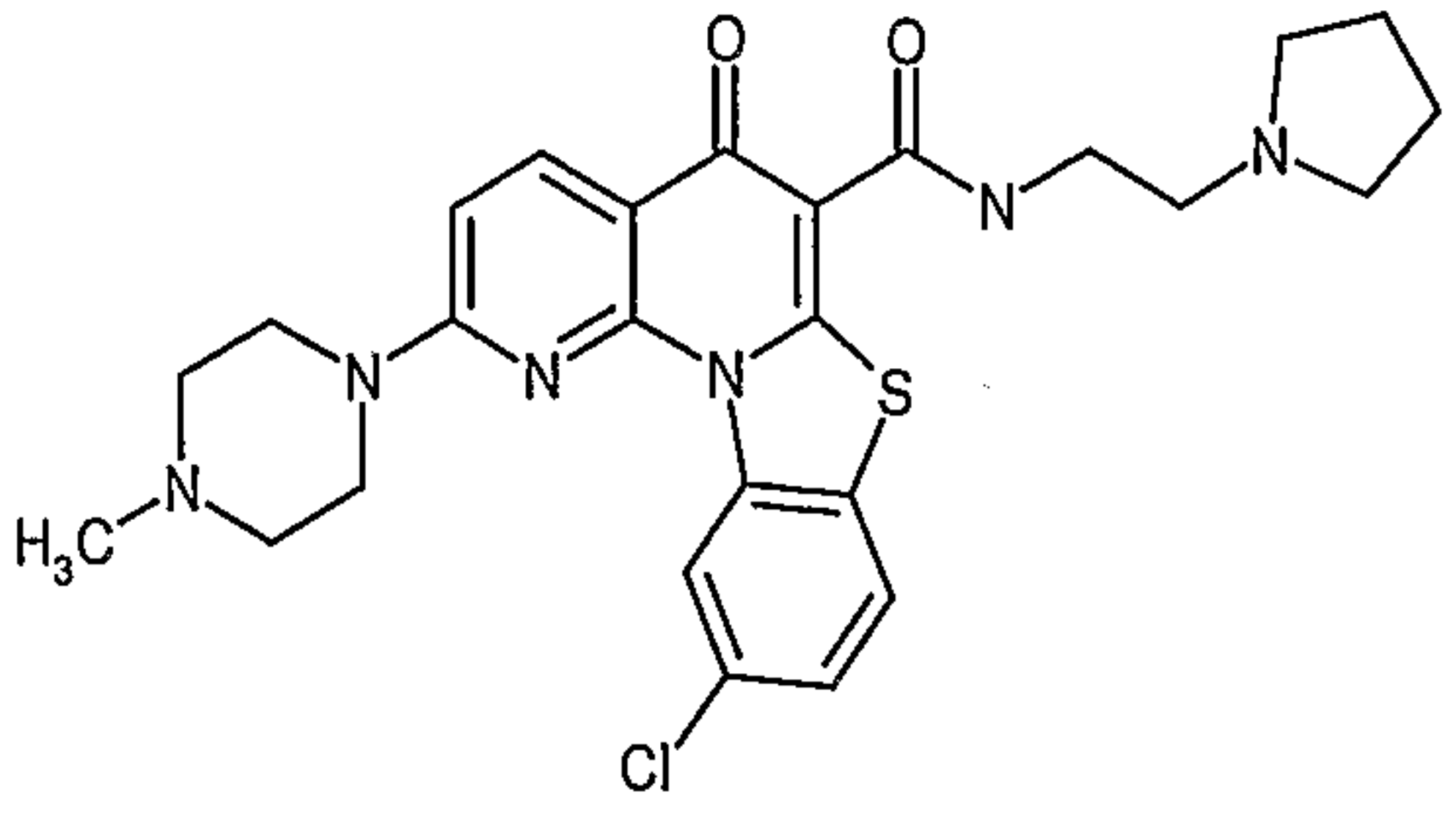
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     |         |        |
|   | HCT-116 | 0.03   |
|   | HCT-116 | 2.10   |
|  | HCT-116 | 0.02   |
|  | HCT-116 | 2.30   |
|   | HCT-116 | <0.01  |

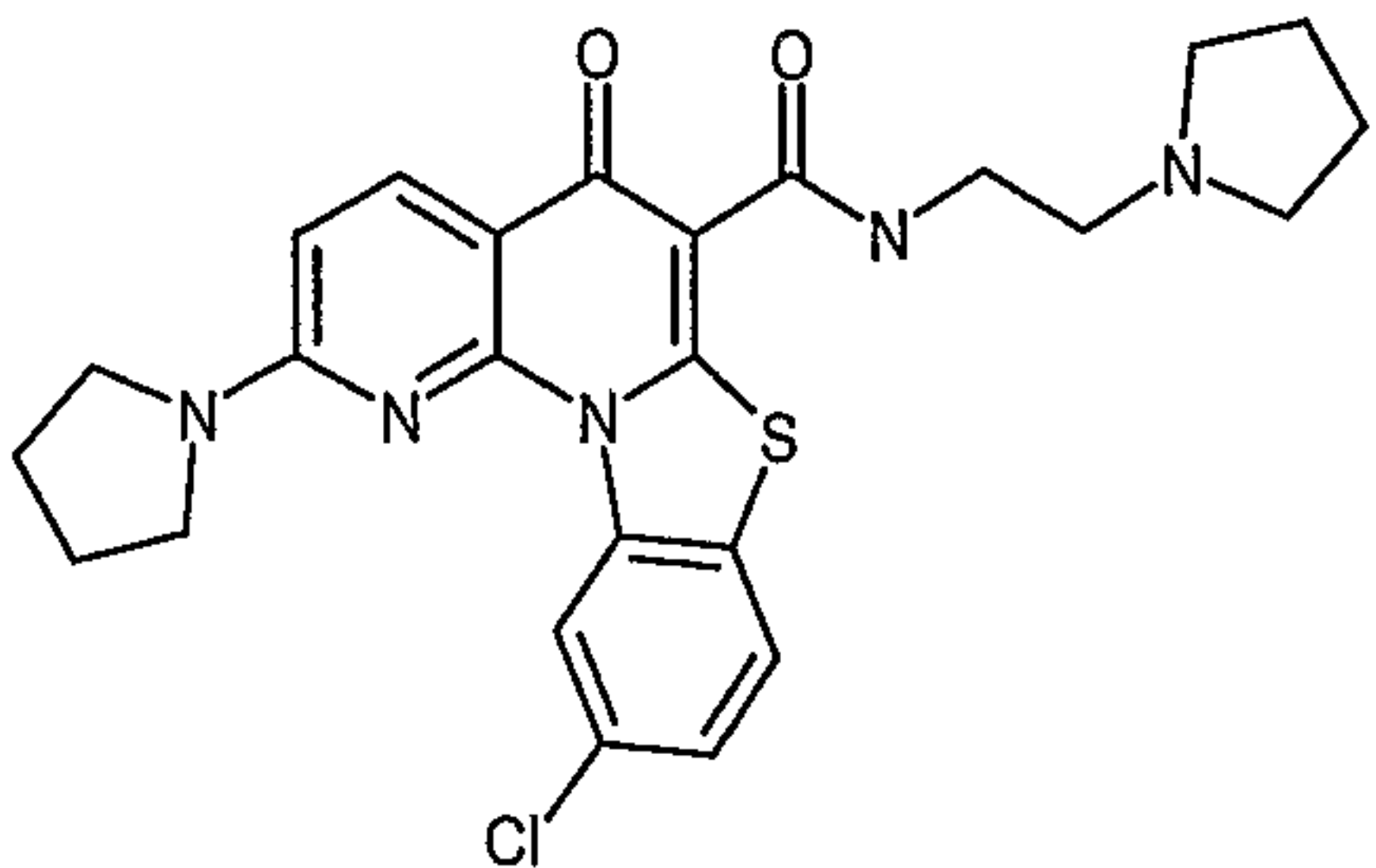
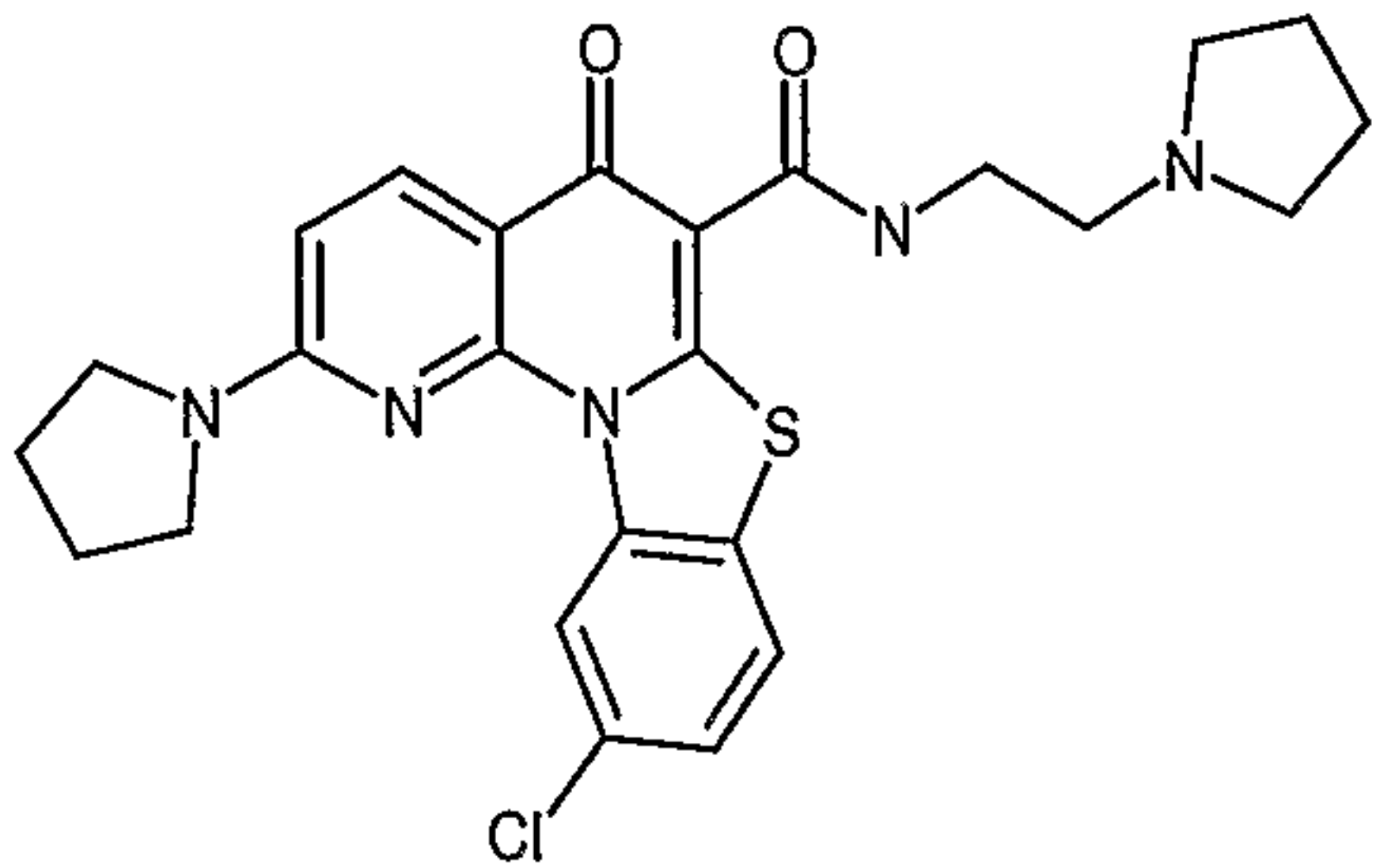
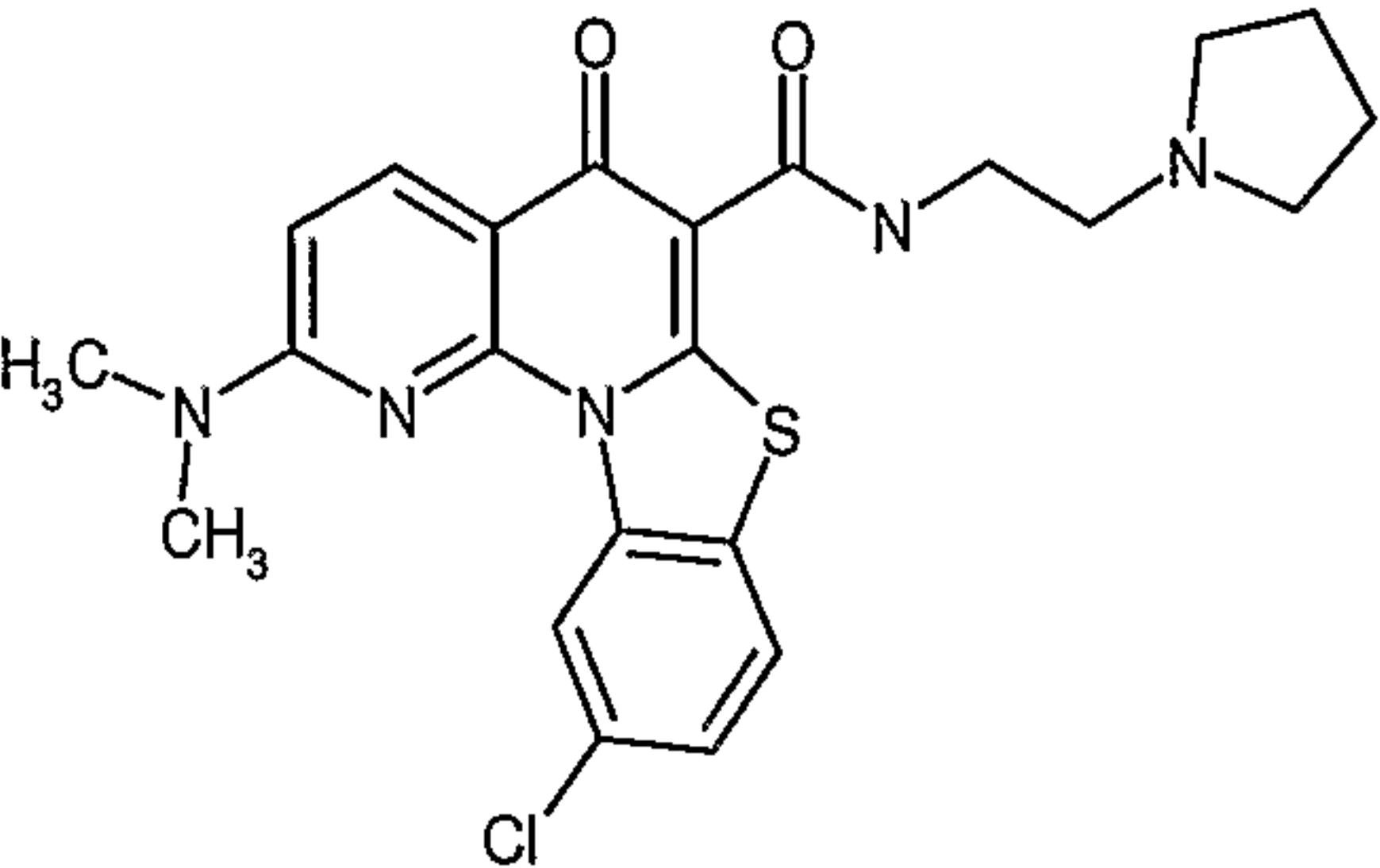
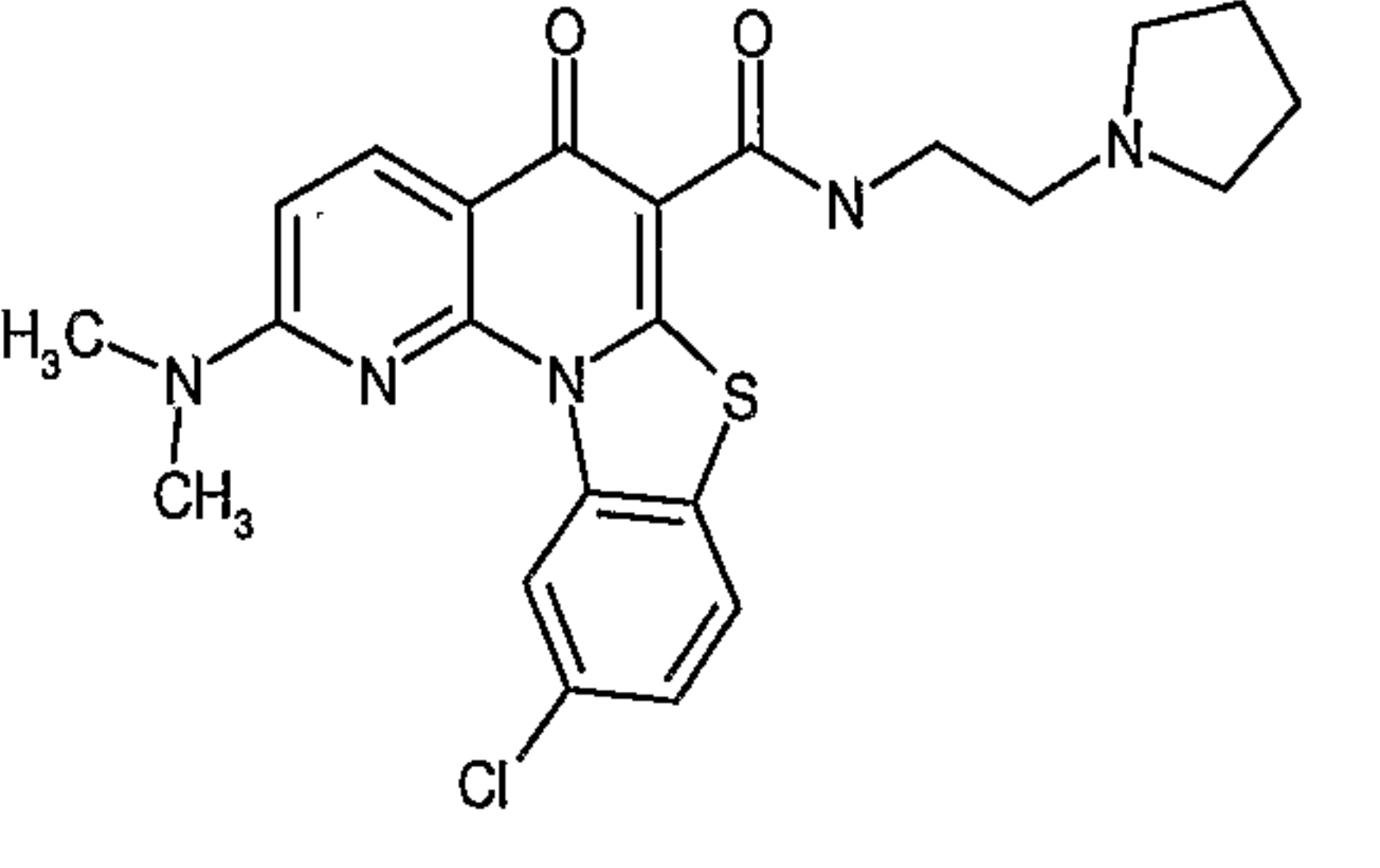
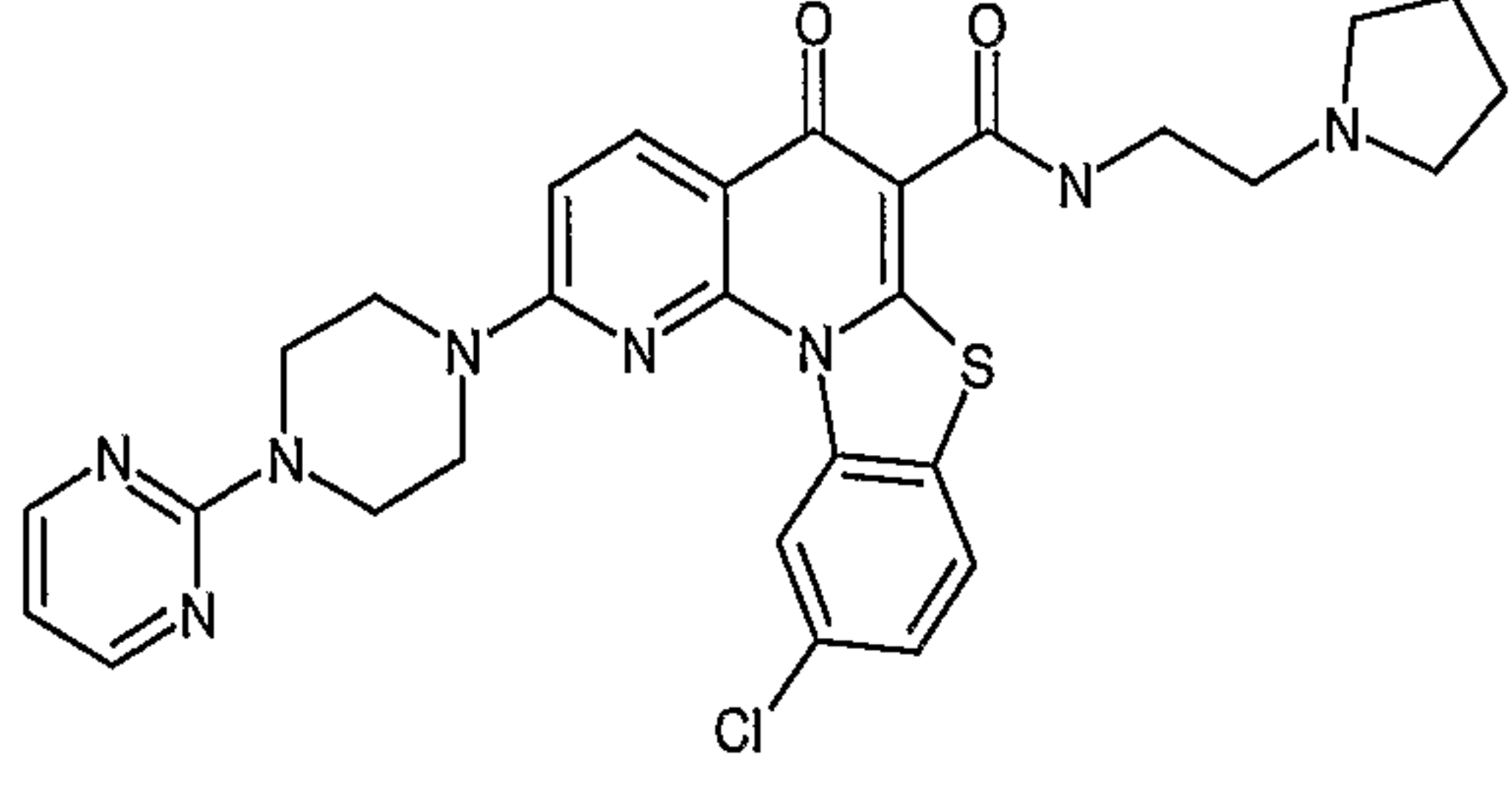
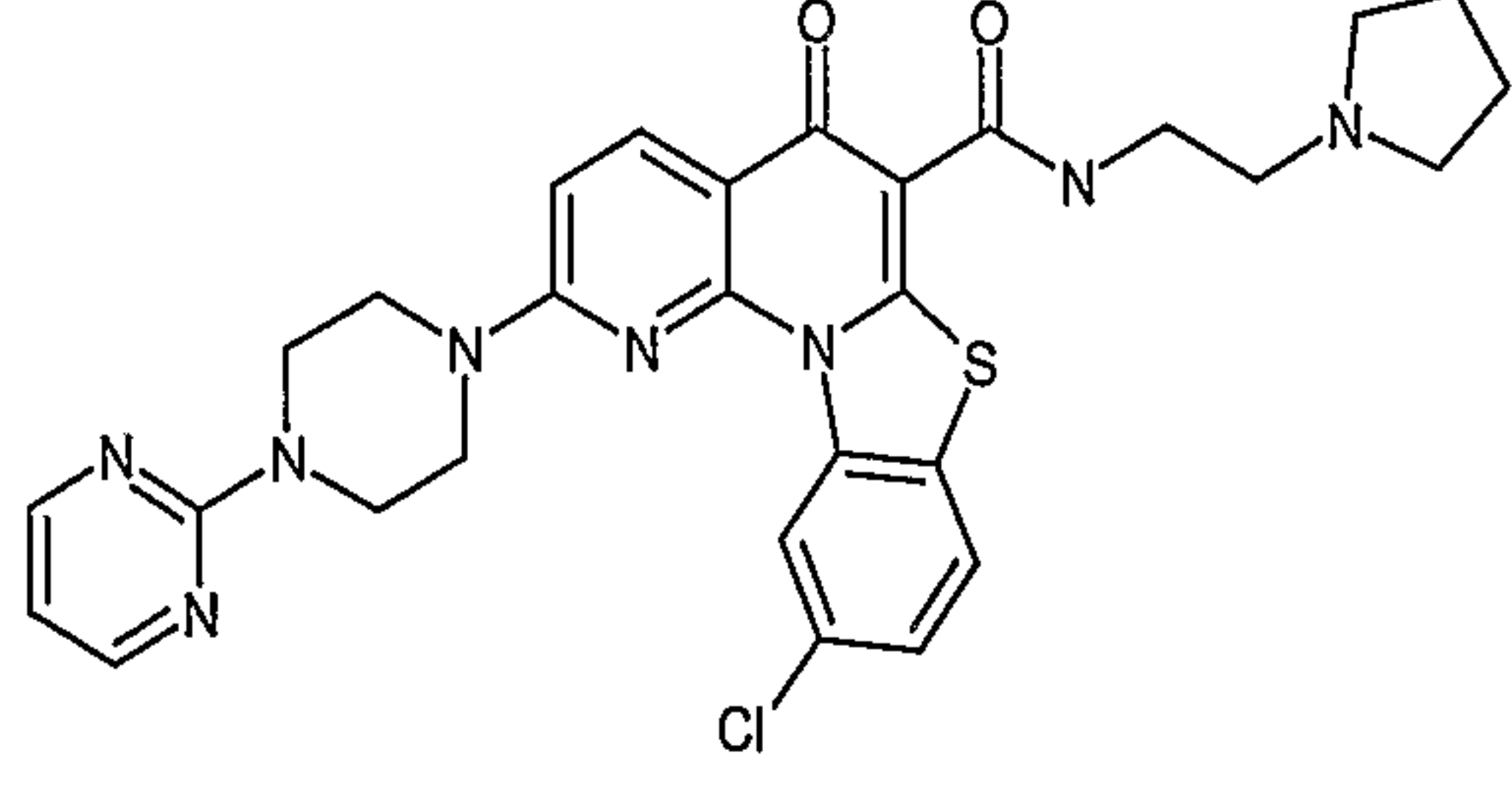
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | MiaPaca | <0.01  |
|   | HCT-116 | <0.01  |
|  | MiaPaca | <0.01  |
|   | HCT-116 | 0.02   |
|   | MiaPaca | 0.01   |
|   | HCT-116 | 0.115  |

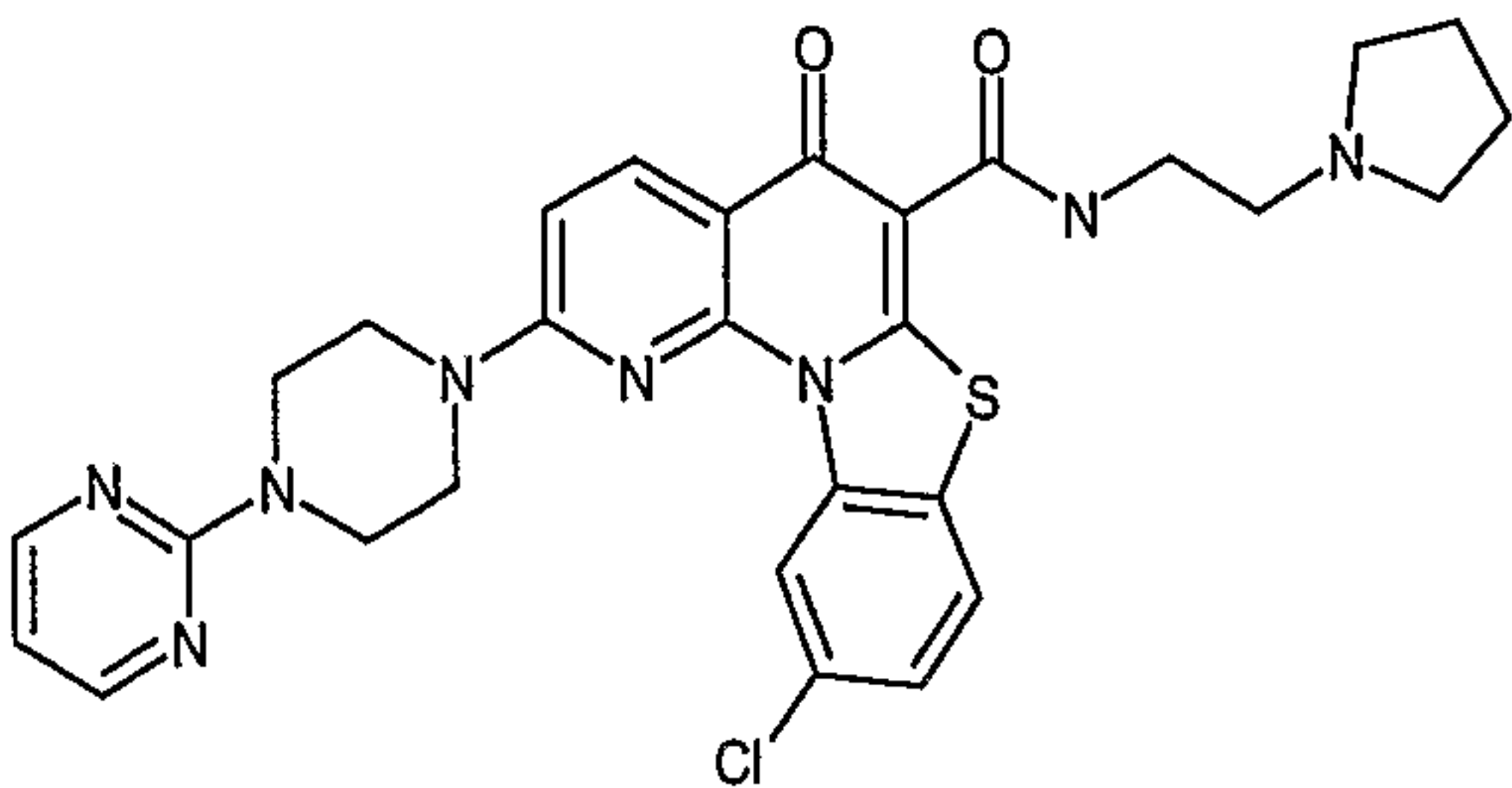
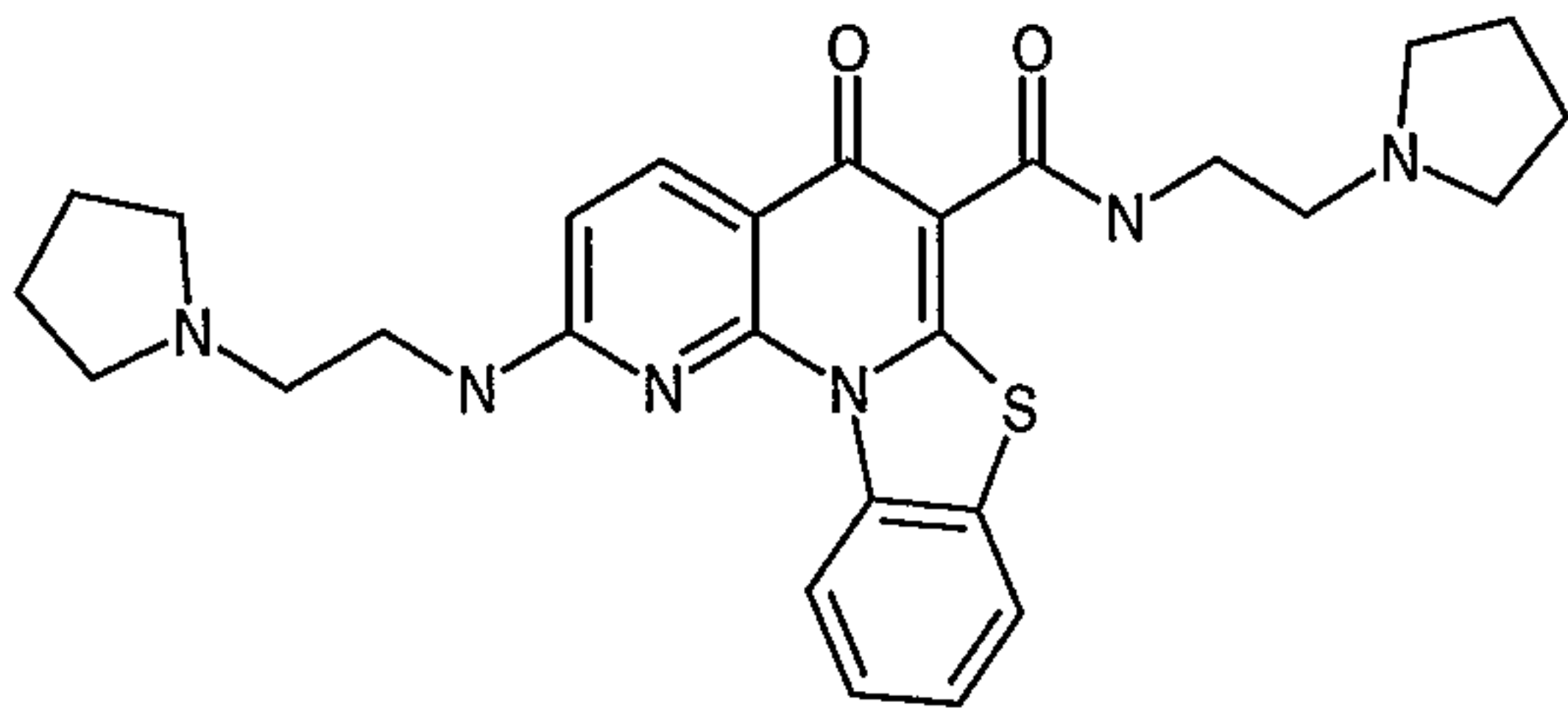
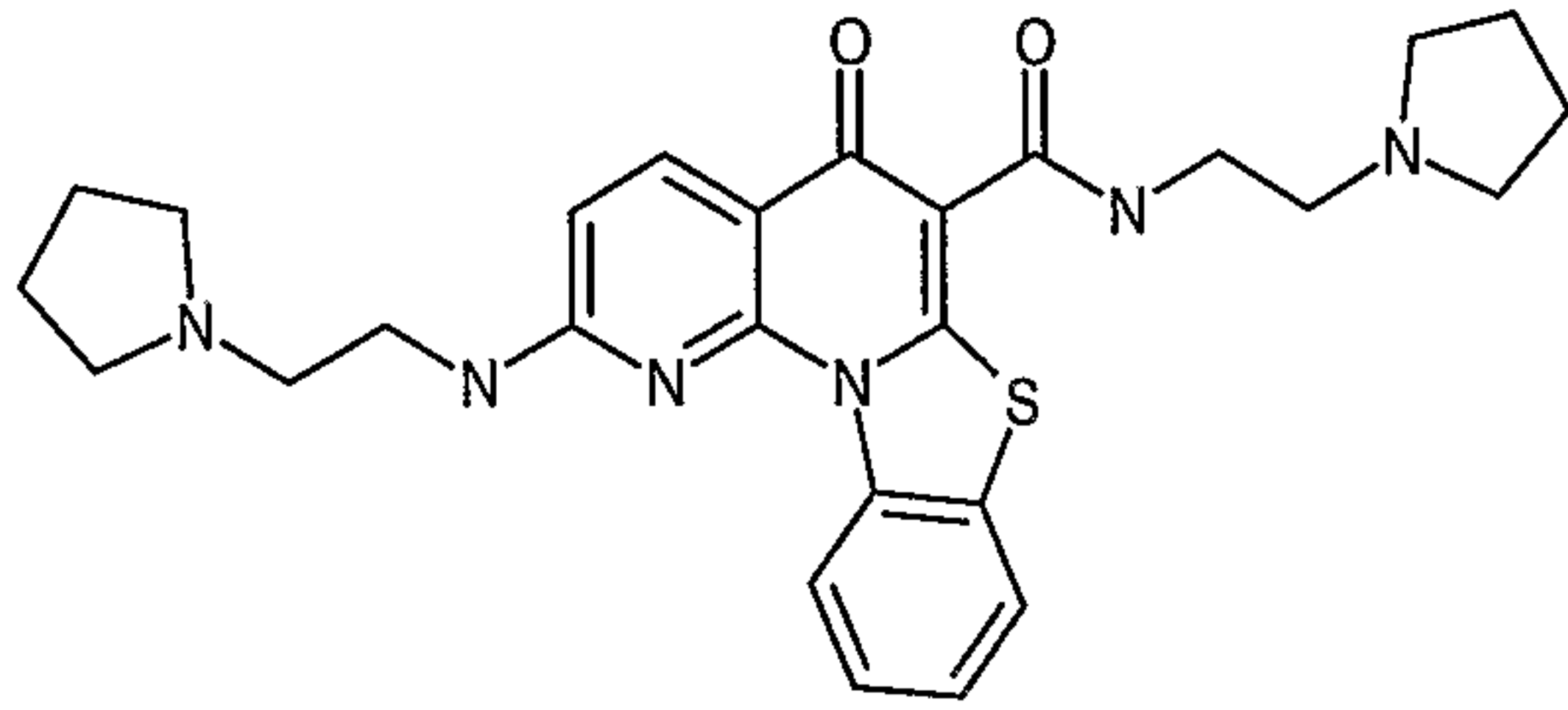
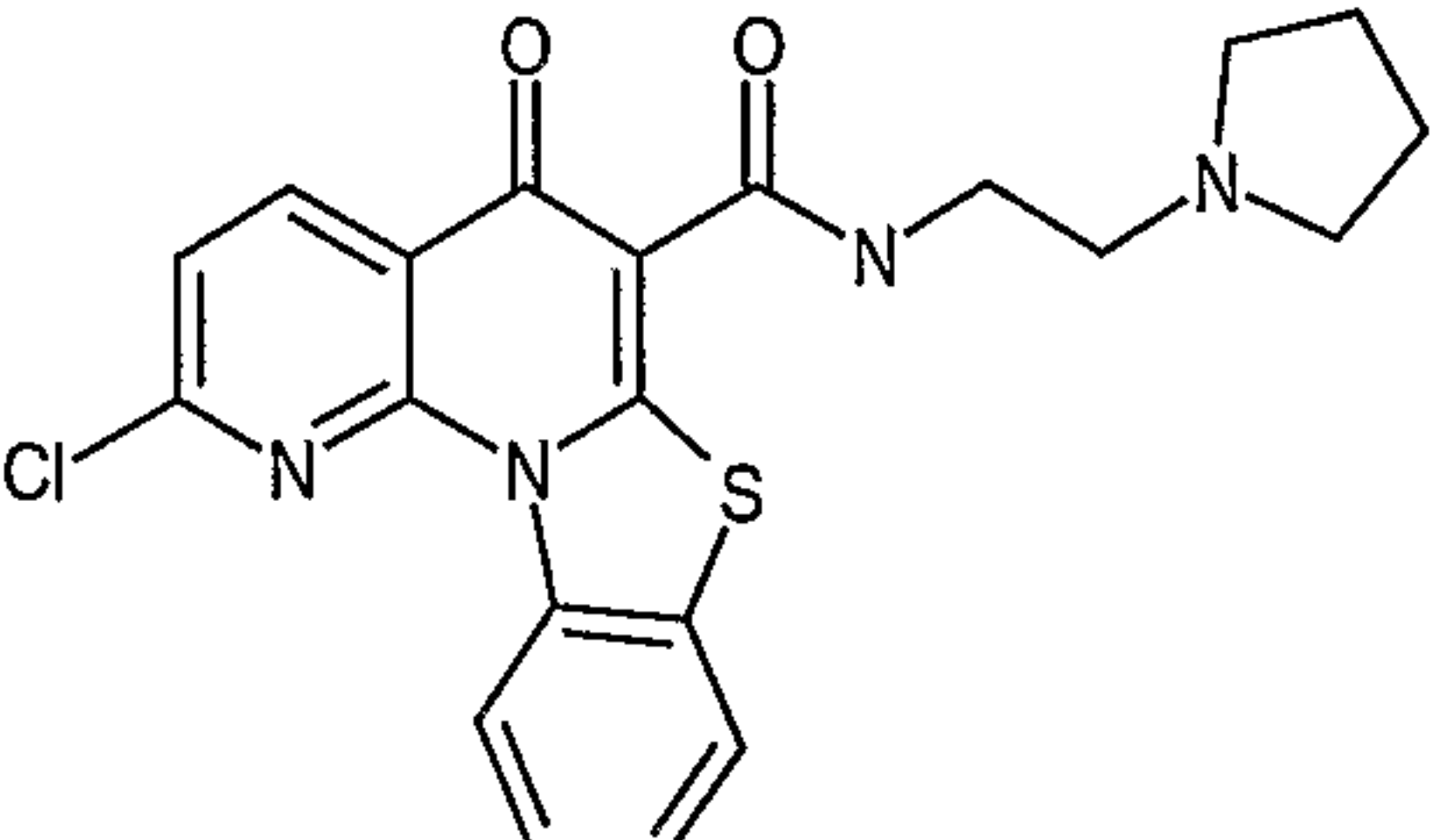
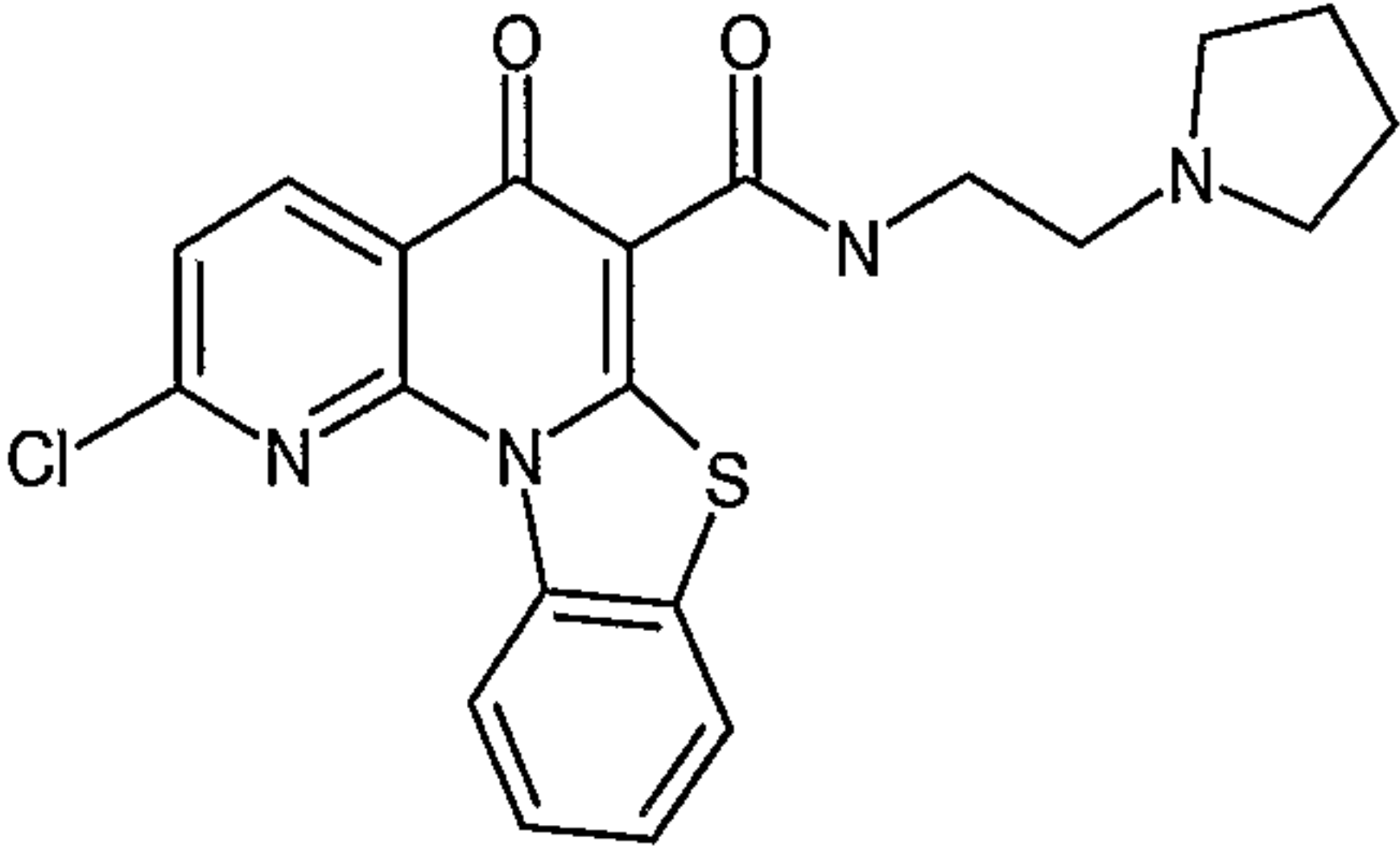
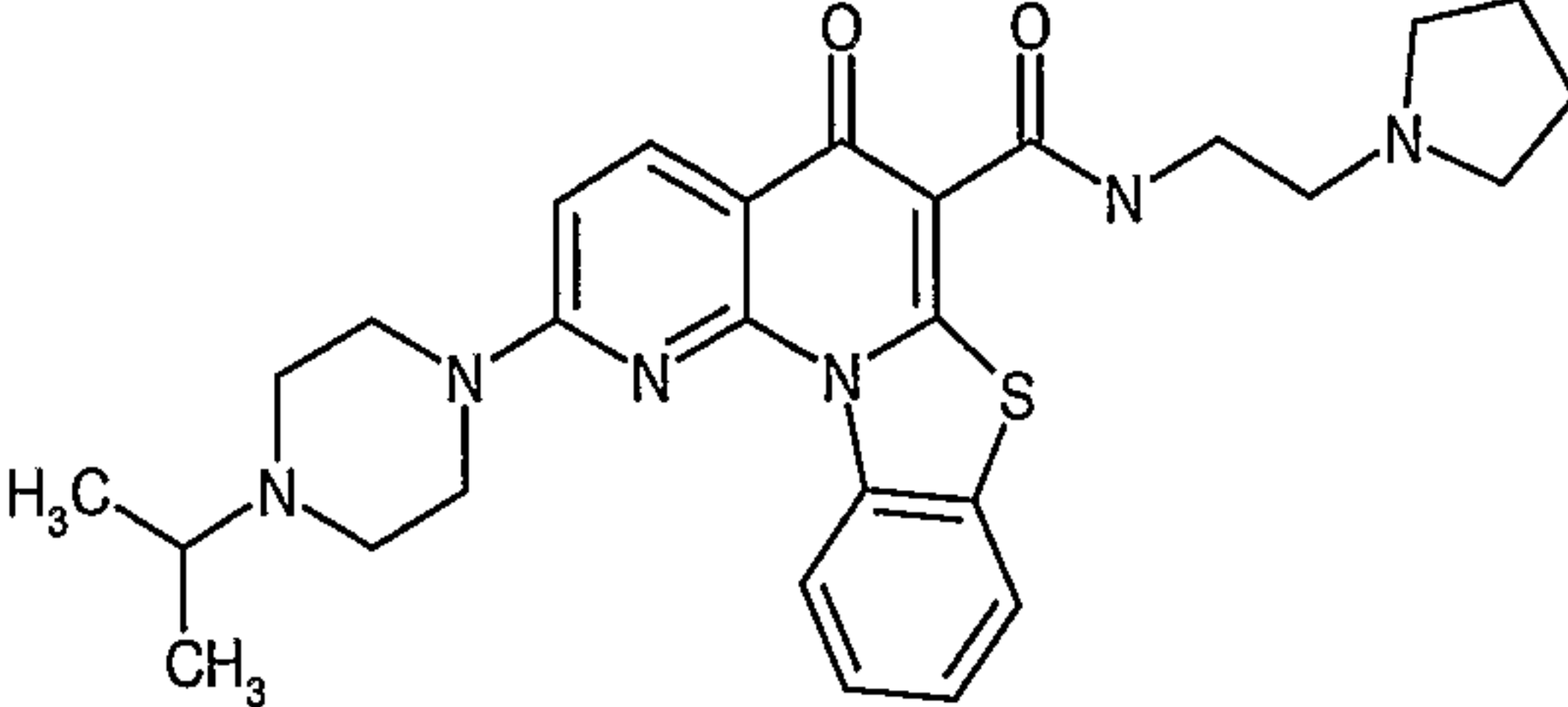
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | MiaPaca | 0.03   |
|    | Miapaca | 0.65   |
|   | HCT-116 | 1.3    |
|   | HCT-116 | <0.01  |
|  | MiaPaca | <0.01  |
|   | HCT-116 | <0.01  |

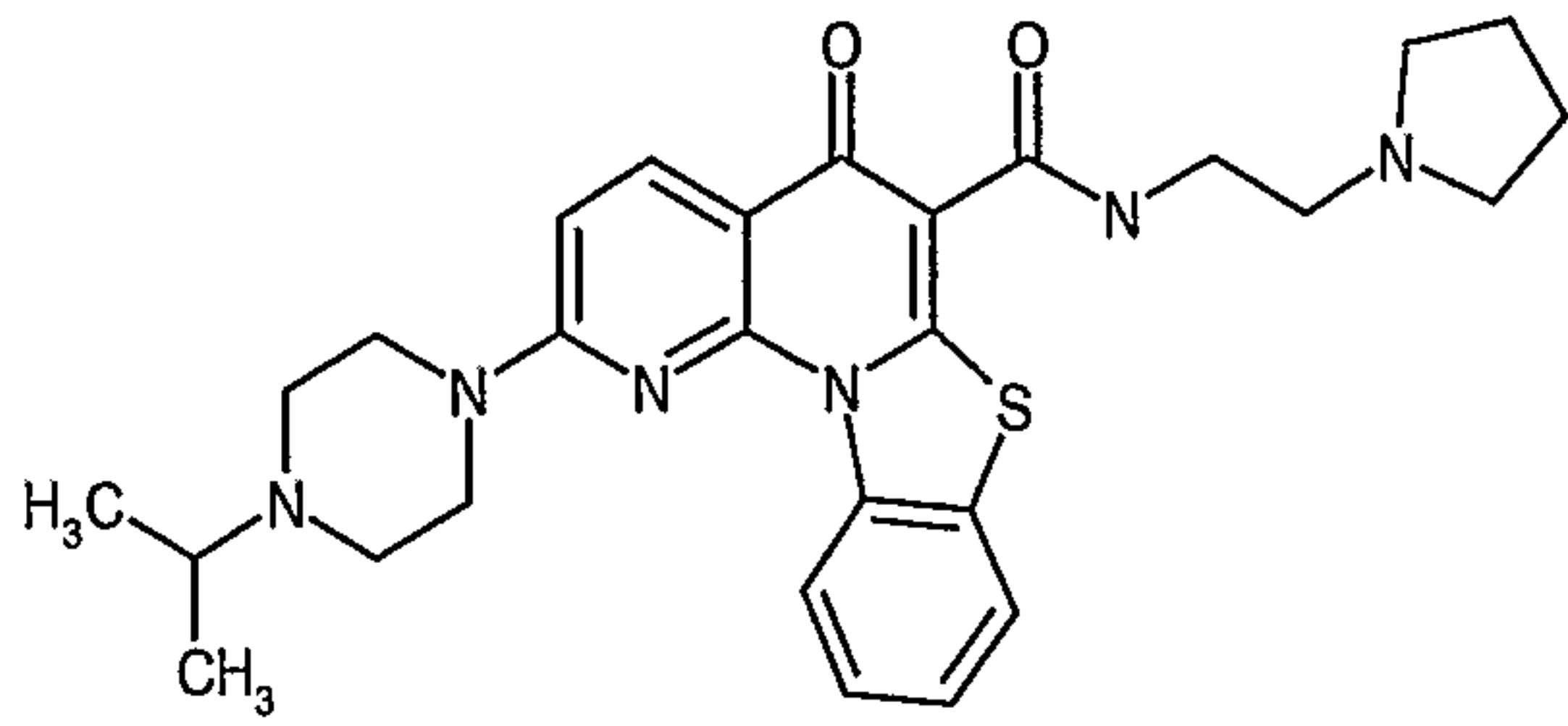
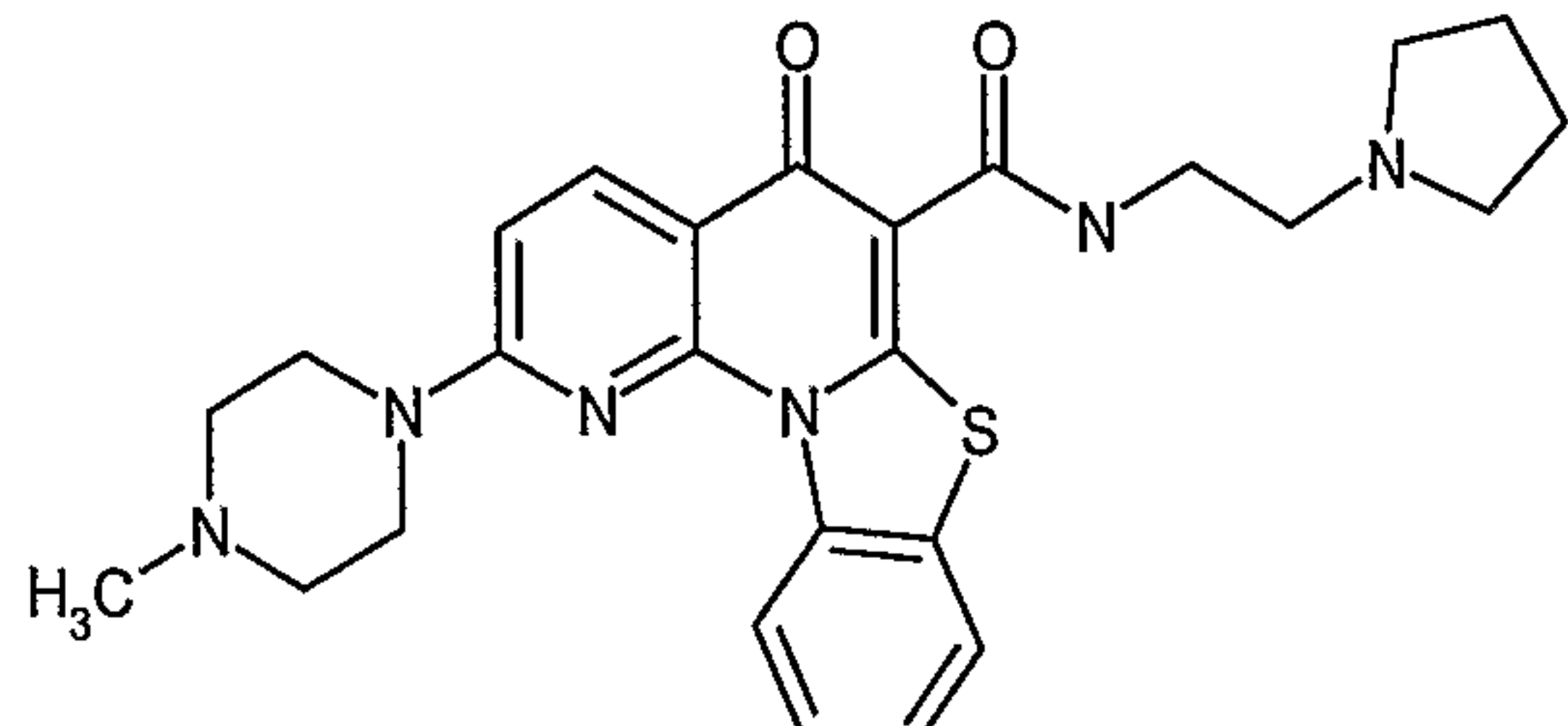
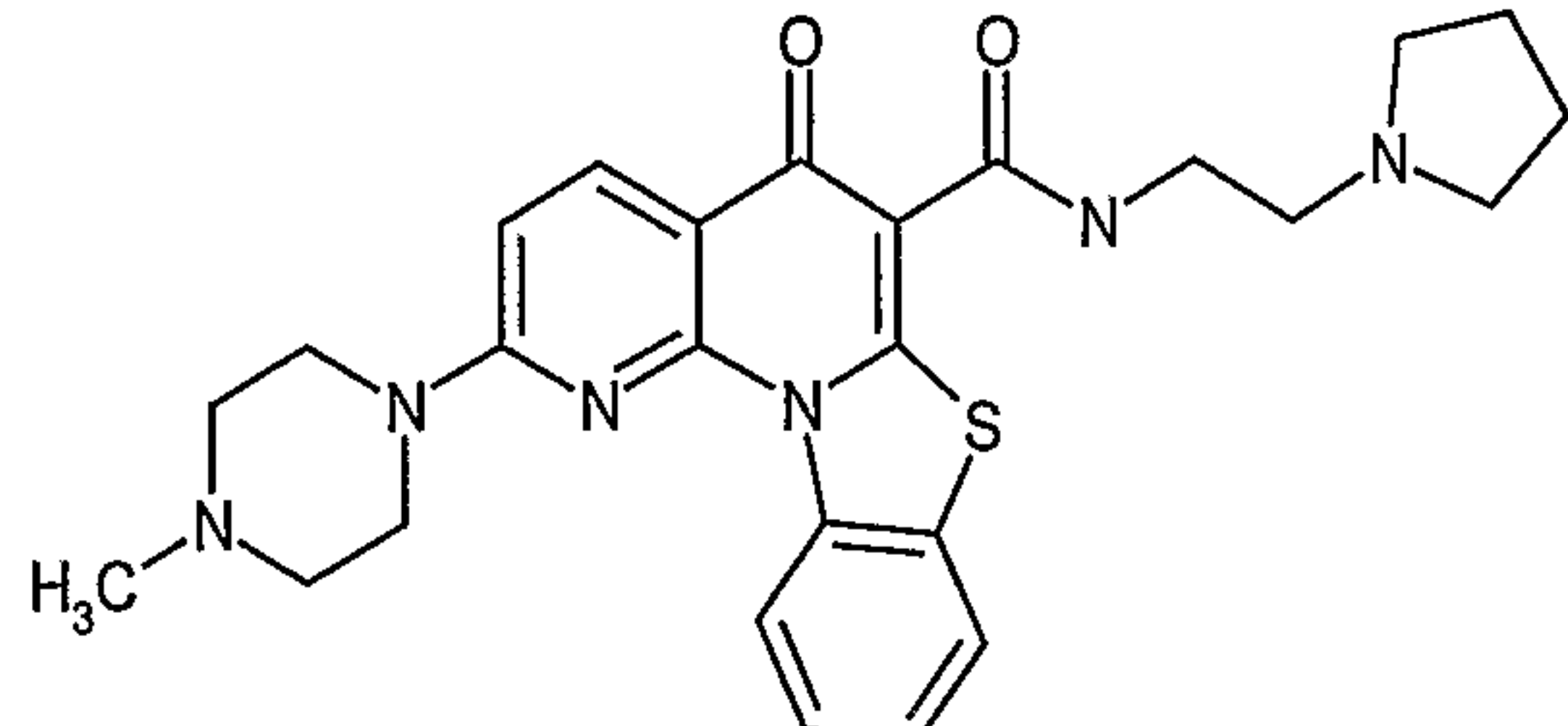
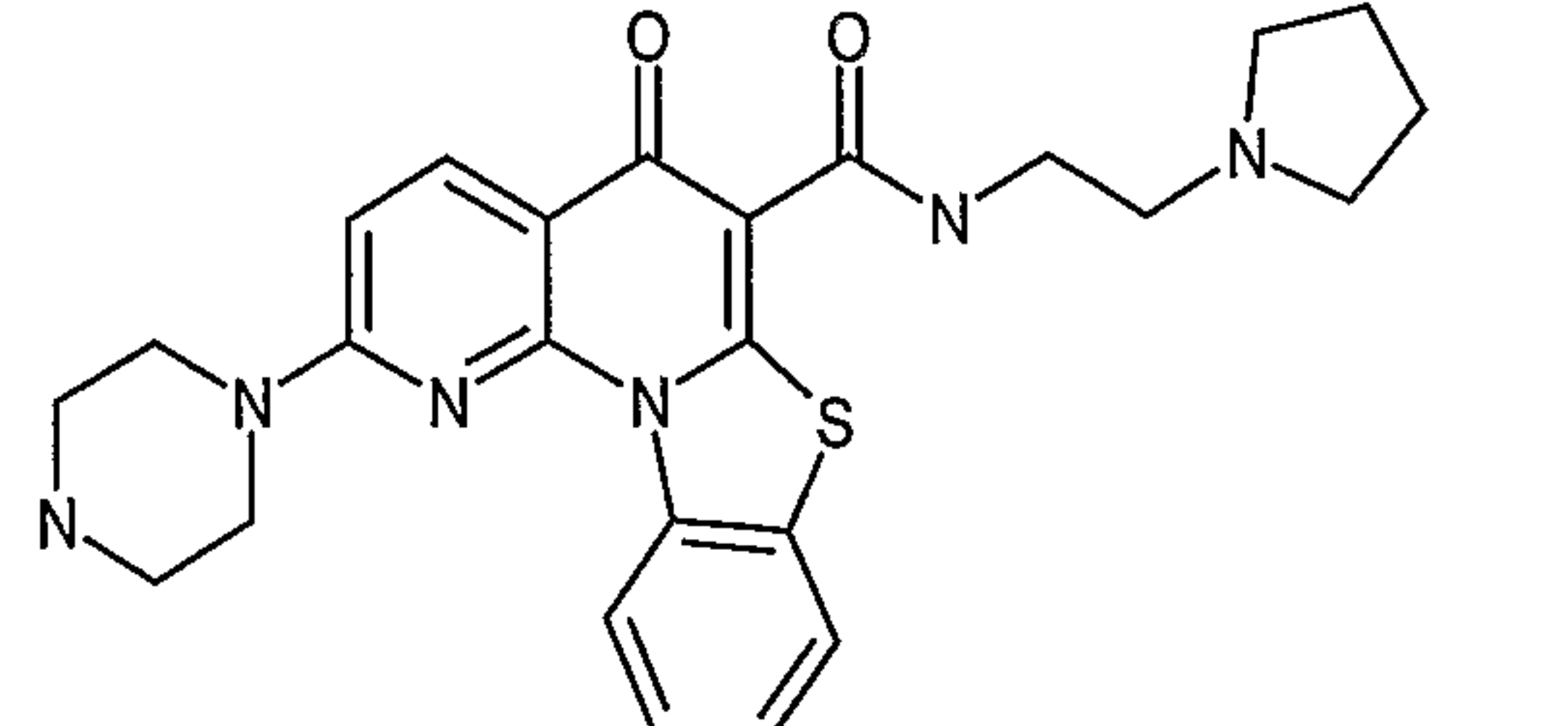
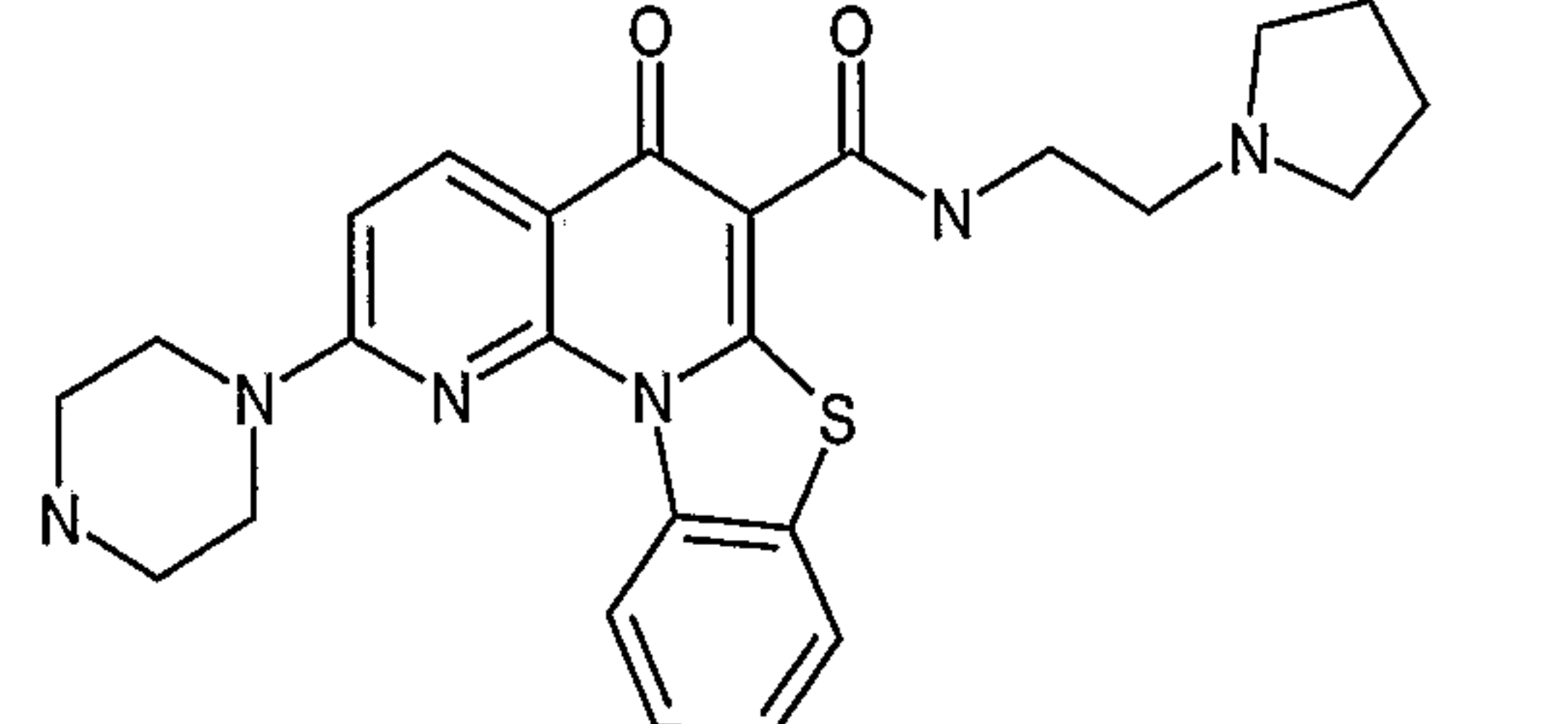
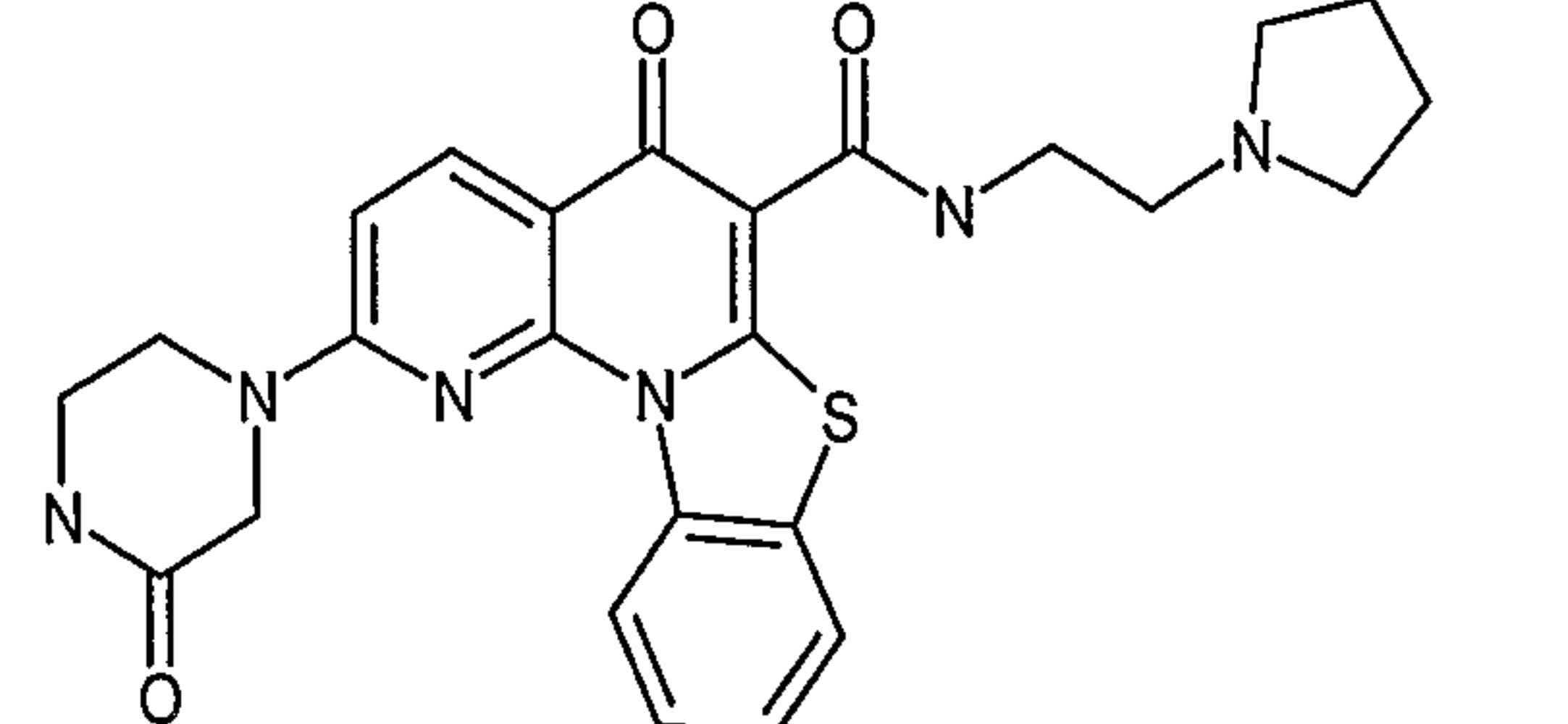
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | <0.01  |
|   | HCT-116 | 0.03   |
|  | MiaPaca | 0.04   |
|  | Miapaca | 0.01   |
|  | HCT-116 | 0.16   |
|  | MiaPaca | 0.08   |

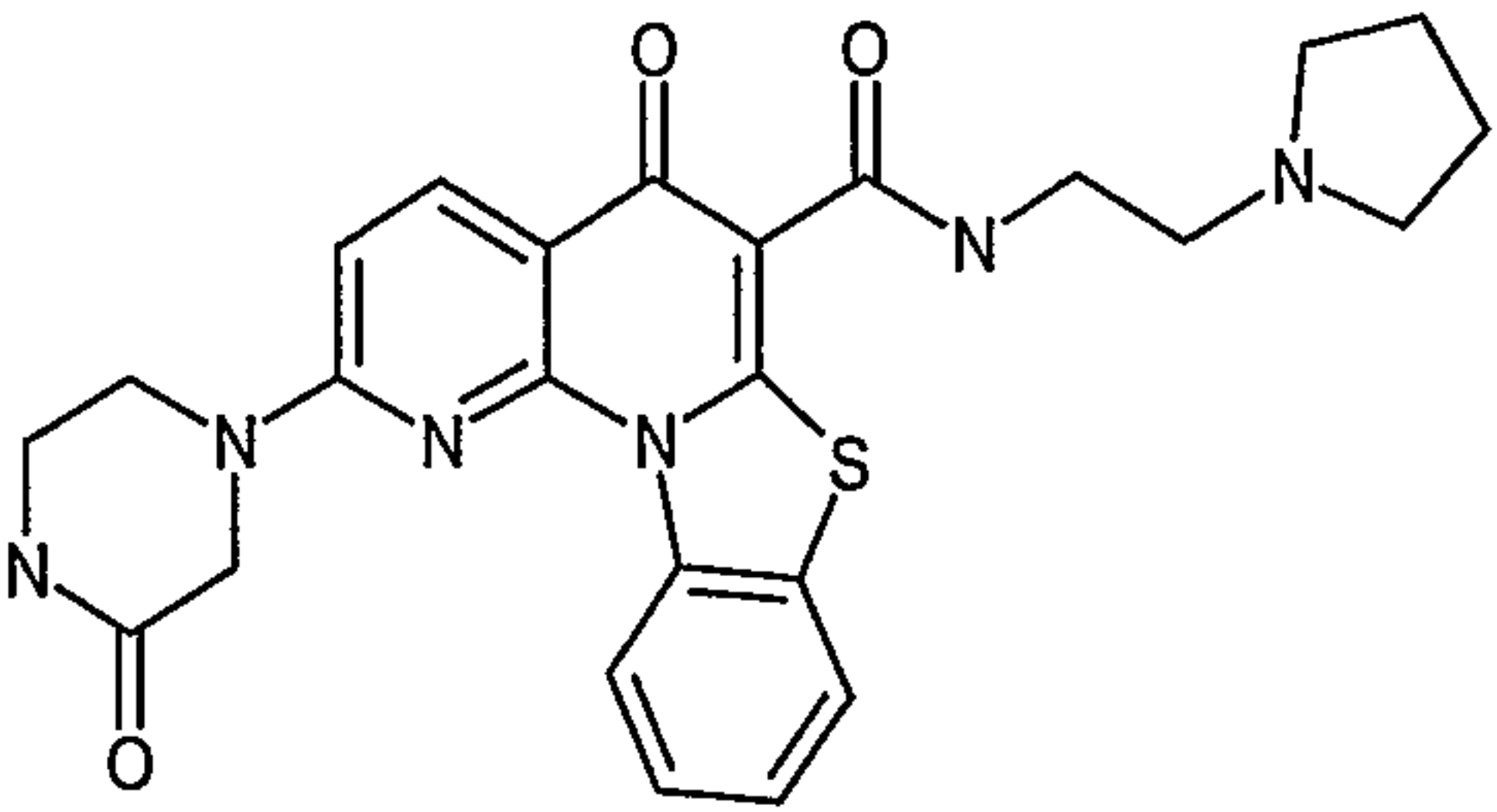
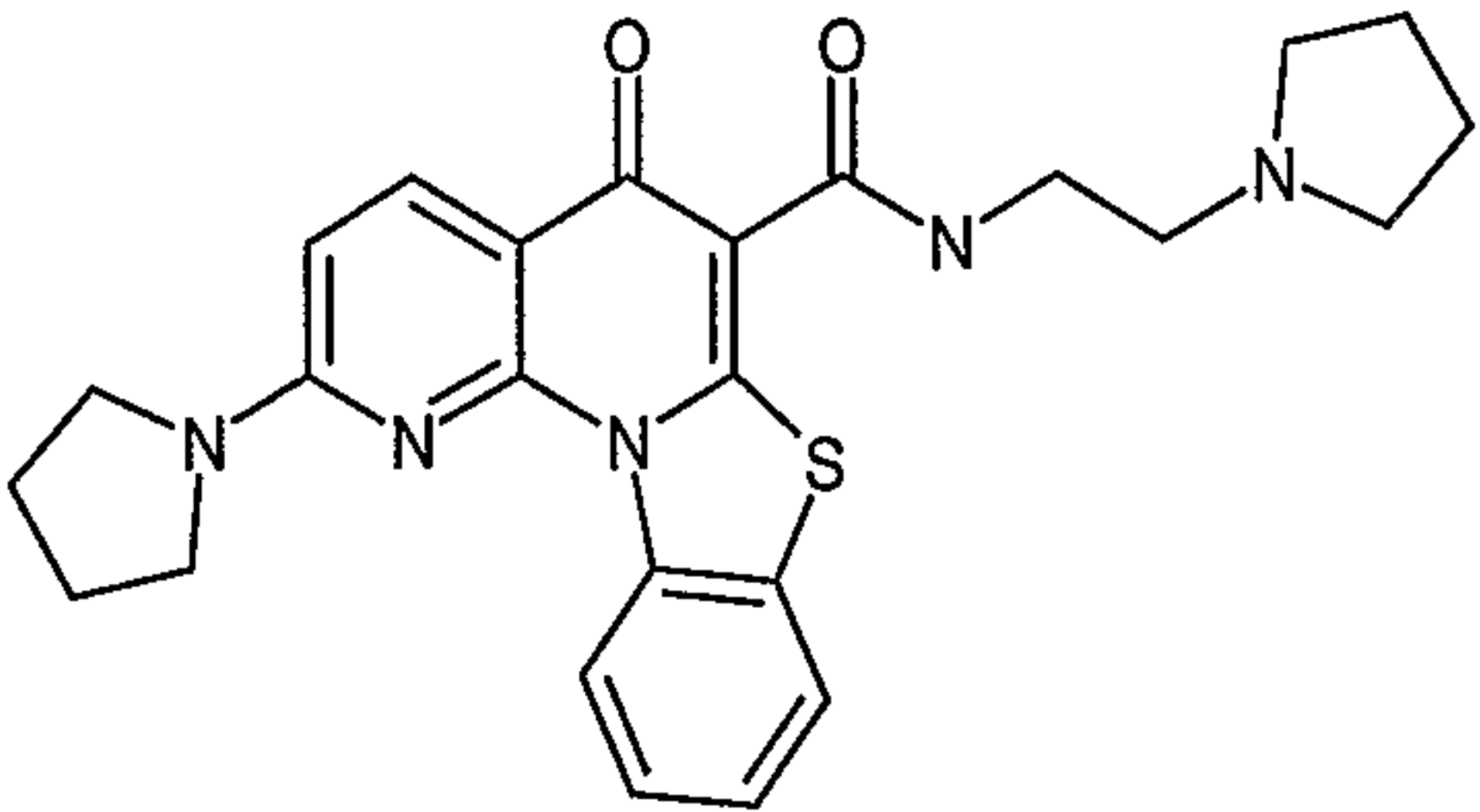
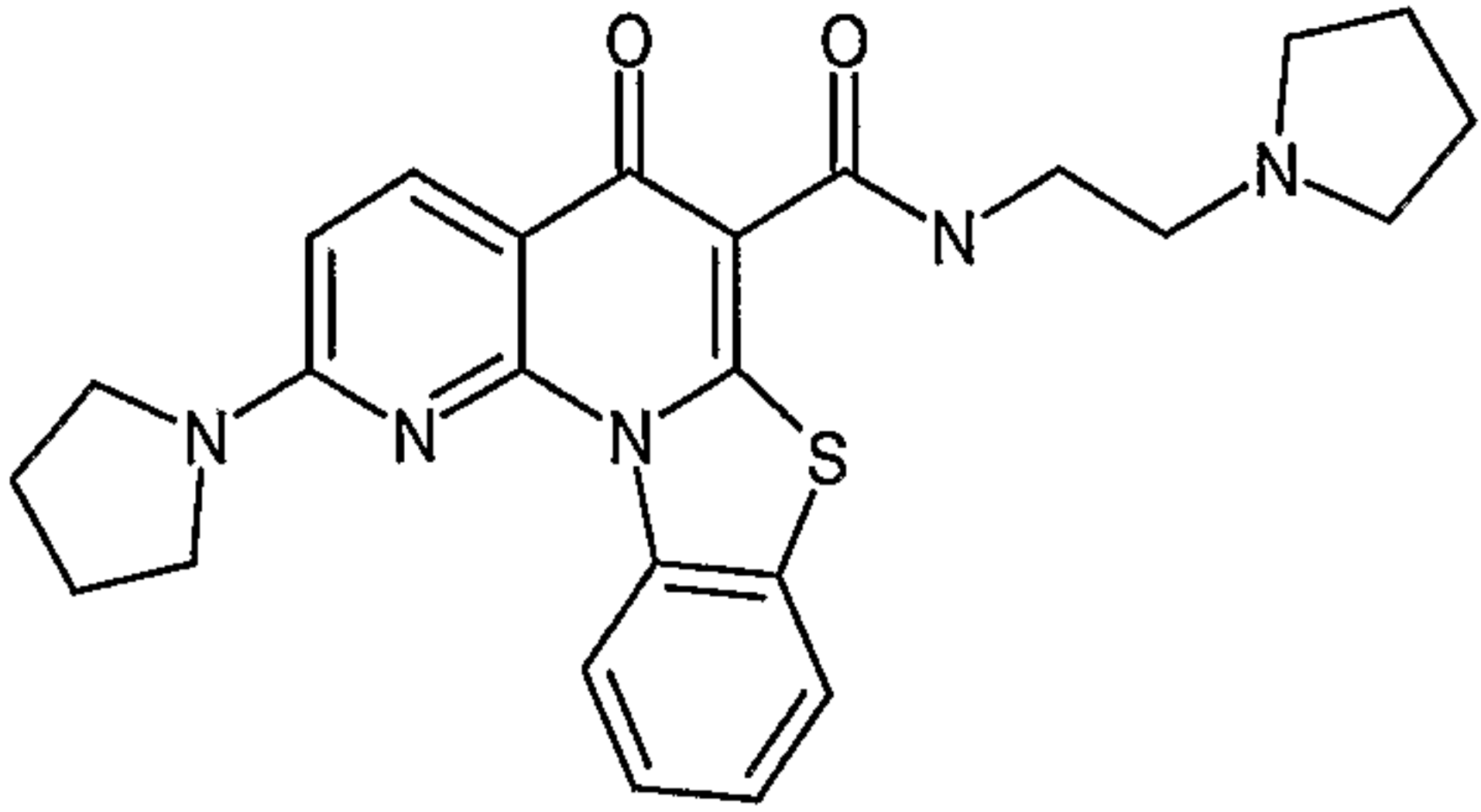
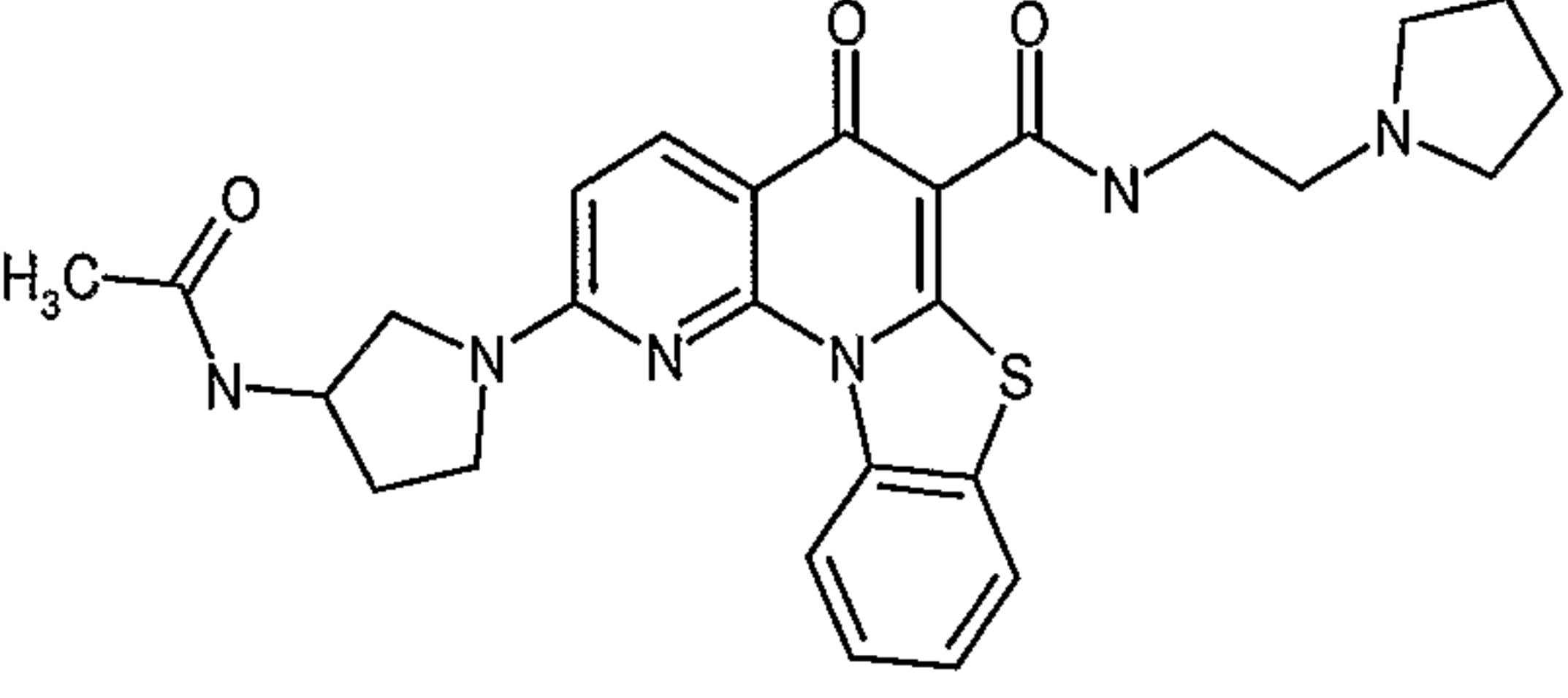
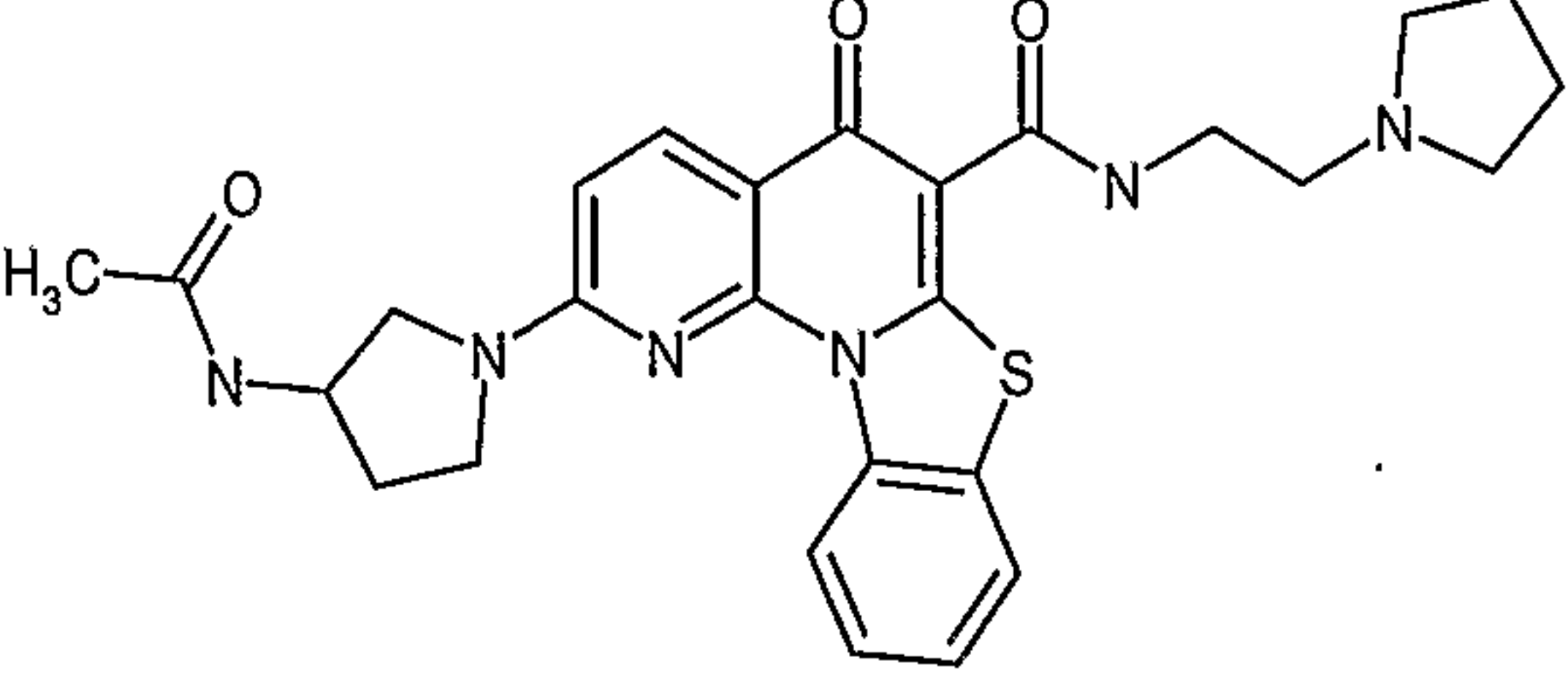
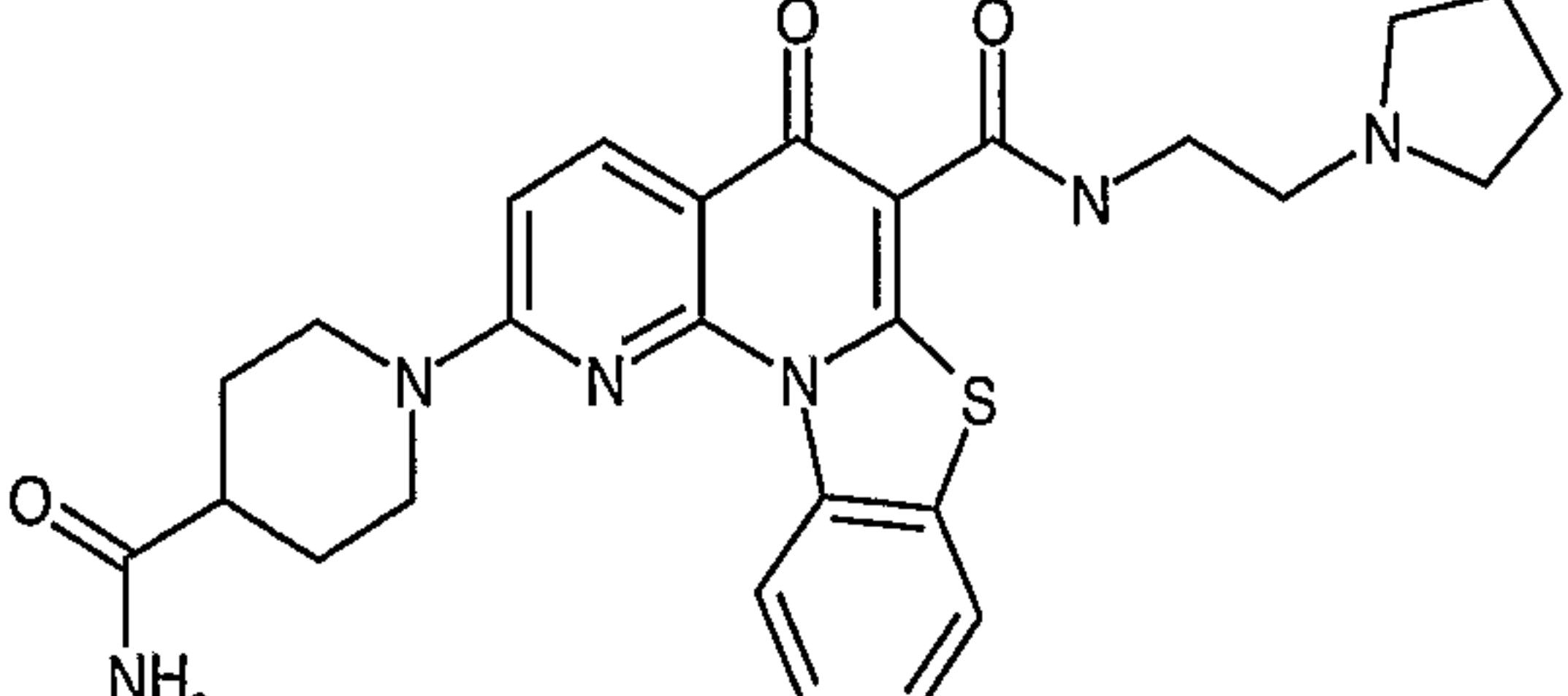
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | HCT-116 | 0.04   |
|    | MiaPaca | 0.02   |
|   | HCT-116 | 0.34   |
|   | MiaPaca | 0.73   |
|  | HCT-116 | 0.01   |
|  | MiaPaca | <0.01  |

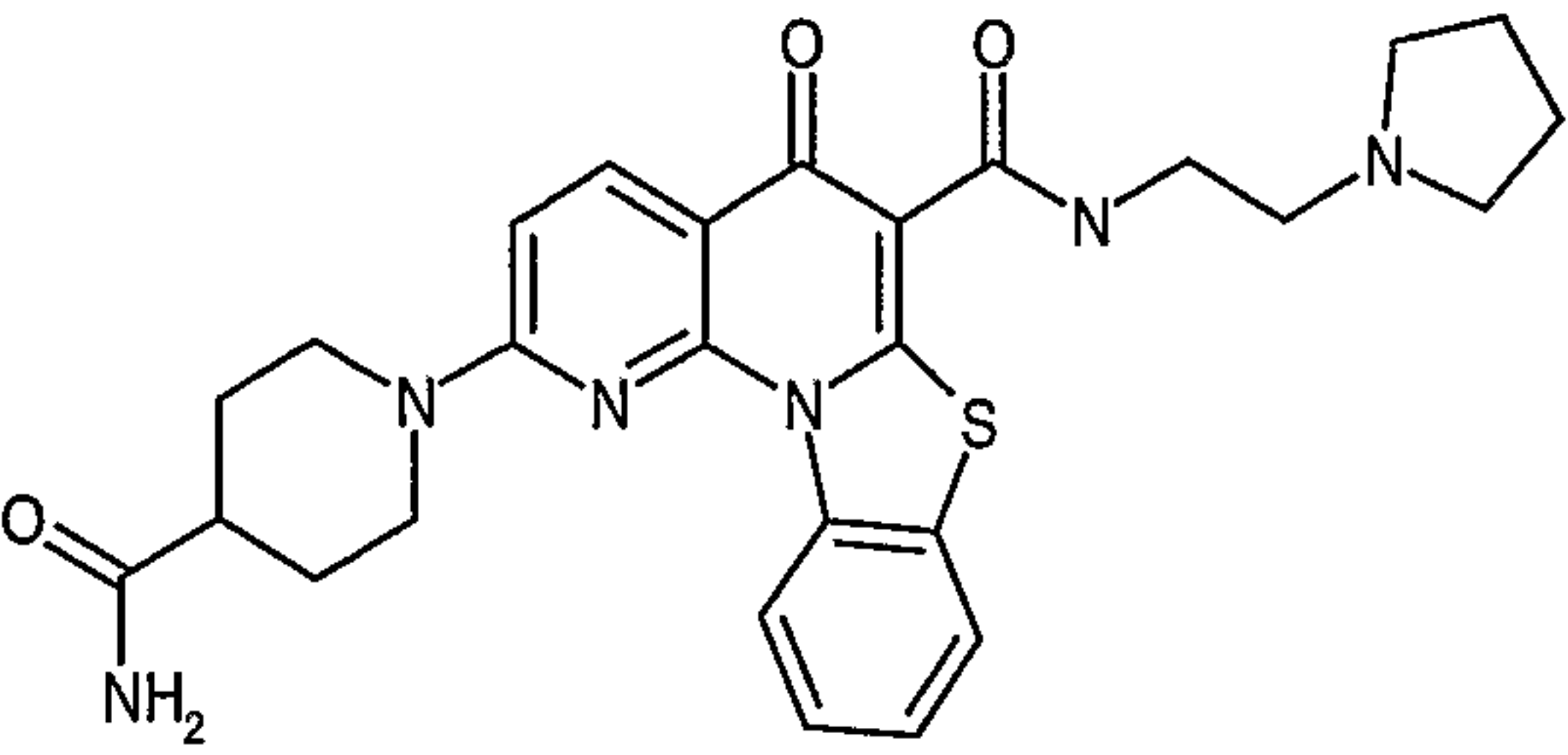
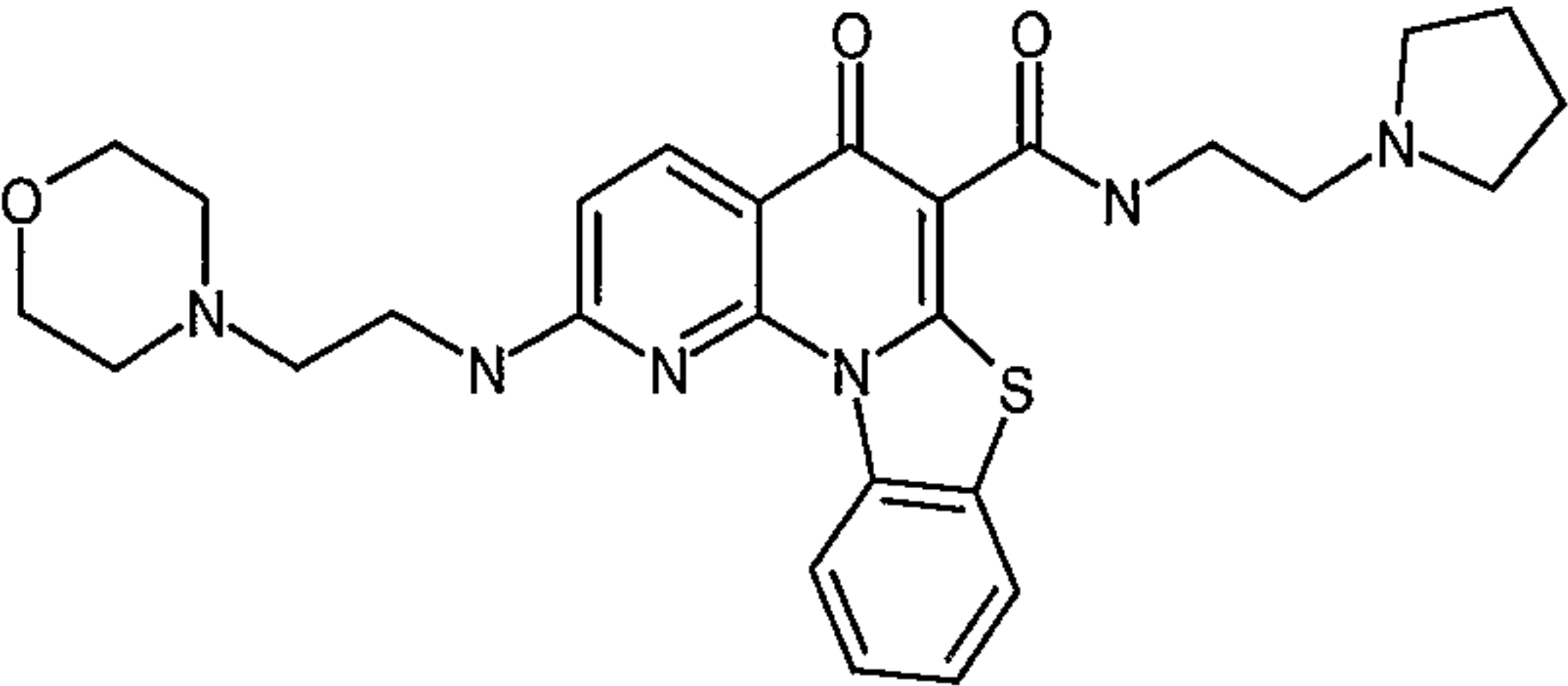
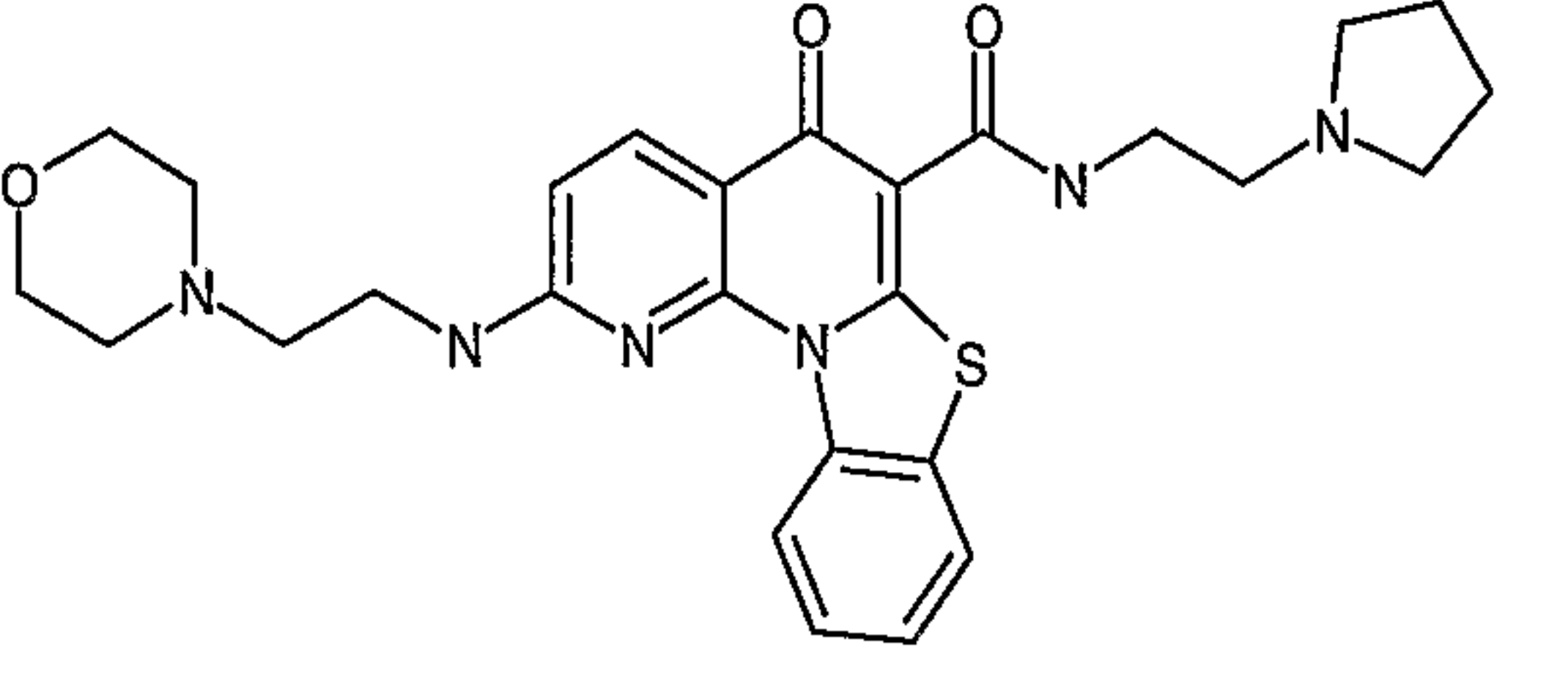
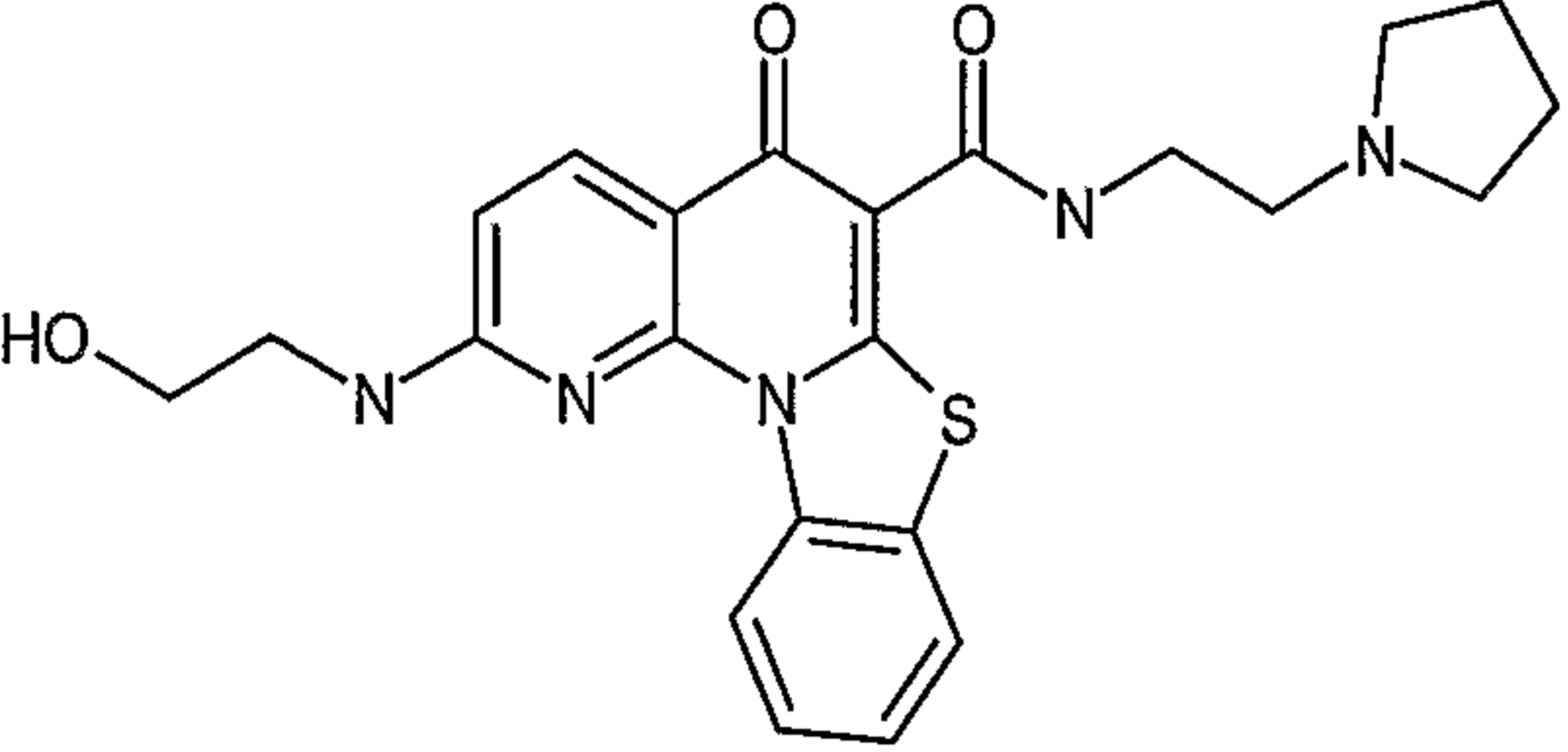
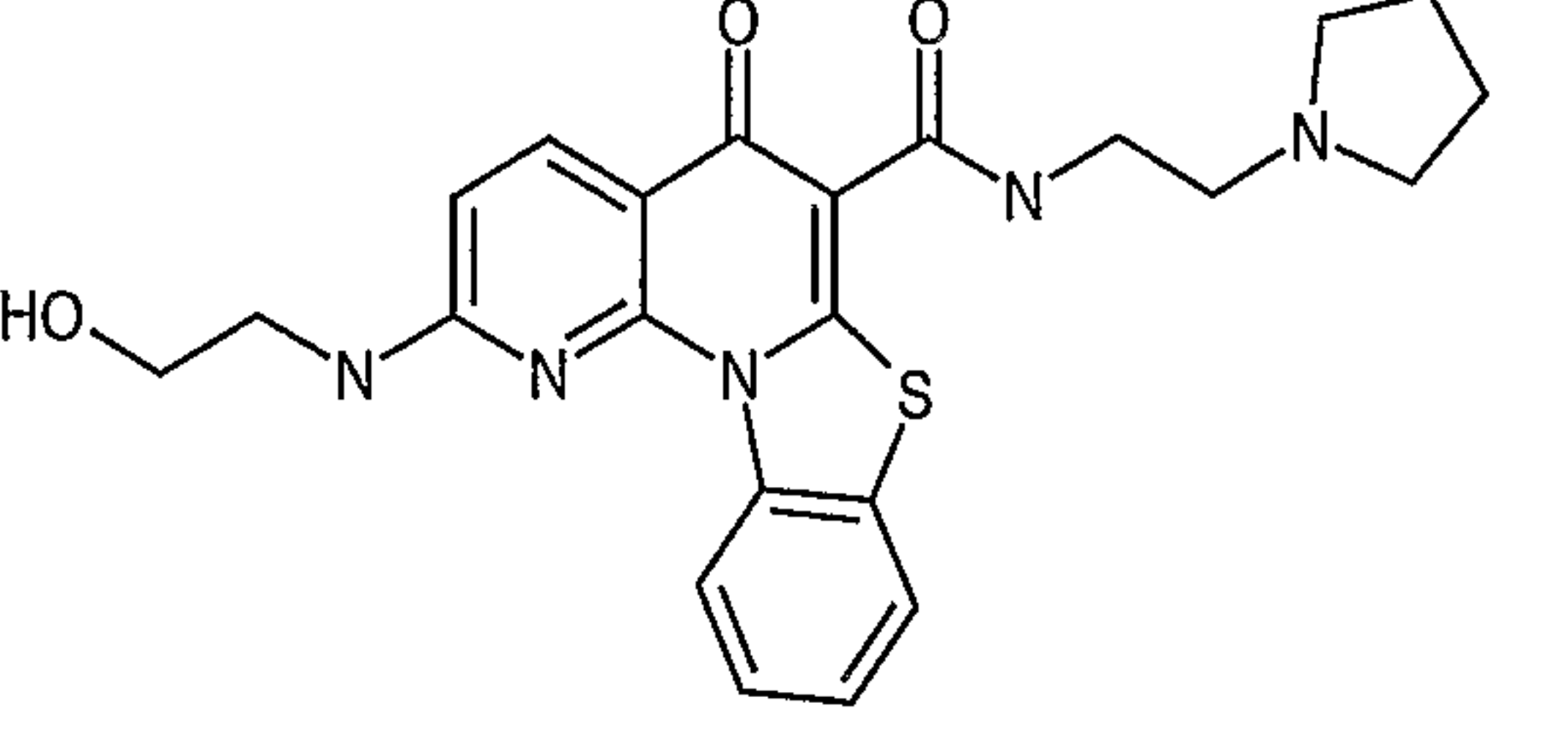
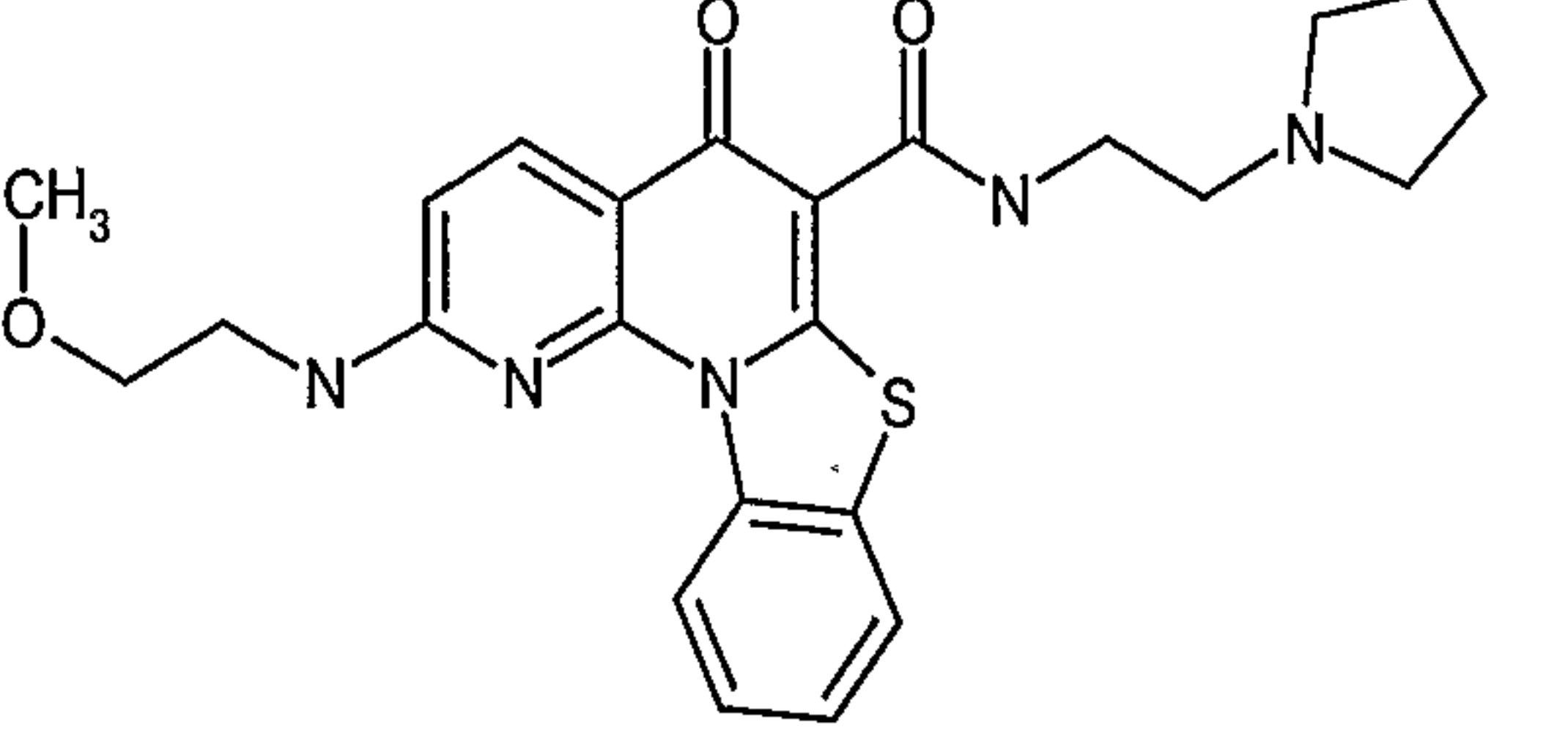
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | <0.01  |
|   | HCT-116 | 0.03   |
|   | HCT-116 | 0.34   |
|   | MiaPaca | 0.48   |
|  | MiaPaca | 0.04   |
|  | HCT-116 | 0.10   |

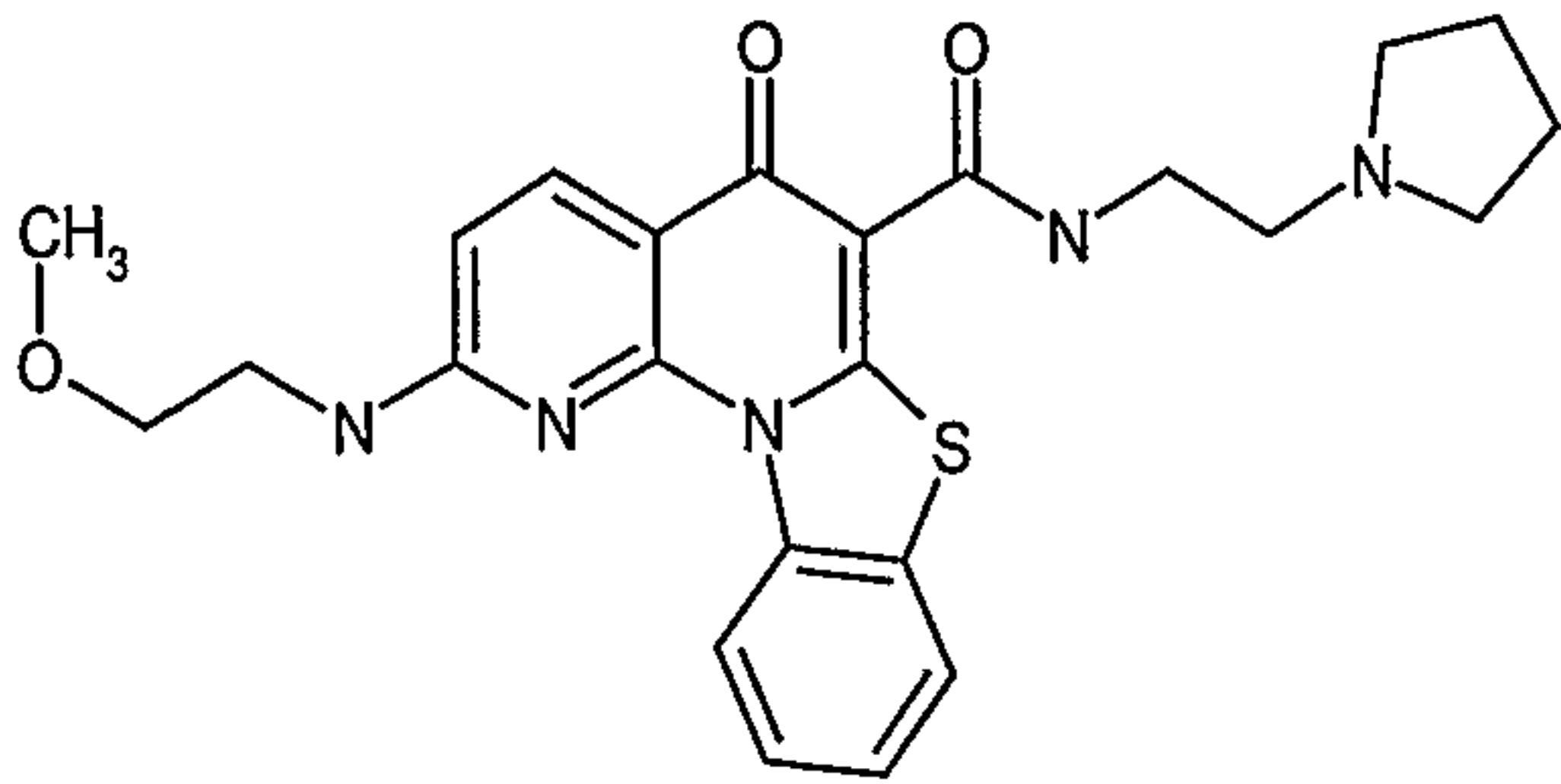
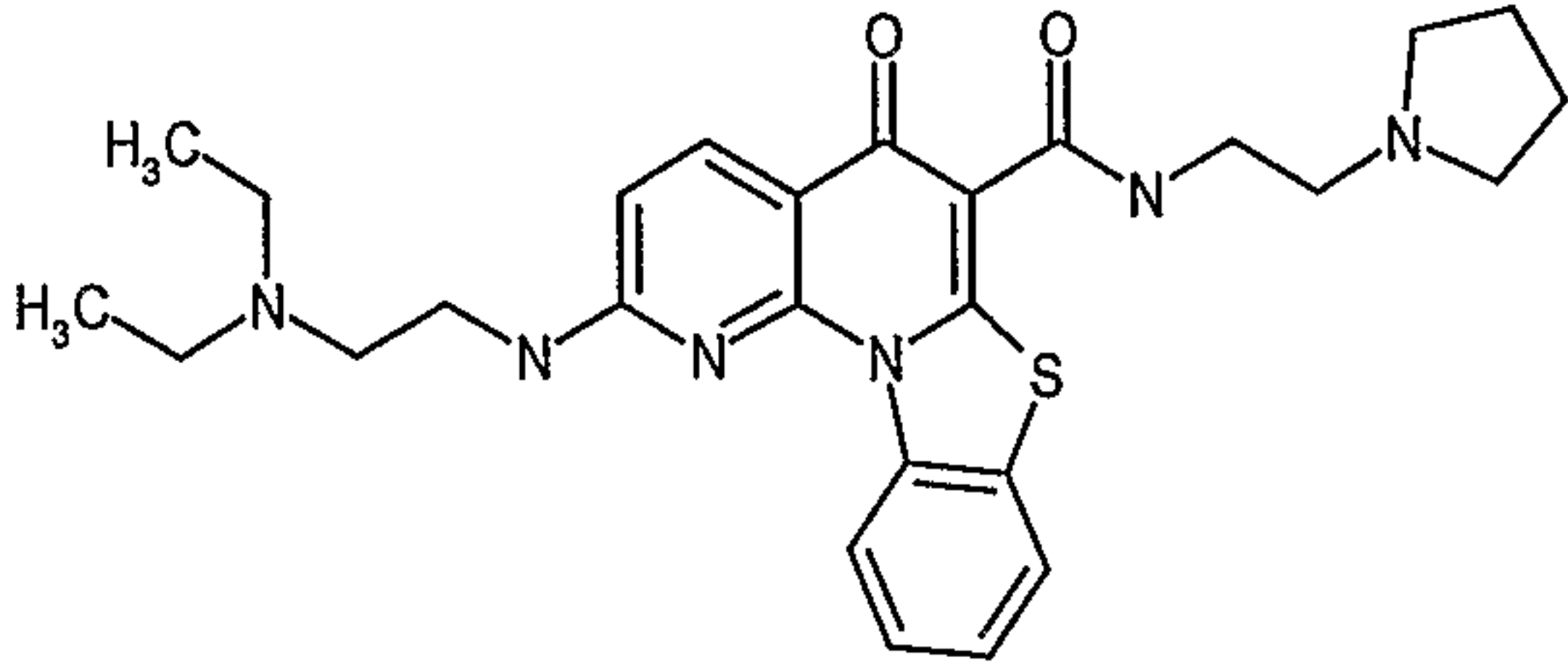
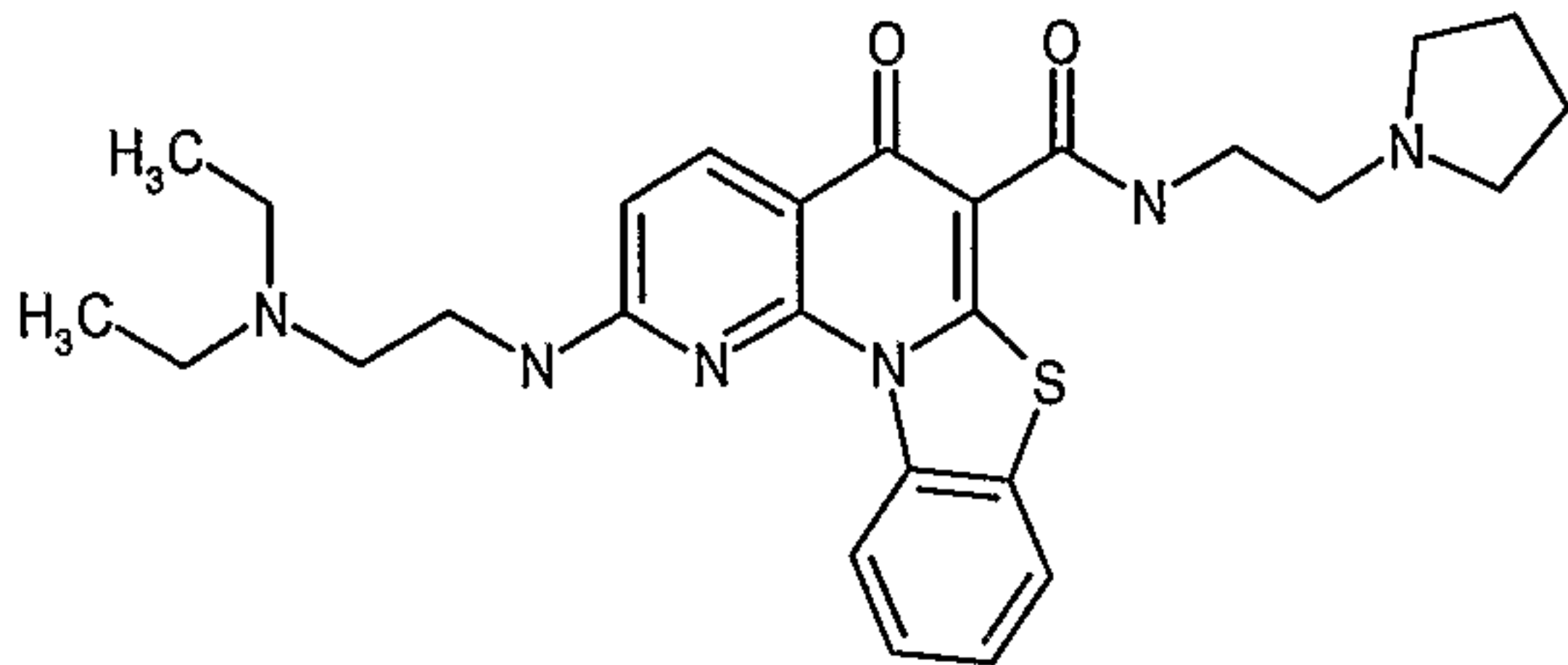
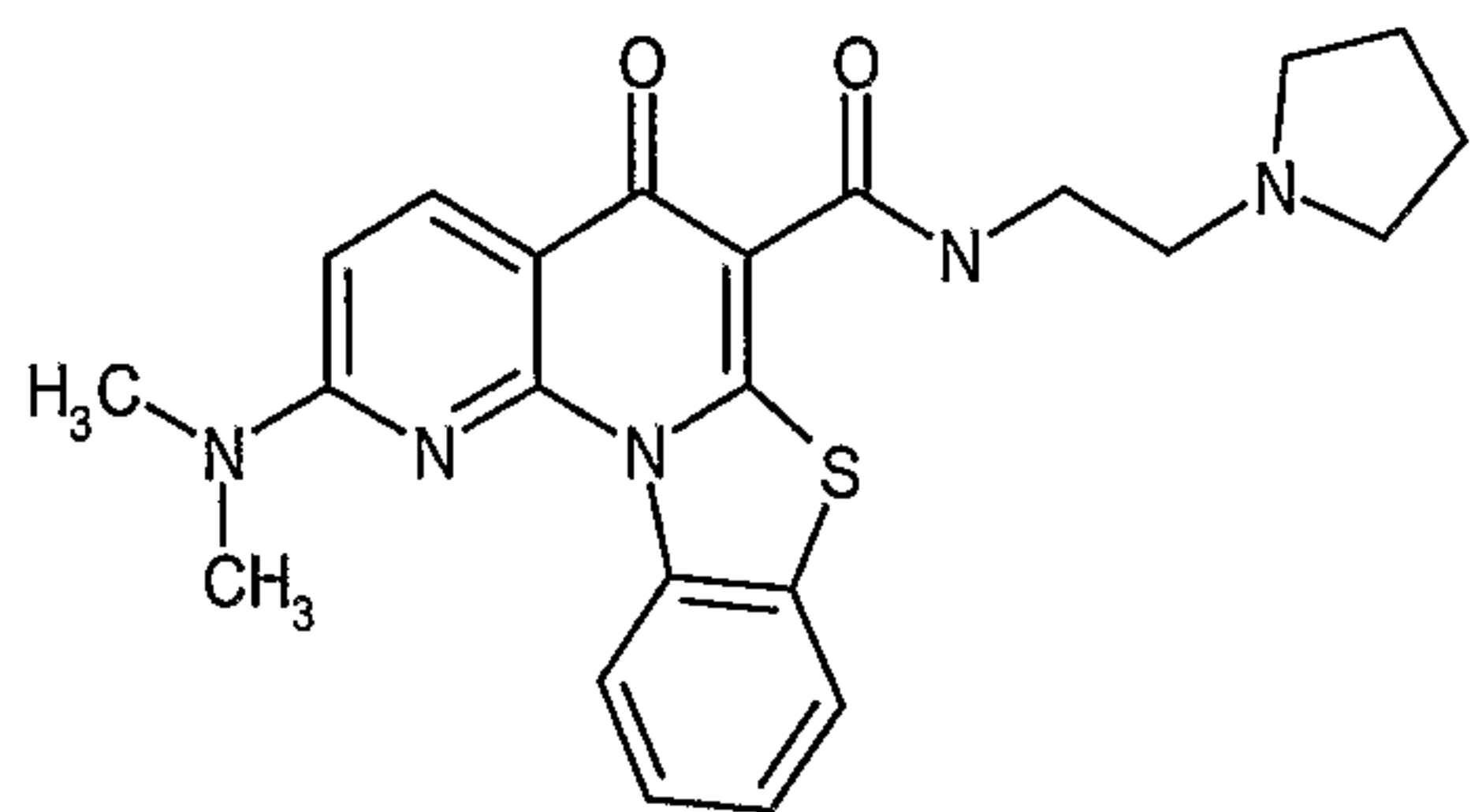
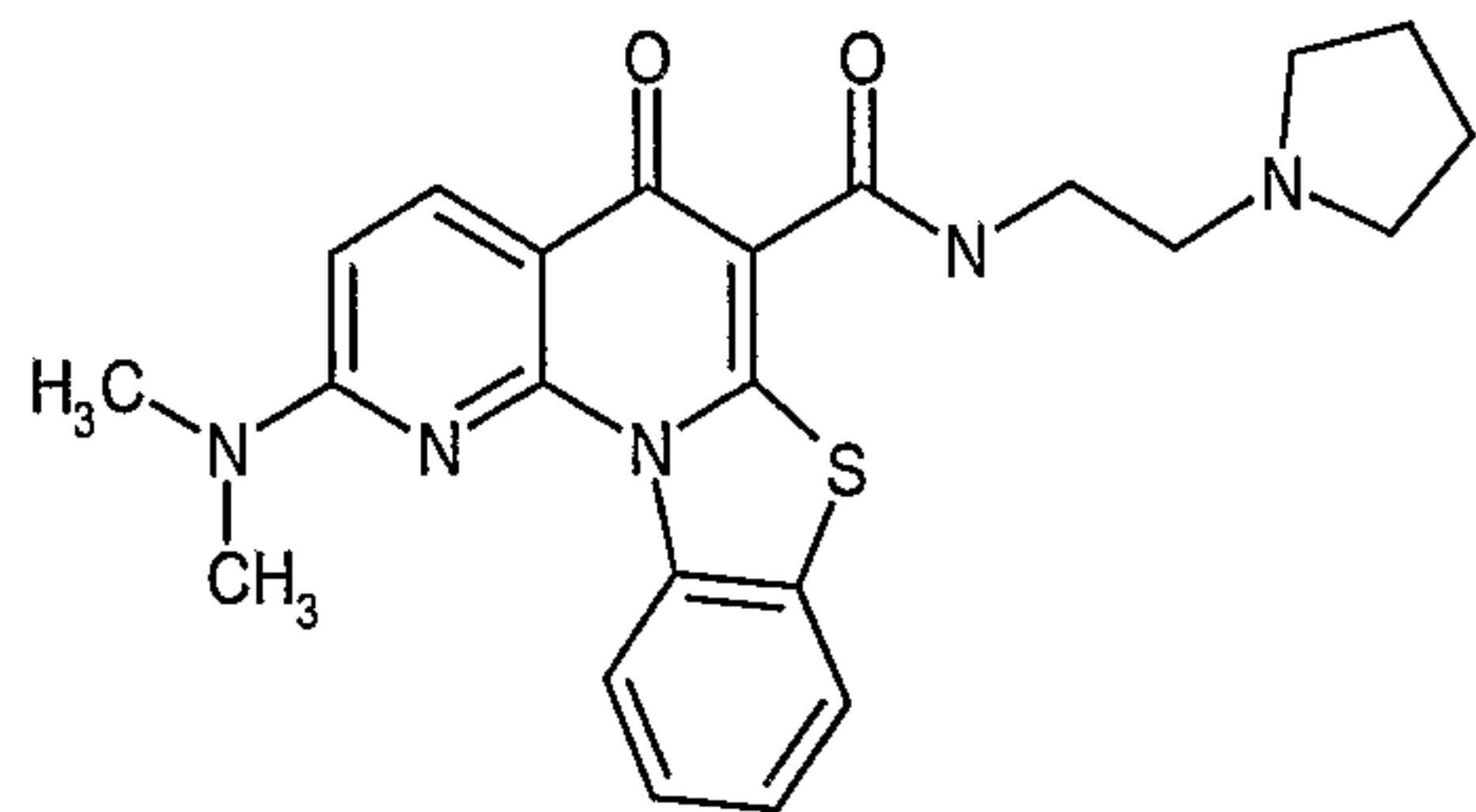
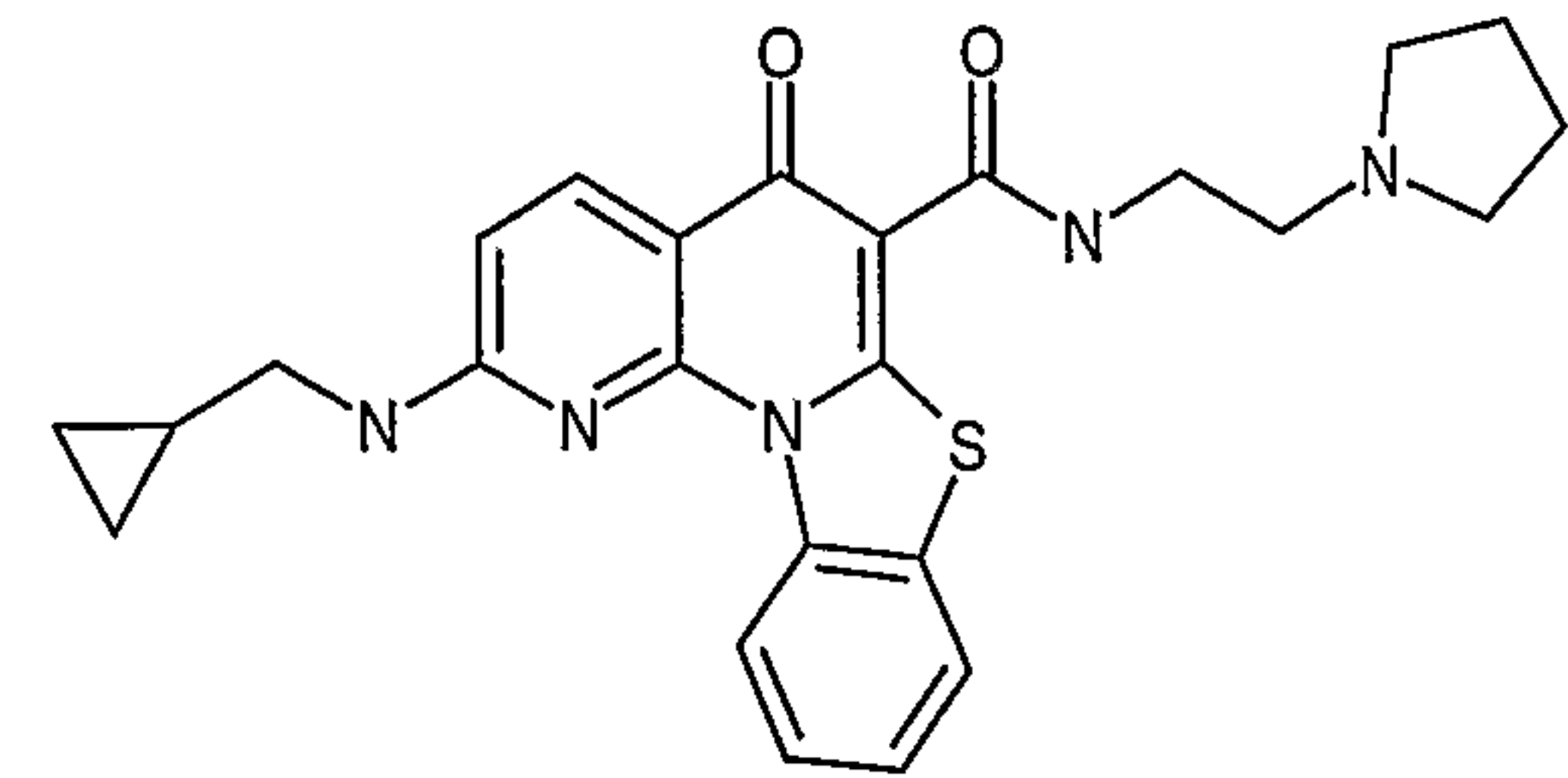
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | HCT-116 | 0.24   |
|    | Miapaca | 0.17   |
|   | HCT-116 | 0.2    |
|   | MiaPaca | 0.45   |
|  | HCT-116 | 0.33   |
|  | MiaPaca | 2.50   |

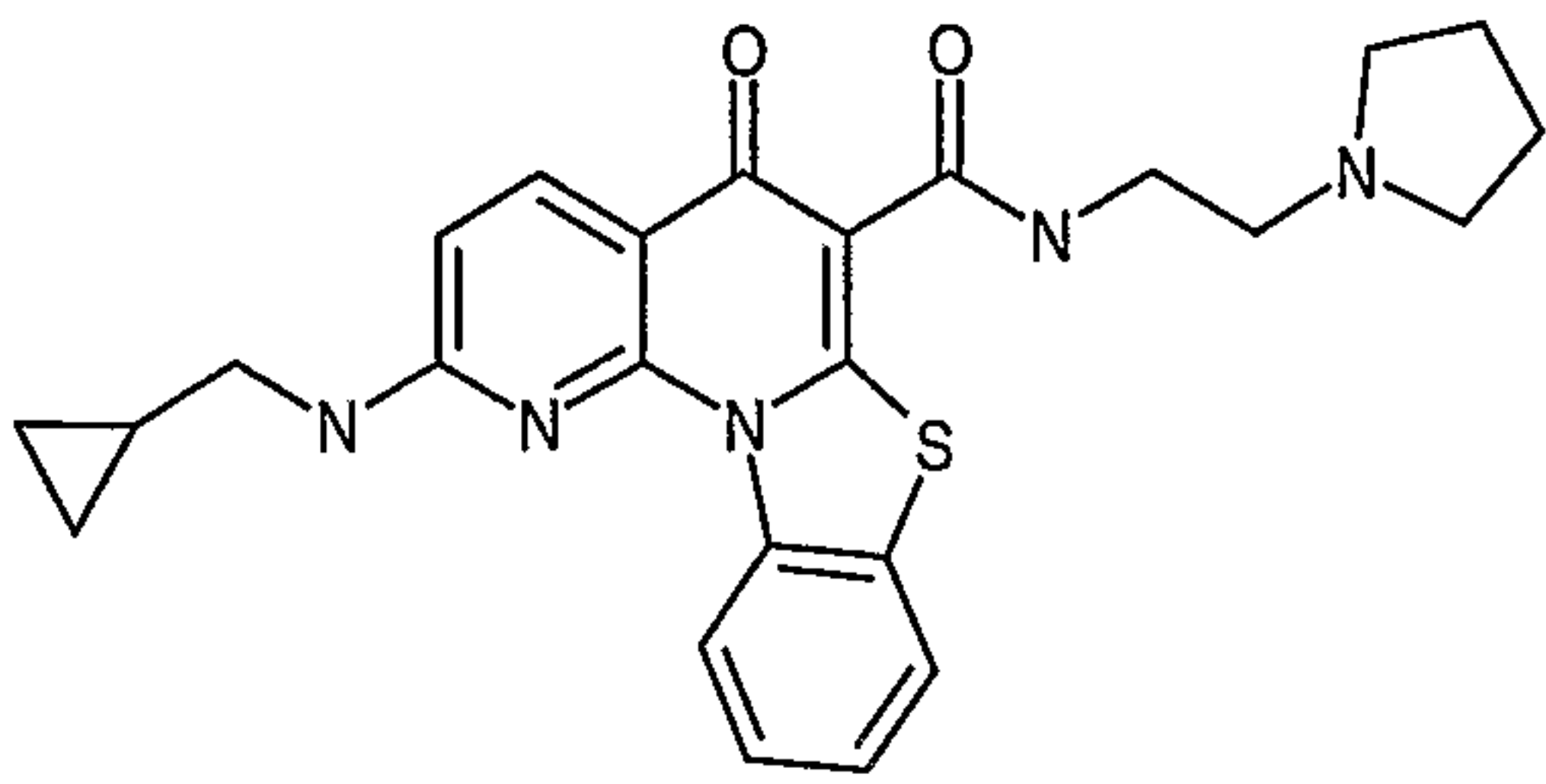
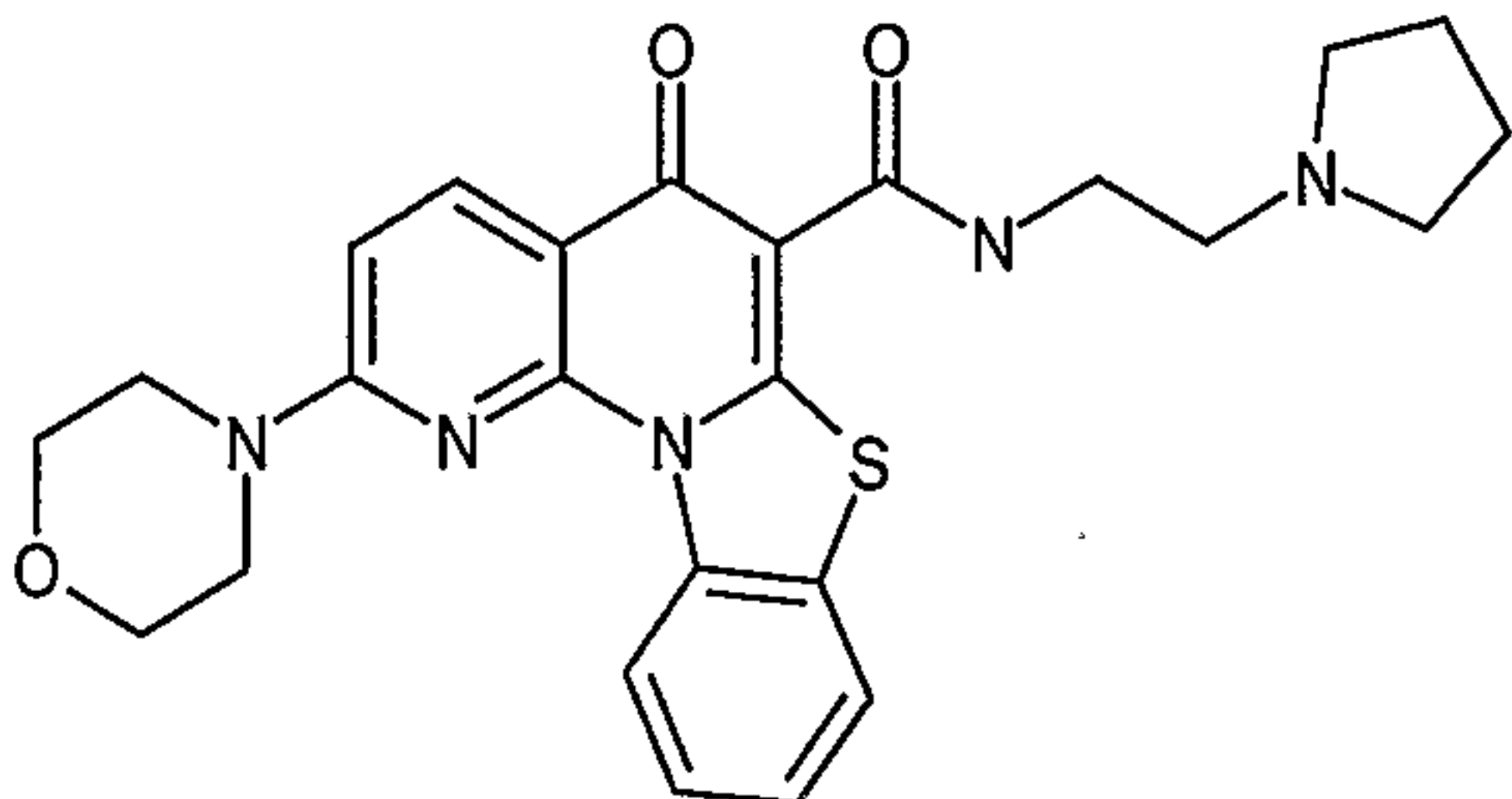
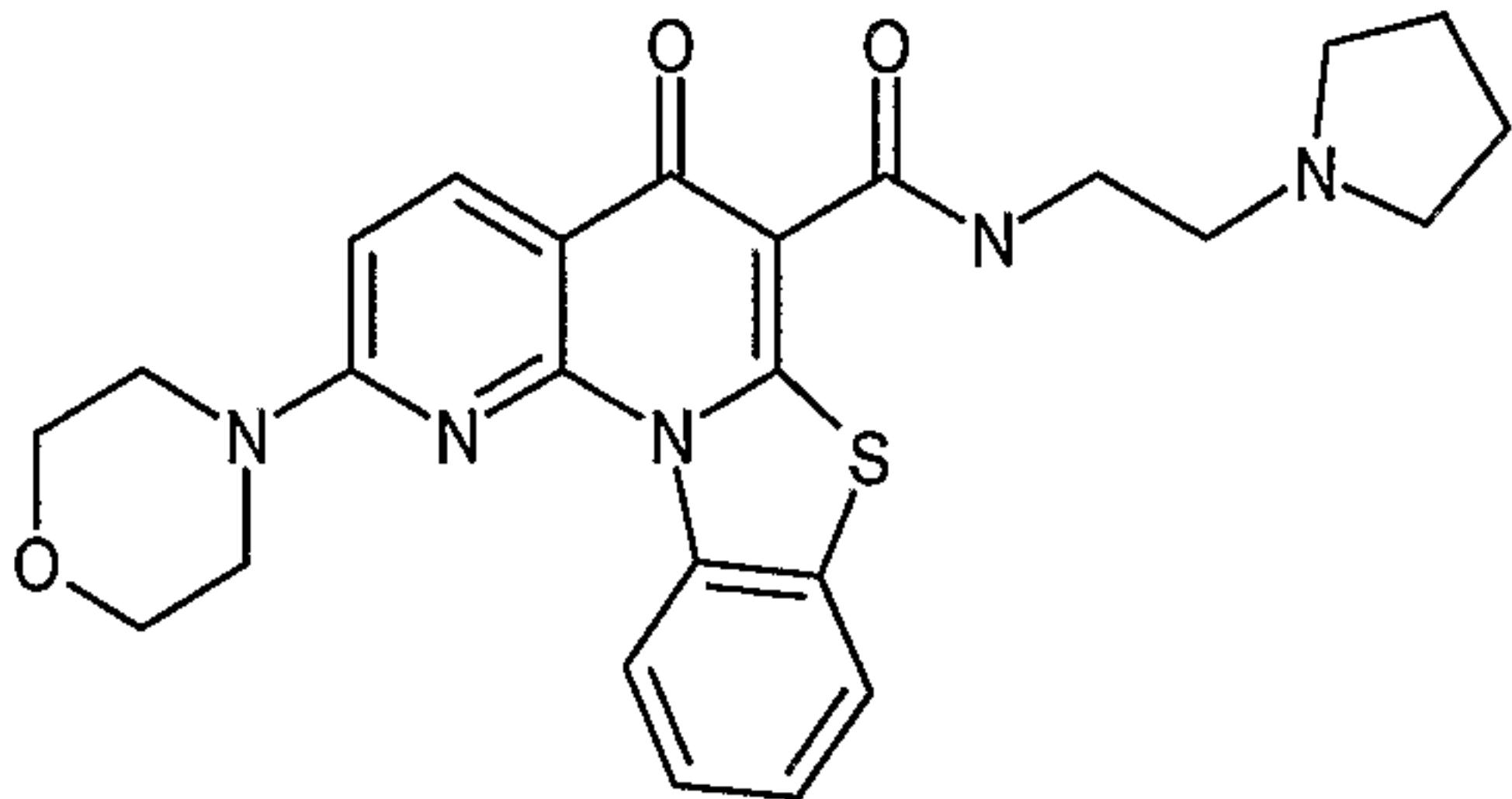
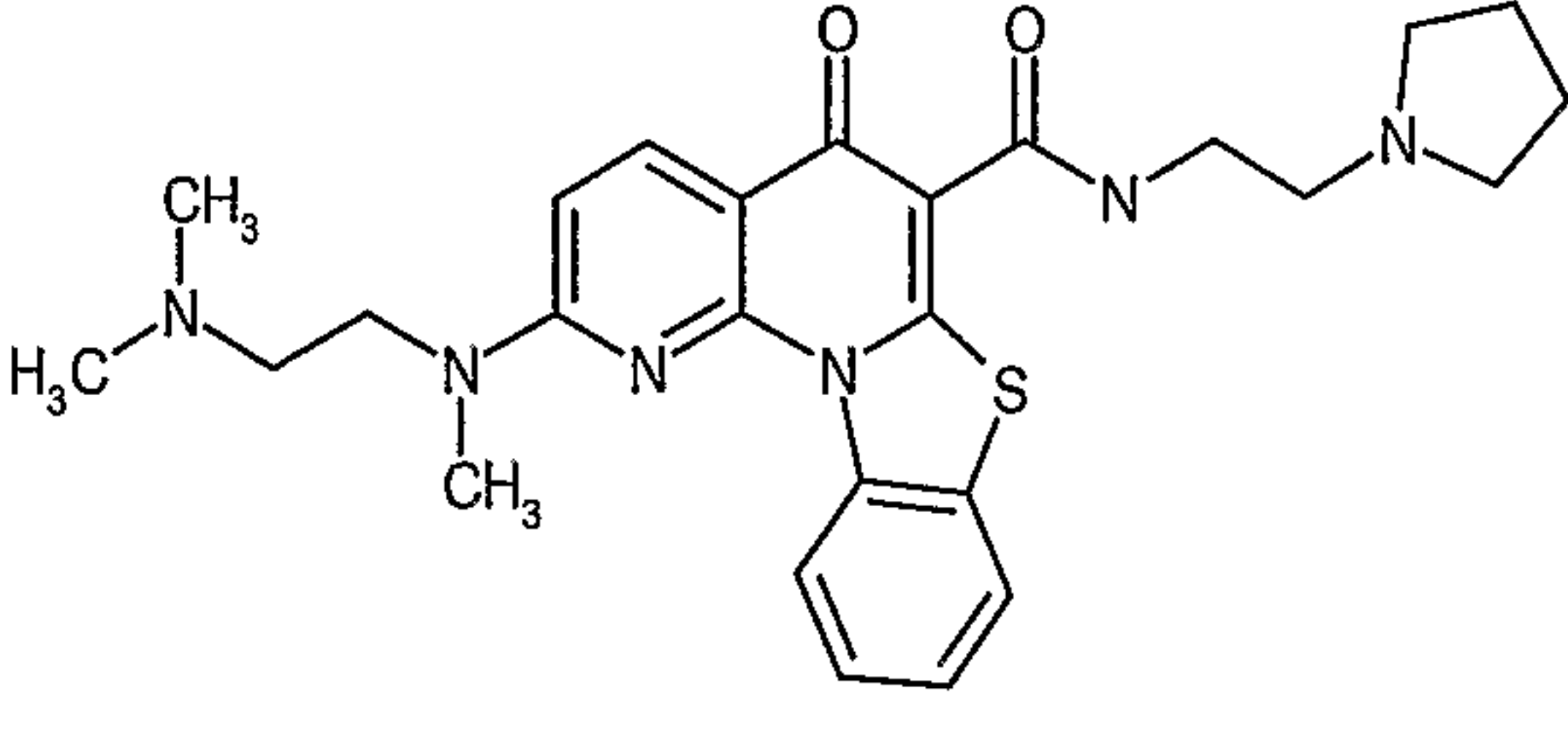
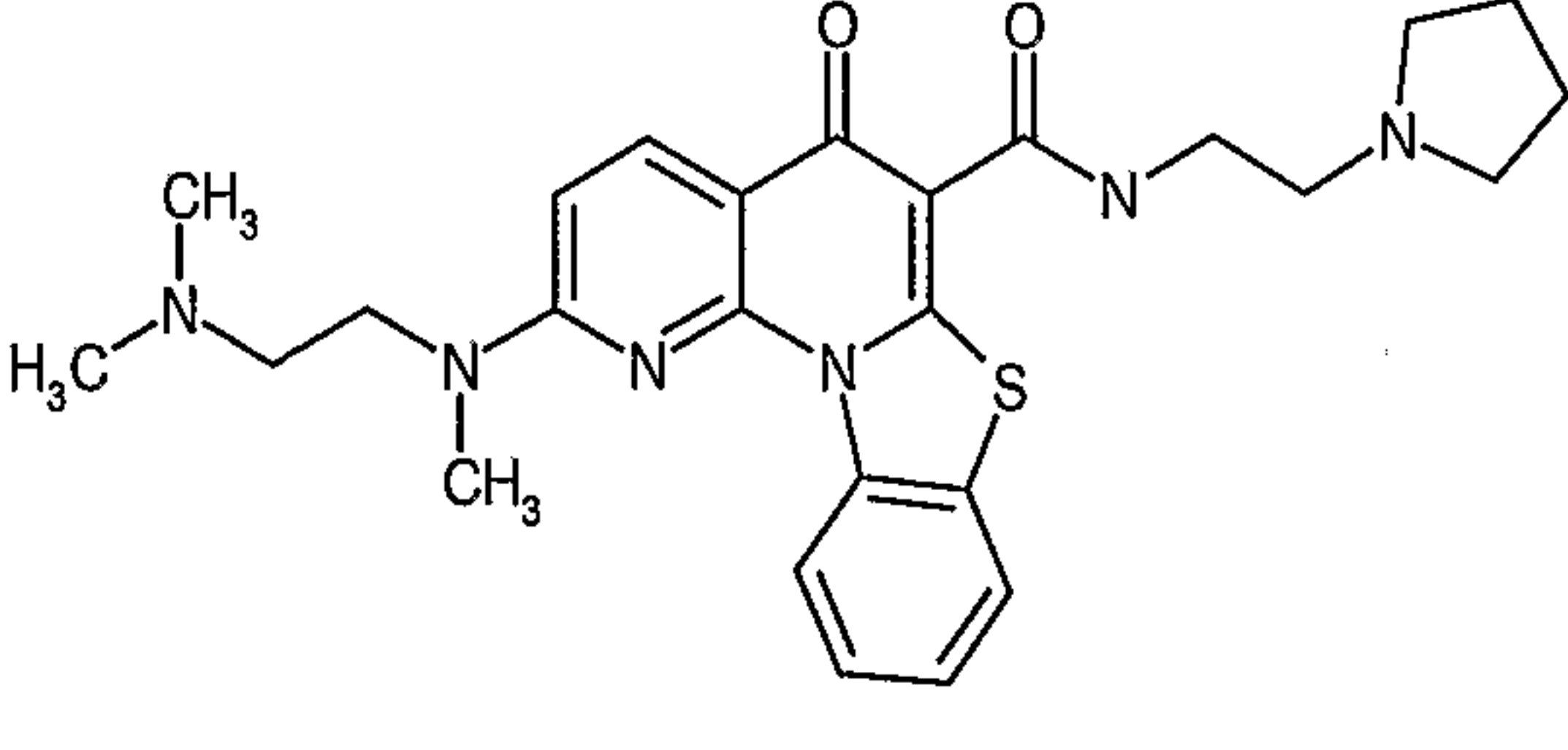
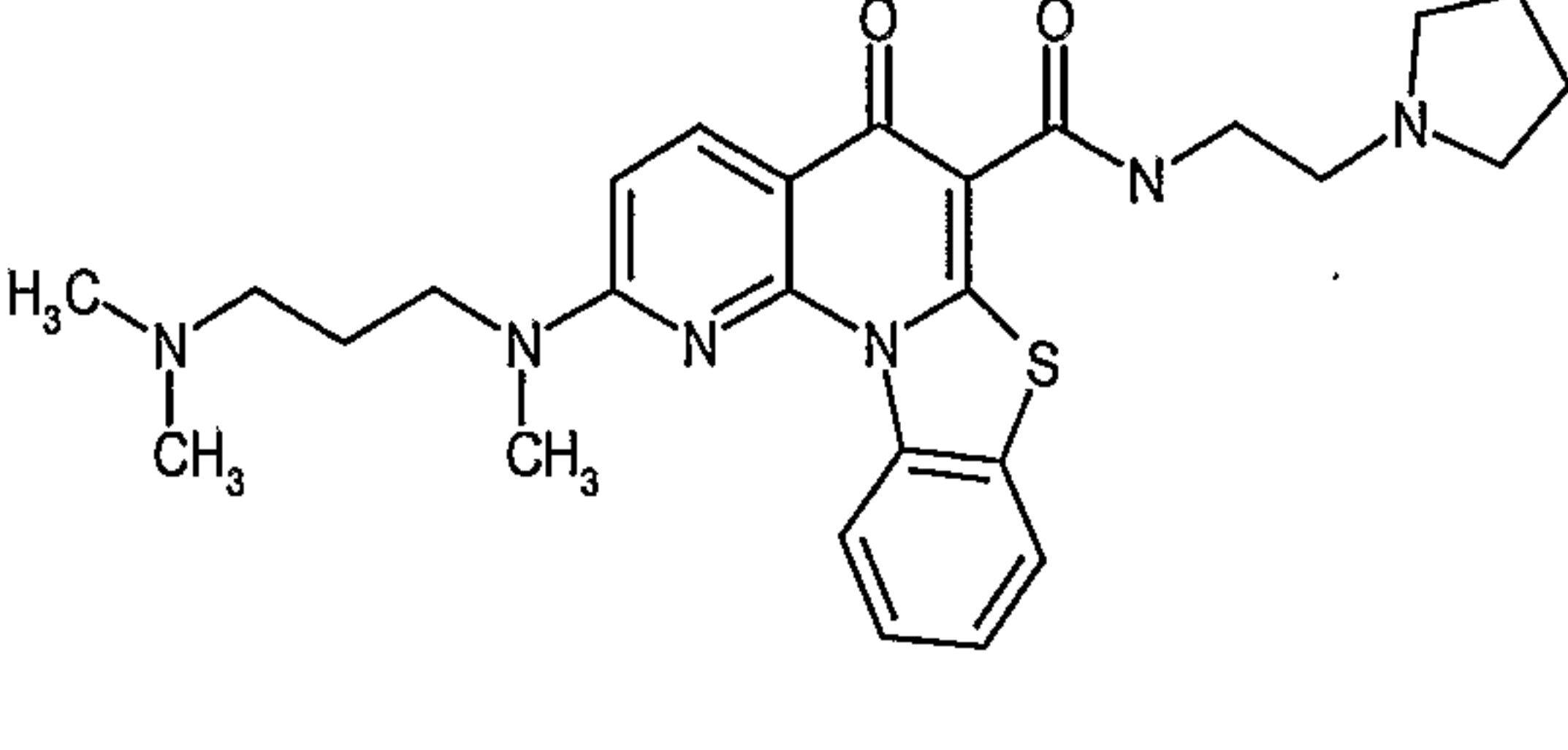
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-126 | 0.34   |
|   | MiaPaca | 0.02   |
|  | HCT-116 | 0.01   |
|   | HCT-116 | 0.35   |
|   | Miapaca | 0.43   |
|  | MiaPaca | <0.01  |

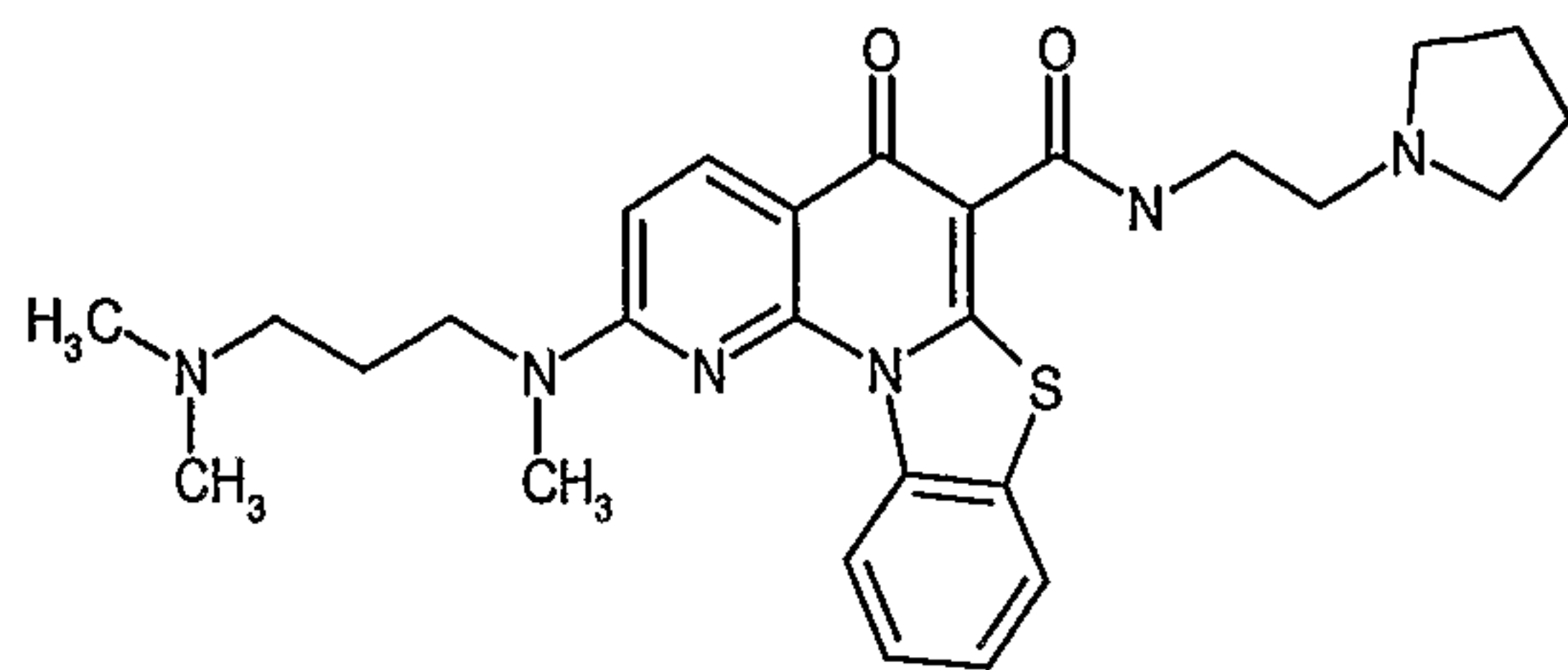
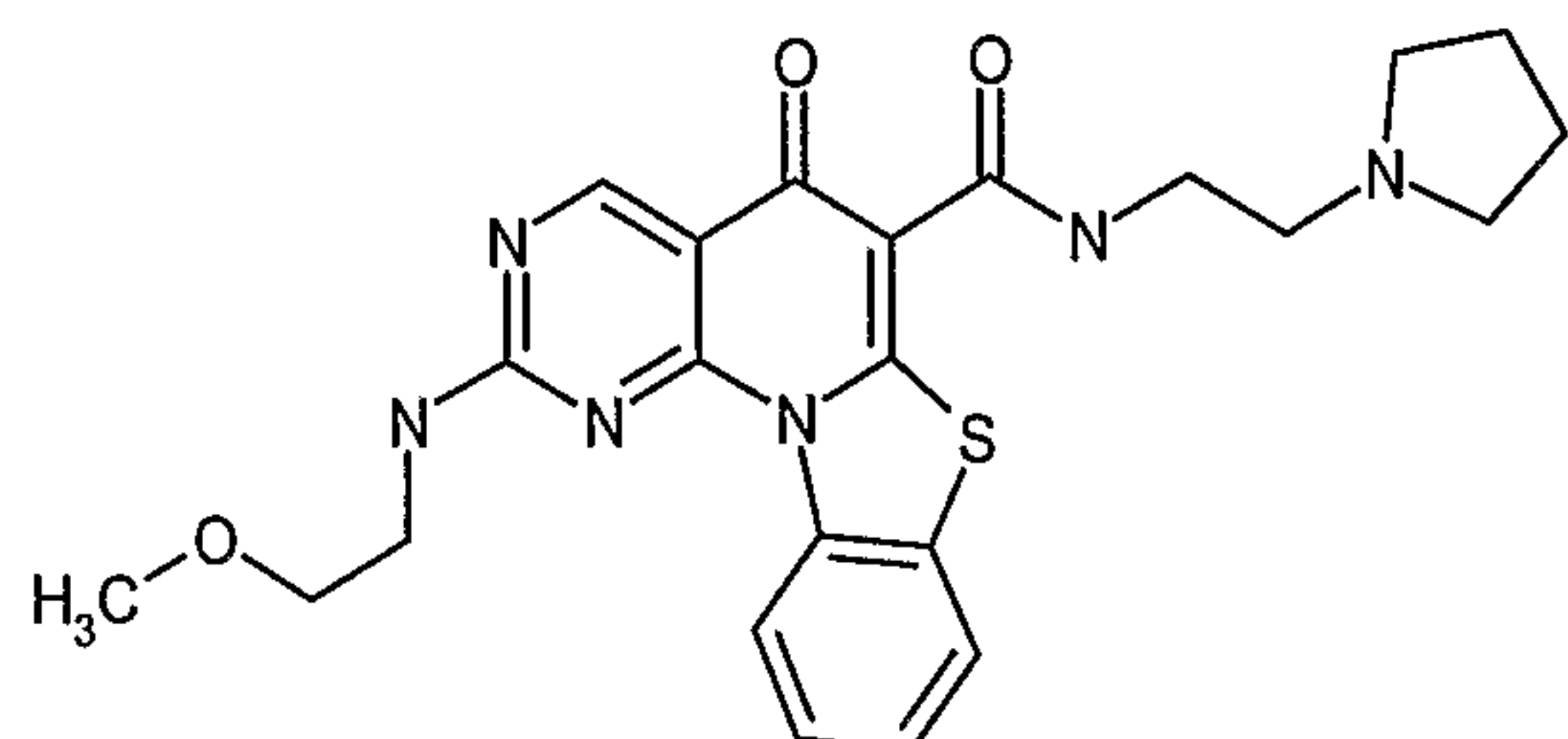
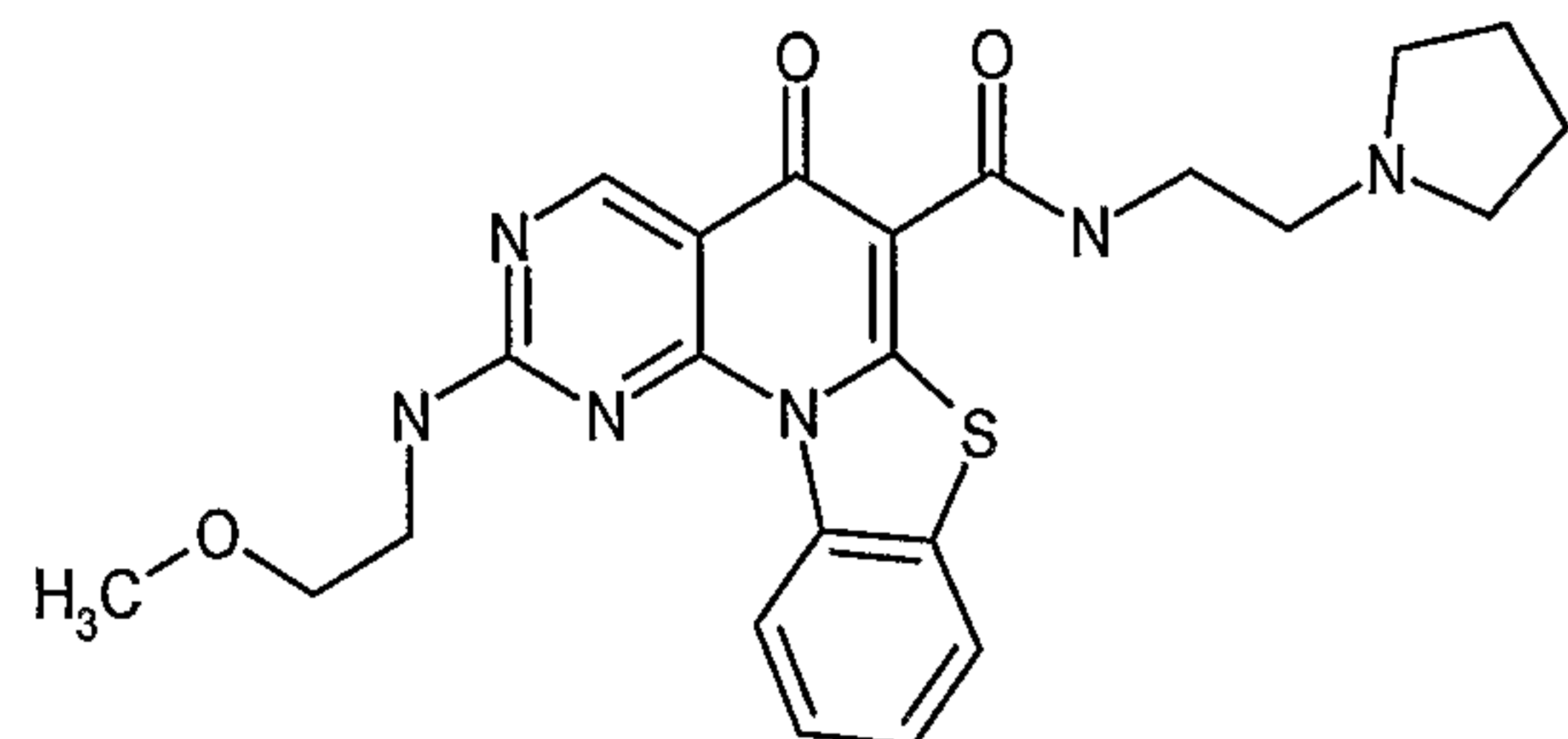
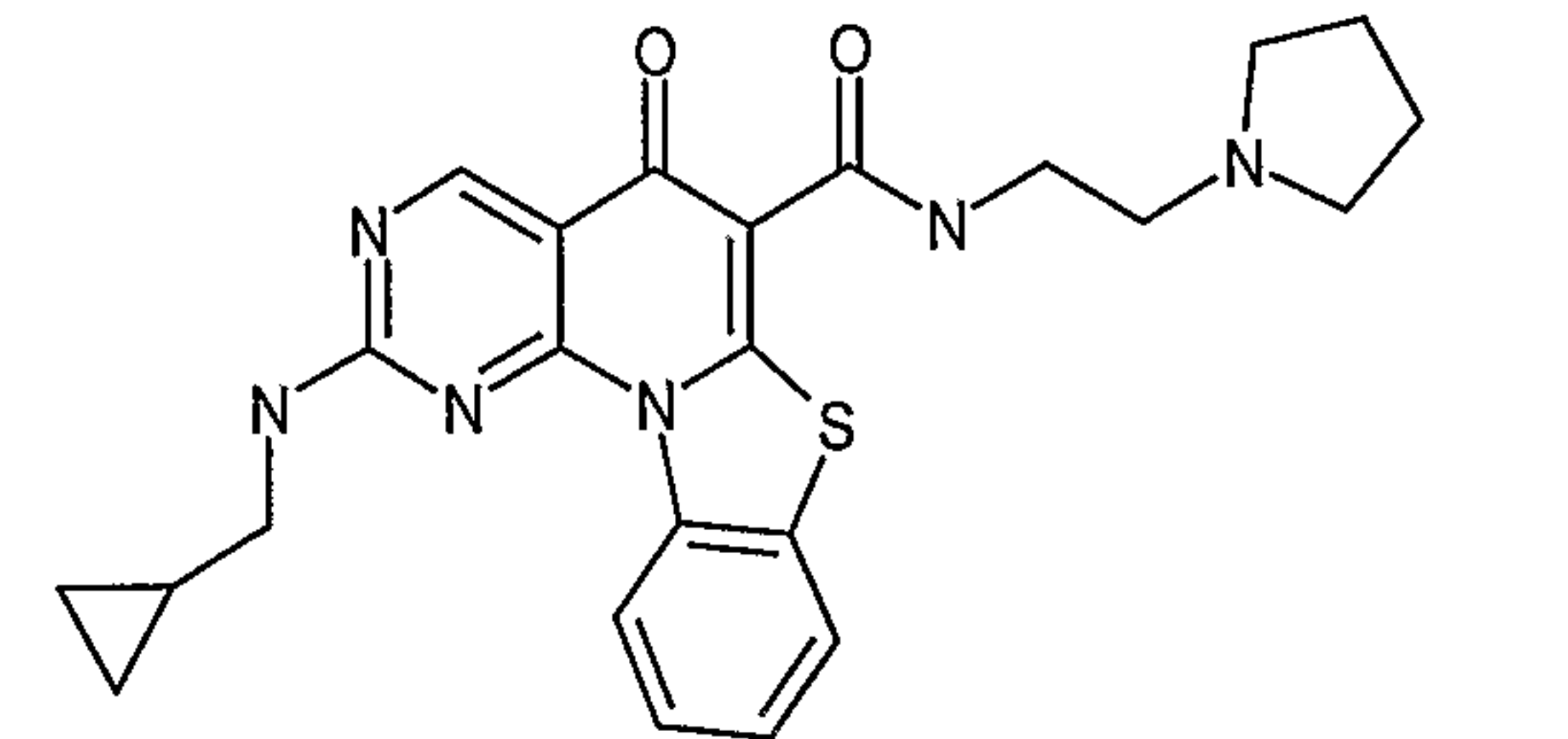
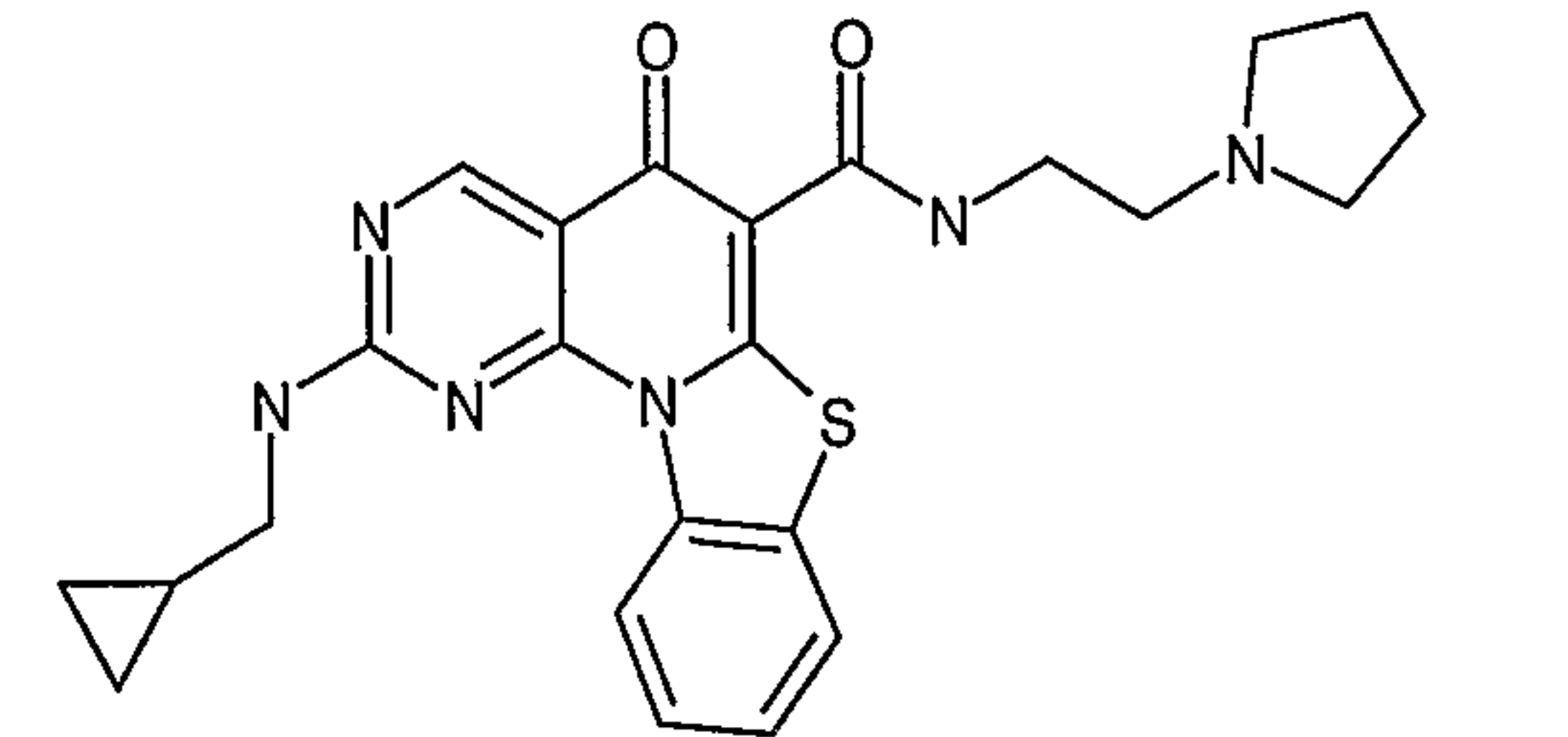
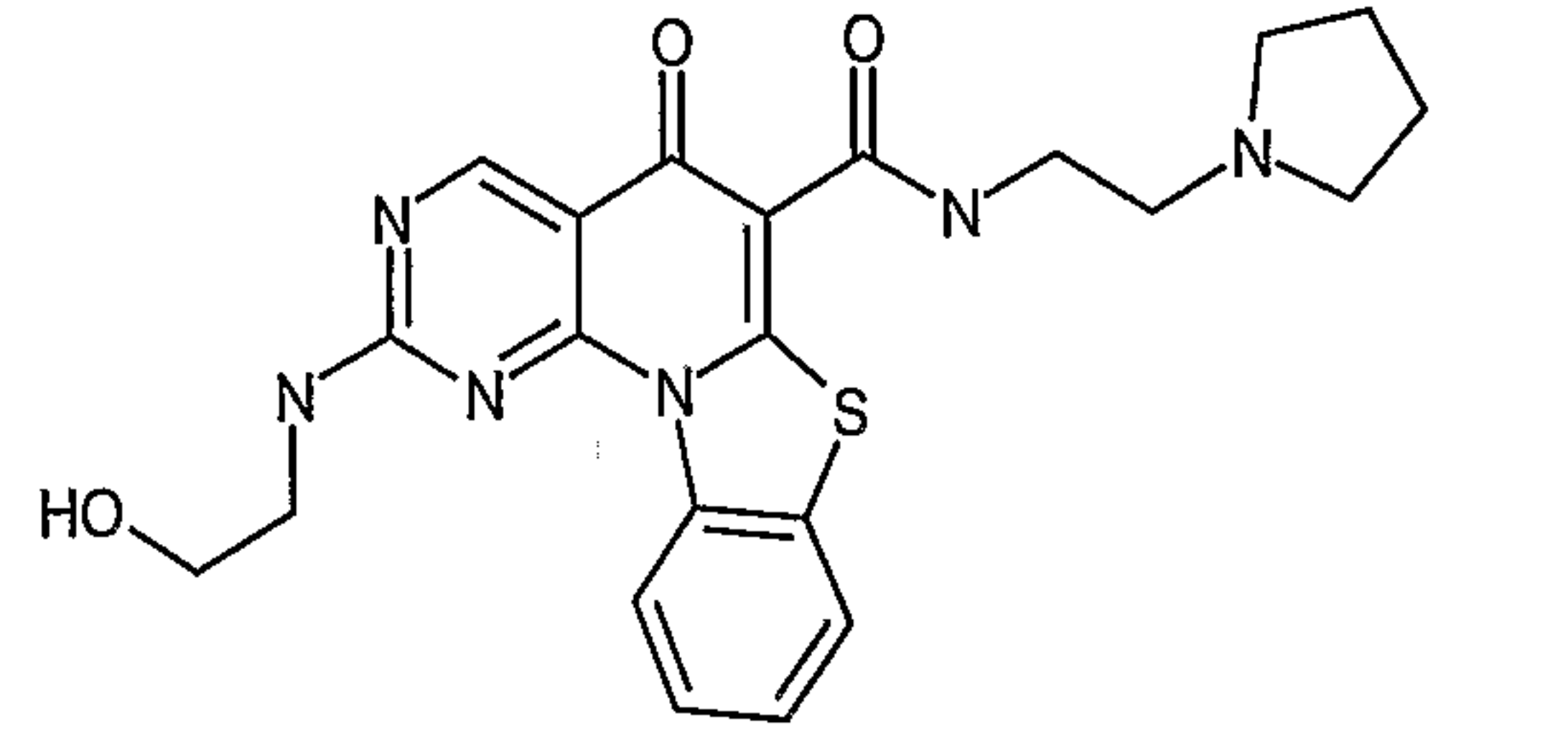
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | <0.01  |
|   | MiaPaca | <0.01  |
|  | HCT-116 | <0.01  |
|  | MiaPaca | 0.01   |
|  | HCT-116 | <0.01  |
|  | MiaPaca | 0.17   |

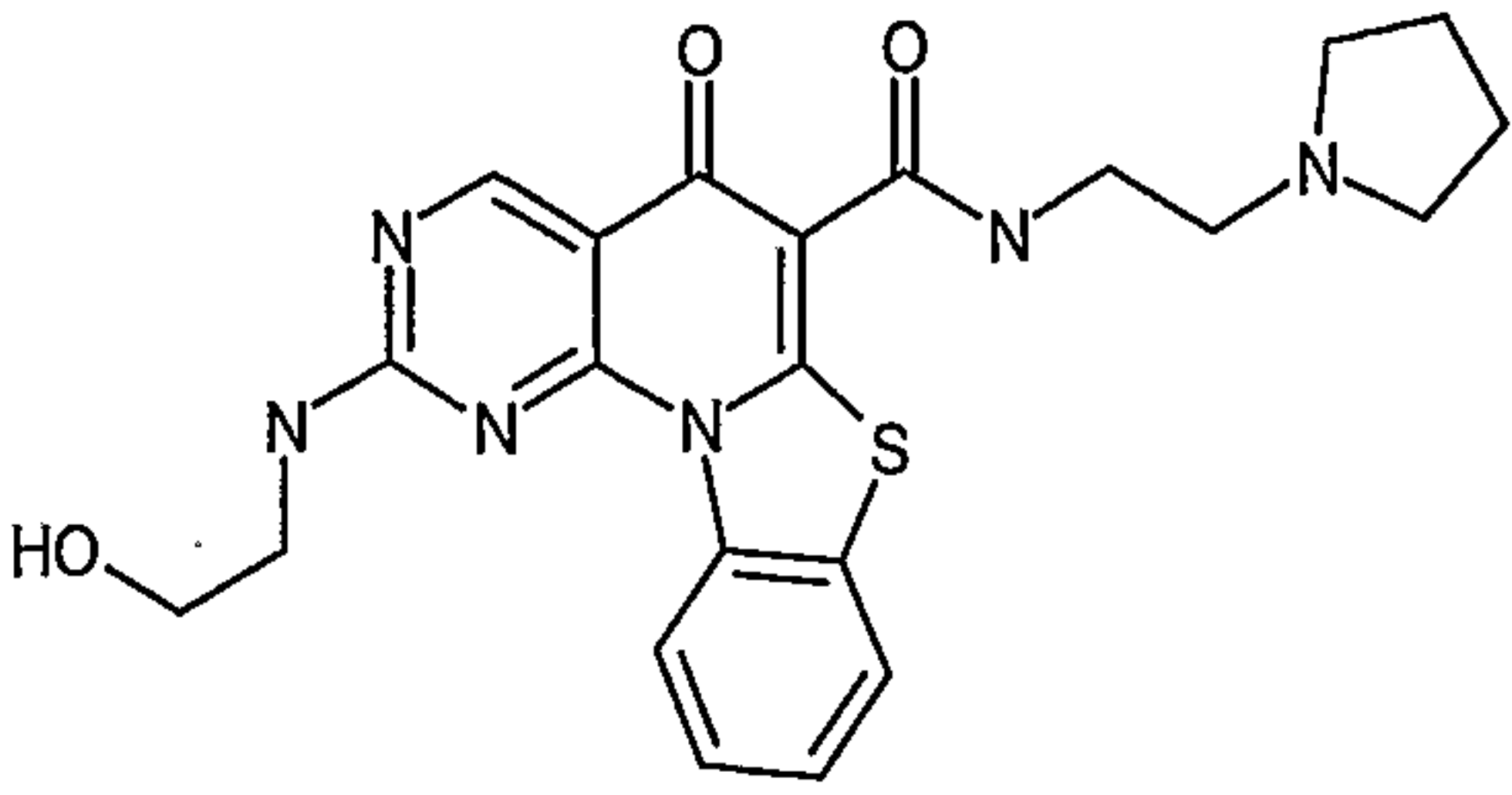
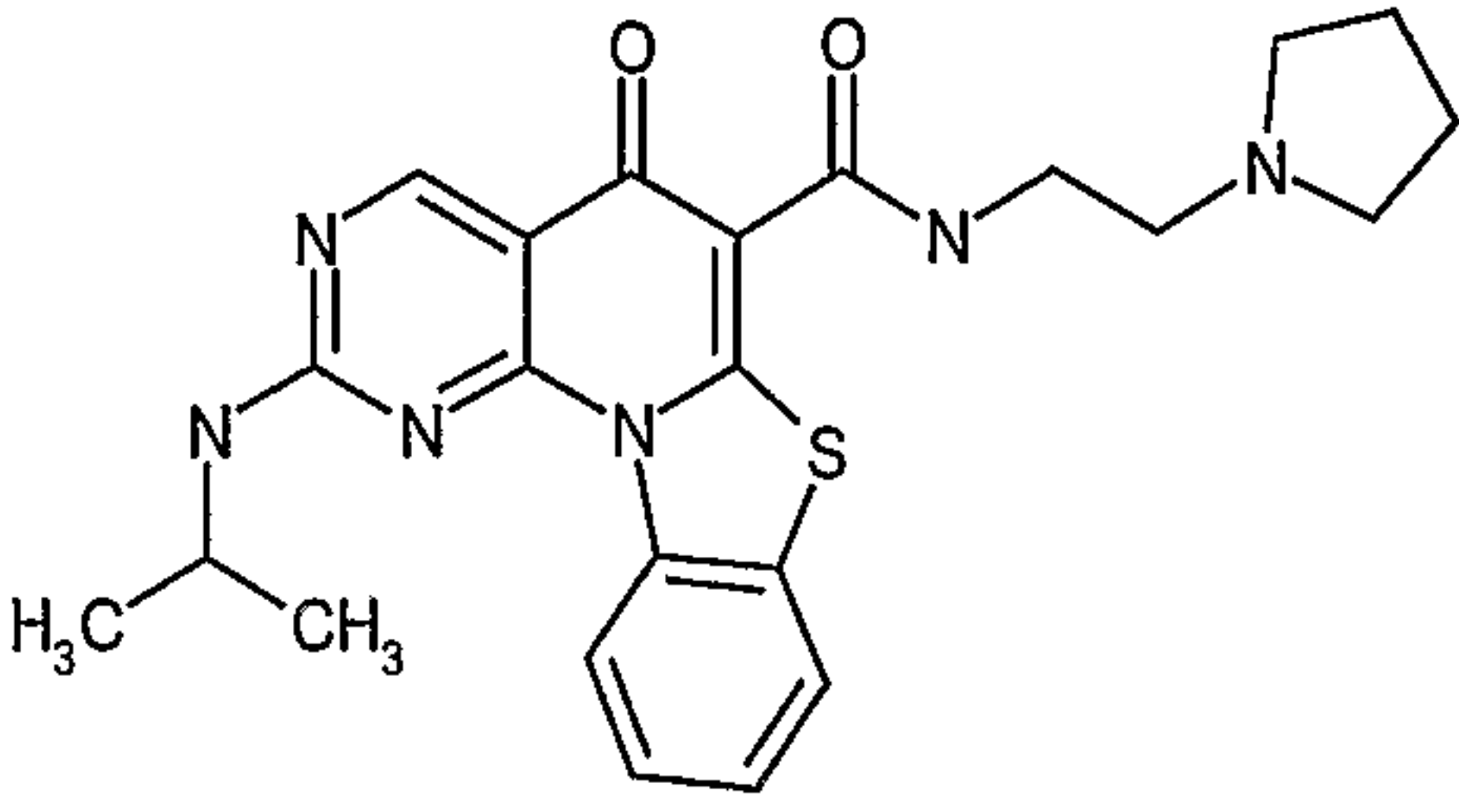
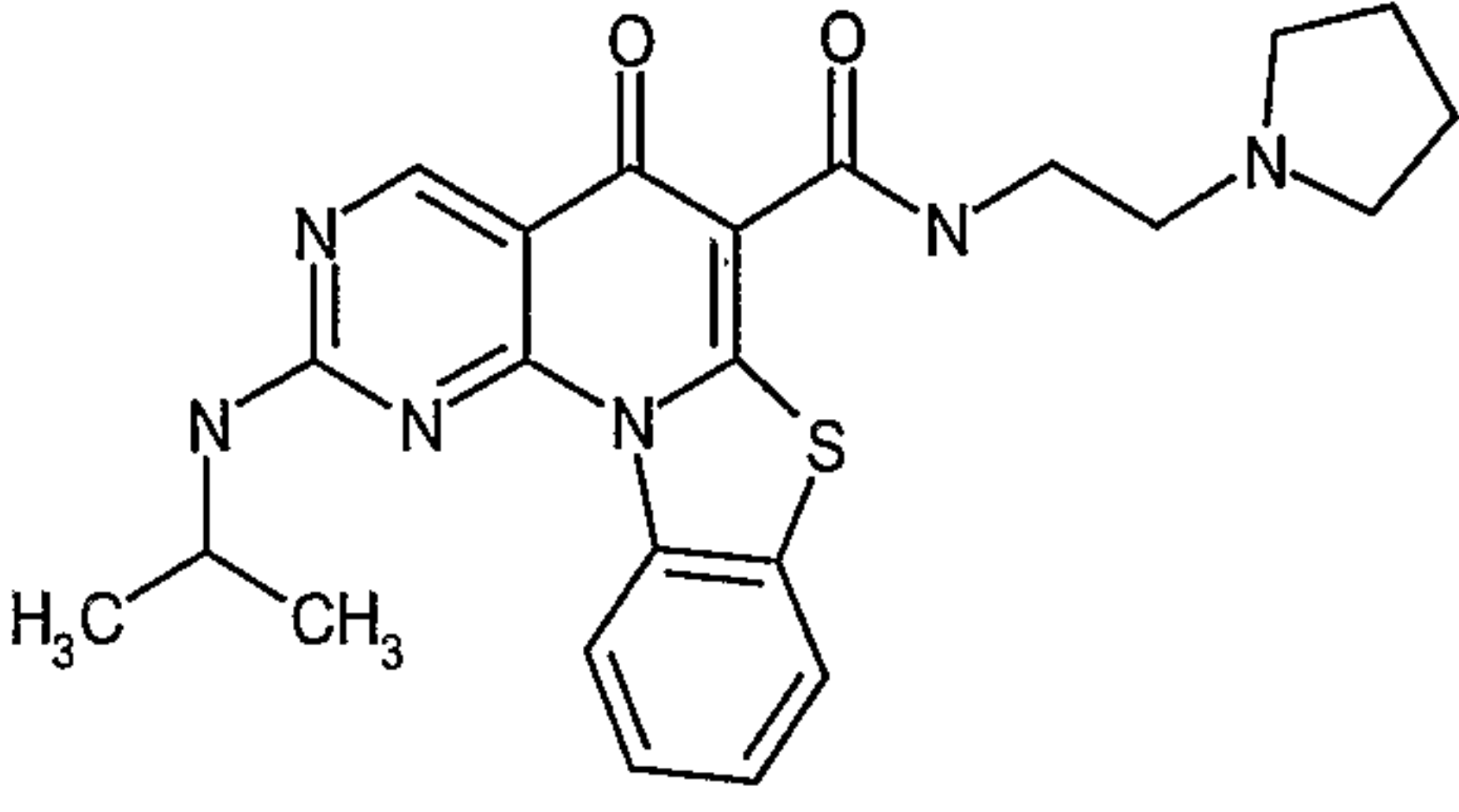
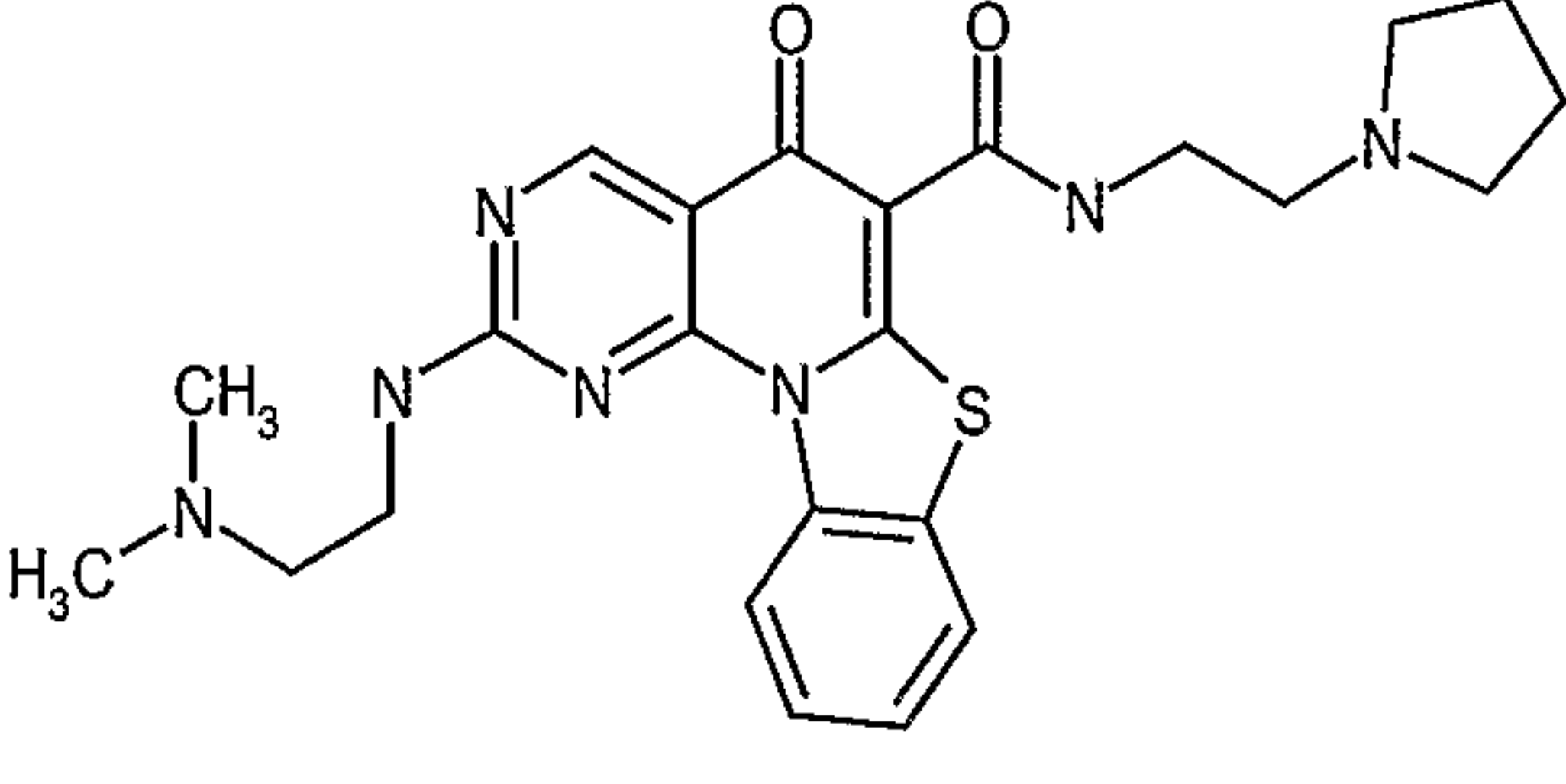
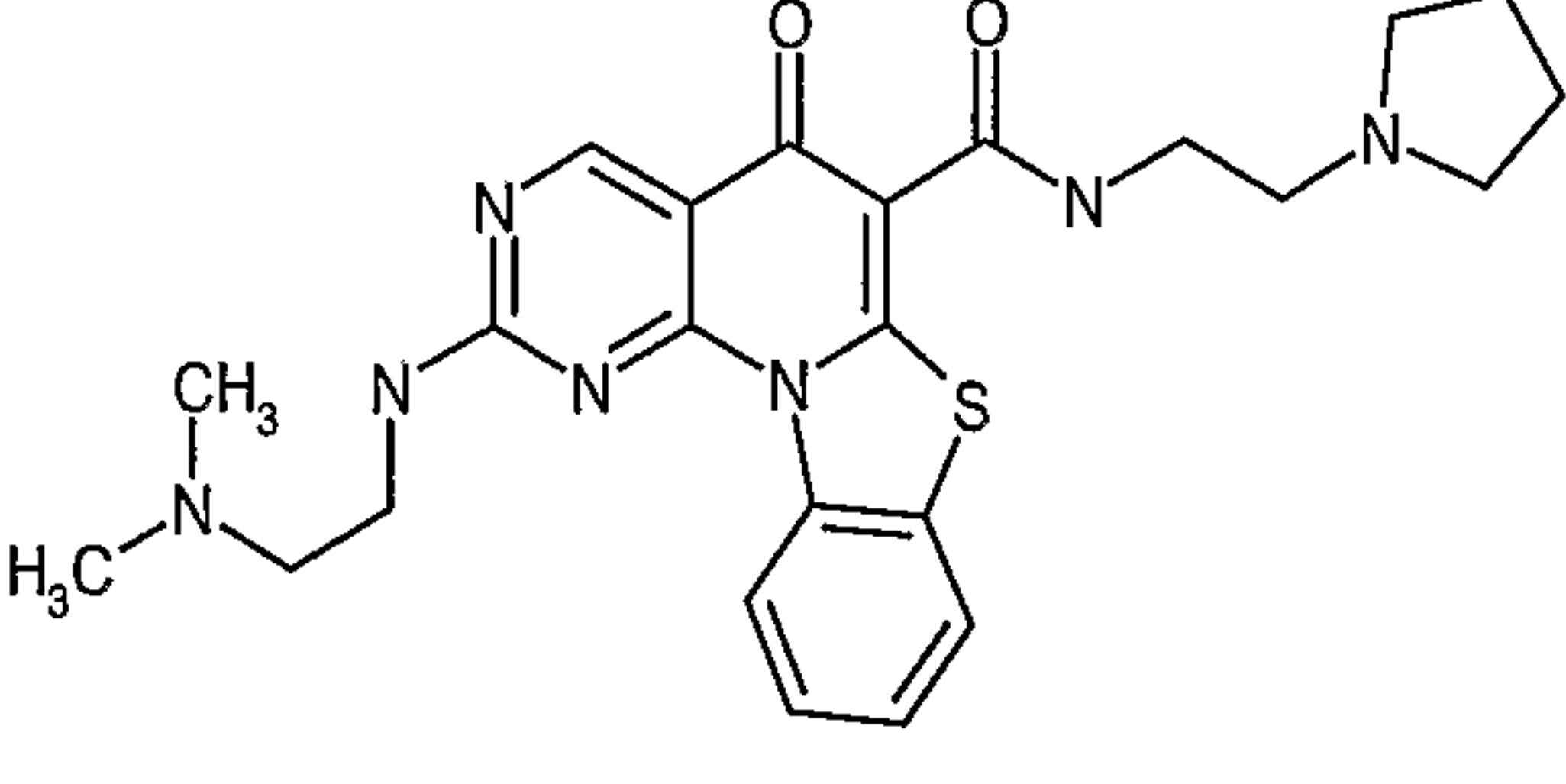
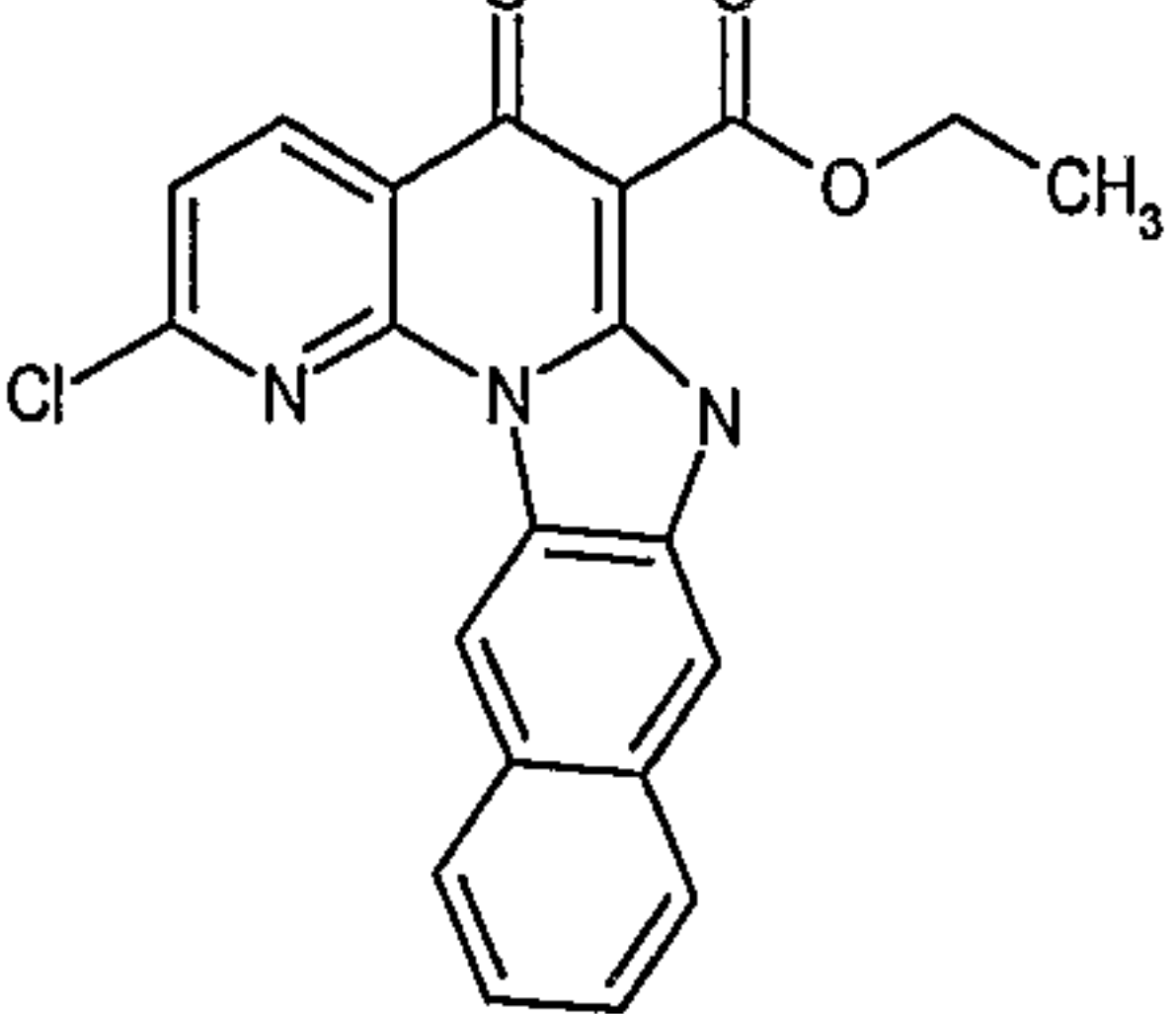
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 0.22   |
|   | MiaPaca | 0.2    |
|  | HCT-116 | 0.11   |
|  | MiaPaca | 0.03   |
|  | HCT-116 | 0.01   |
|  | MiaPaca | 0.05   |

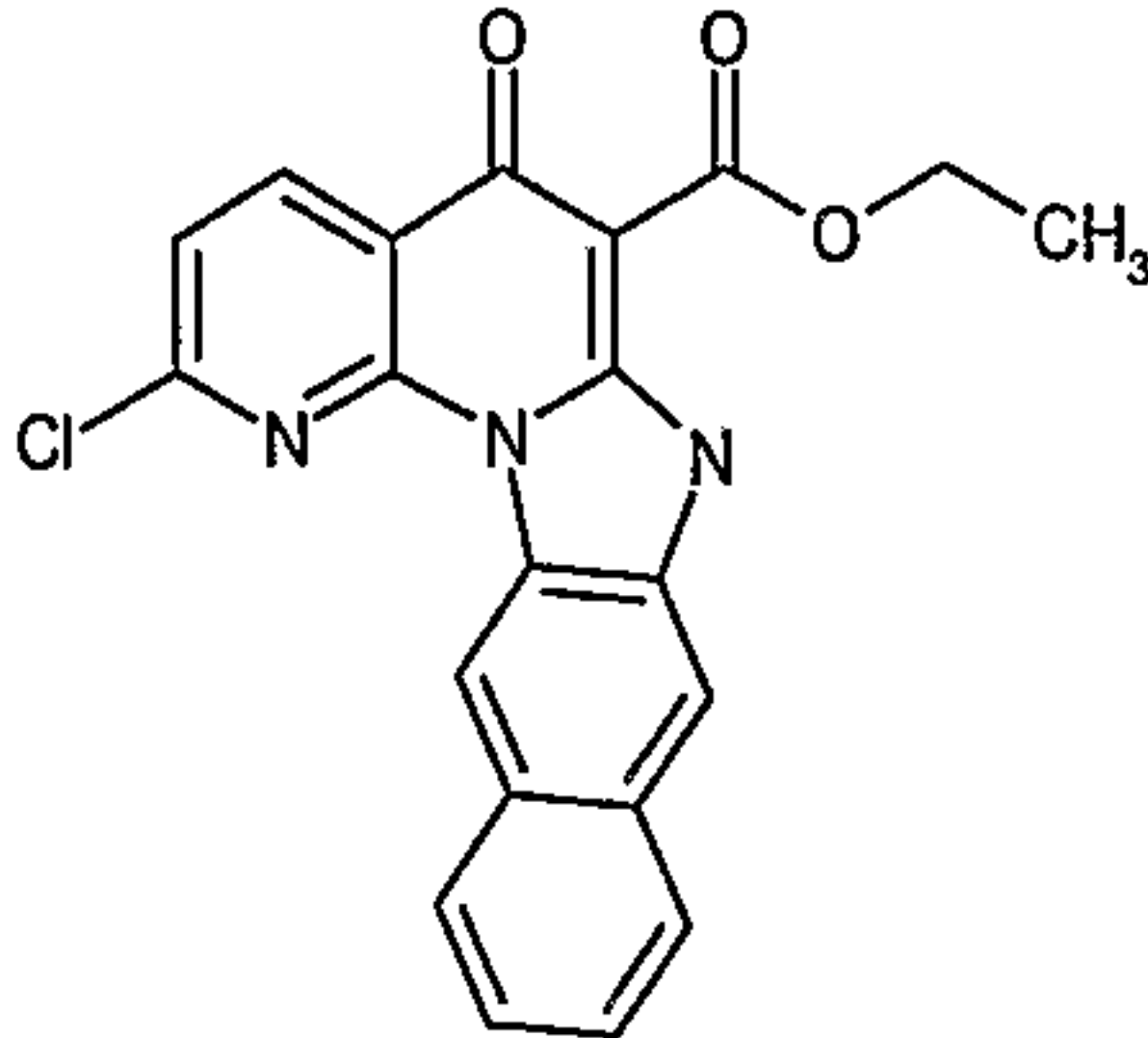
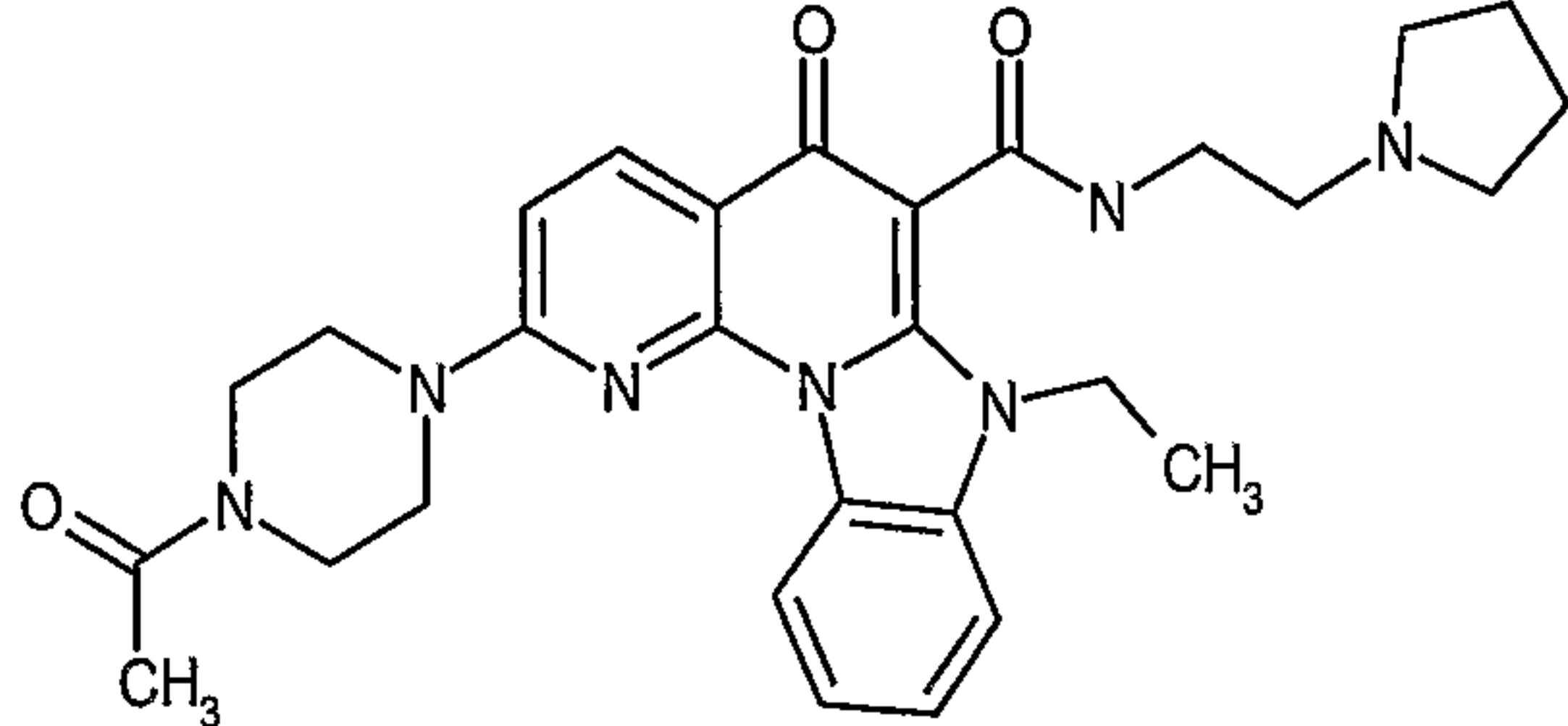
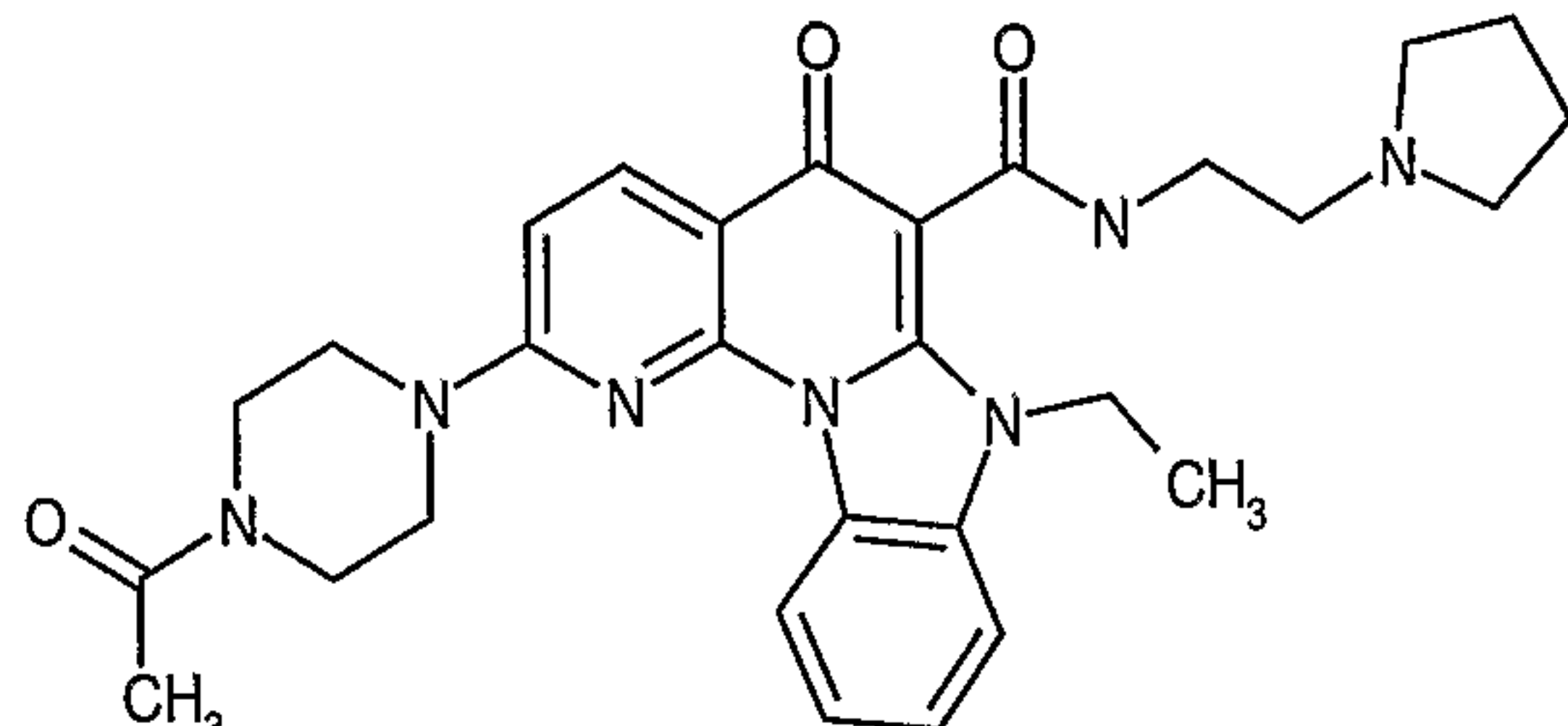
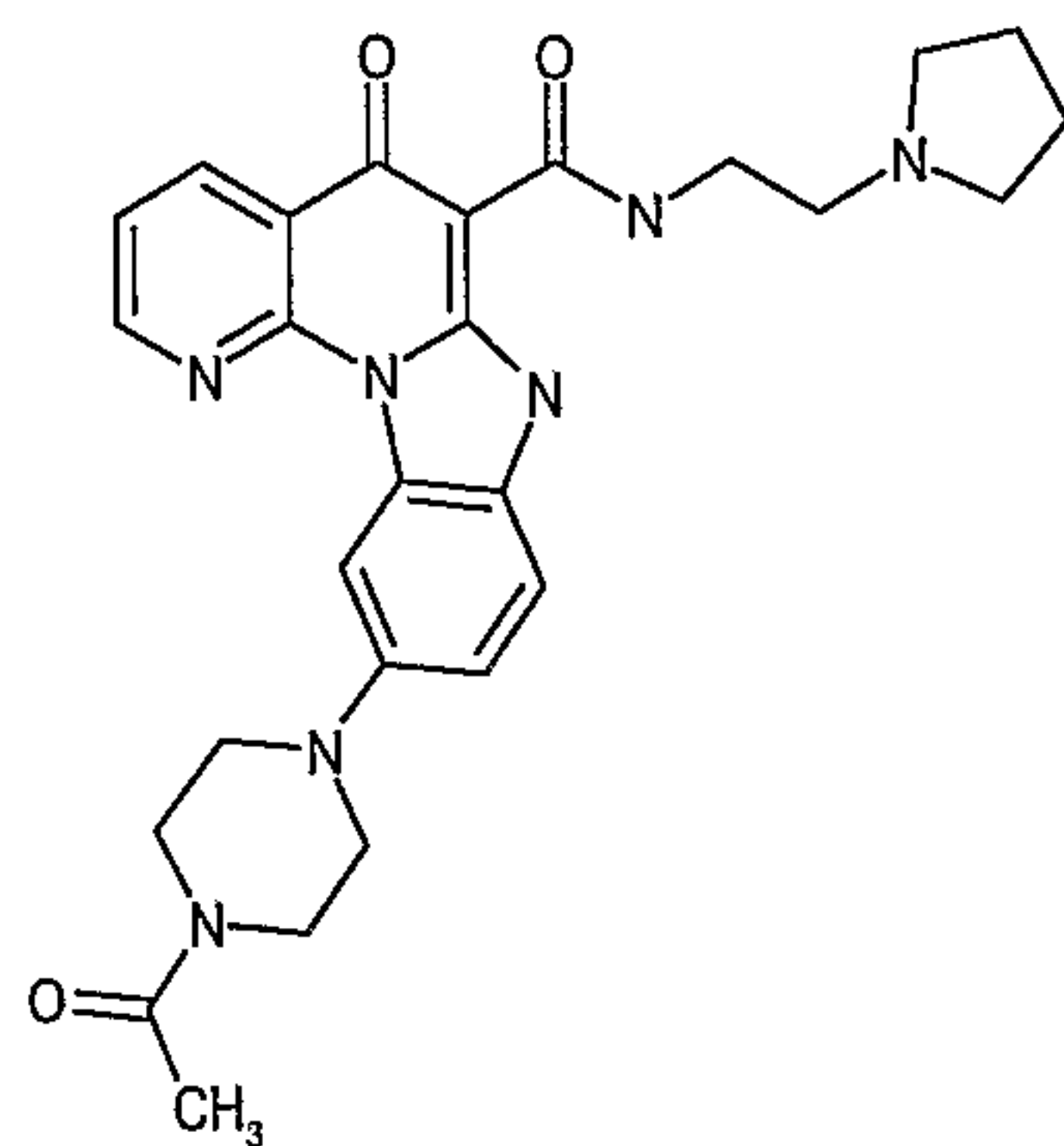
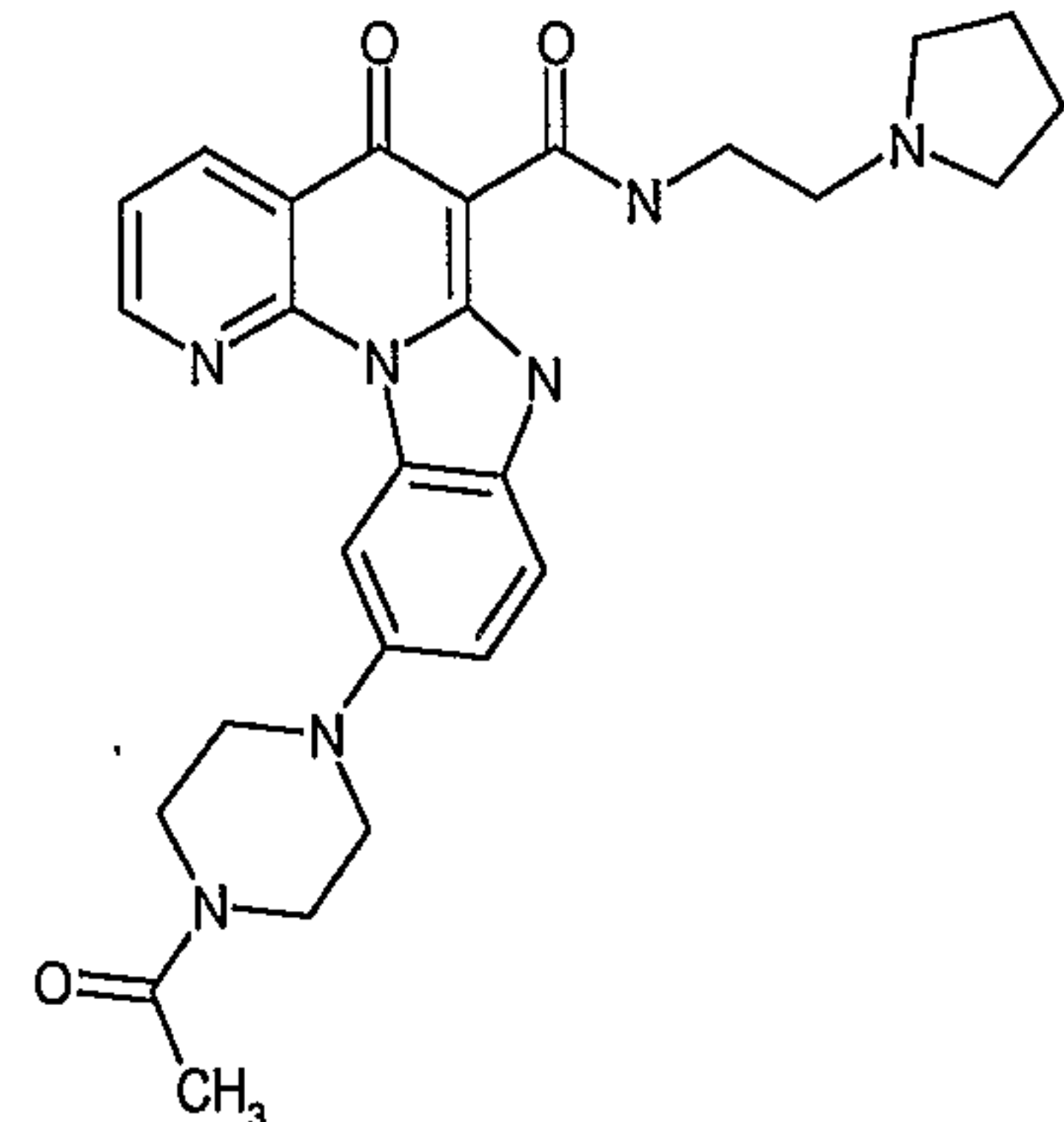
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 0.02   |
|   | MiaPaca | 0.03   |
|  | HCT-116 | 0.02   |
|  | MiaPaca | 0.04   |
|  | HCT-116 | 0.02   |
|  | MiaPaca | 0.05   |

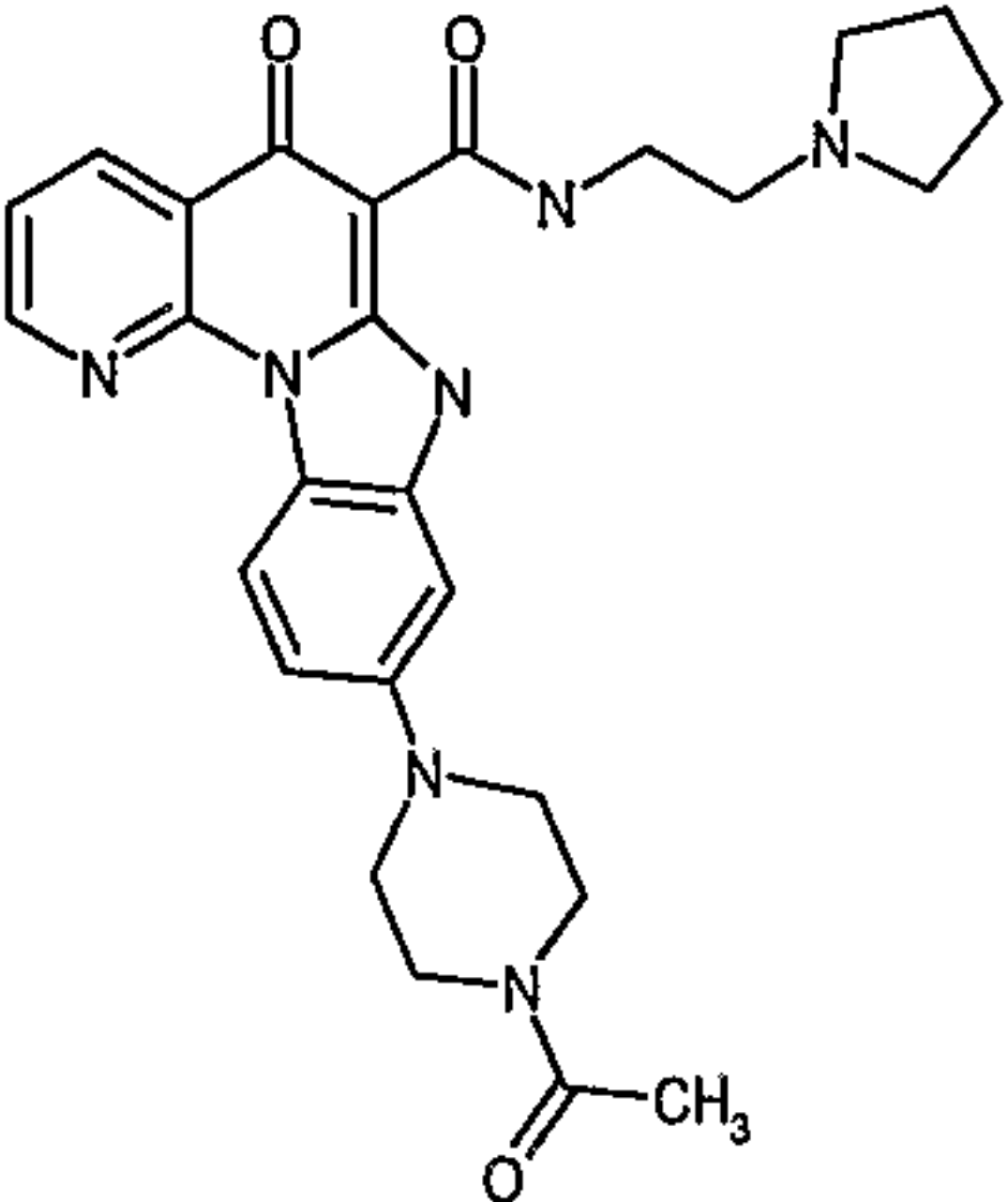
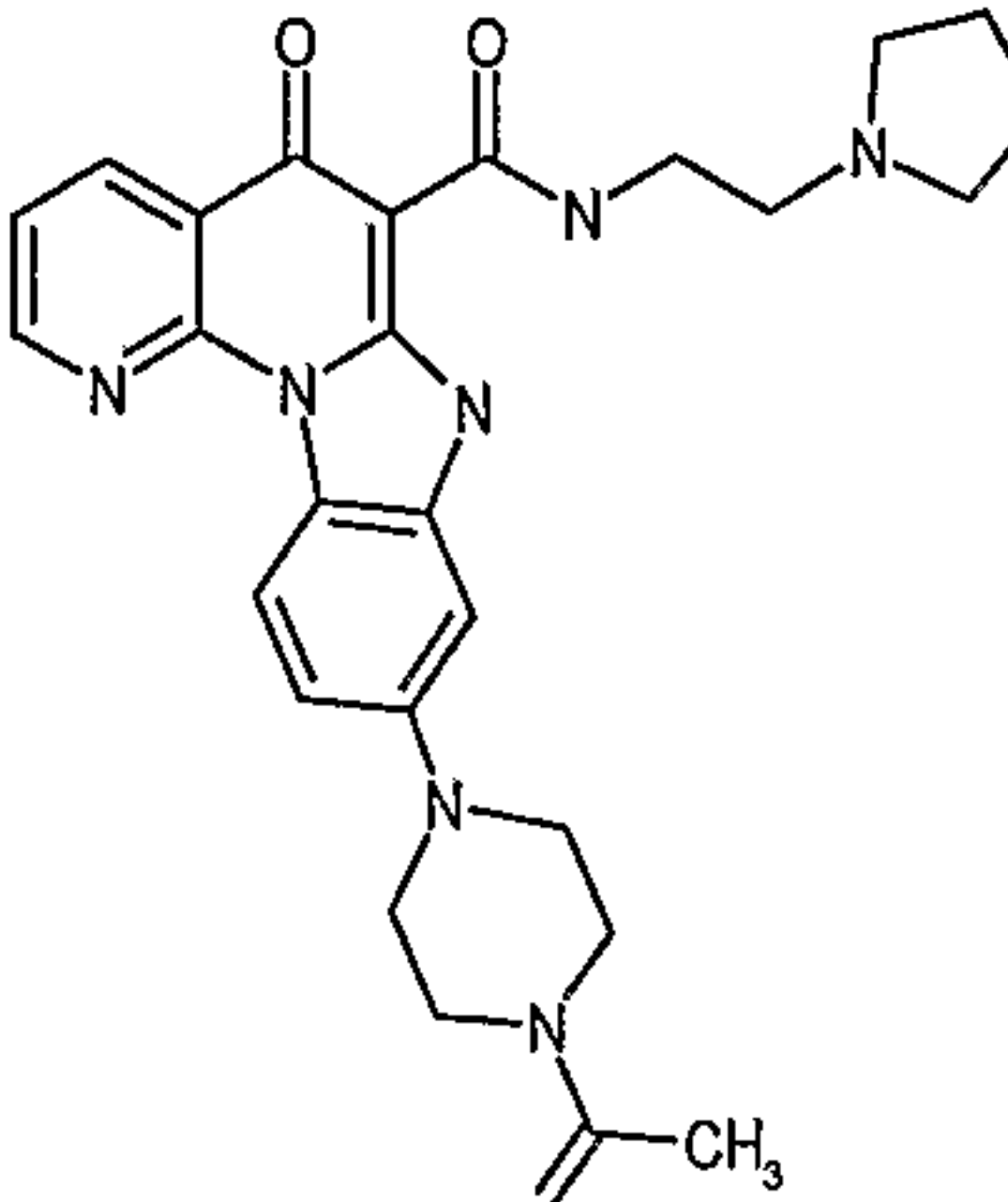
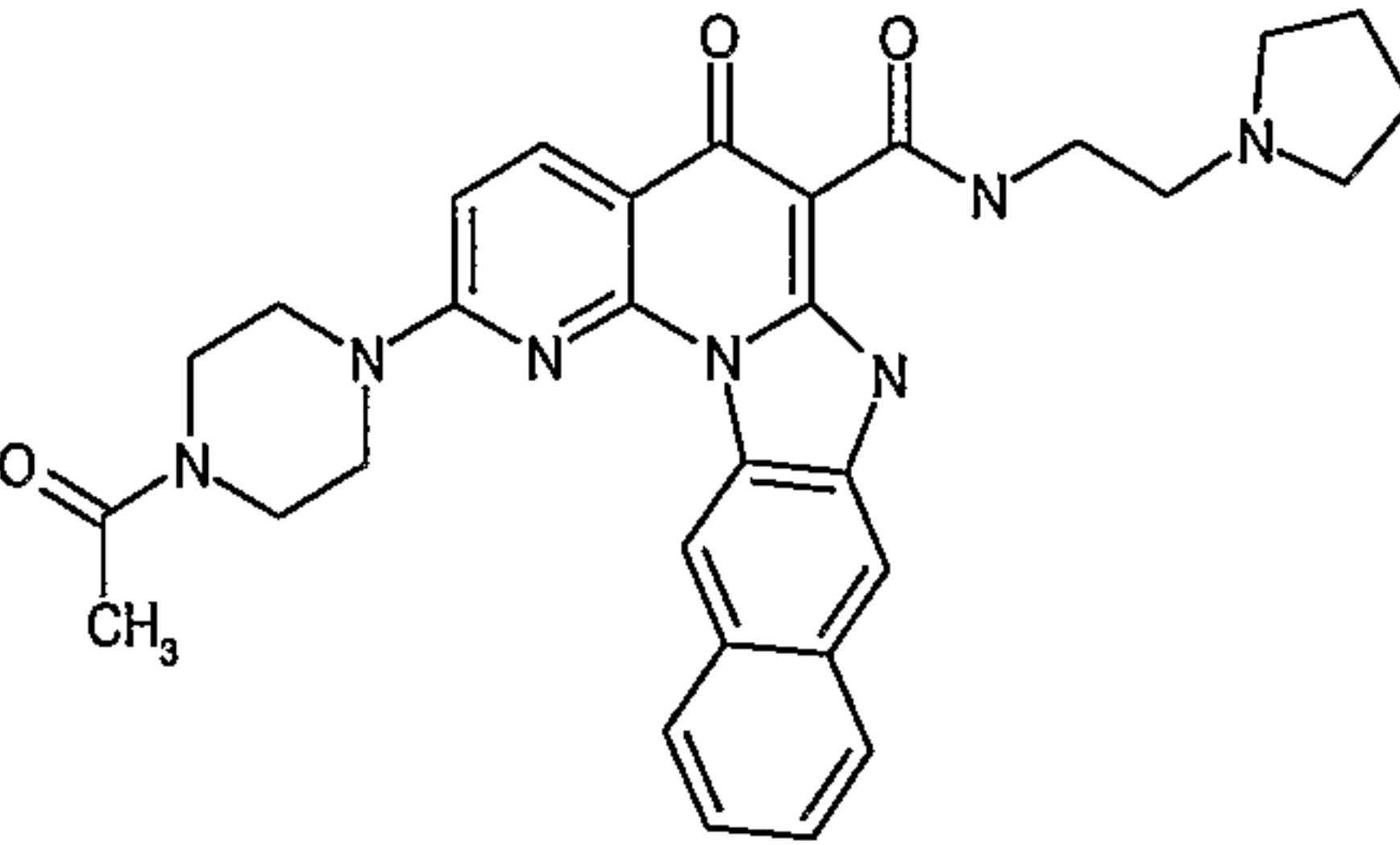
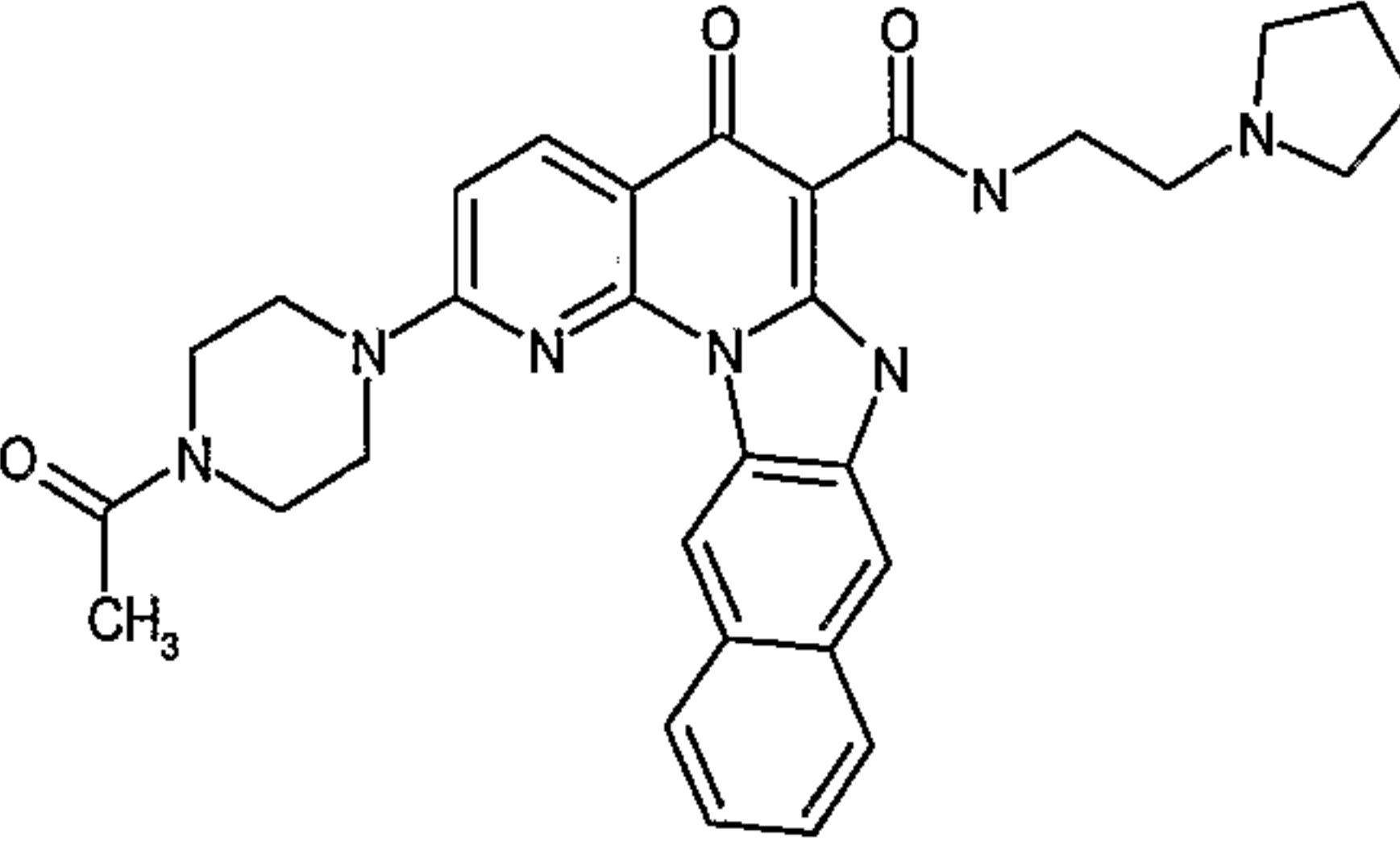
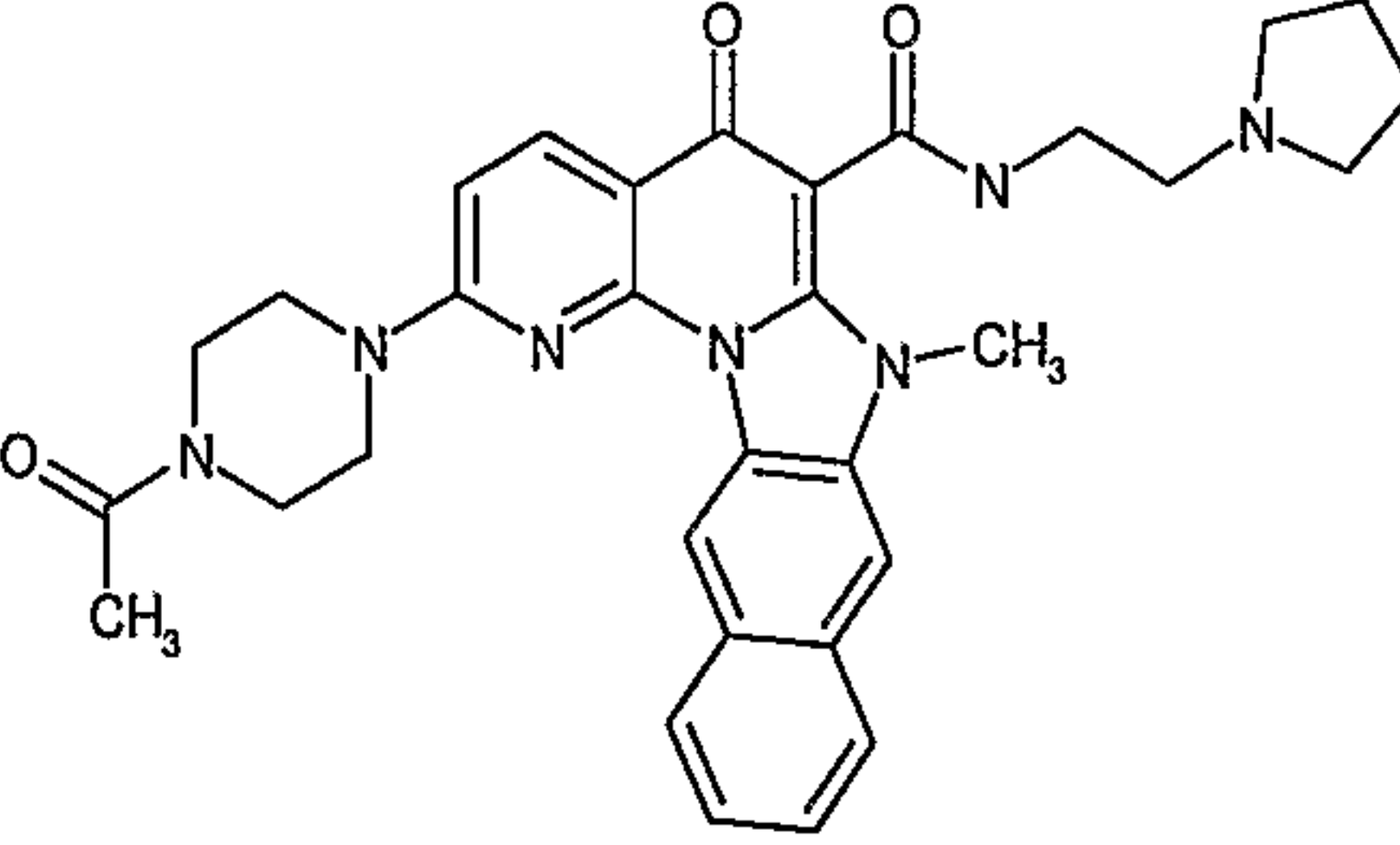
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 0.03   |
|   | MiaPaca | <0.01  |
|  | HCT-116 | 0.02   |
|   | MiaPaca | 0.07   |
|   | HCT-116 | 0.03   |
|  | MiaPaca | 0.24   |

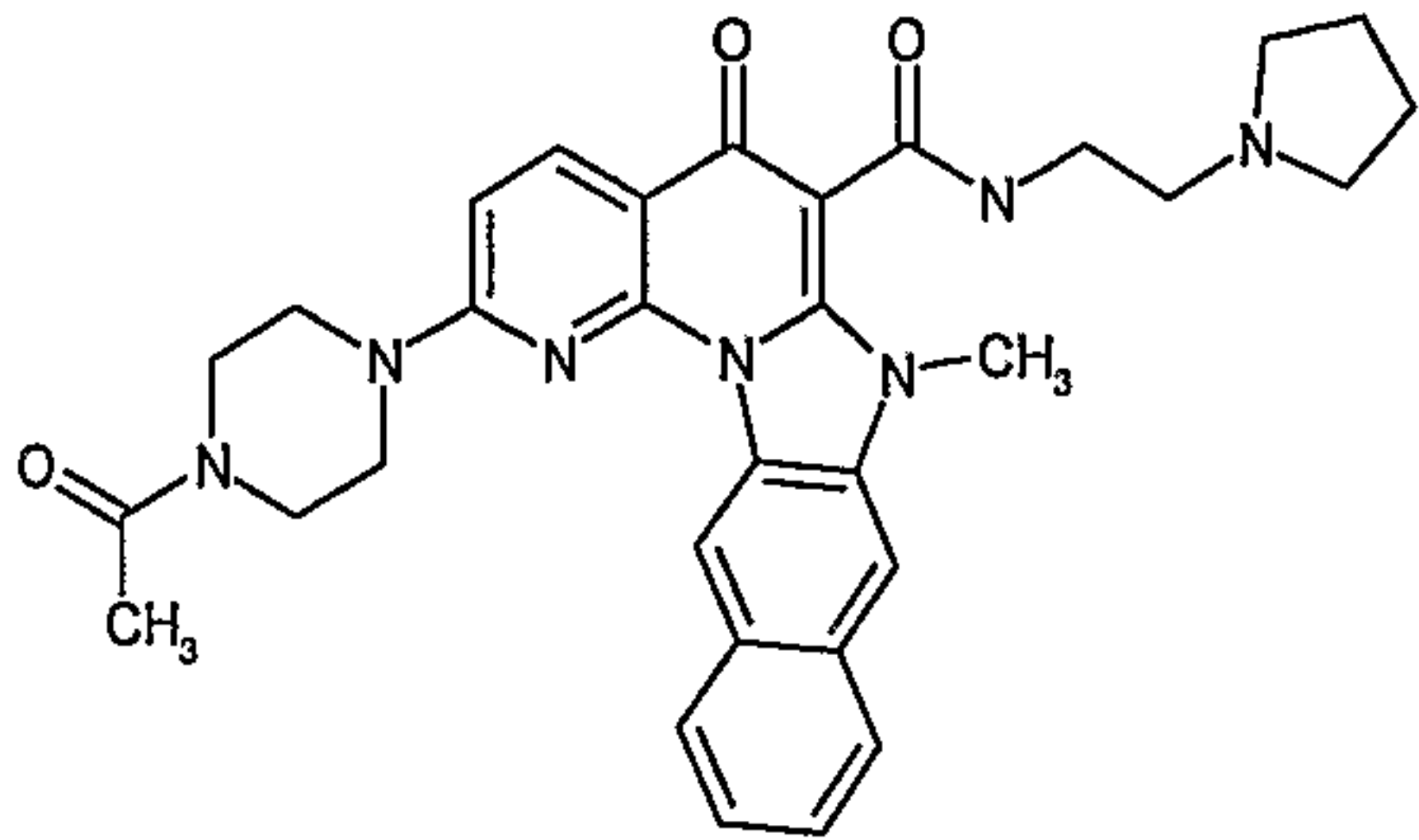
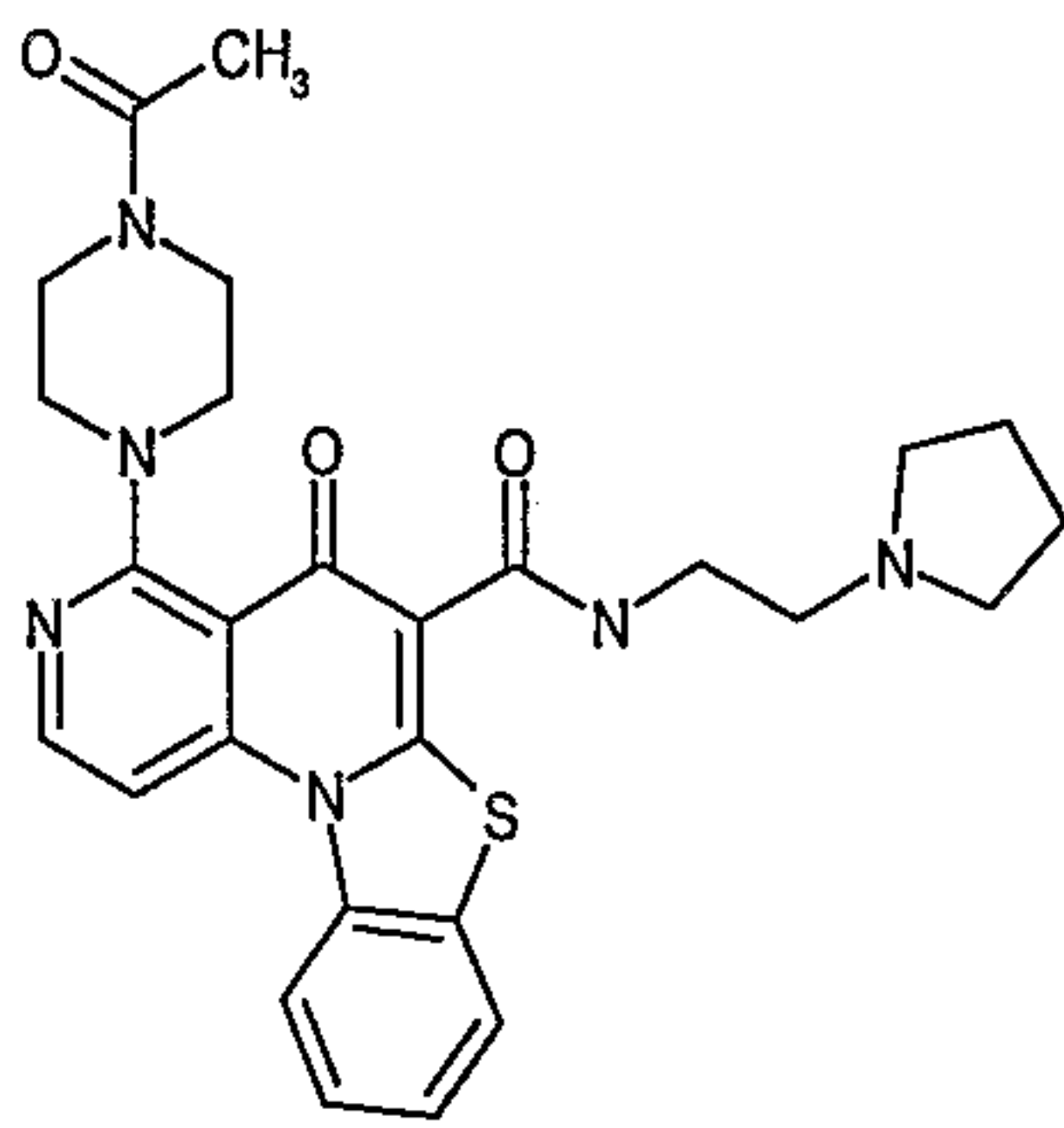
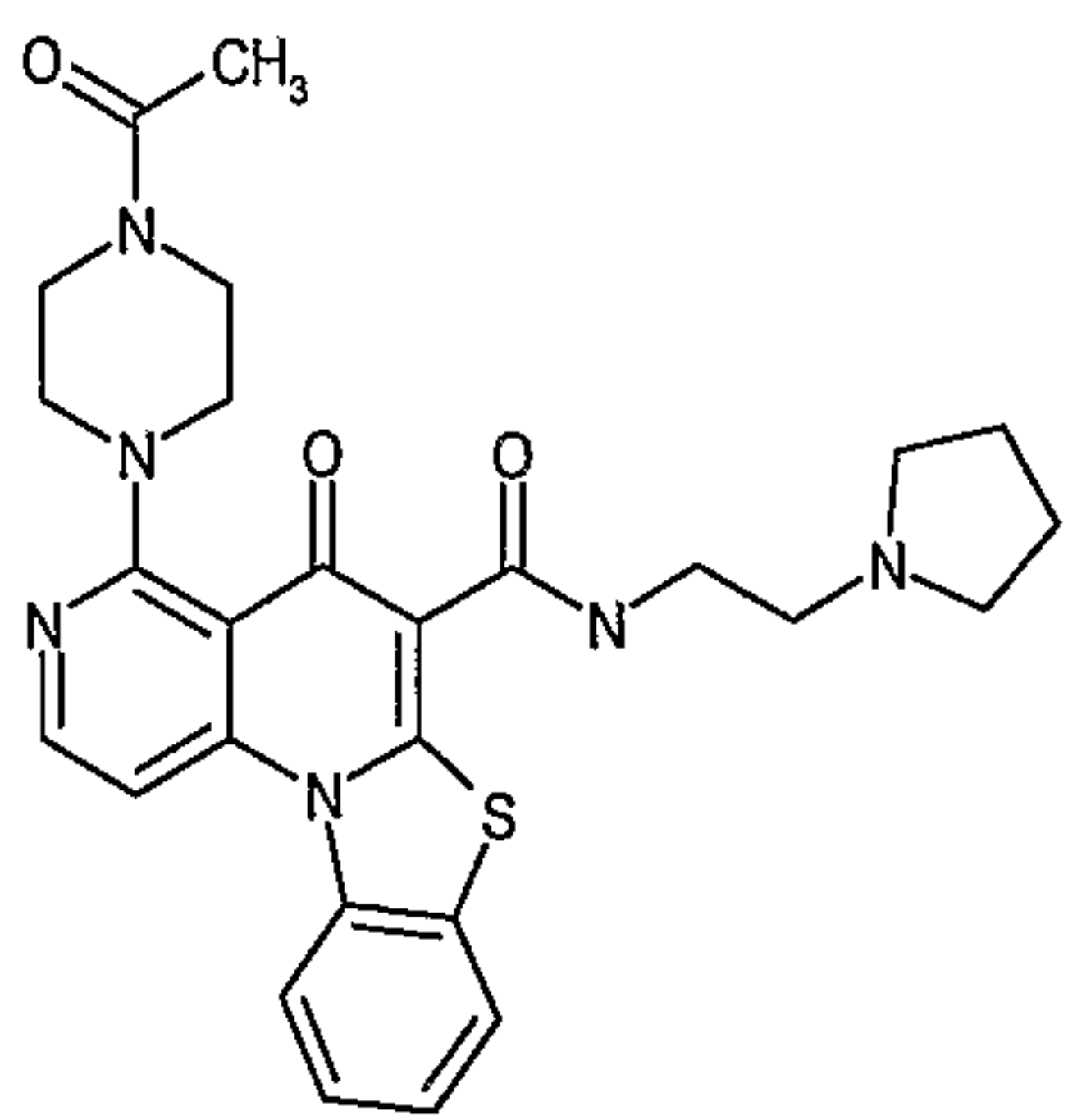
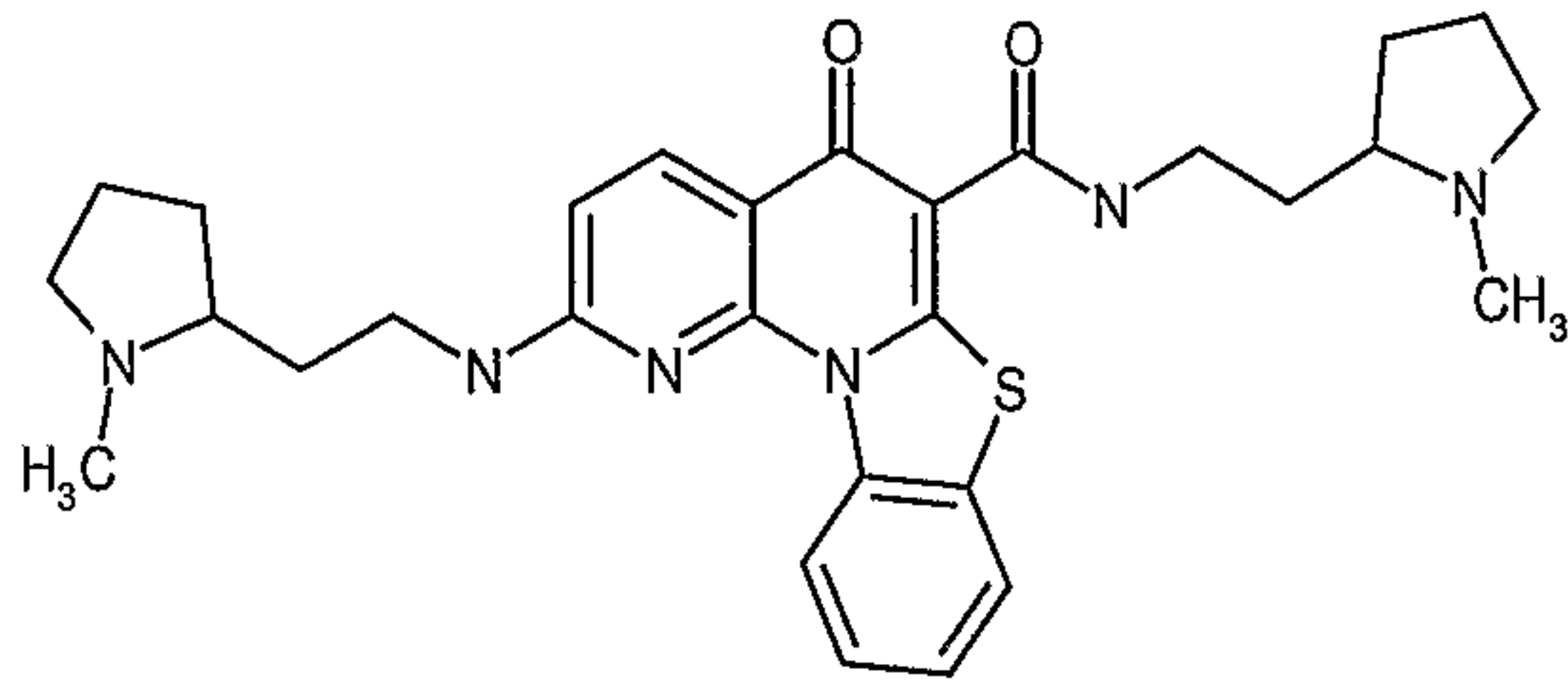
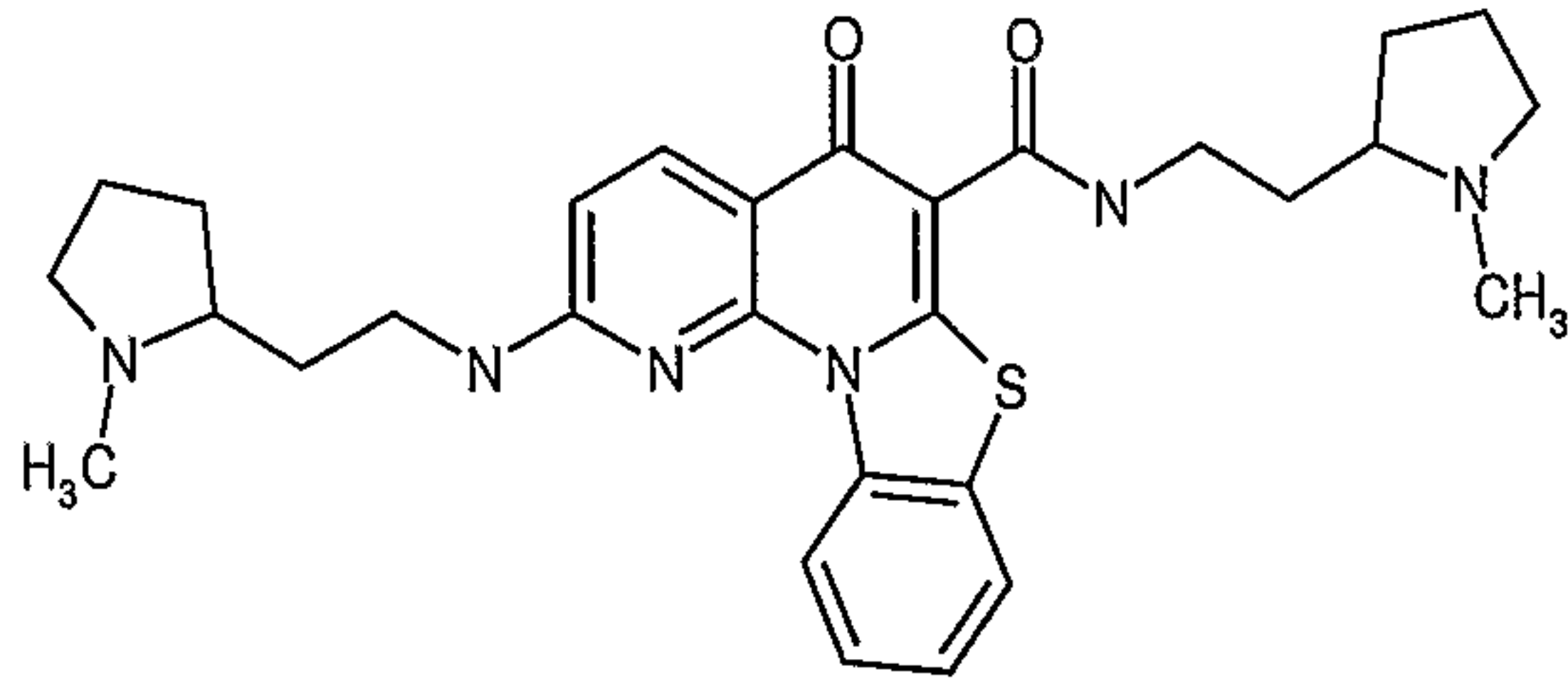
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 0.16   |
|   | MiaPaca | 0.03   |
|  | HCT-116 | 0.01   |
|  | MiaPaca | <0.01  |
|  | HCT-116 | <0.01  |
|  | MiaPaca | 0.03   |

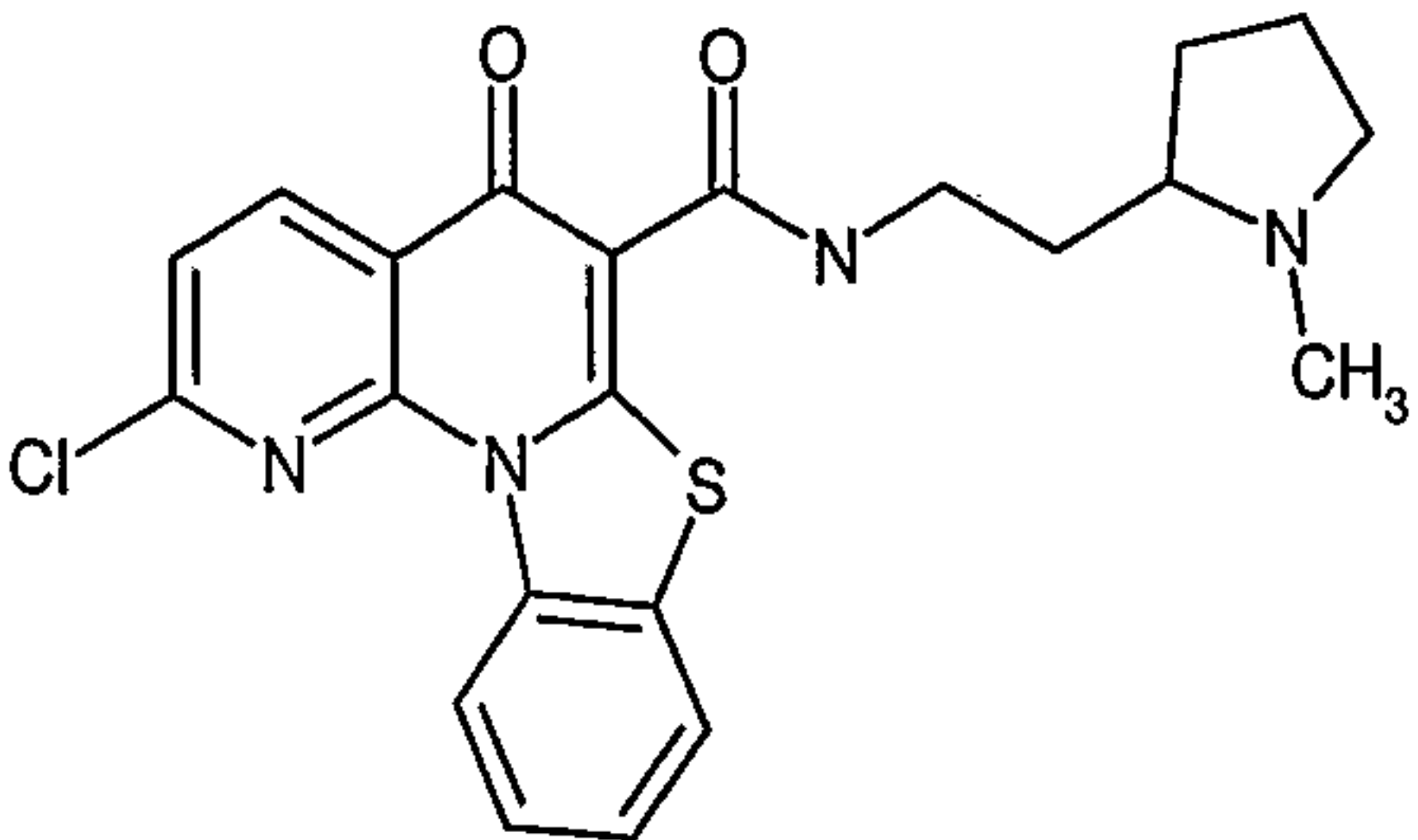
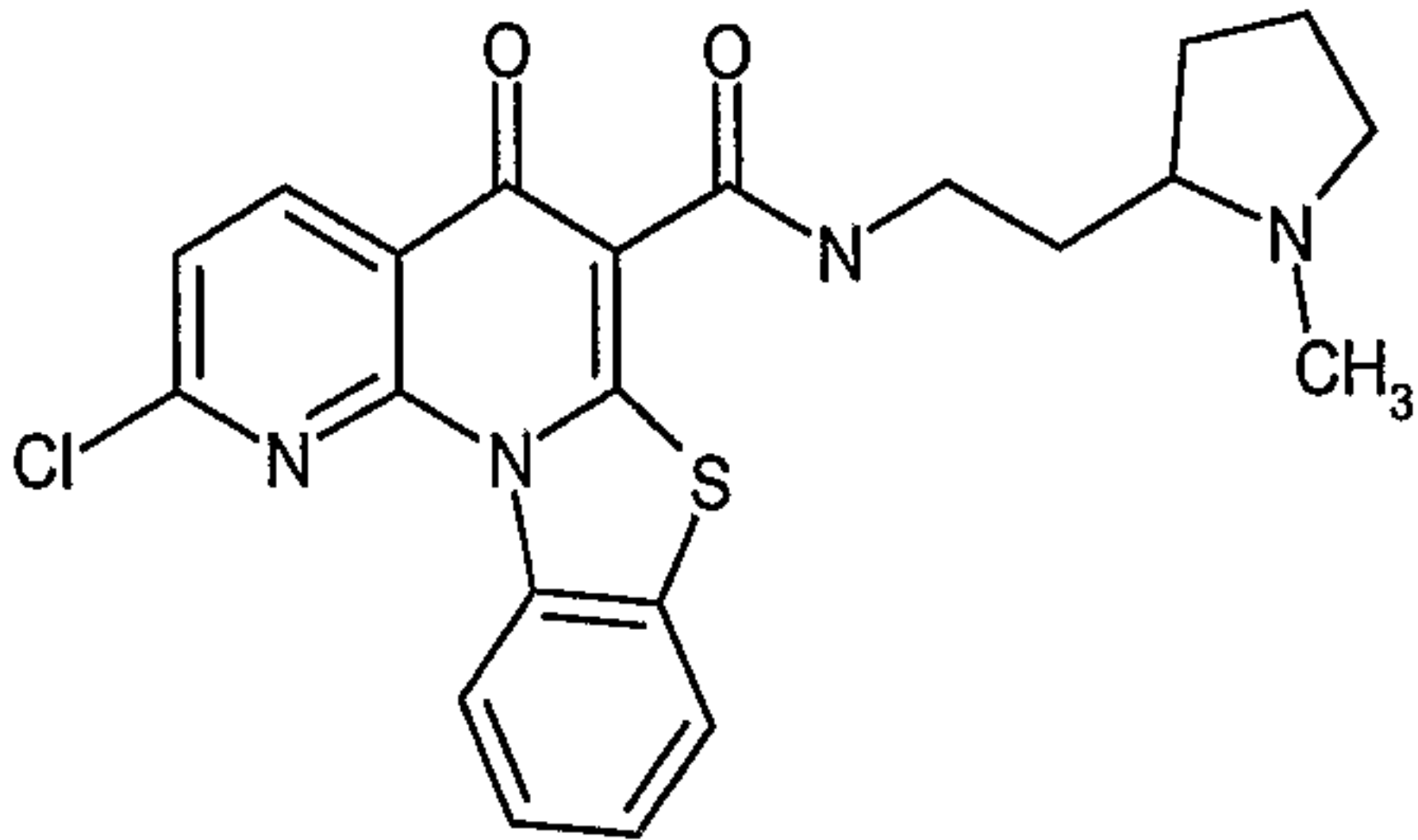
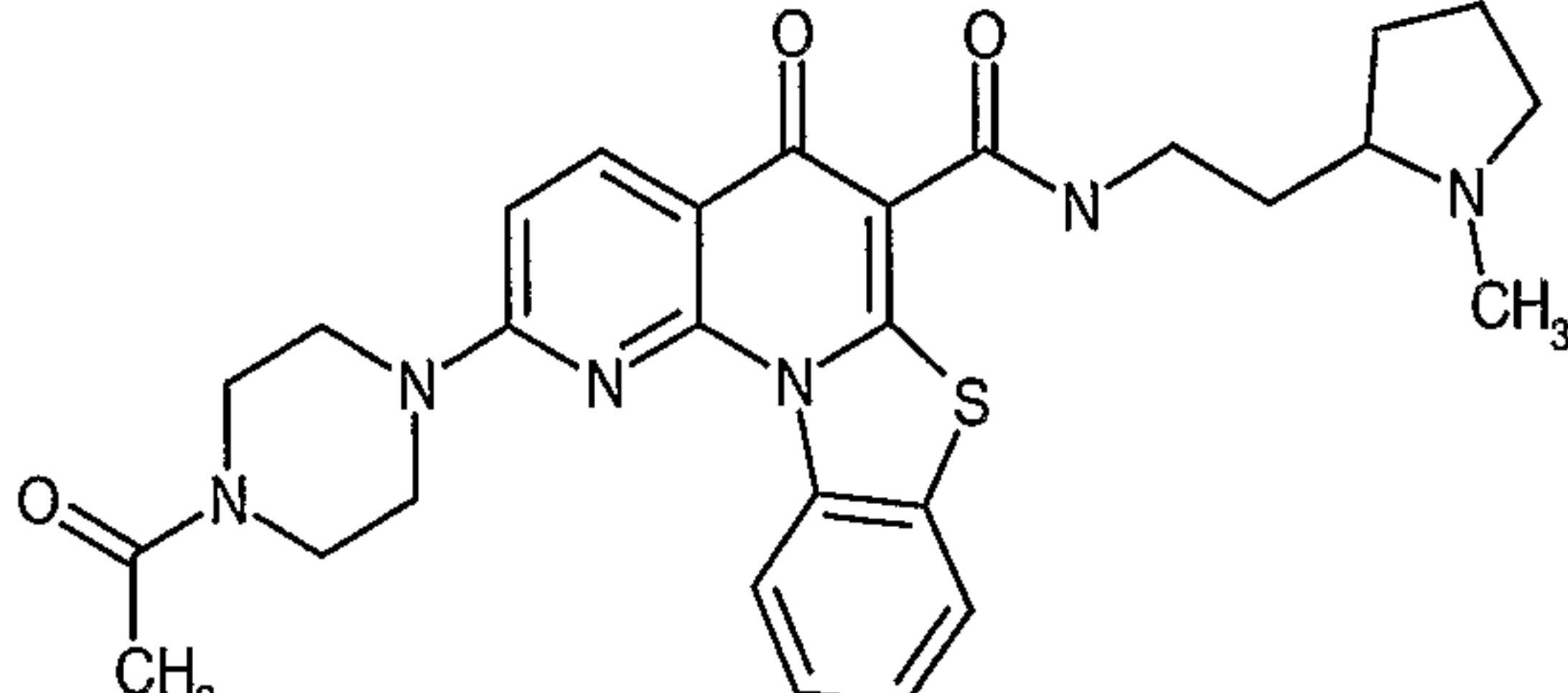
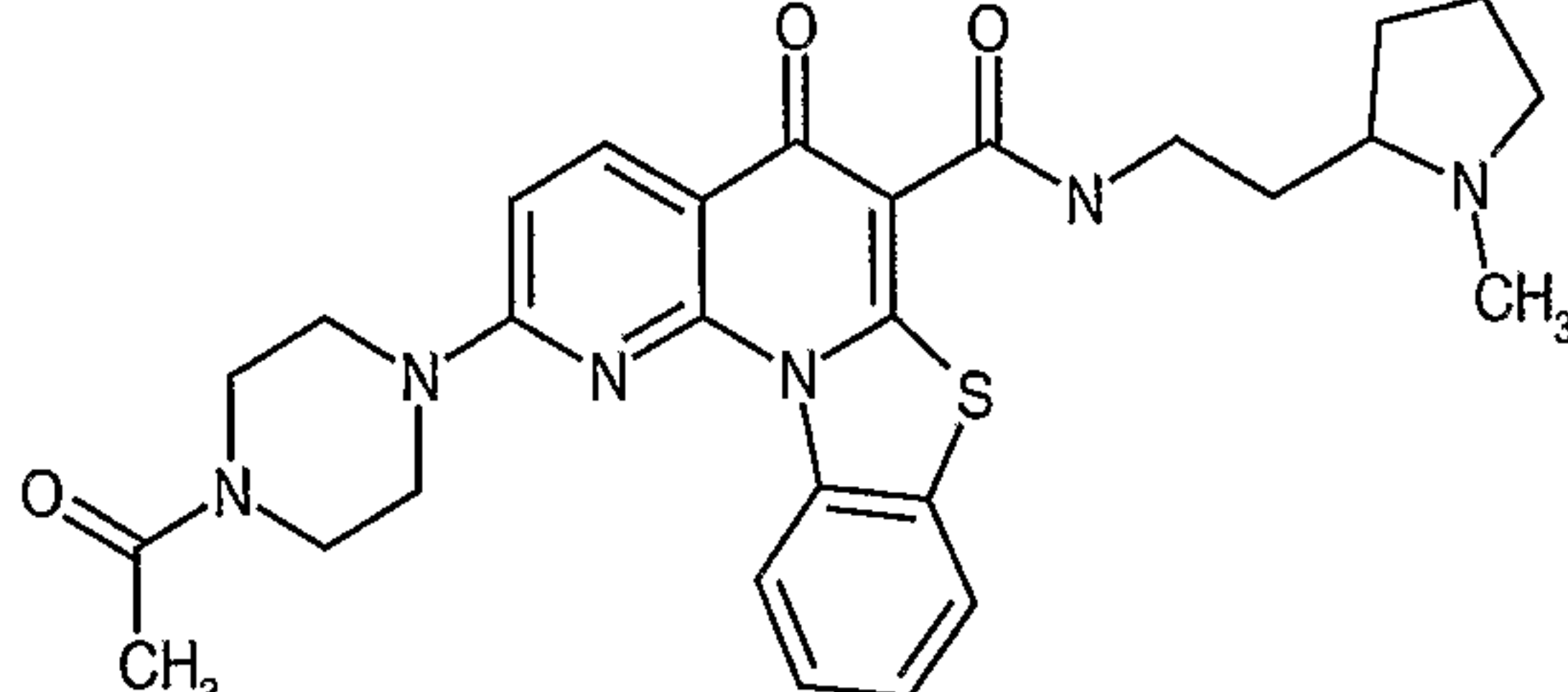
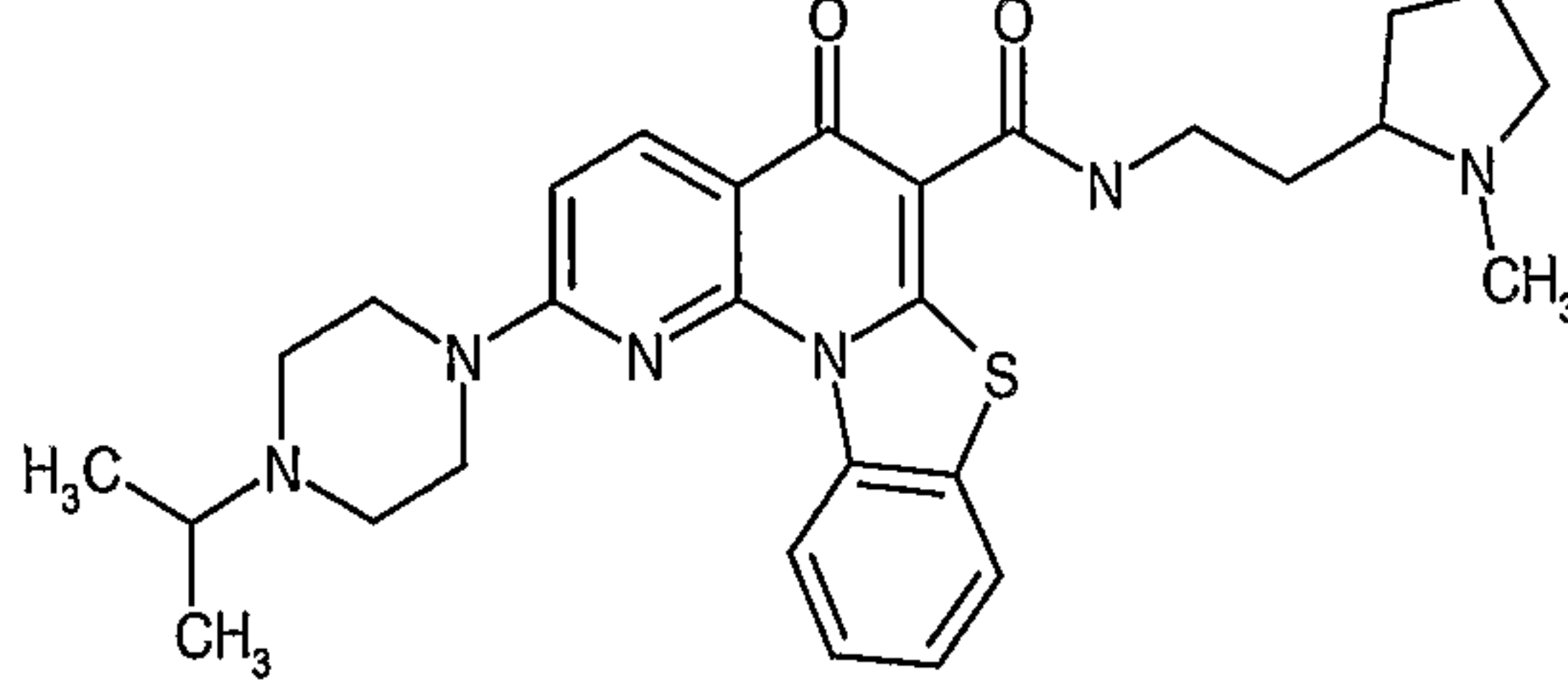
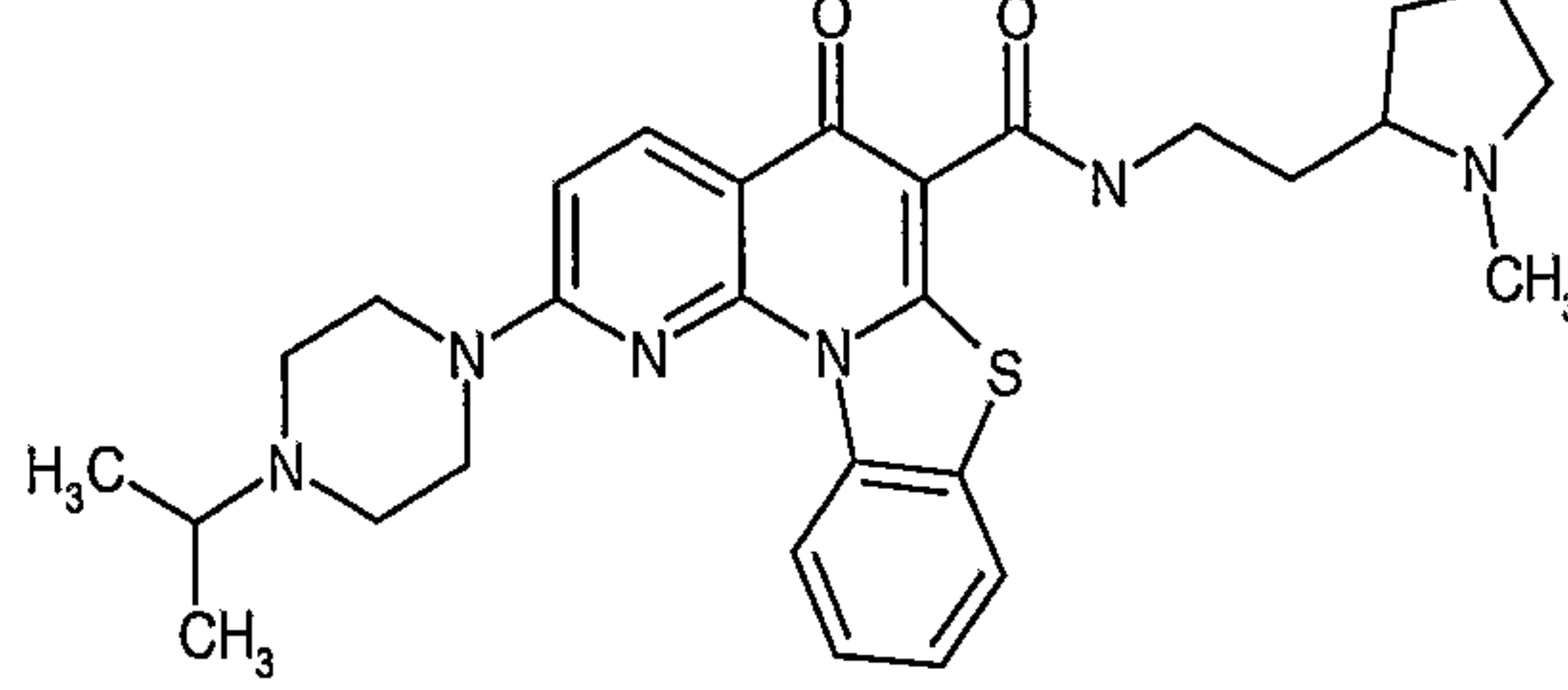
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 0.01   |
|   | MiaPaca | 0.04   |
|  | HCT-116 | 0.02   |
|  | MiaPaca | 0.05   |
|  | HCT-116 | 0.03   |
|  | MiaPaca | 0.04   |

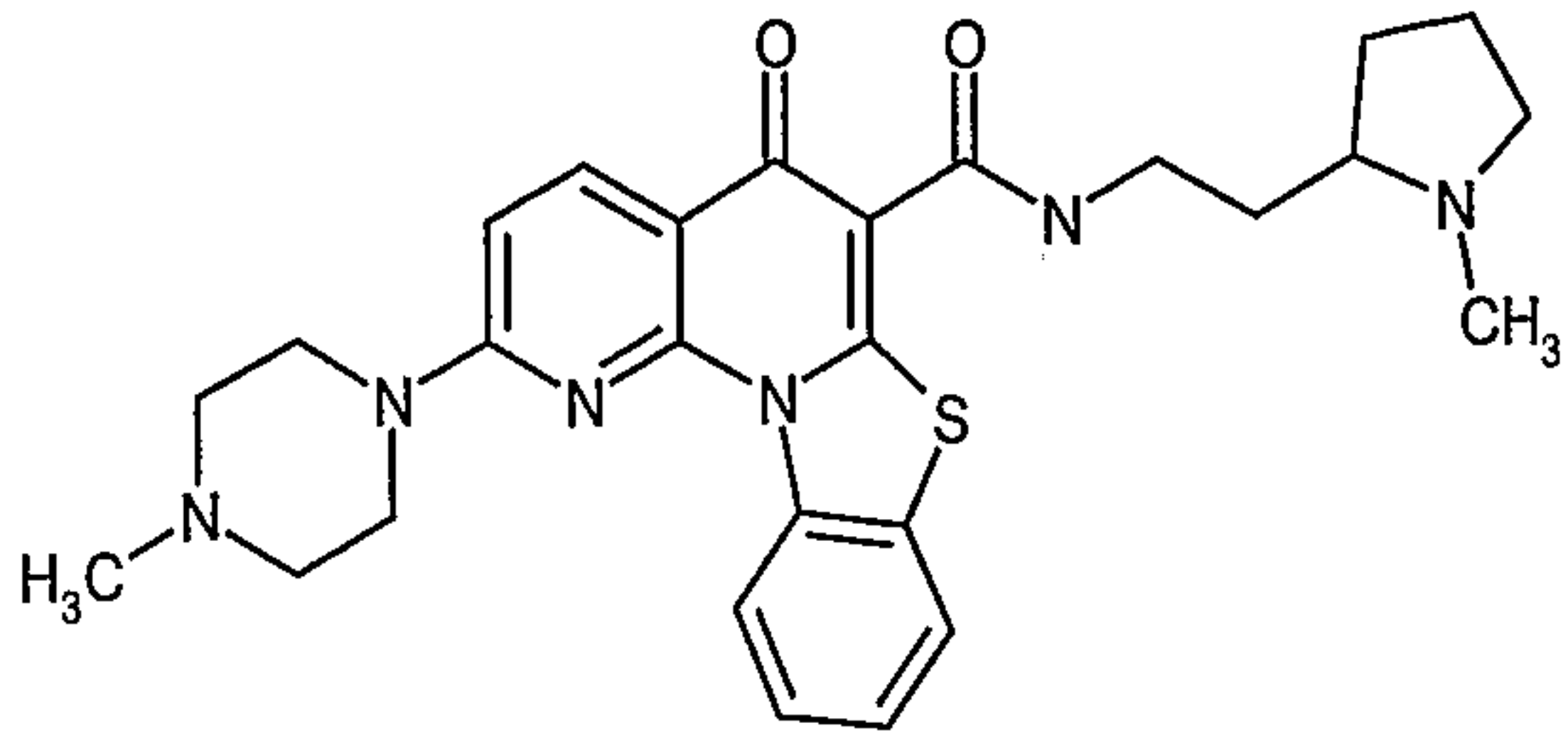
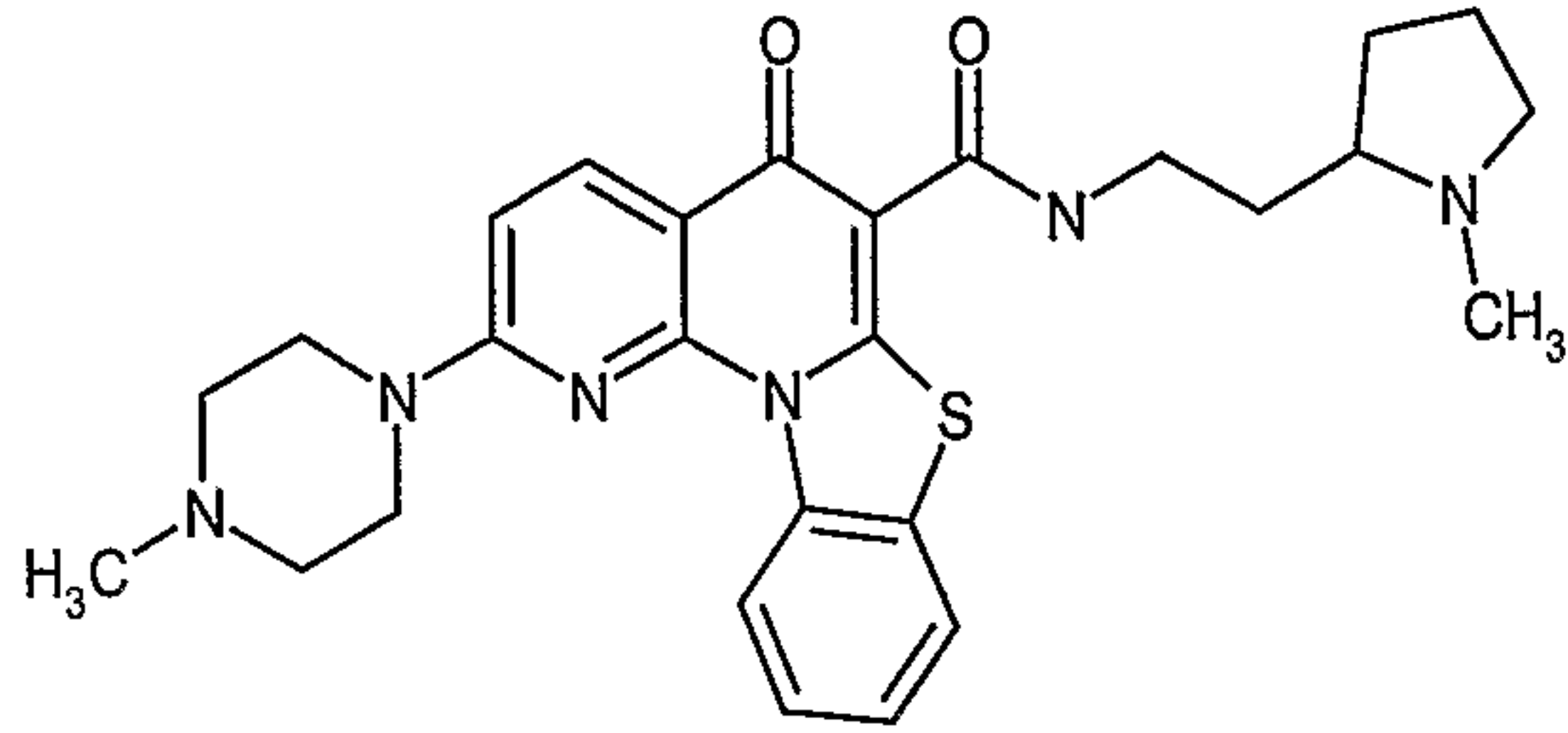
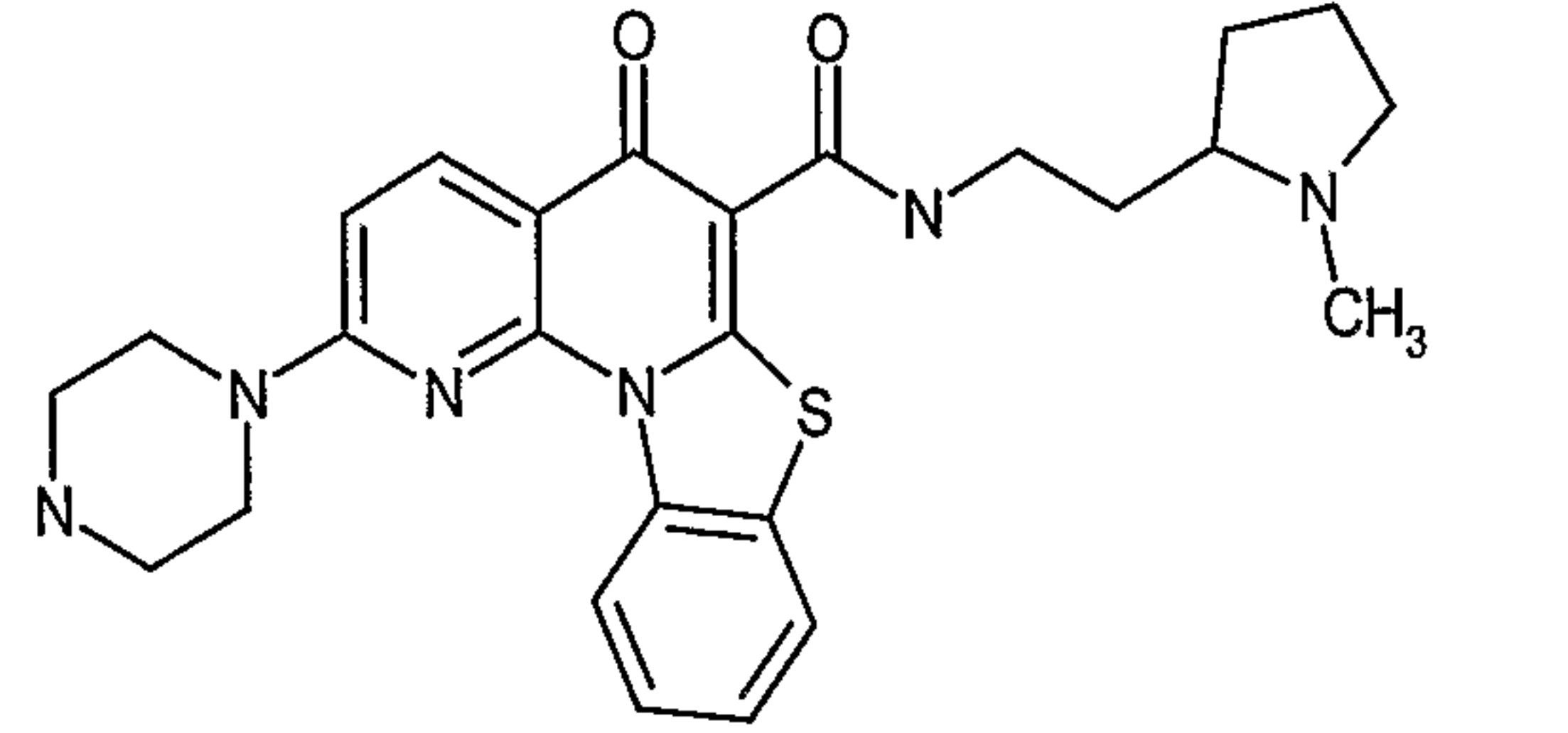
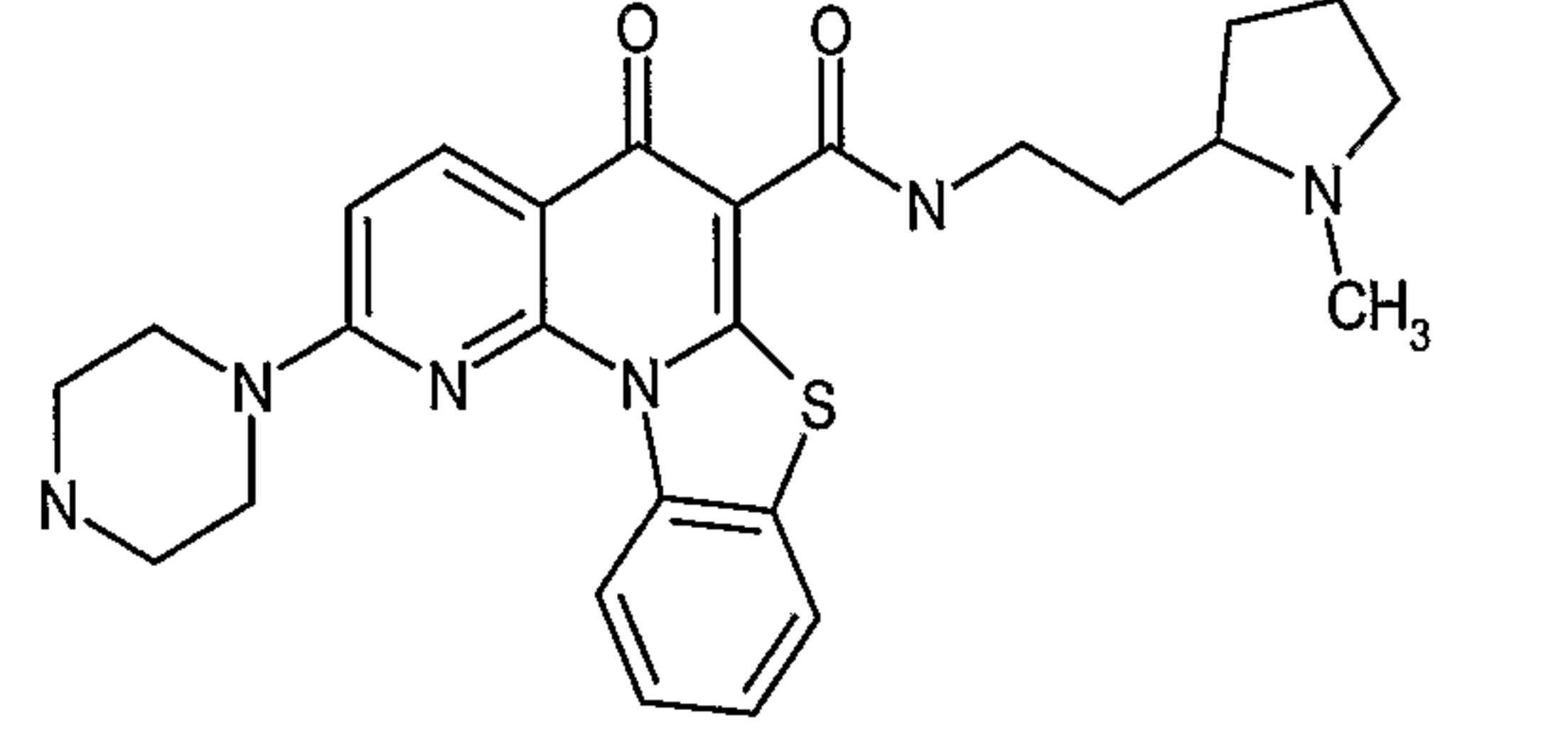
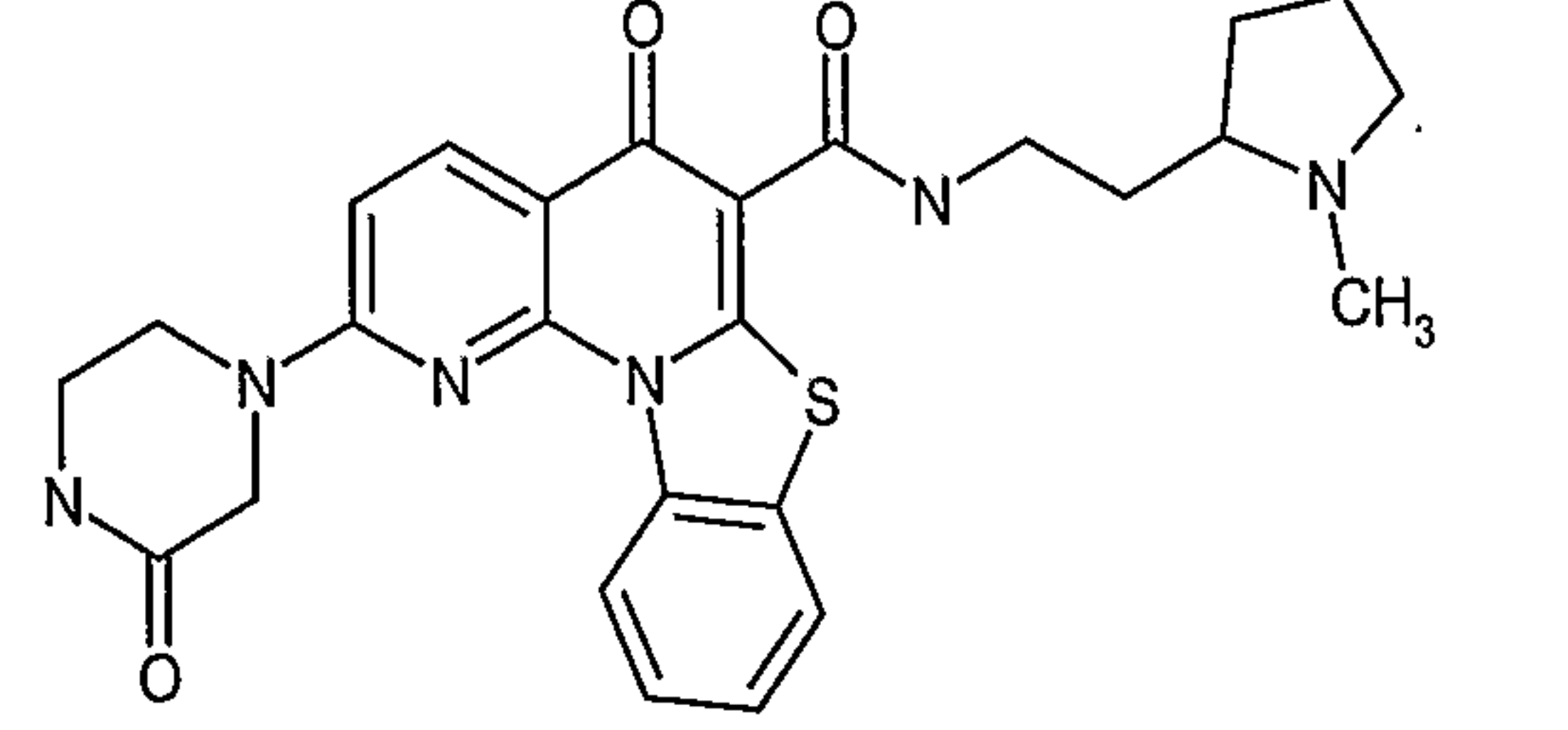
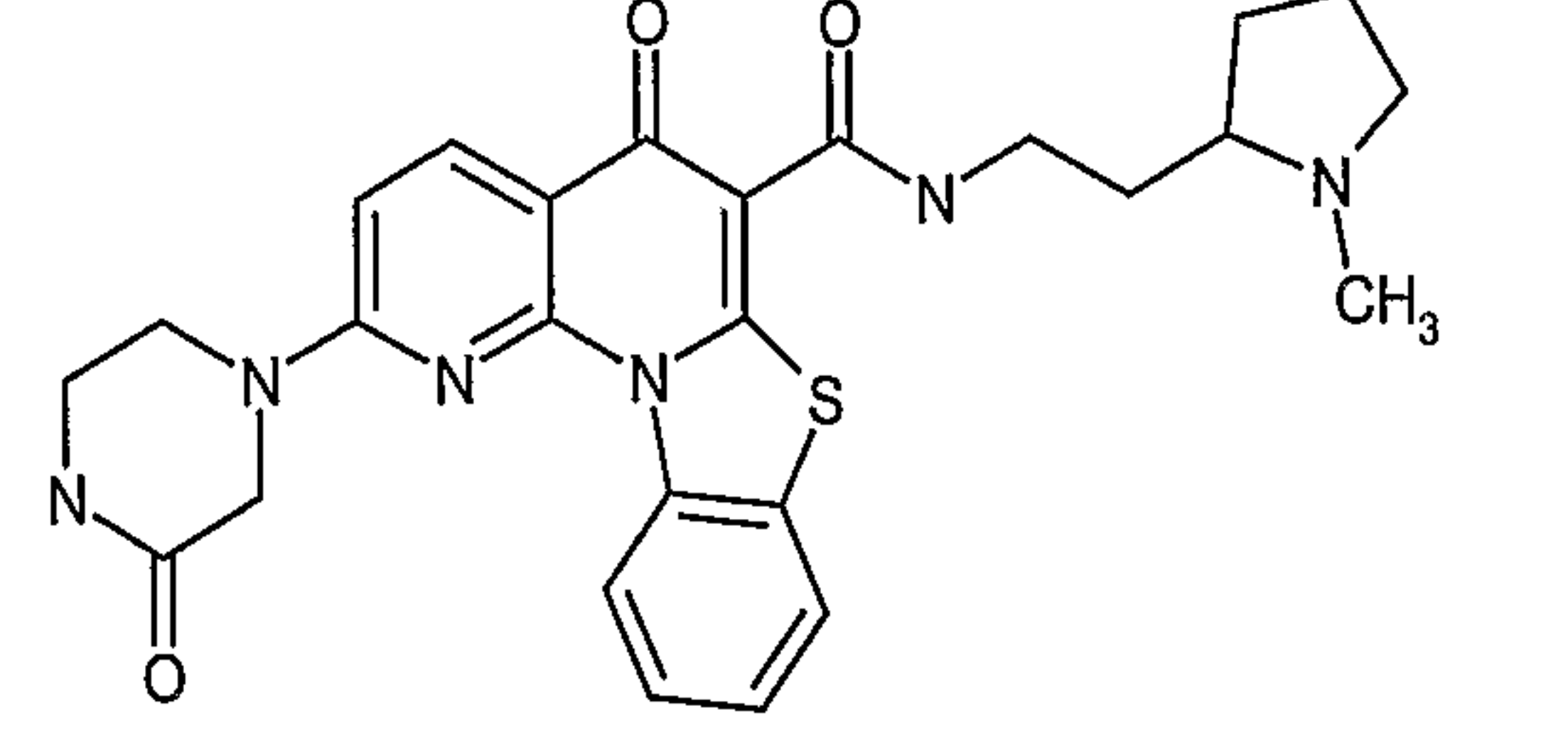
| MOLSTRUCTURE                                                                                                                                  | M CELL  | M DATA |
|-----------------------------------------------------------------------------------------------------------------------------------------------|---------|--------|
|  <chem>OCCNc1nc2c(nc(=O)c1c3ccccc3s2)nc4ccccc4</chem>       | HCT-116 | 0.04   |
|  <chem>CC(C)Nc1nc2c(nc(=O)c1c3ccccc3s2)nc4ccccc4</chem>     | MiaPaca | 0.11   |
|  <chem>CC(C)Nc1nc2c(nc(=O)c1c3ccccc3s2)nc4ccccc4</chem>    | HCT-116 | 0.03   |
|  <chem>CN(C)CCNc1nc2c(nc(=O)c1c3ccccc3s2)nc4ccccc4</chem> | MiaPaca | <0.01  |
|  <chem>CN(C)CCNc1nc2c(nc(=O)c1c3ccccc3s2)nc4ccccc4</chem> | HCT-116 | <0.01  |
|  <chem>CCOC(=O)c1c2c(nc3cc(Cl)ccc3c2=O)n4c5ccccc5n4</chem> | MiaPaca | >10    |

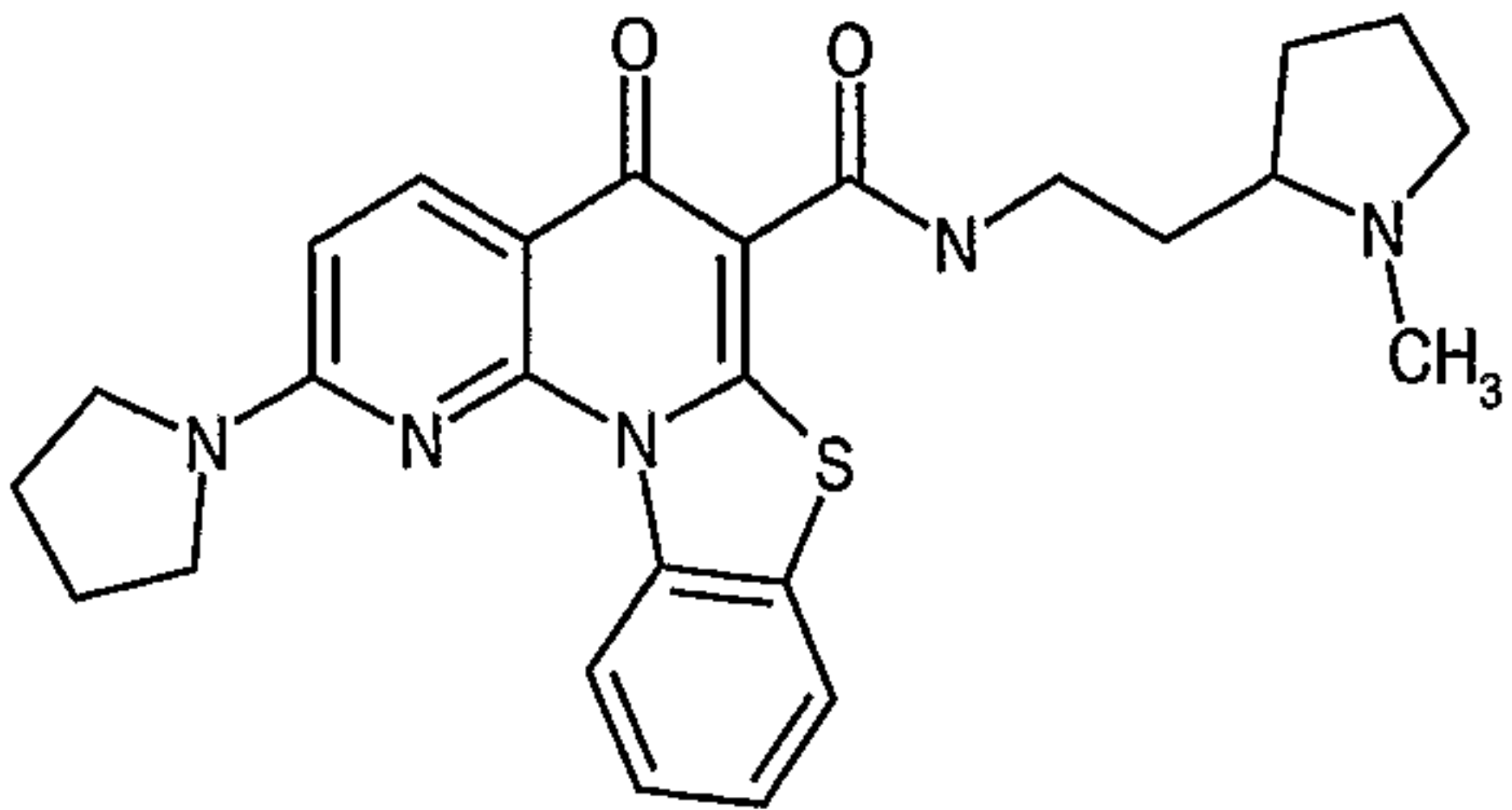
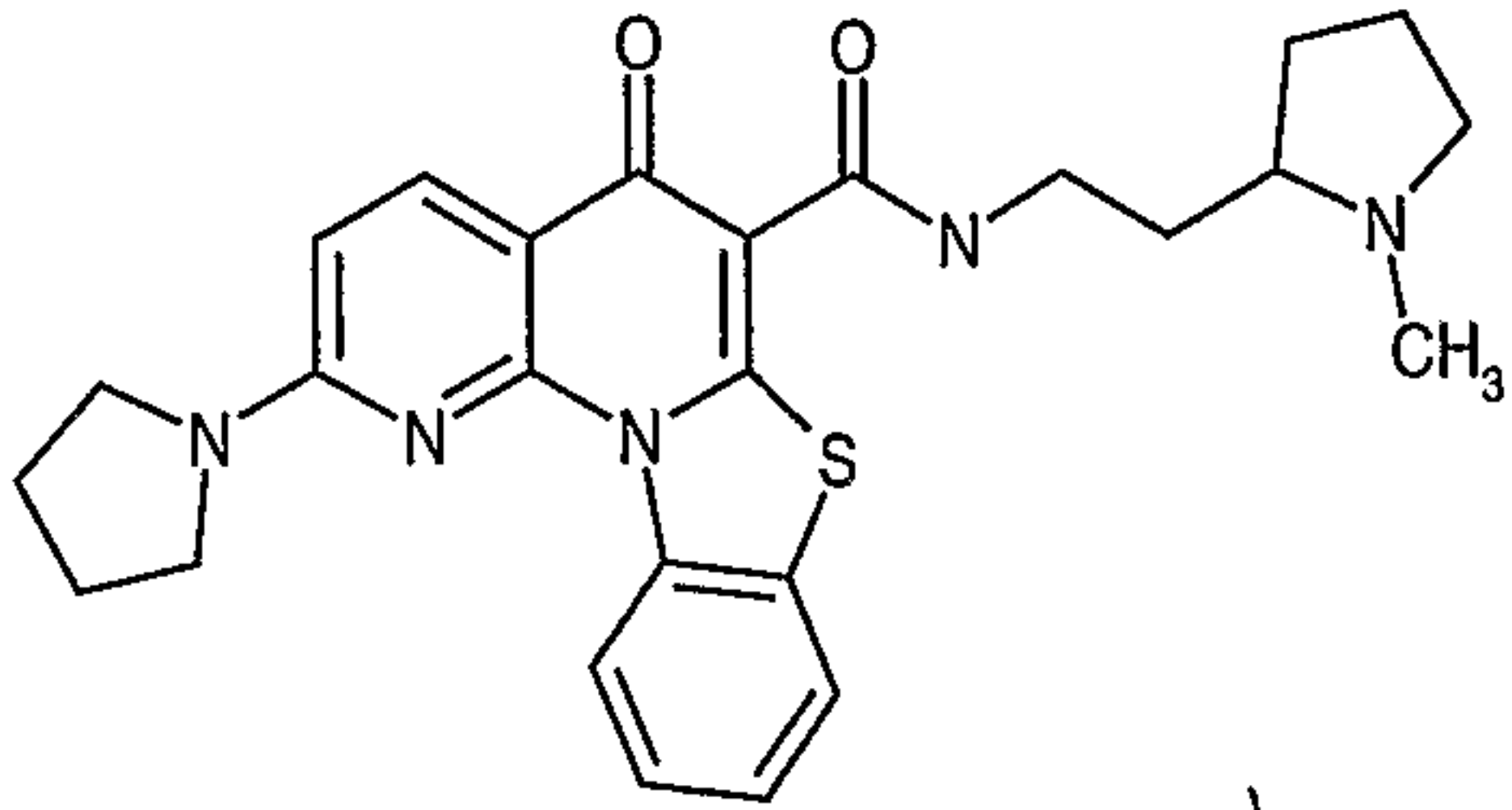
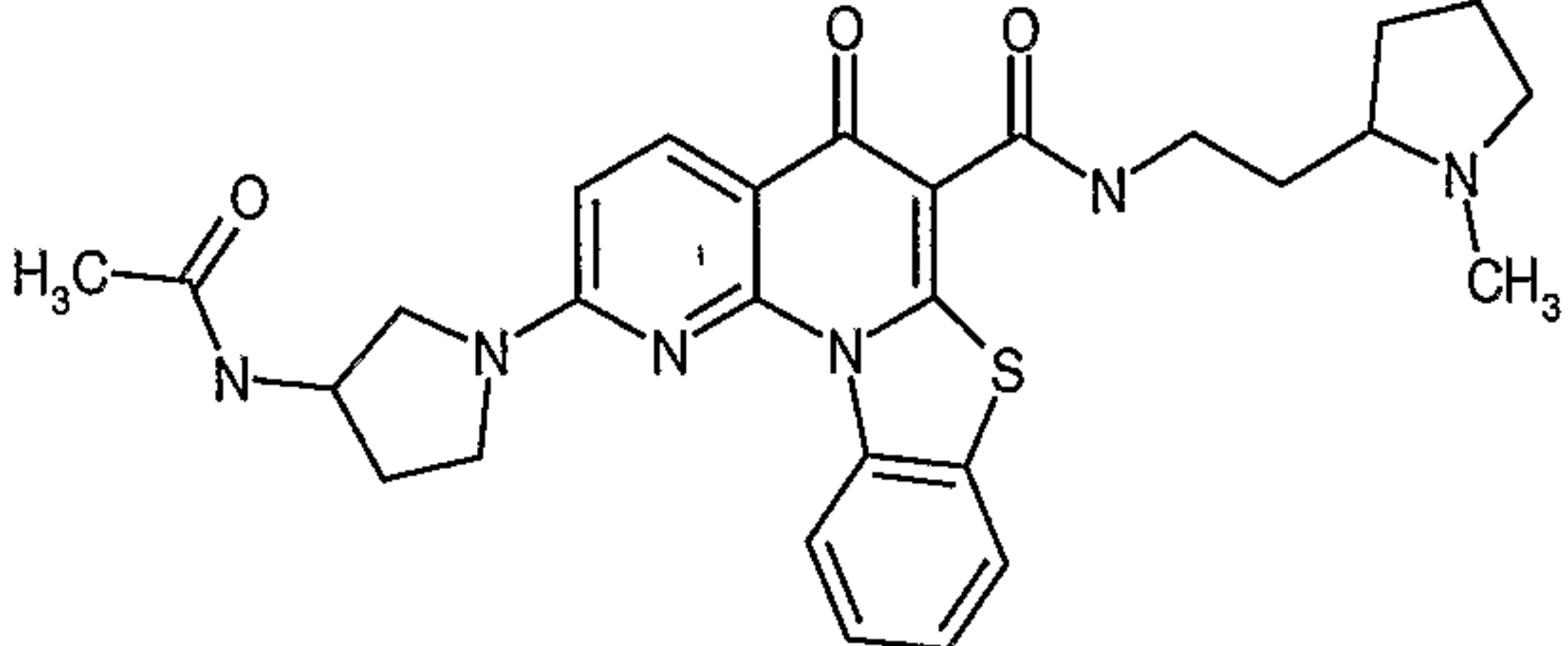
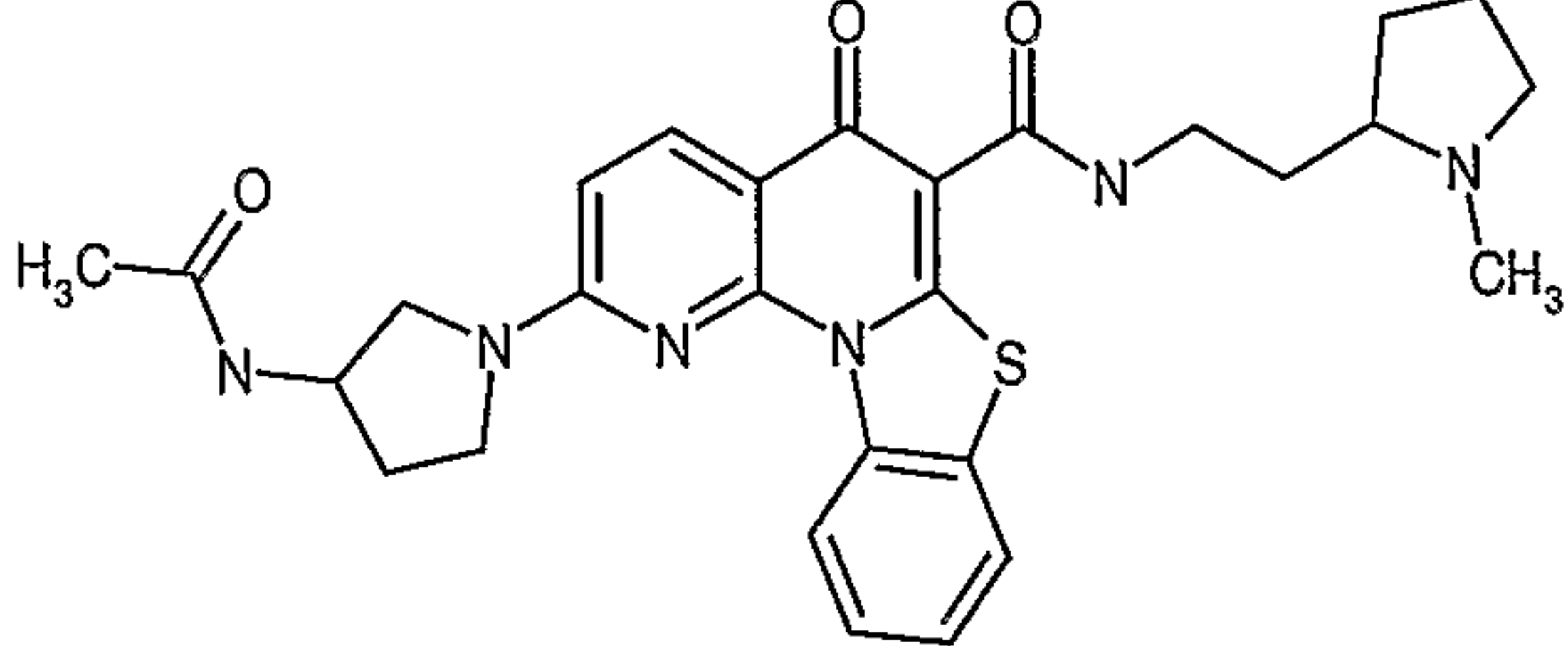
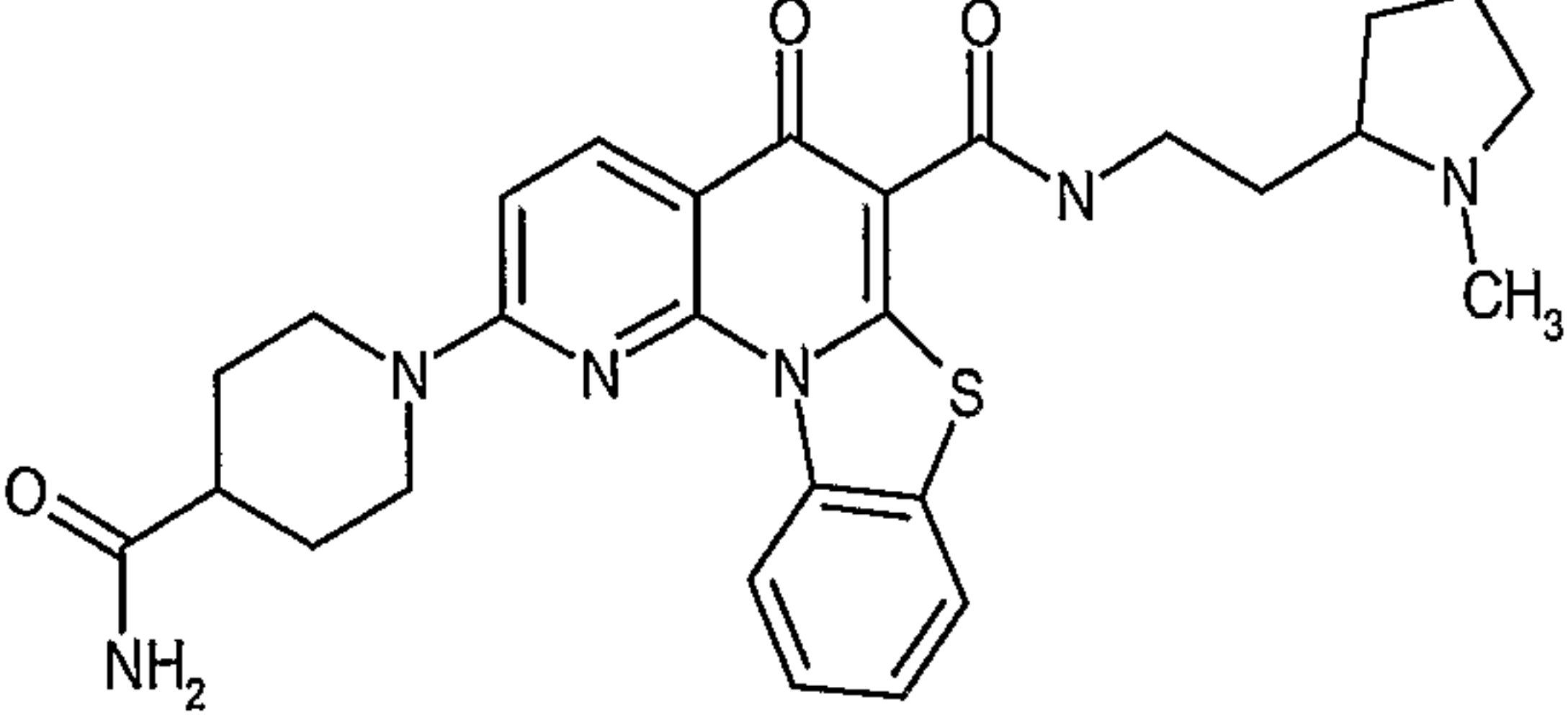
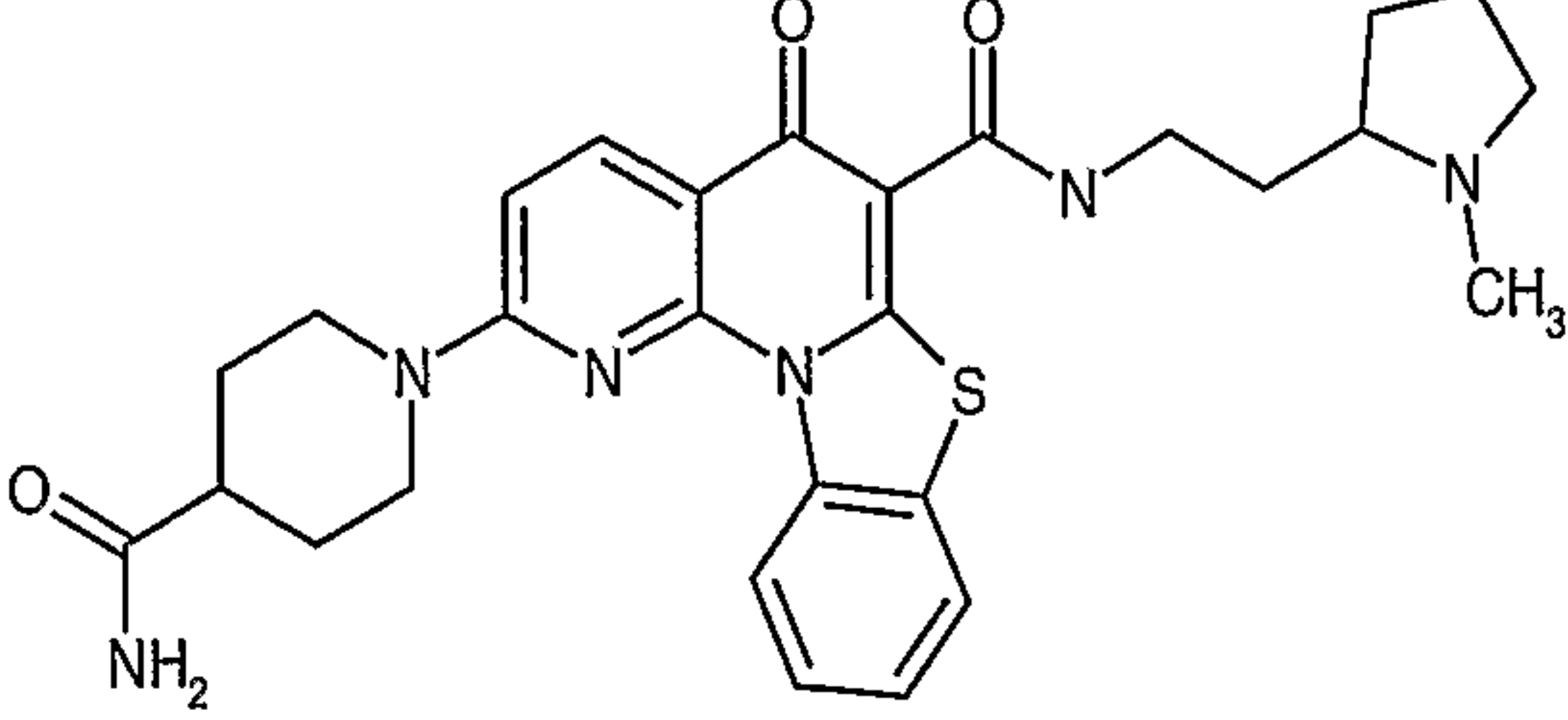
| MOLSTRUCTURE                                                                                                                                     | M CELL  | M DATA |
|--------------------------------------------------------------------------------------------------------------------------------------------------|---------|--------|
|  <chem>CCOC(=O)c1c2c(c3ccccc3n2)c4ccc(Cl)cn41</chem>            | HCT-116 | 6.00   |
|  <chem>CCN1c2c(c3ccccc3n2)c4ccc(N5CCN(CC5)C(=O)C)cc41</chem>  | MiaPaca | 0.27   |
|  <chem>CCN1c2c(c3ccccc3n2)c4ccc(N5CCN(CC5)C(=O)C)cc41</chem> | HCT-116 | 0.26   |
|  <chem>CC(=O)N1CCN(CC1)c2ccc3c(c2)n4c(=O)c5cccnc5n43</chem>   | MiaPaca | 0.3    |
|  <chem>CC(=O)N1CCN(CC1)c2ccc3c(c2)n4c(=O)c5cccnc5n43</chem>   | HCT-116 | 0.32   |

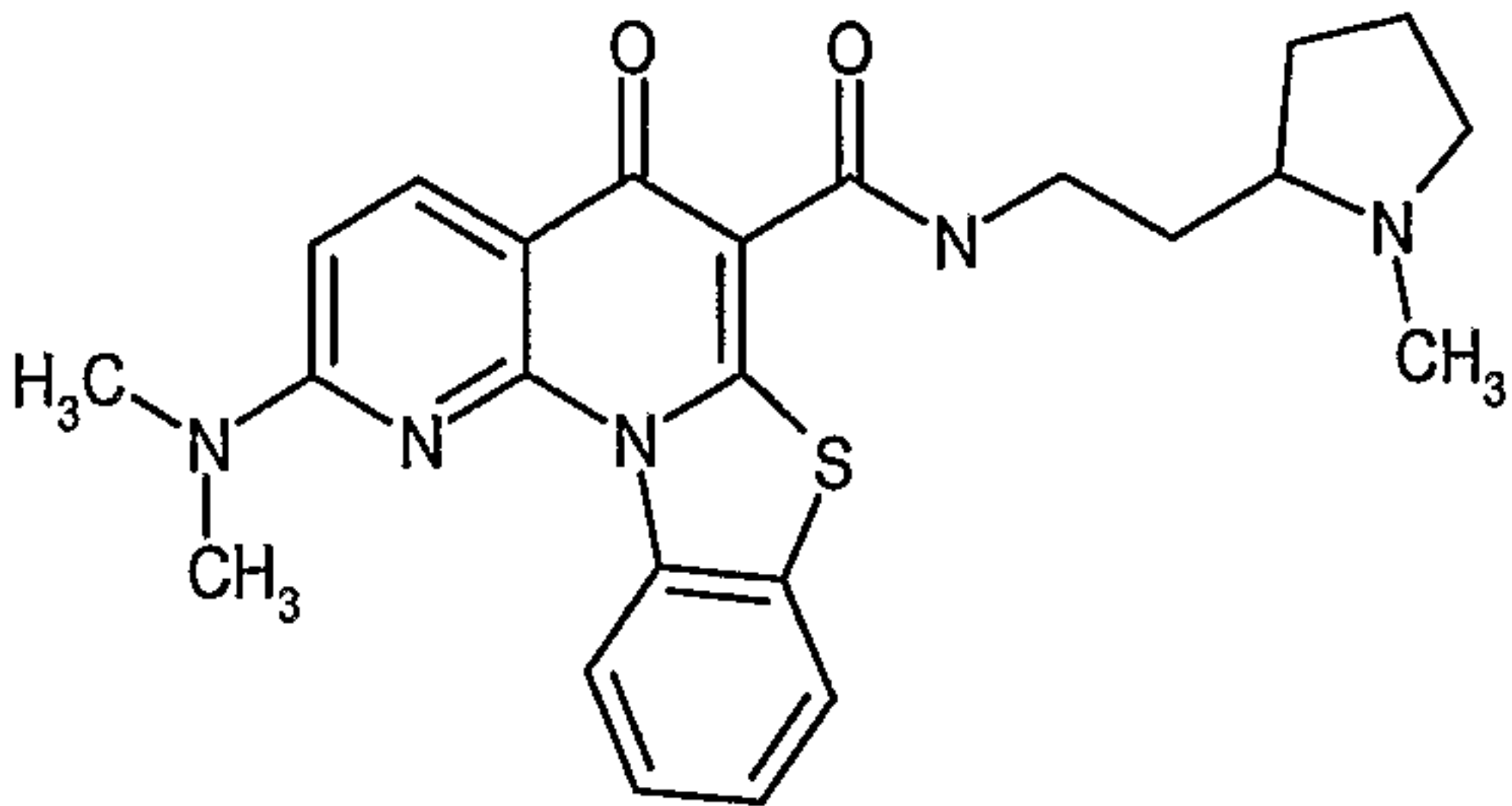
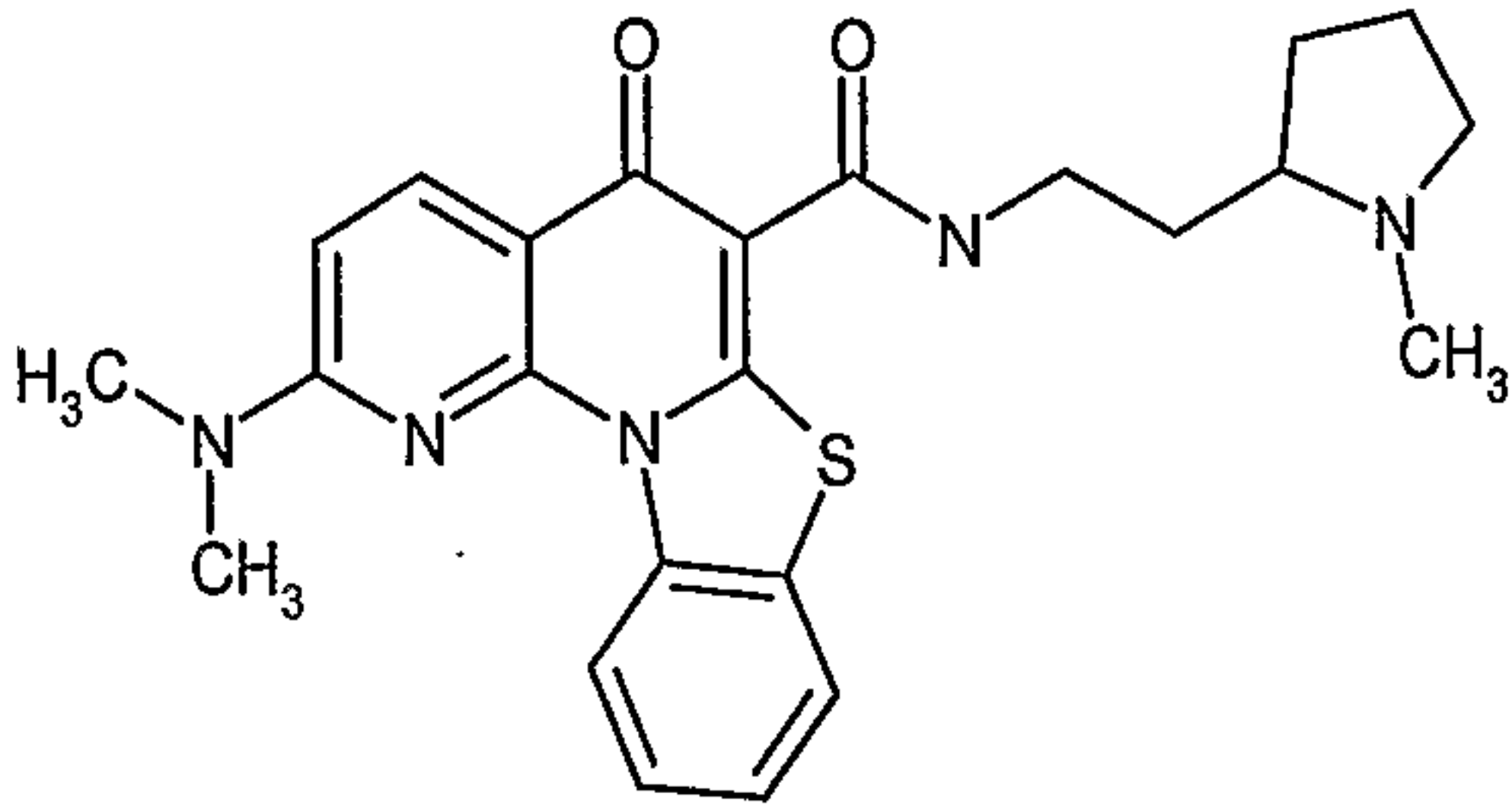
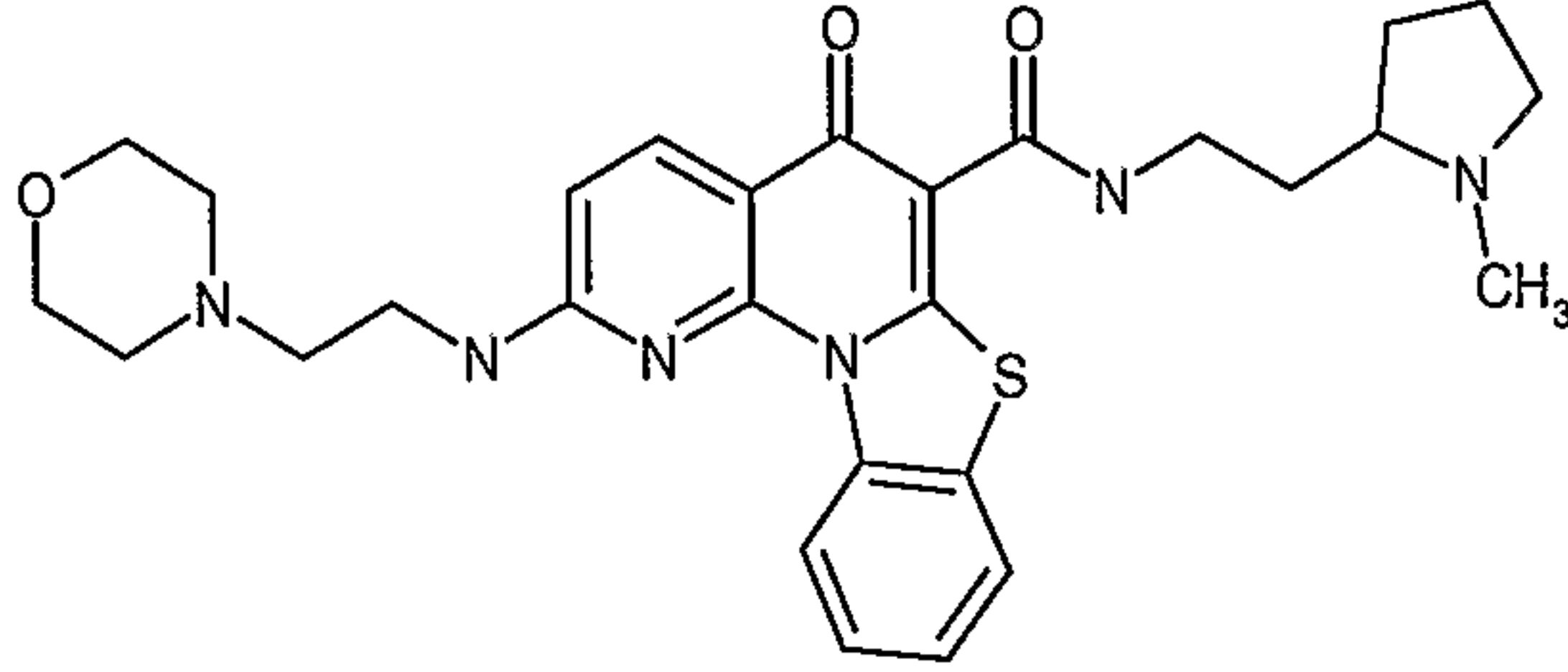
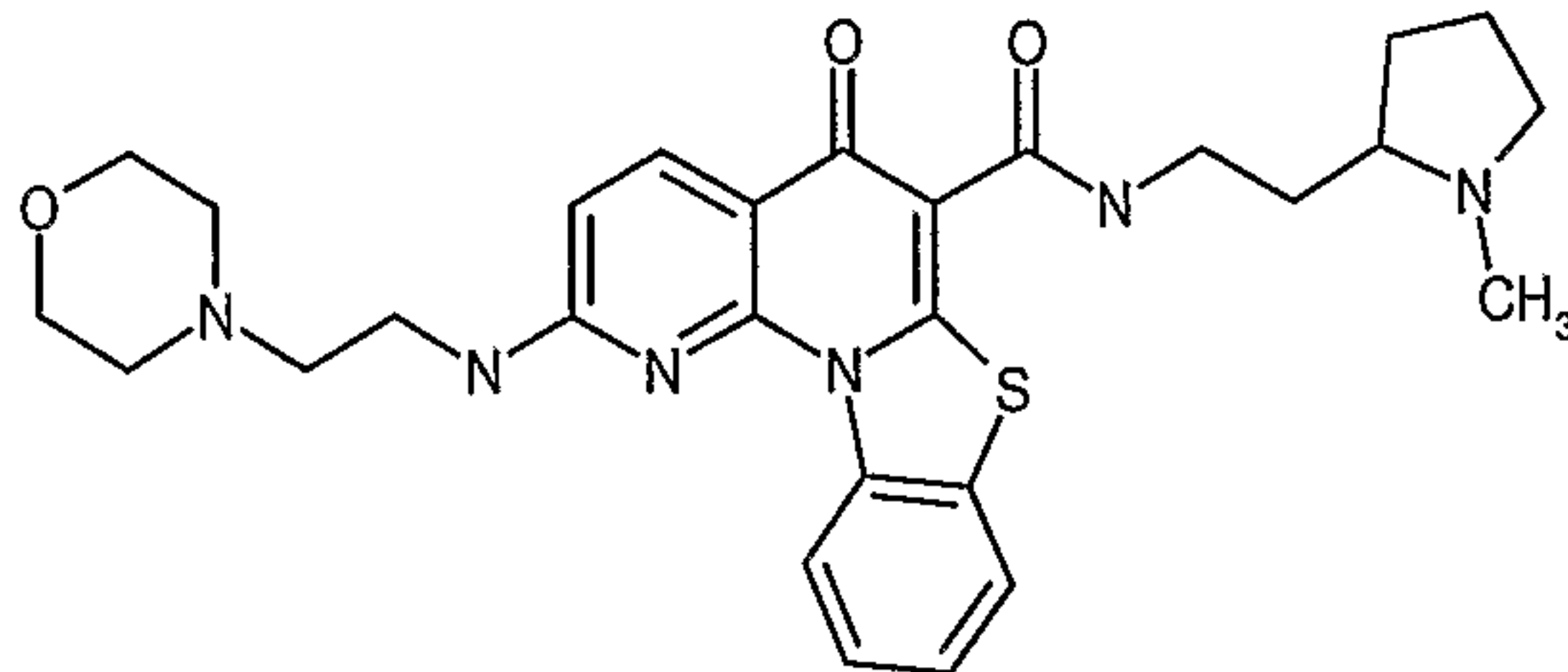
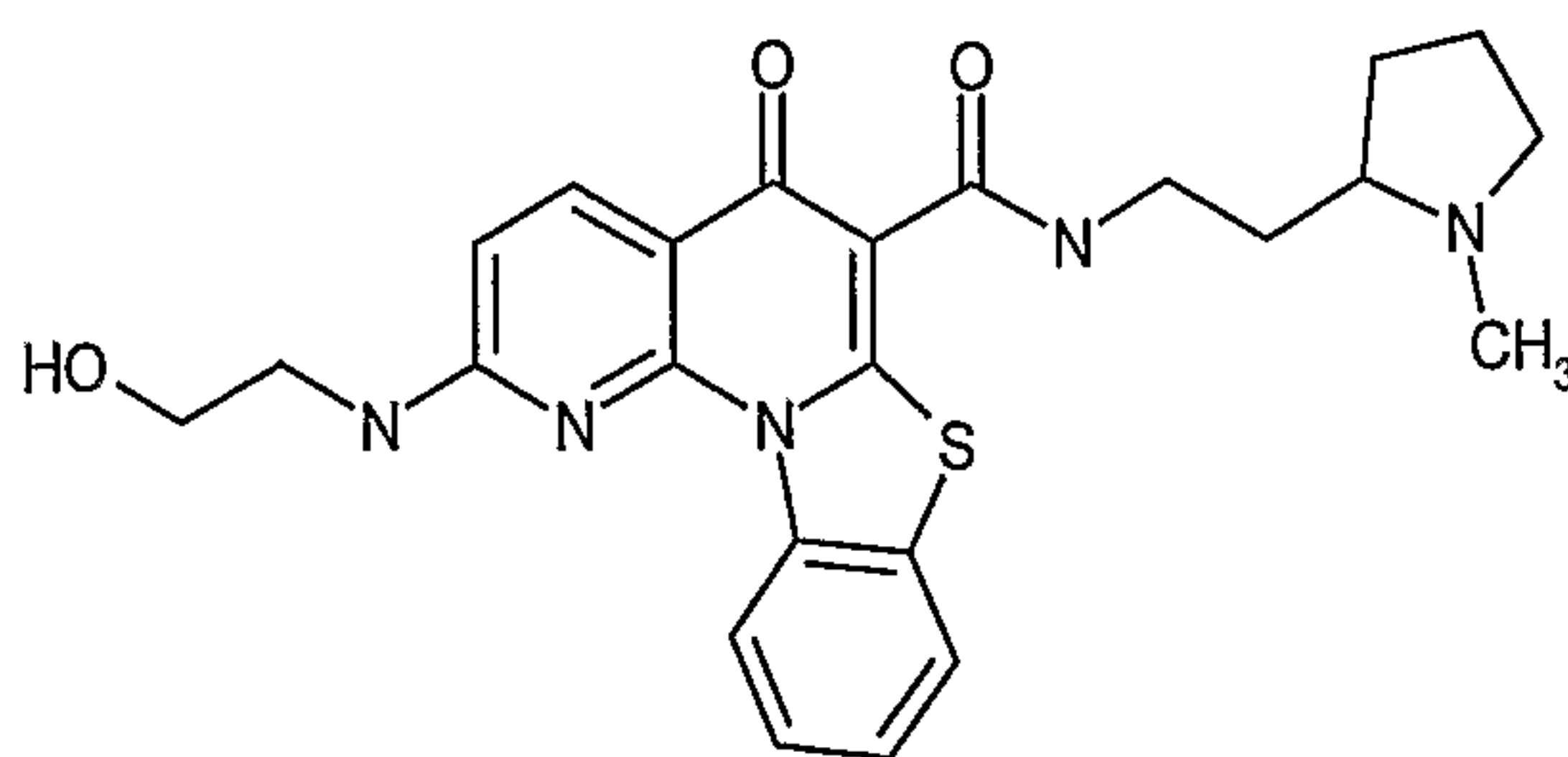
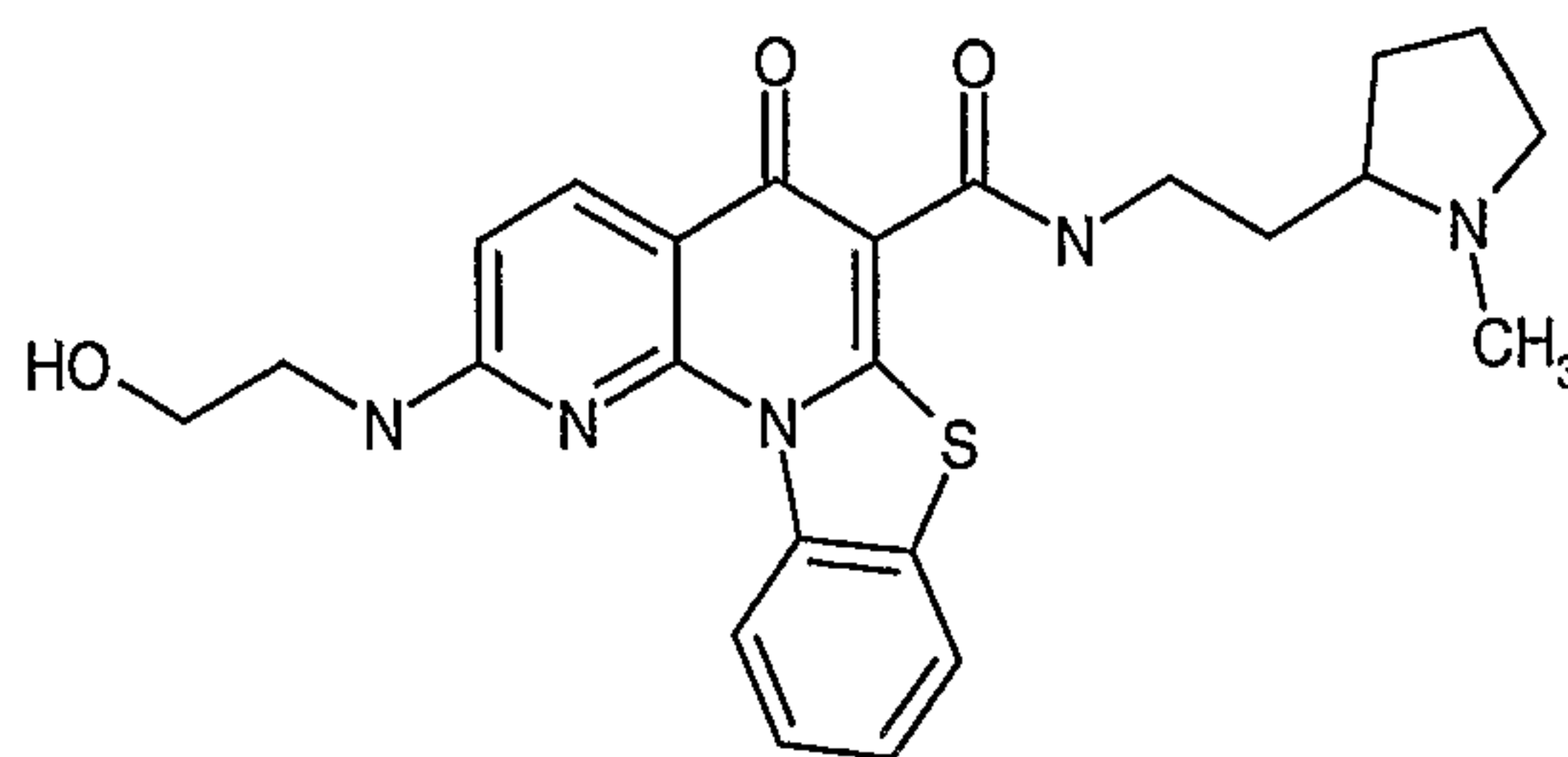
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | MiaPaca | 0.27   |
|   | HCT-116 | 0.26   |
|  | HCT-116 | 0.07   |
|  | Miapaca | 0.04   |
|  | MiaPaca | 0.08   |

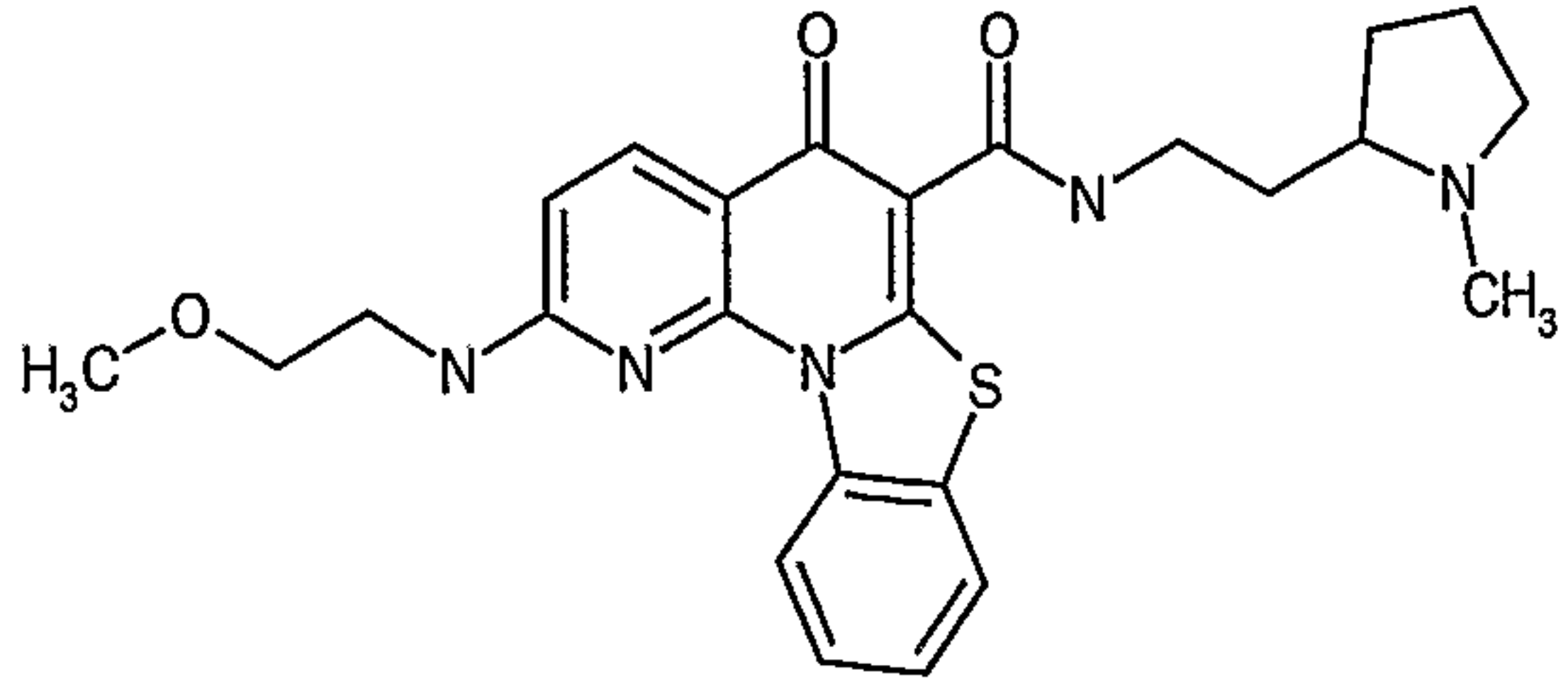
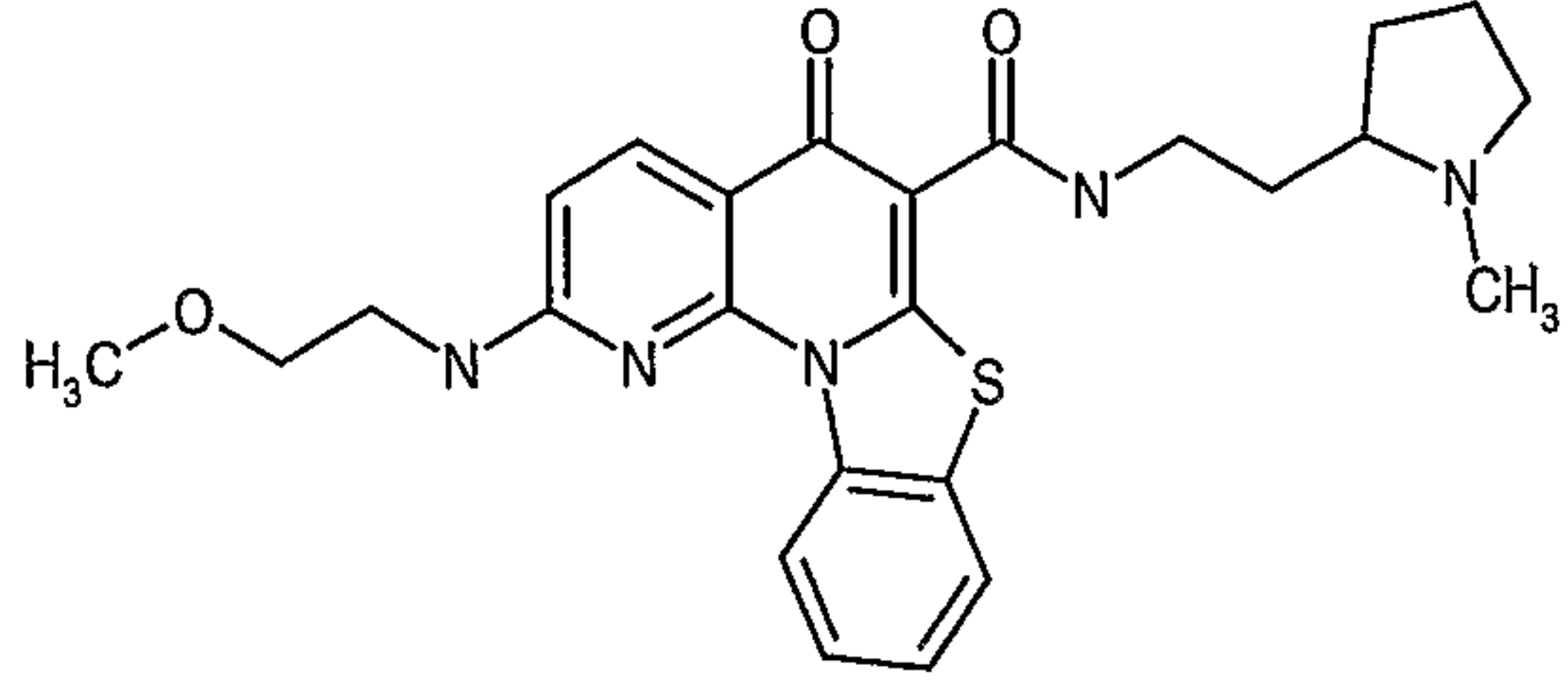
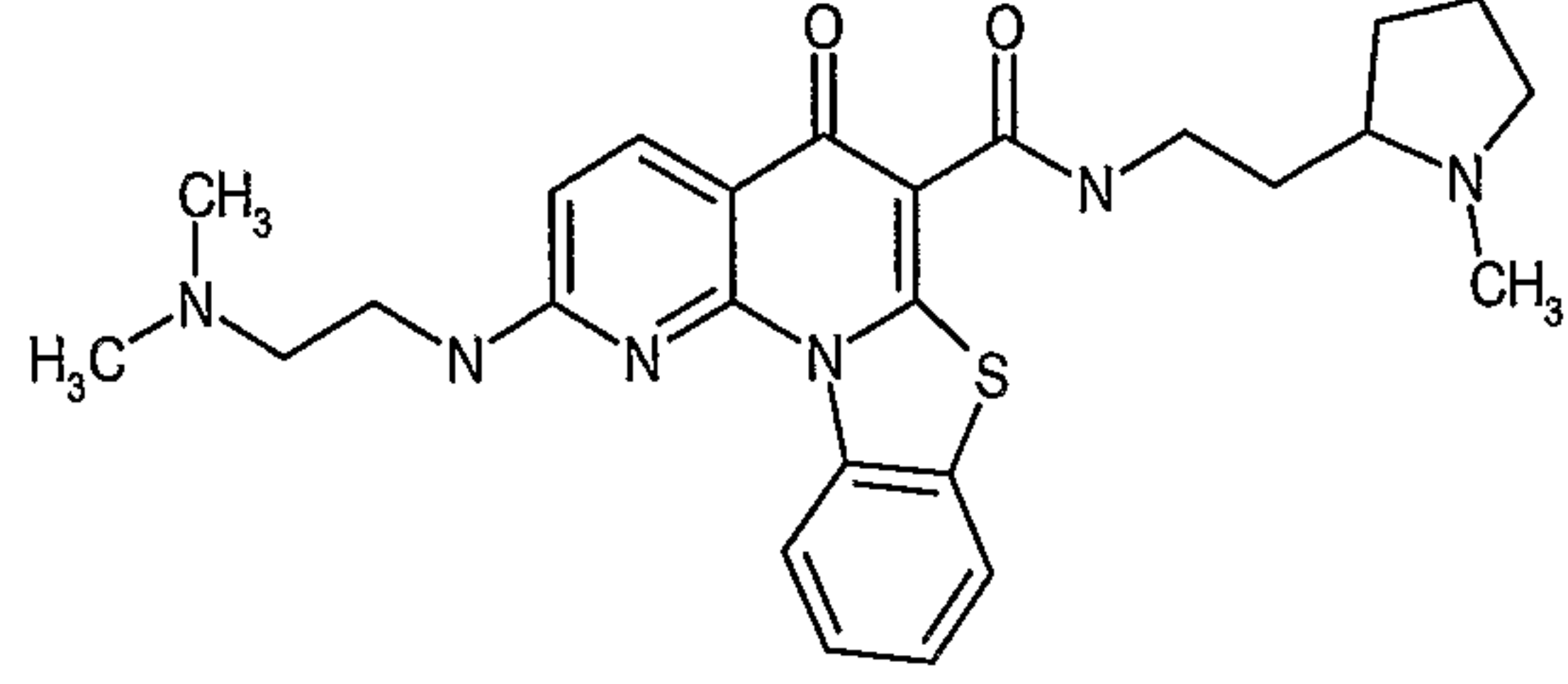
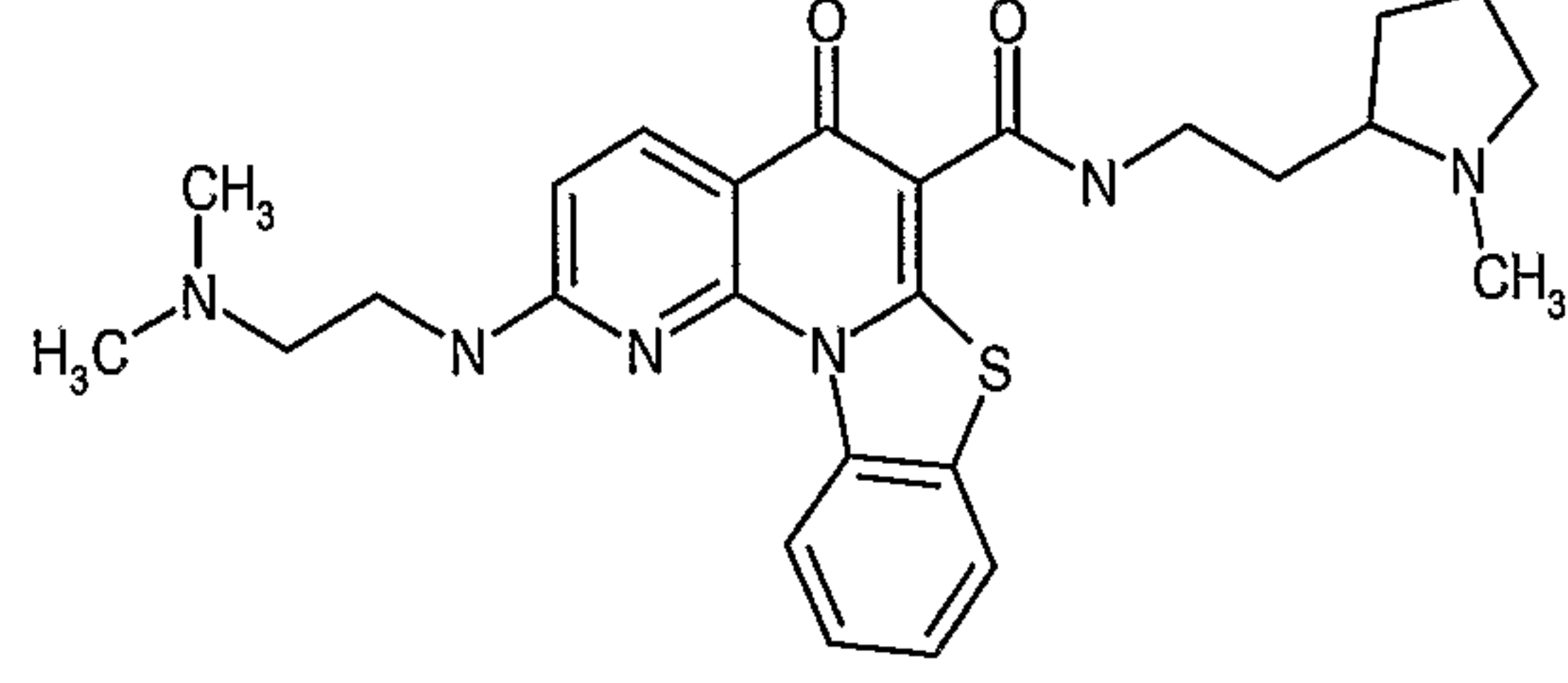
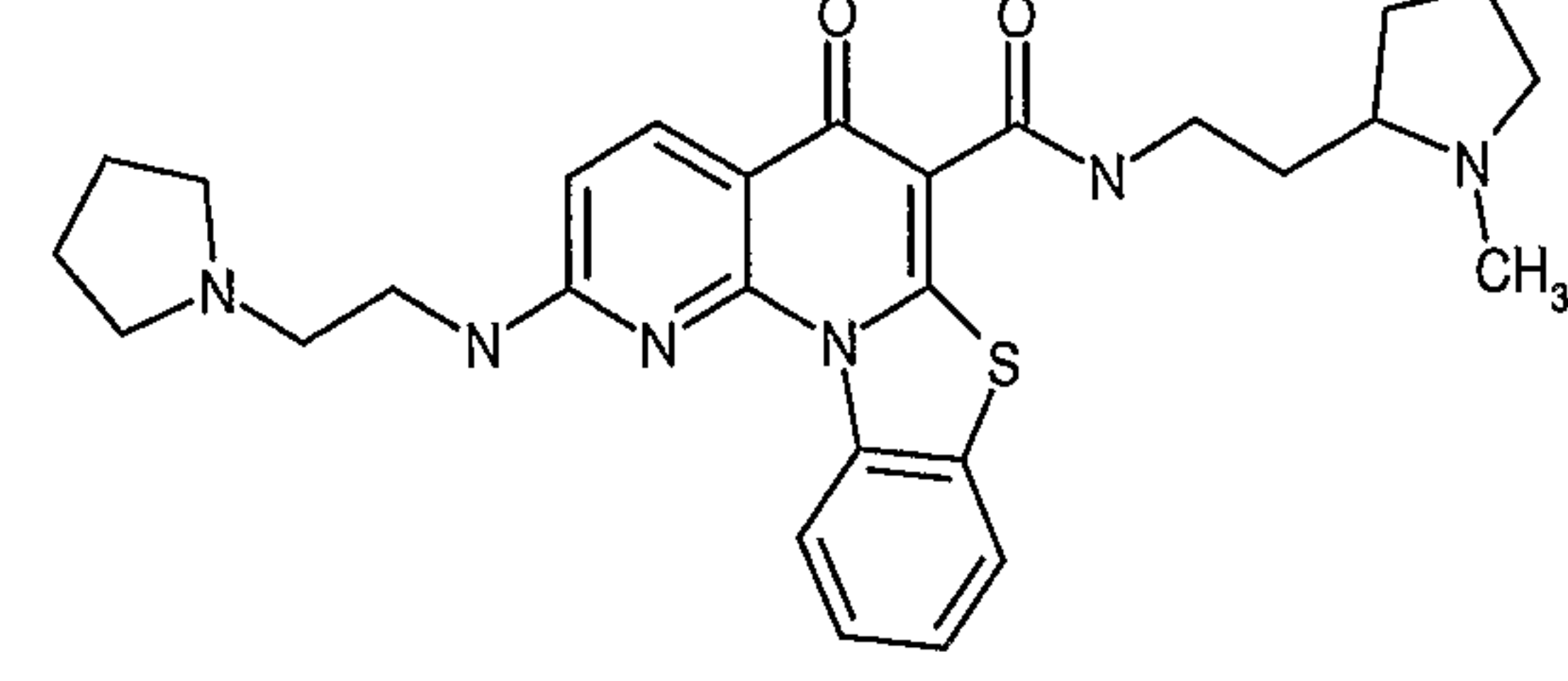
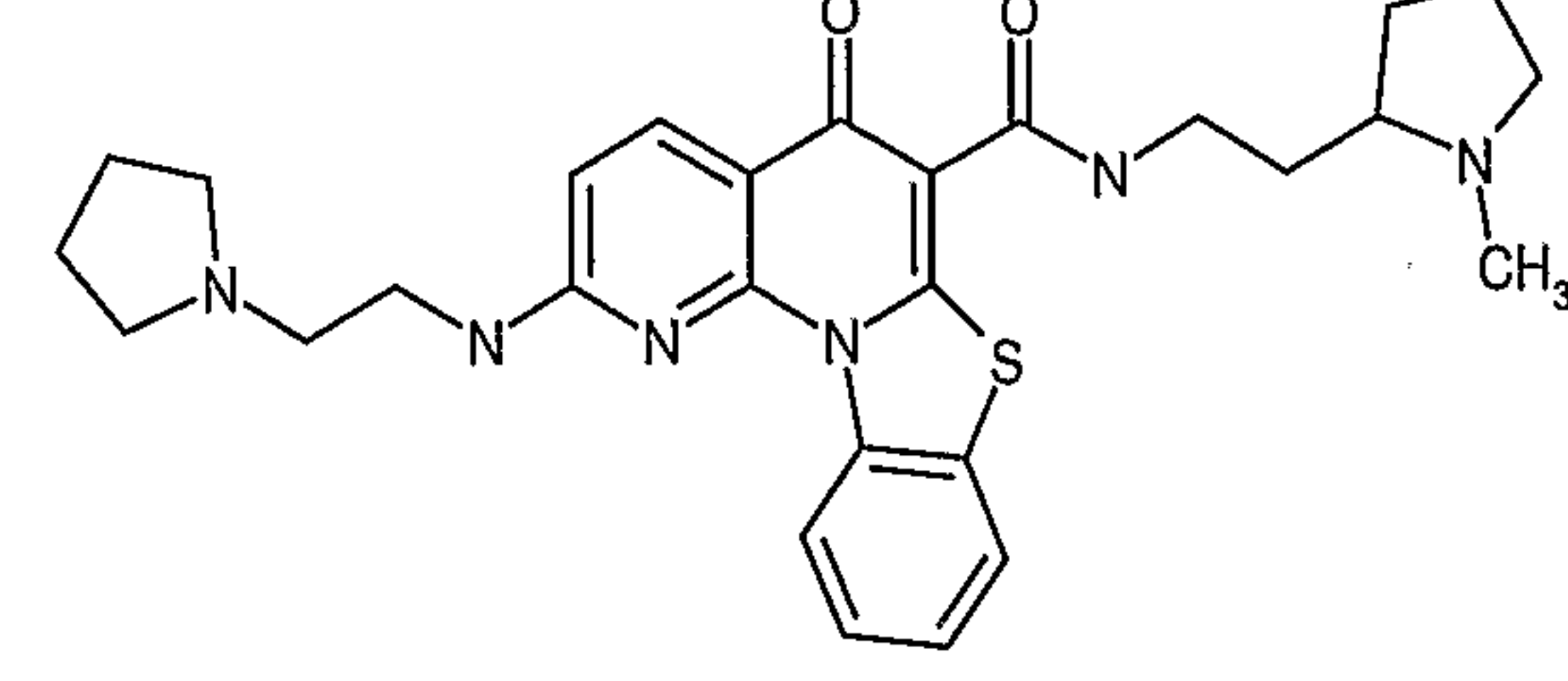
| MOLSTRUCTURE                                                                                                                                                                  | M CELL  | M DATA |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------|--------|
|  <chem>CC(=O)N1CCN(C1)C2=CC=C3C(=C2)C(=O)N(C)C3c4ccc5c(c4)nc6c5cnc6N(C)C7=CC=CC=C7</chem>    | HCT-116 | 0.04   |
|  <chem>CC(=O)N1CCN(C1)C2=CC=C3C(=C2)C(=O)N(C)C3c4ccc5c(c4)nc6c5cnc6N(C)C7=CC=CC=C7</chem>   | Miapaca | 0.37   |
|  <chem>CC(=O)N1CCN(C1)C2=CC=C3C(=C2)C(=O)N(C)C3c4ccc5c(c4)nc6c5cnc6N(C)C7=CC=CC=C7</chem>  | HCT-116 | 0.27   |
|  <chem>CC(=O)N1CCN(C1)C2=CC=C3C(=C2)C(=O)N(C)C3c4ccc5c(c4)nc6c5cnc6N(C)C7=CC=CC=C7</chem> | Miapaca | 0.02   |
|  <chem>CC(=O)N1CCN(C1)C2=CC=C3C(=C2)C(=O)N(C)C3c4ccc5c(c4)nc6c5cnc6N(C)C7=CC=CC=C7</chem> | HCT-116 | 0.03   |

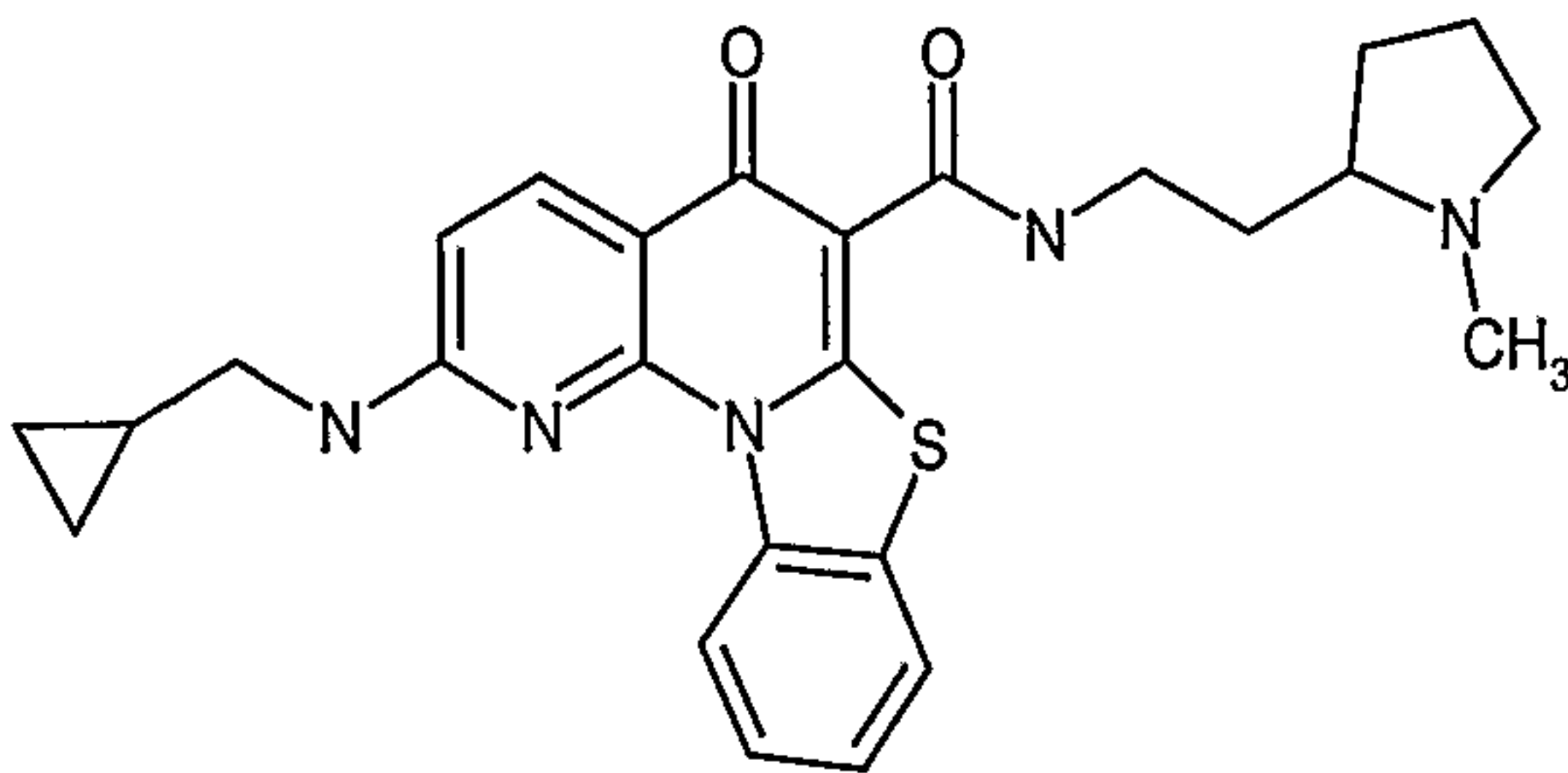
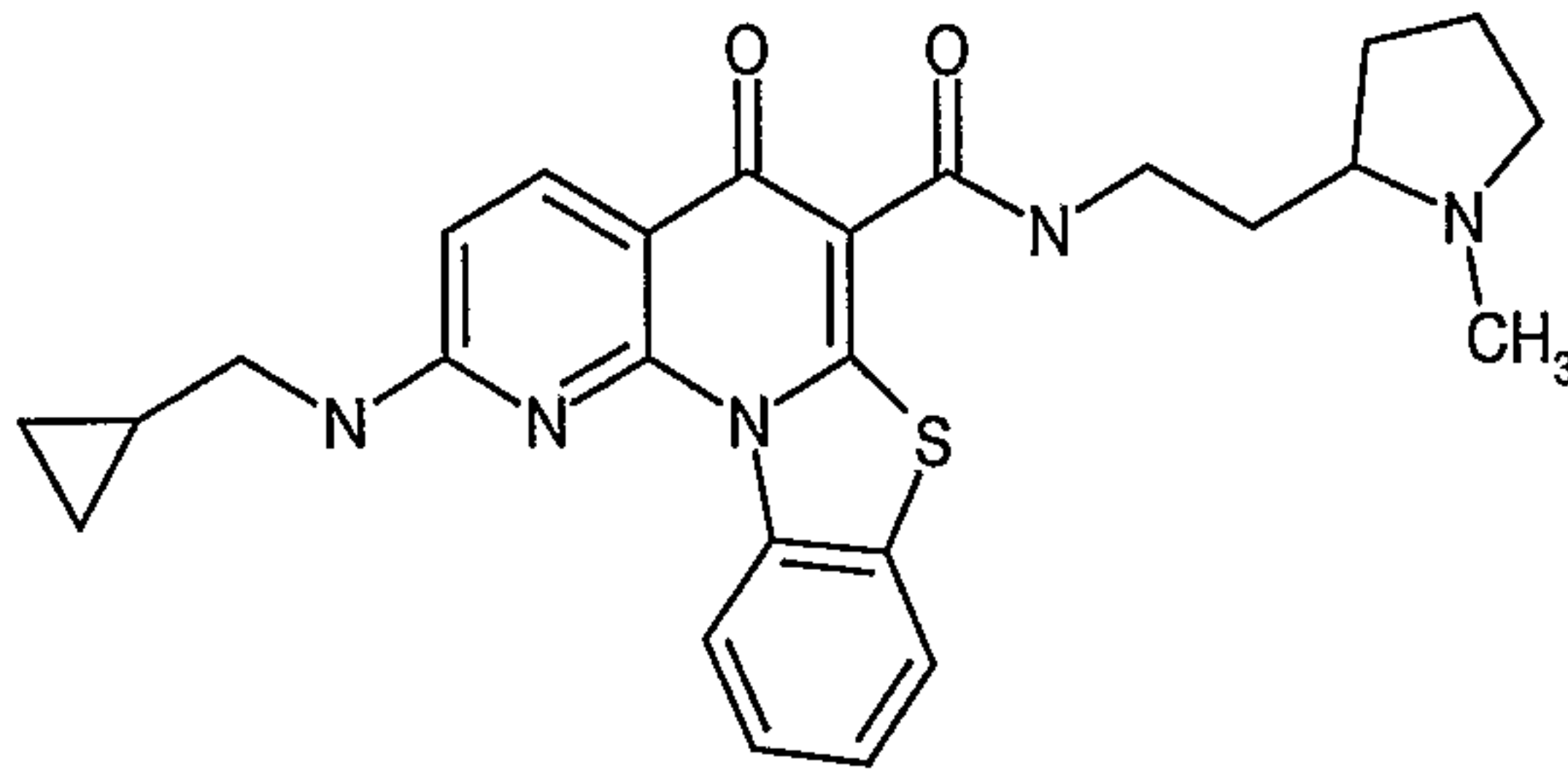
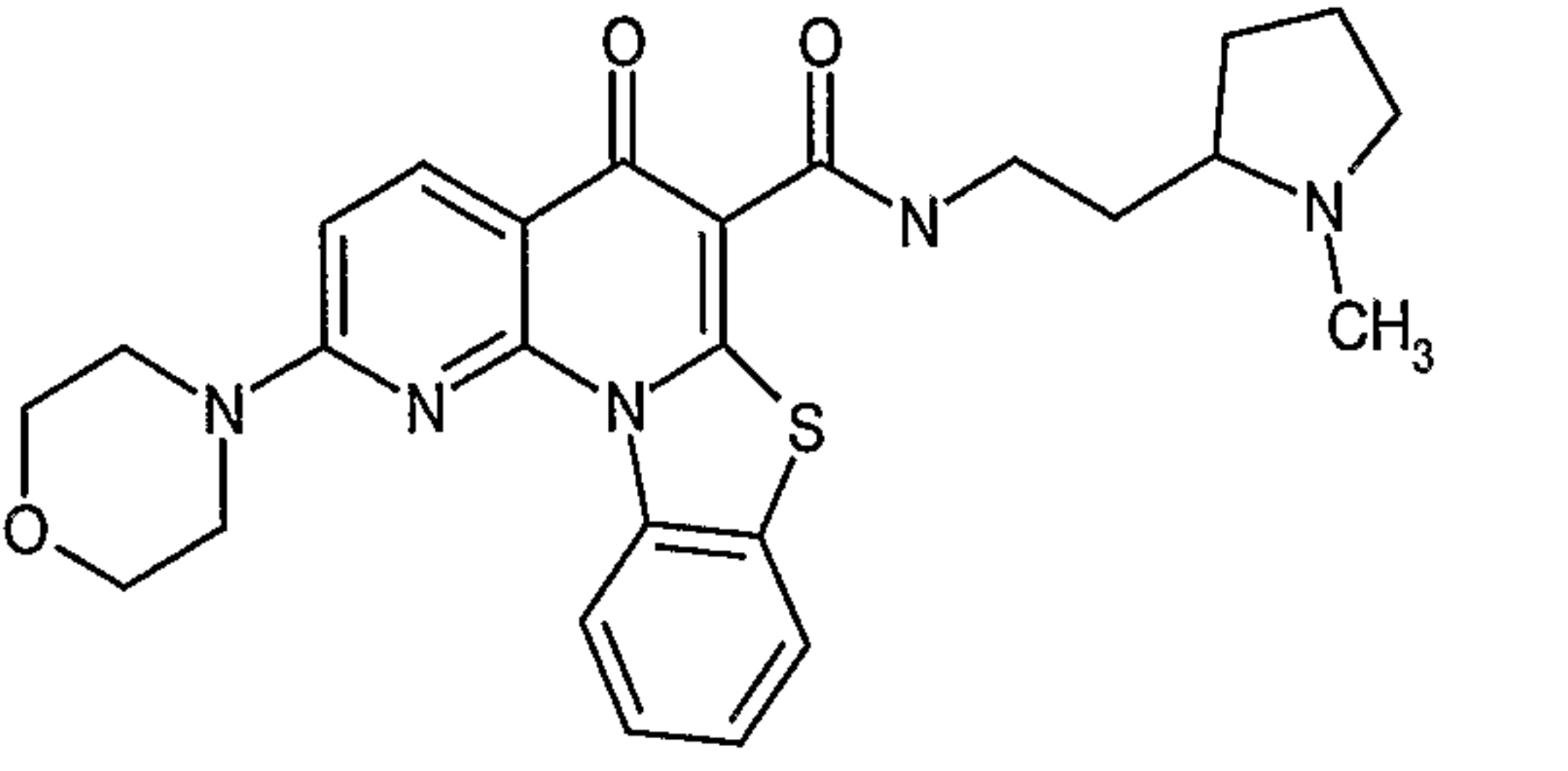
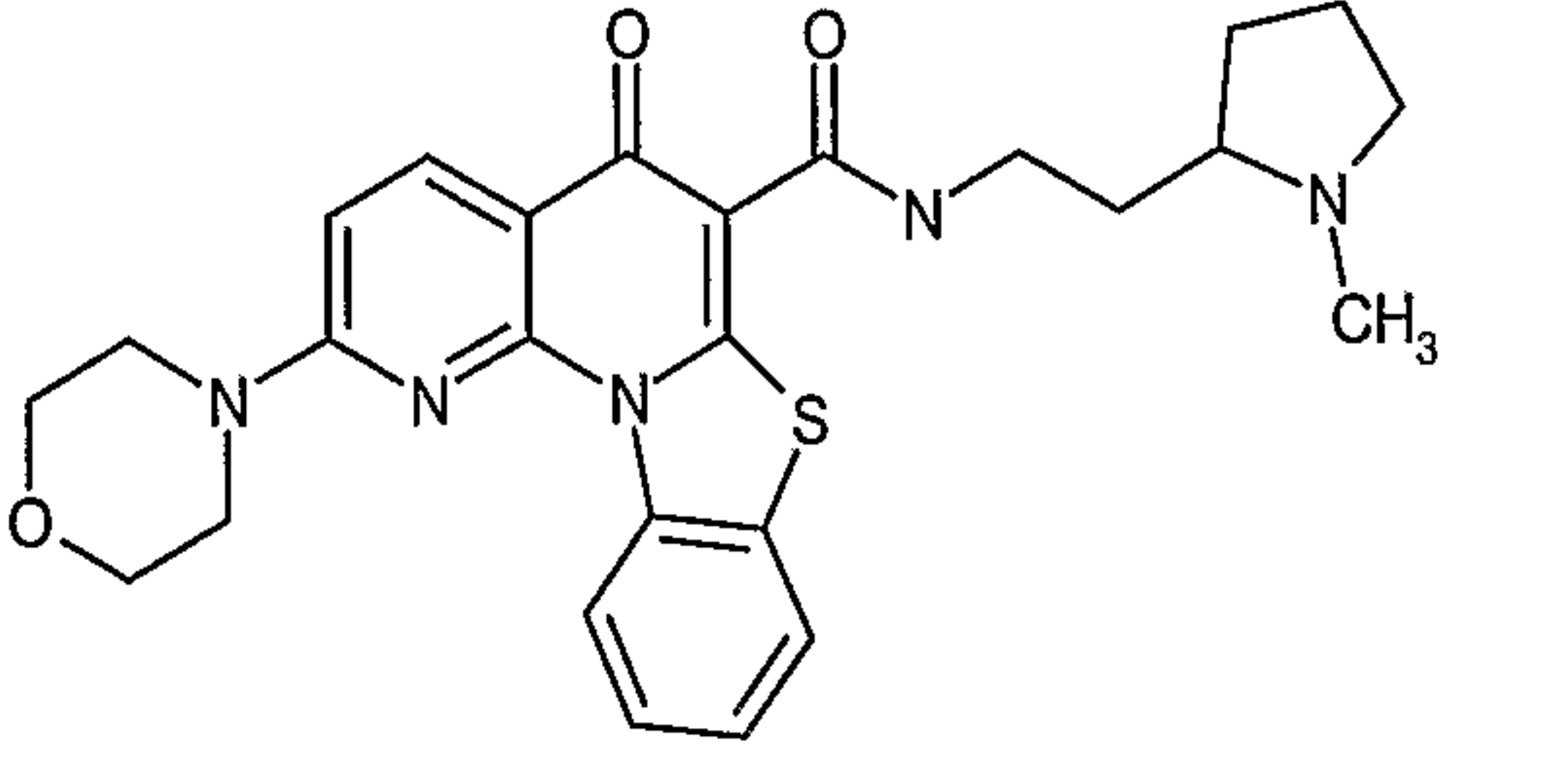
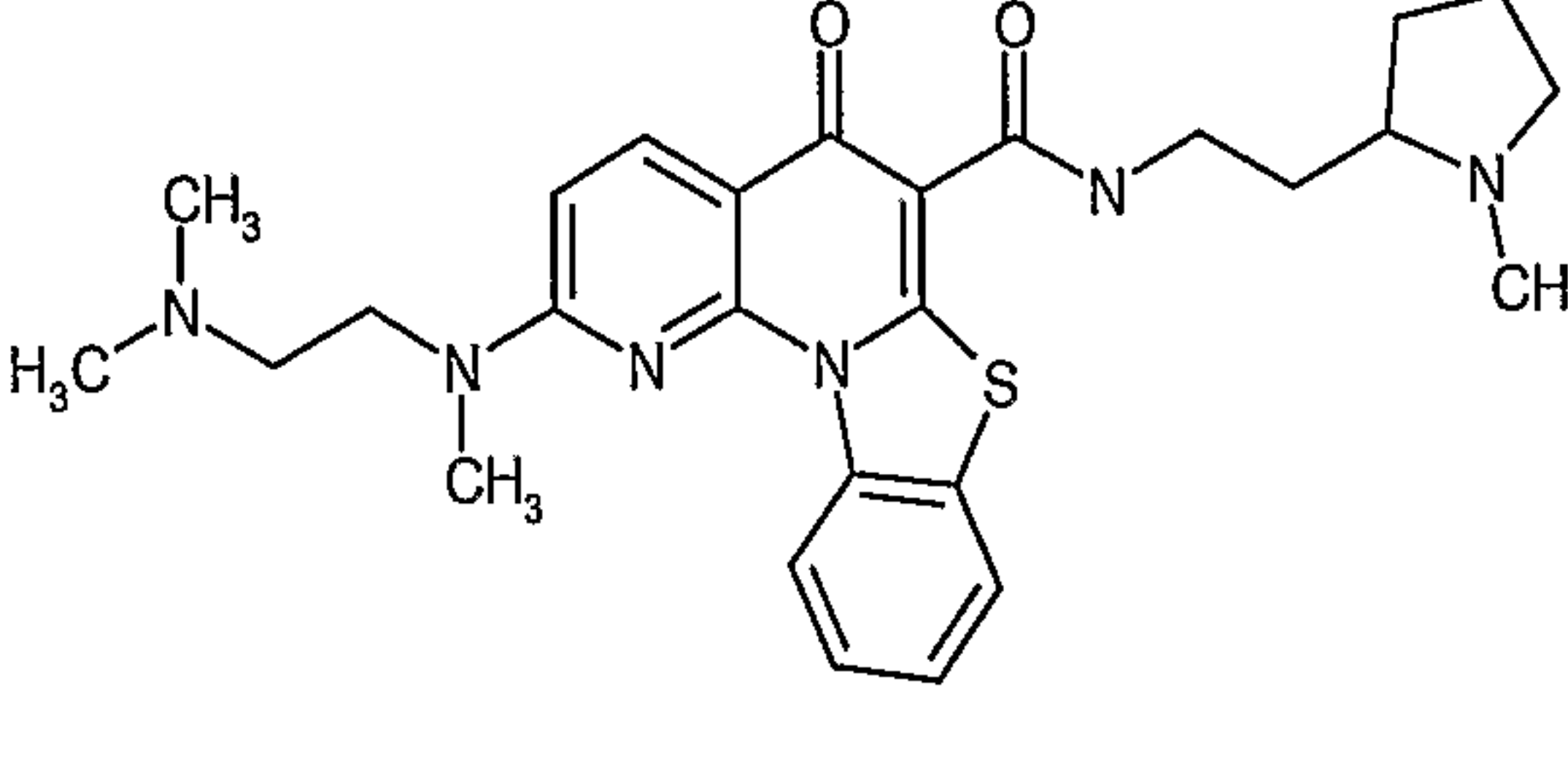
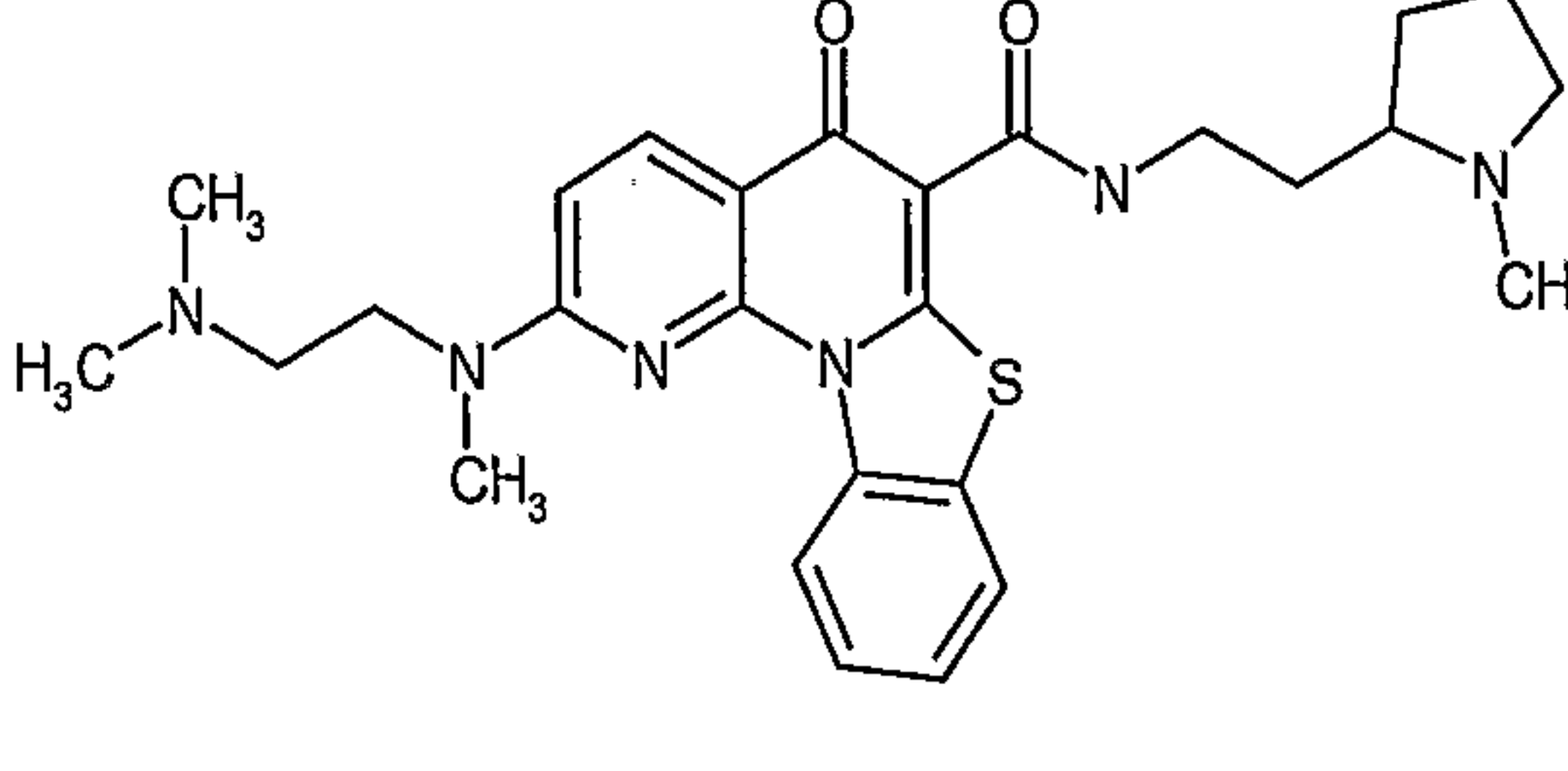
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | Miapaca | 0.52   |
|    | HCT-116 | 0.18   |
|  | Miapaca | 0.04   |
|  | HCT-116 | 0.04   |
|  | Miapaca | 0.01   |
|  | HCT-116 | <0.01  |

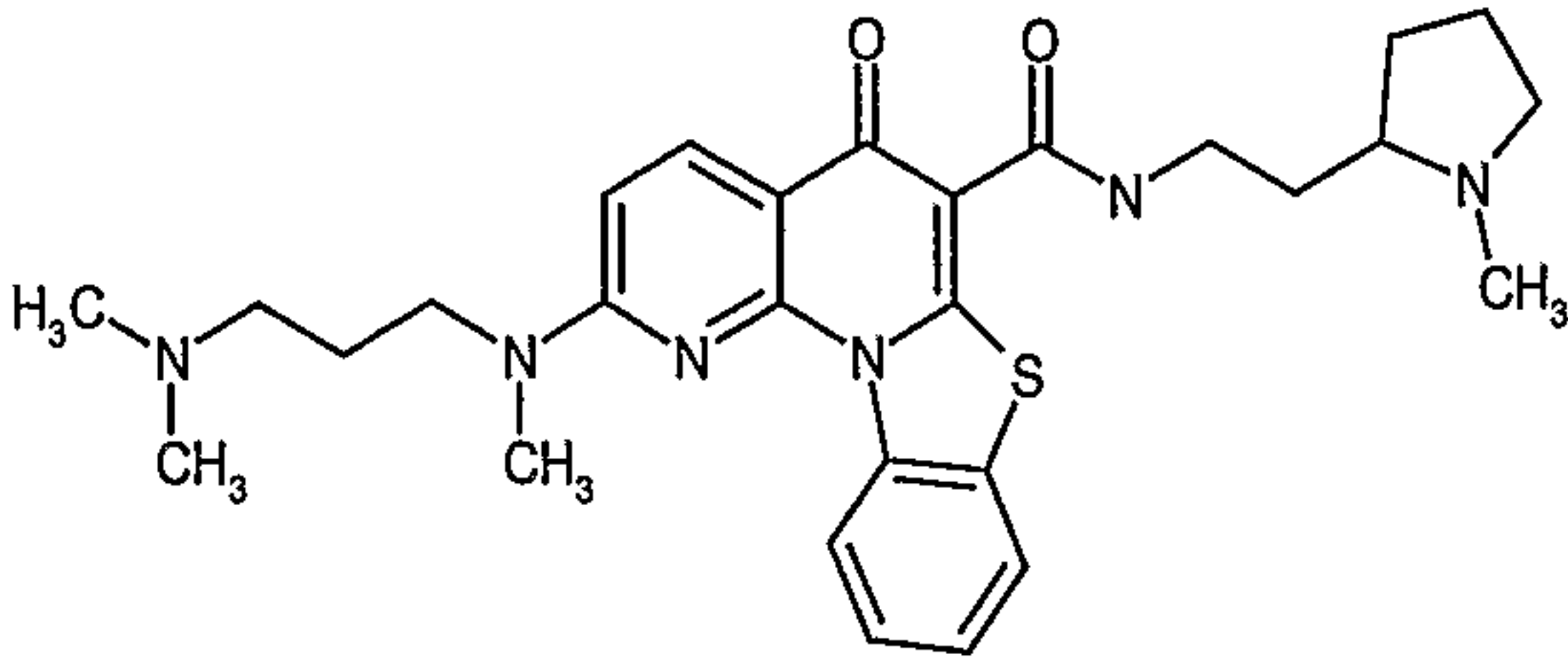
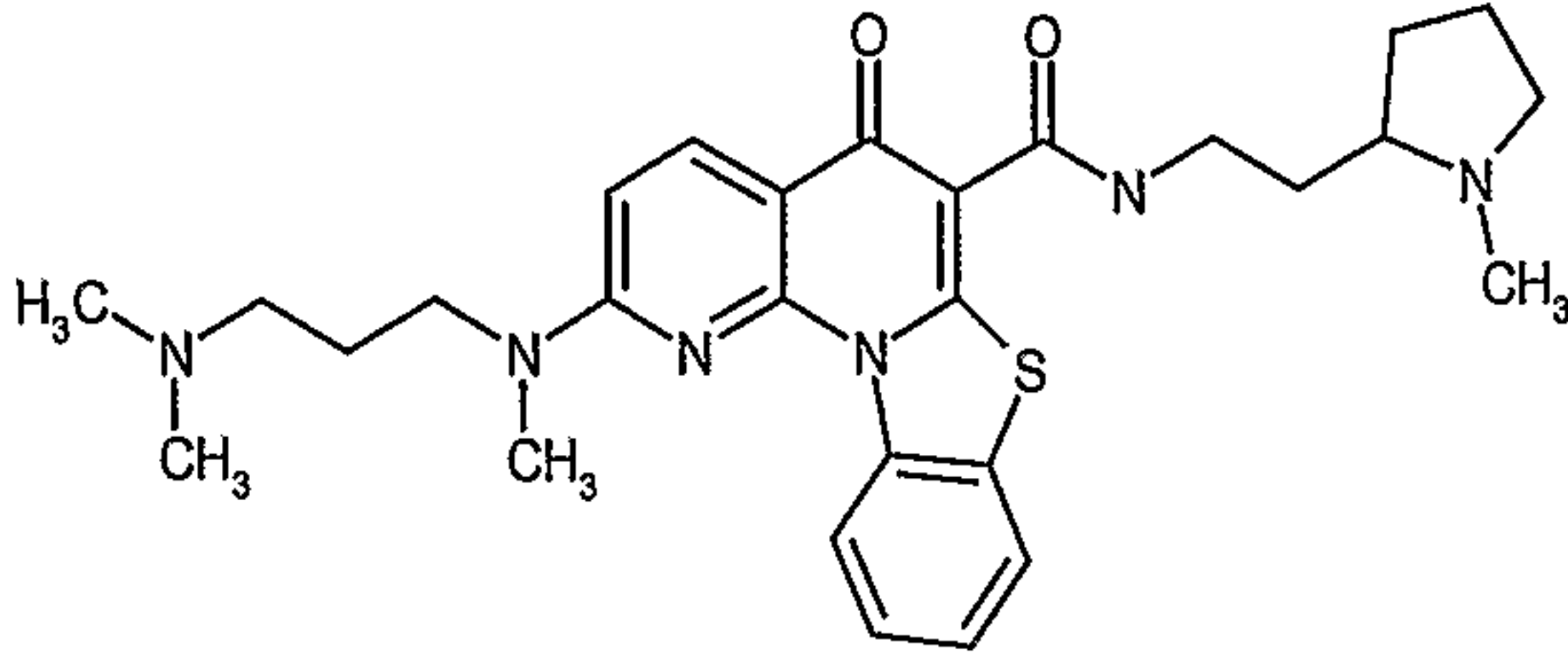
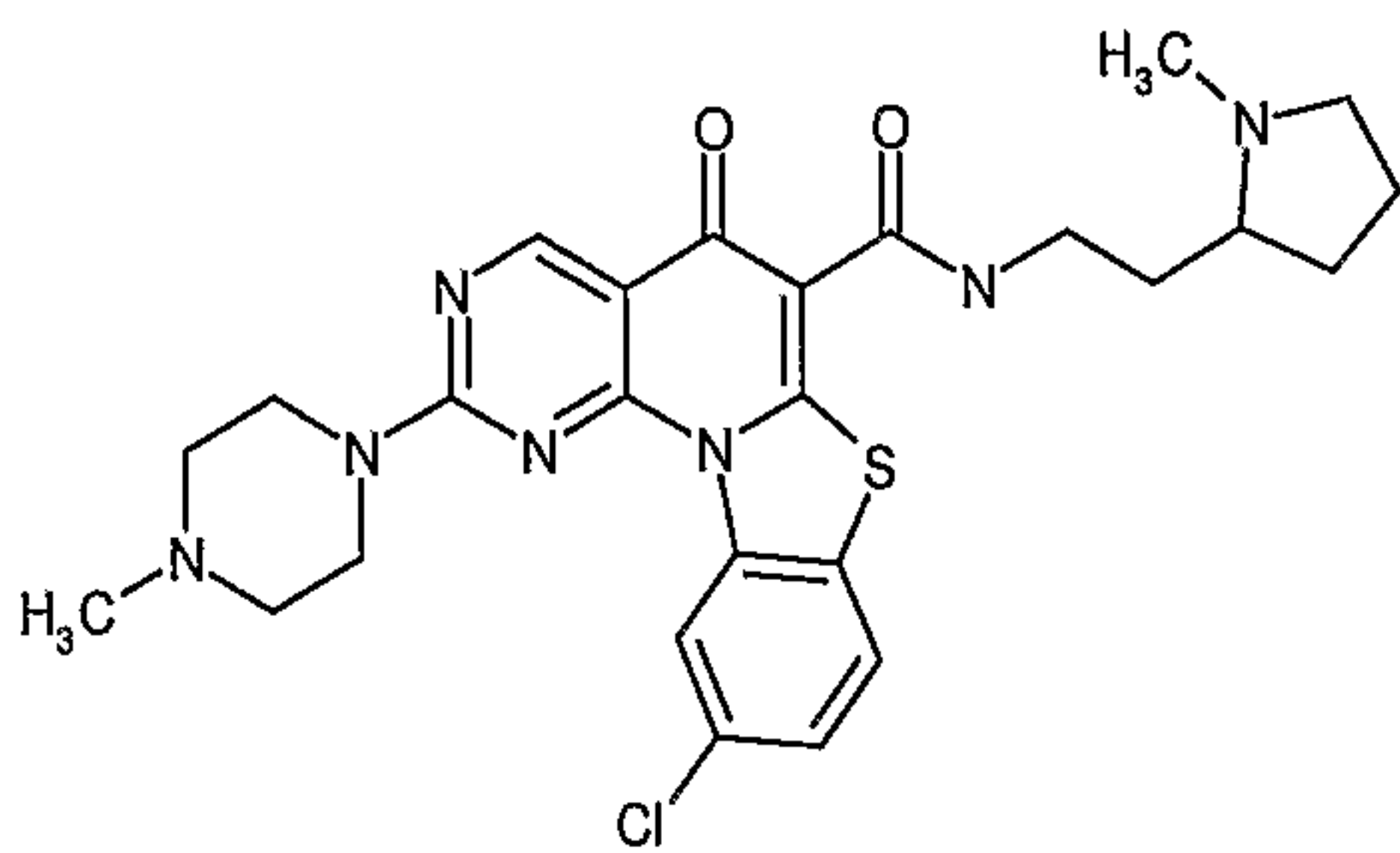
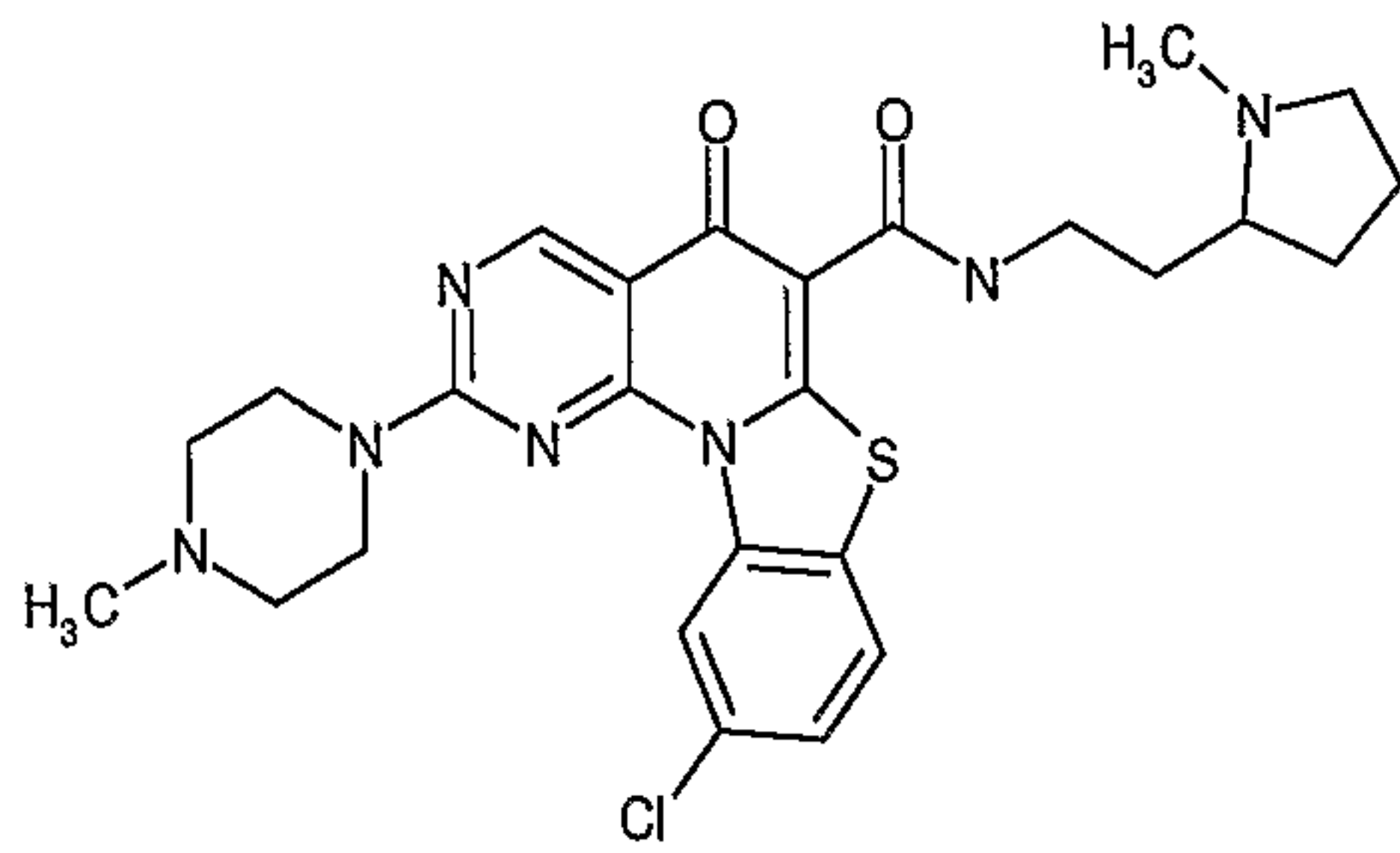
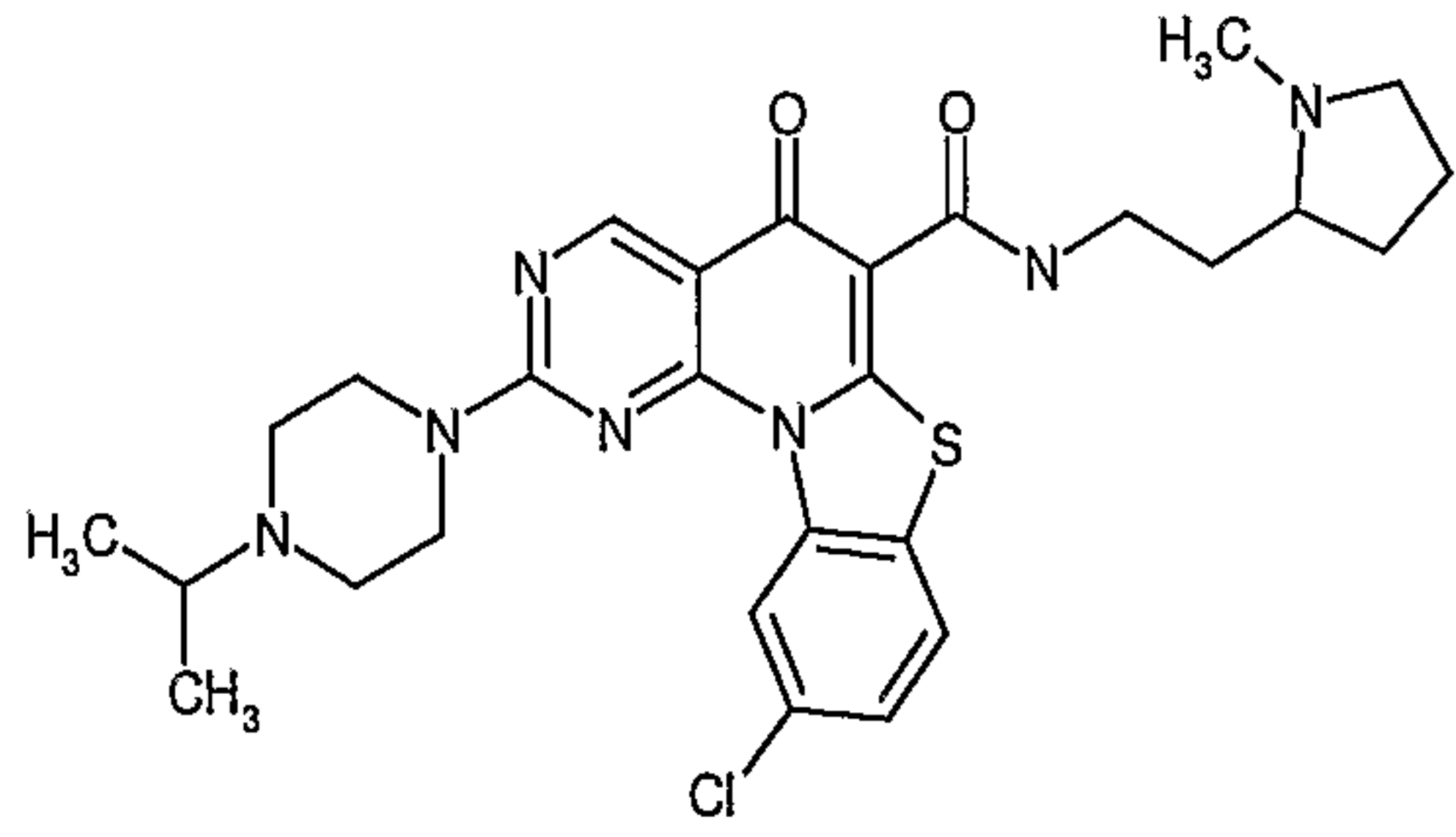
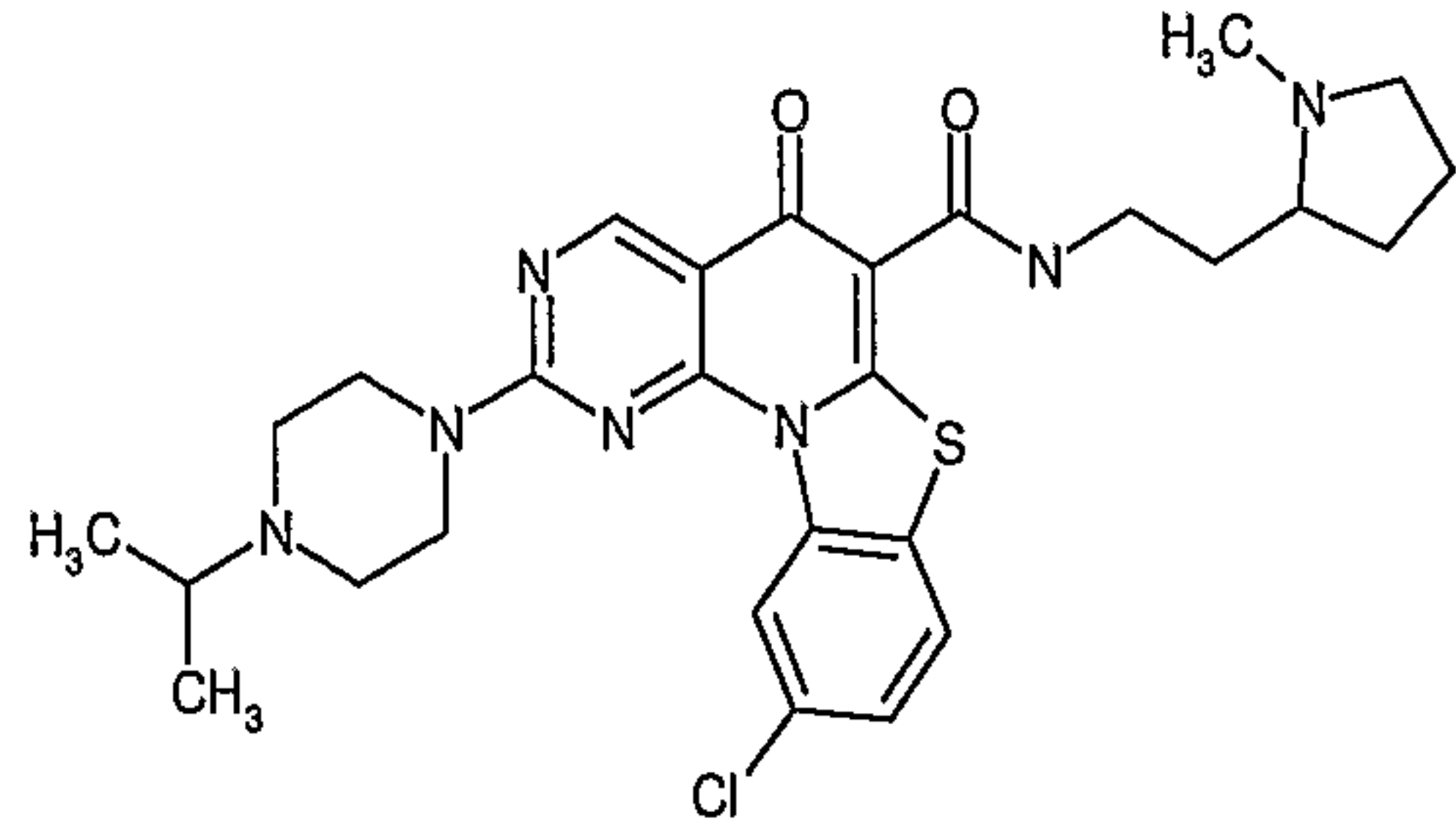
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | 0.03   |
|   | HCT-116 | 0.03   |
|  | Miapaca | 0.01   |
|  | HCT-116 | <0.01  |
|  | Miapaca | 0.20   |
|  | HCT-116 | 0.14   |

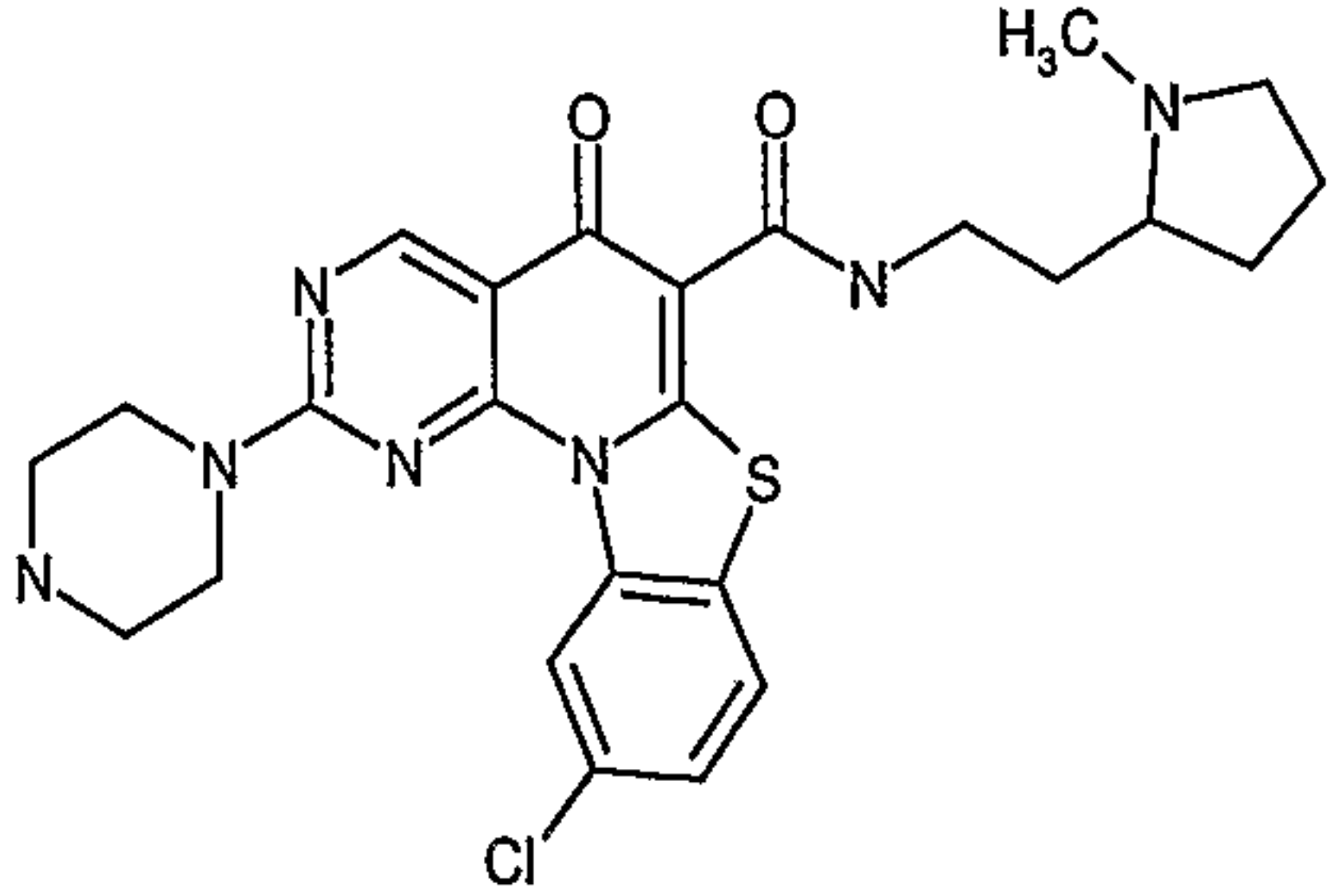
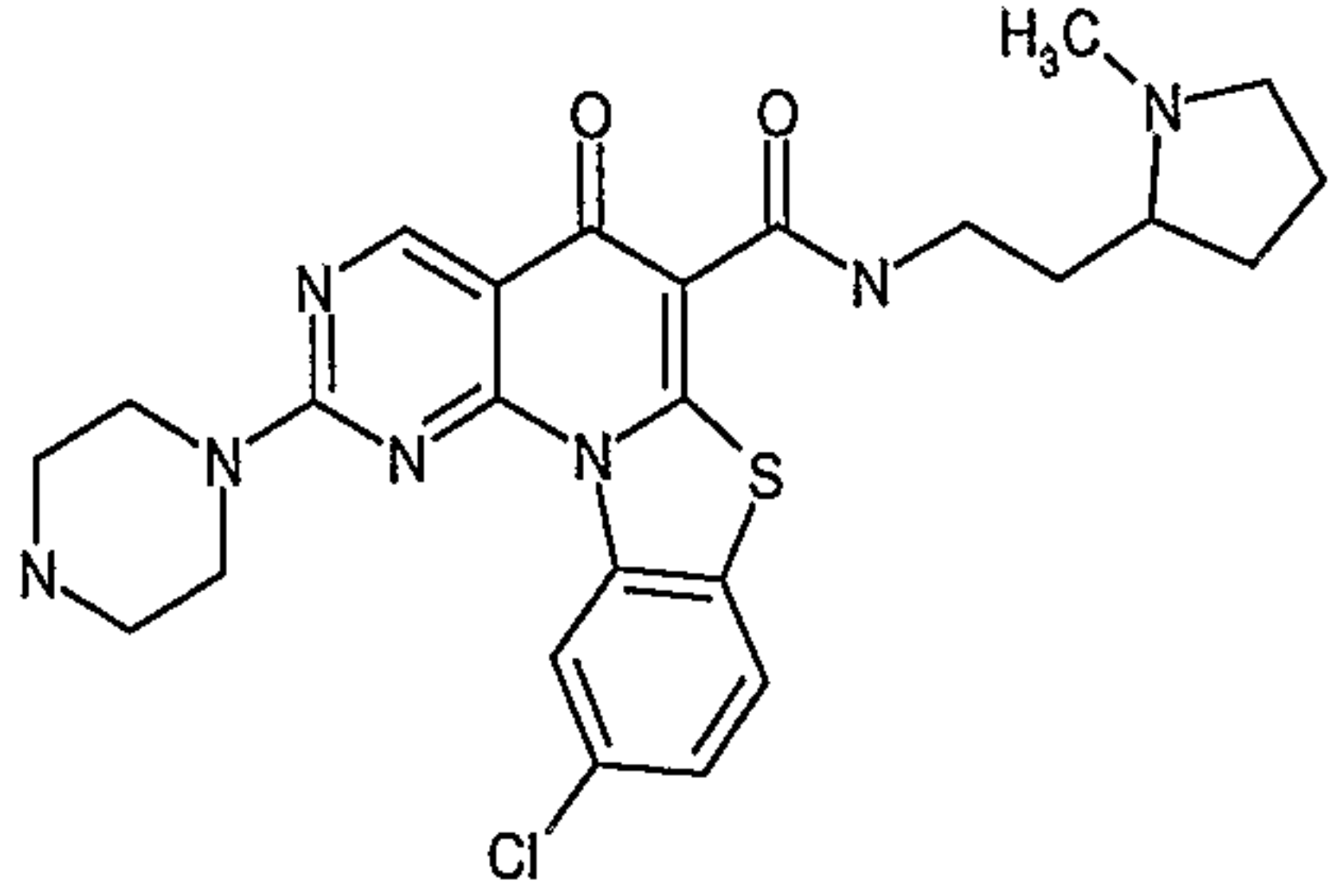
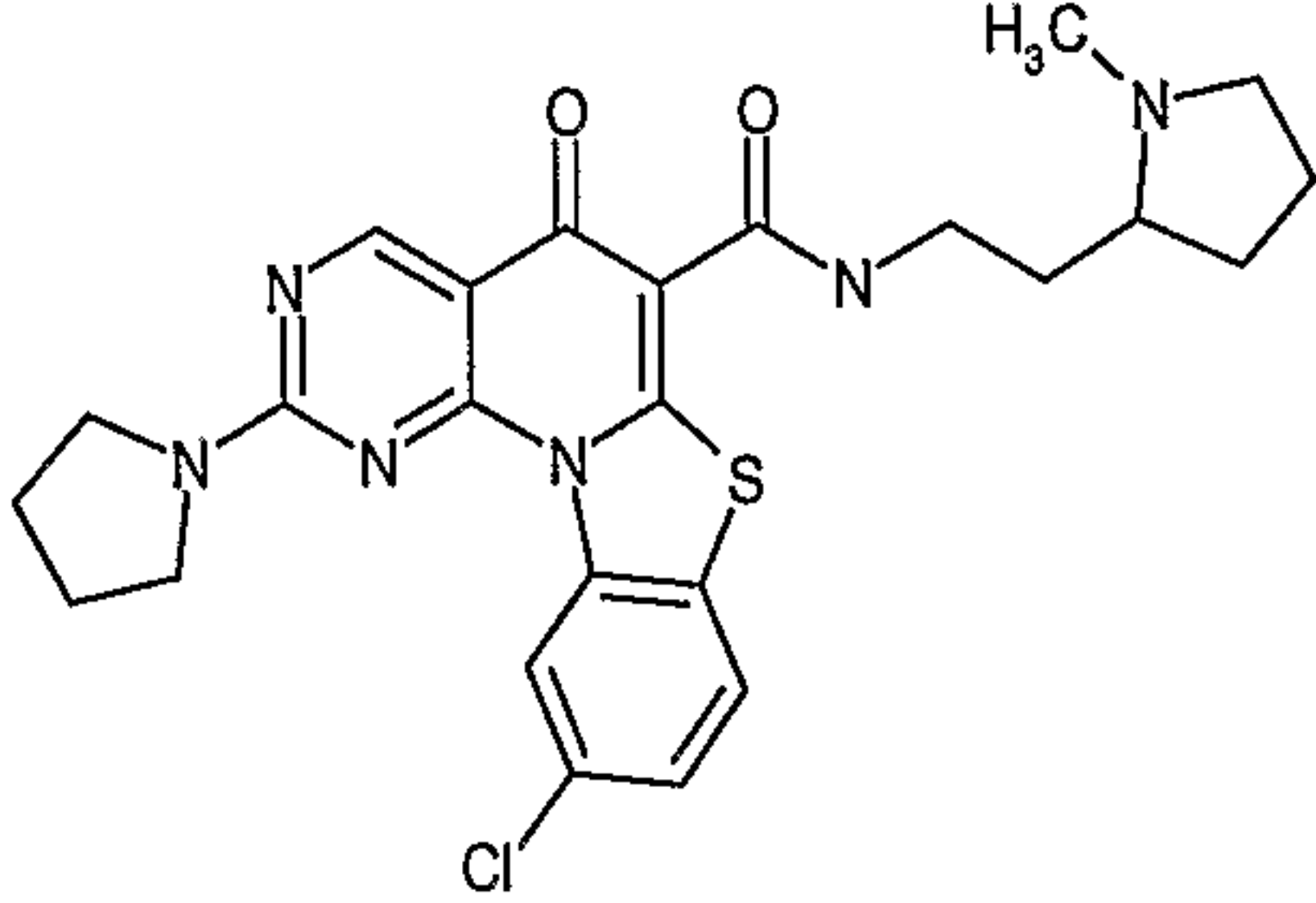
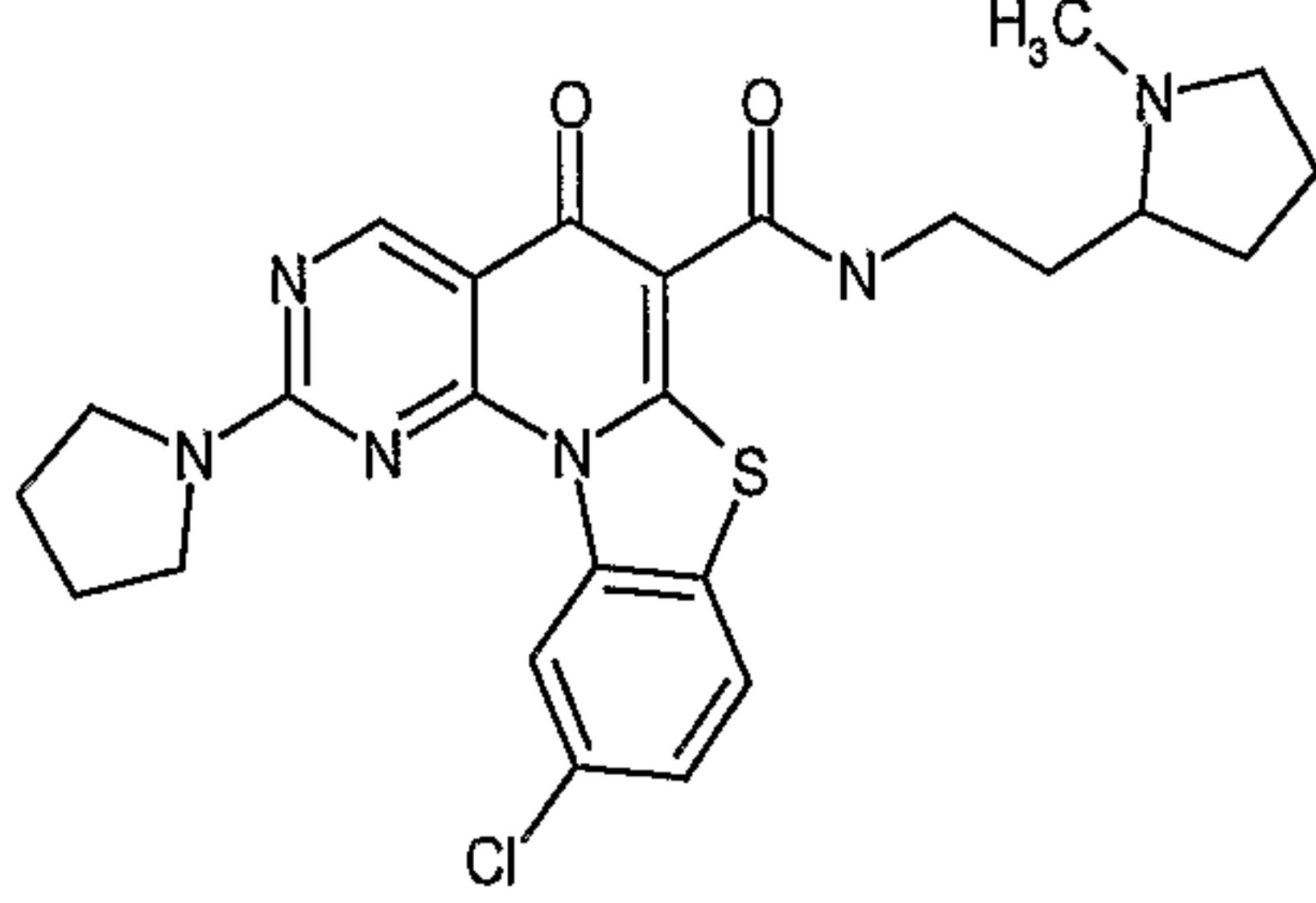
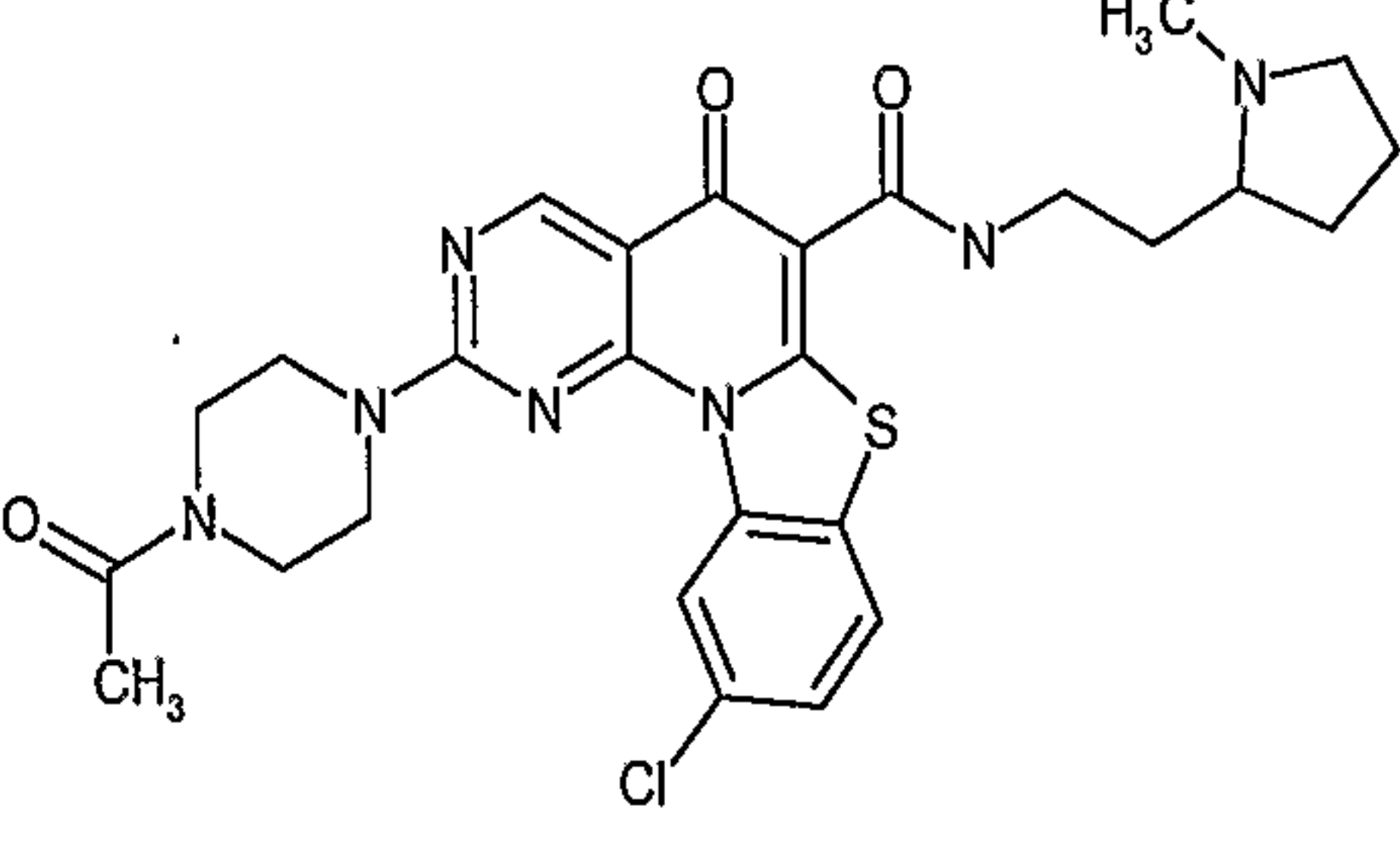
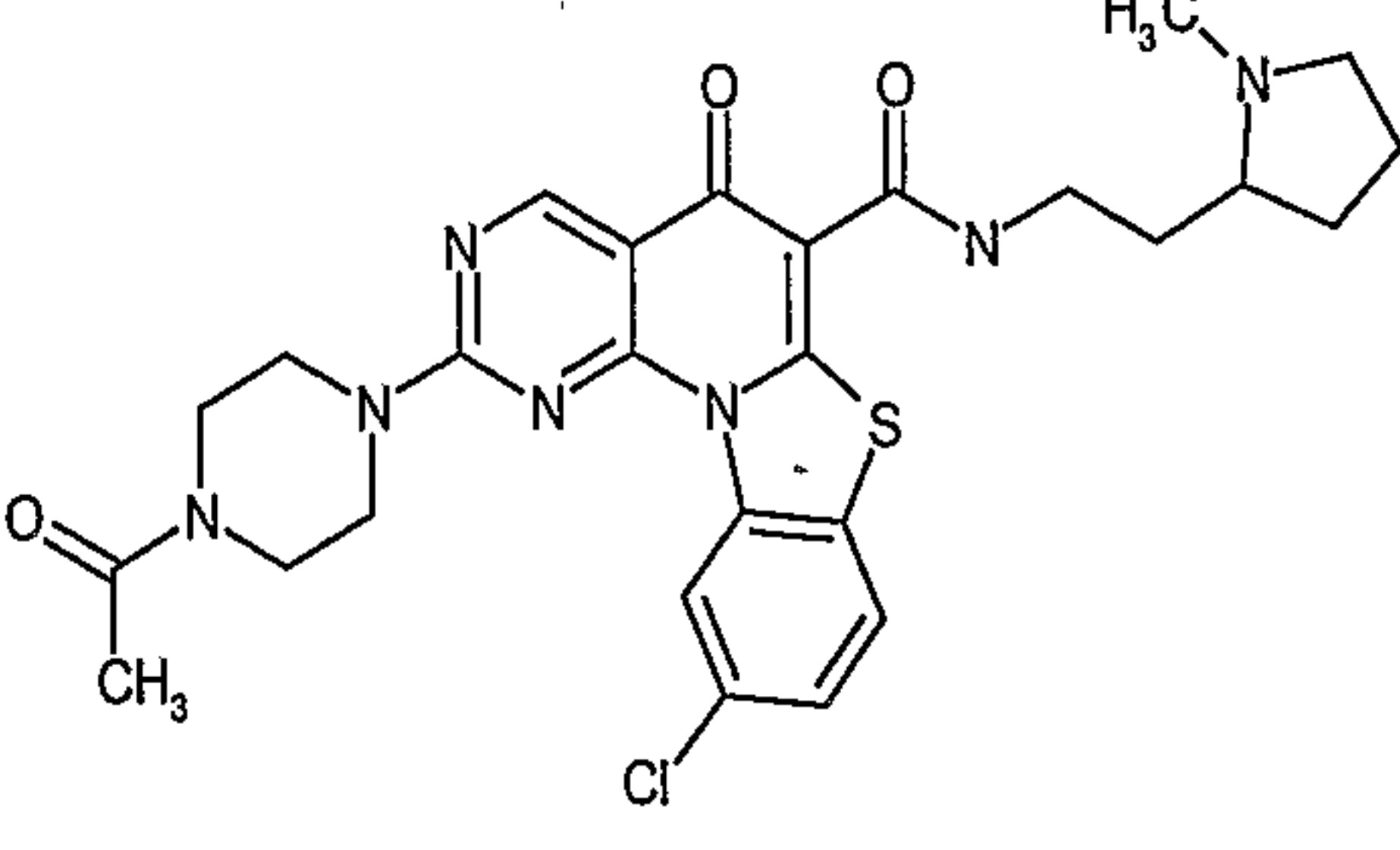
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | Miapaca | 0.70   |
|    | HCT-116 | 0.10   |
|  | Miapaca | 0.03   |
|  | HCT-116 | 0.03   |
|  | Miapaca | 0.04   |
|  | HCT-116 | 0.04   |

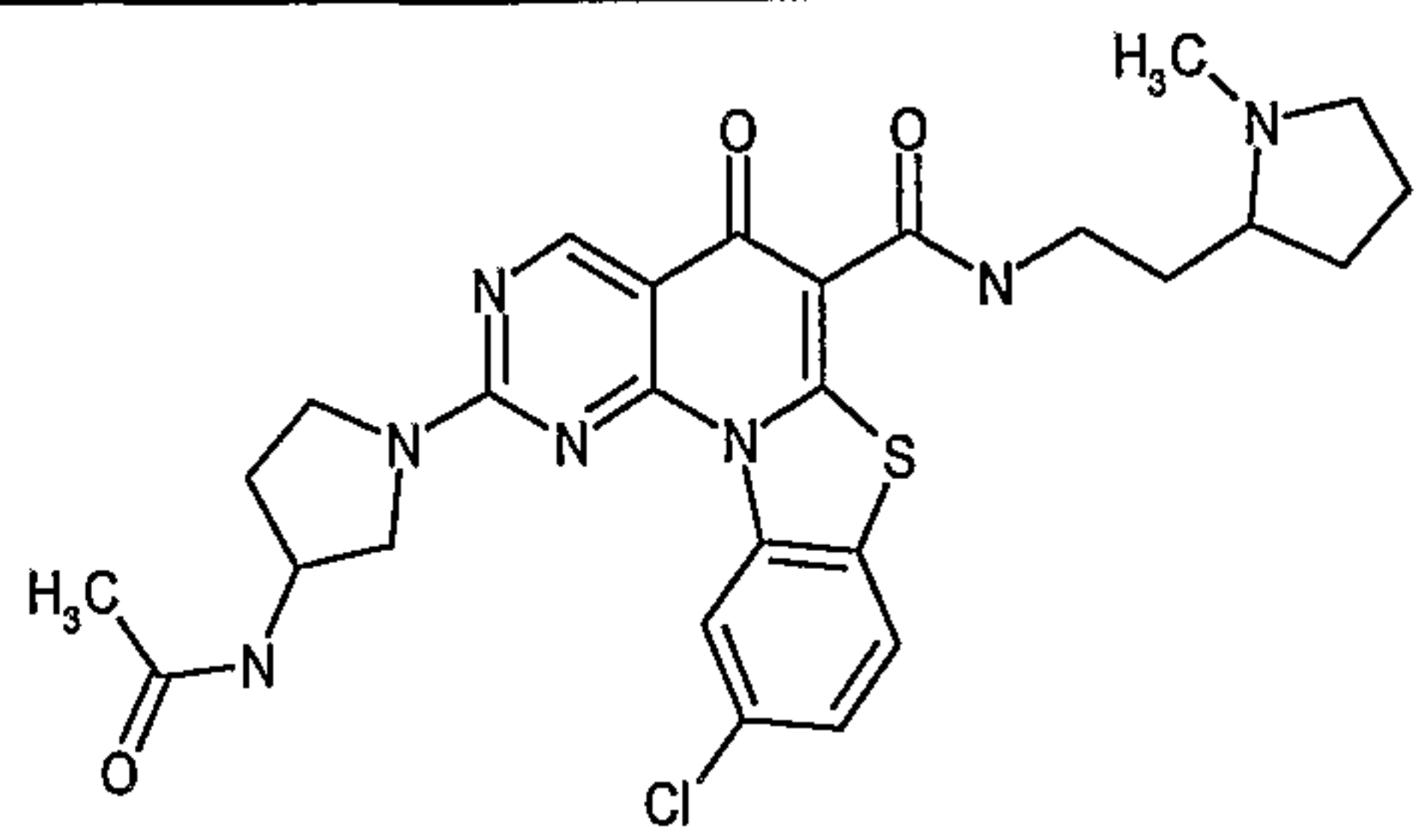
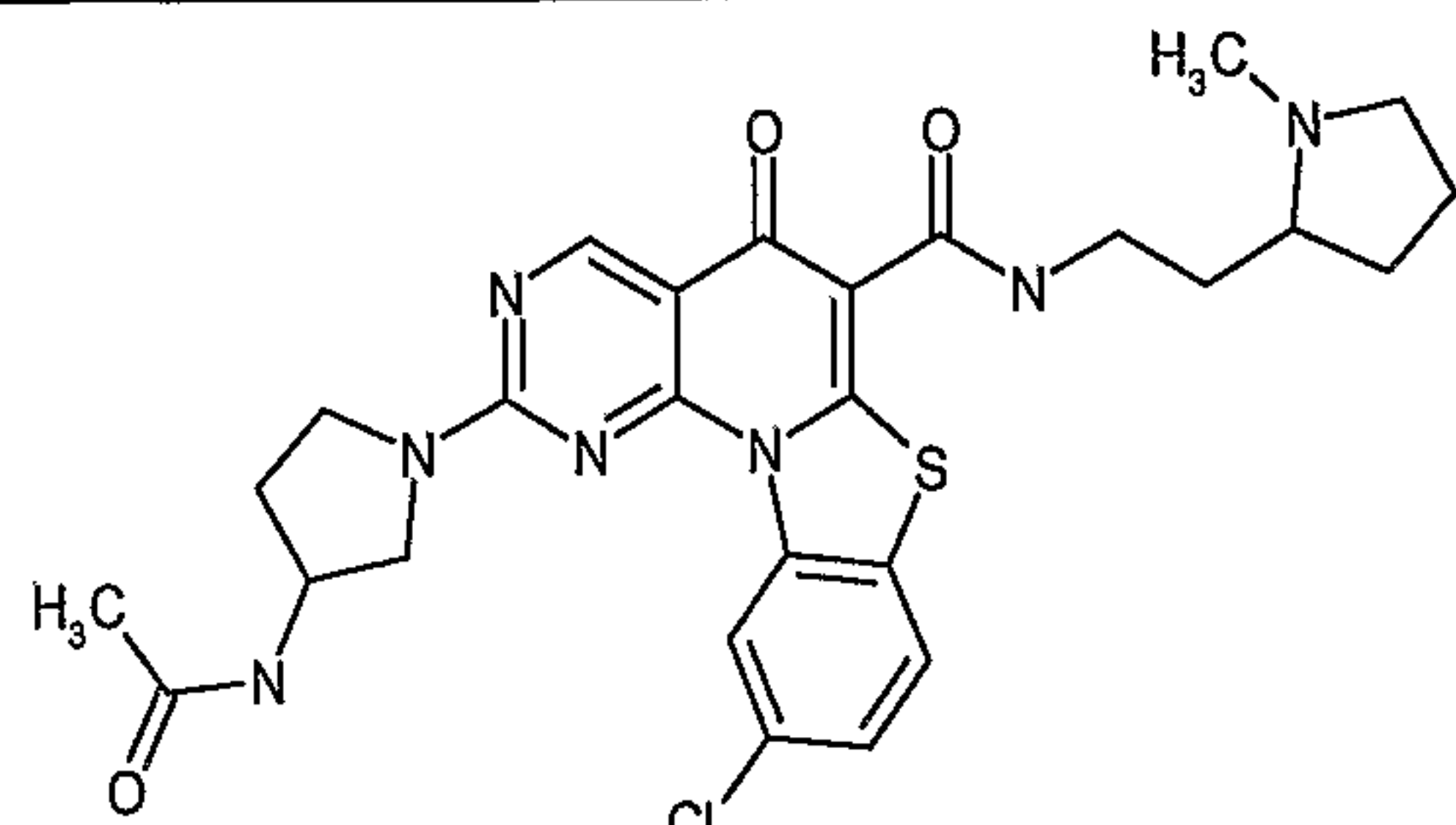
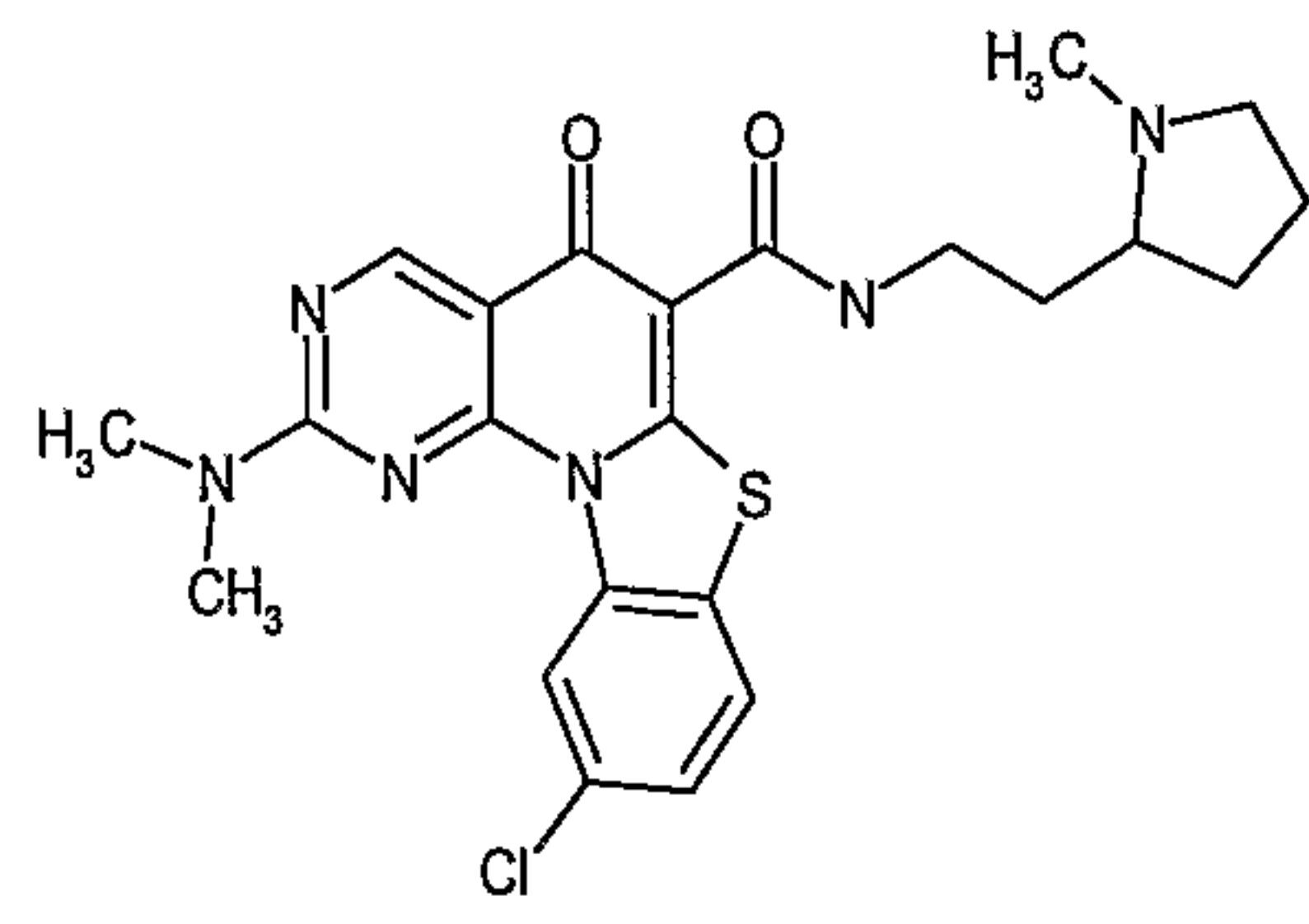
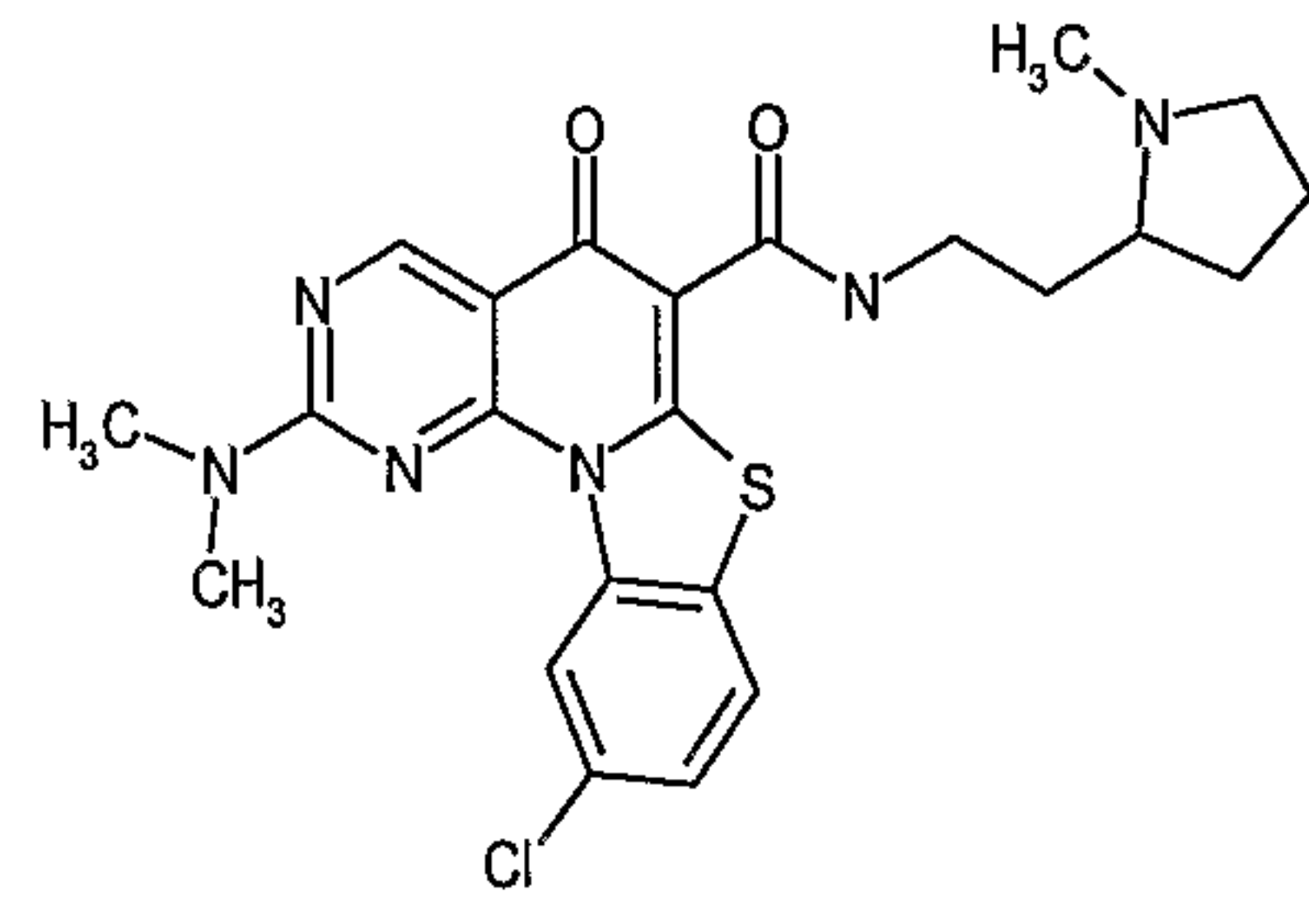
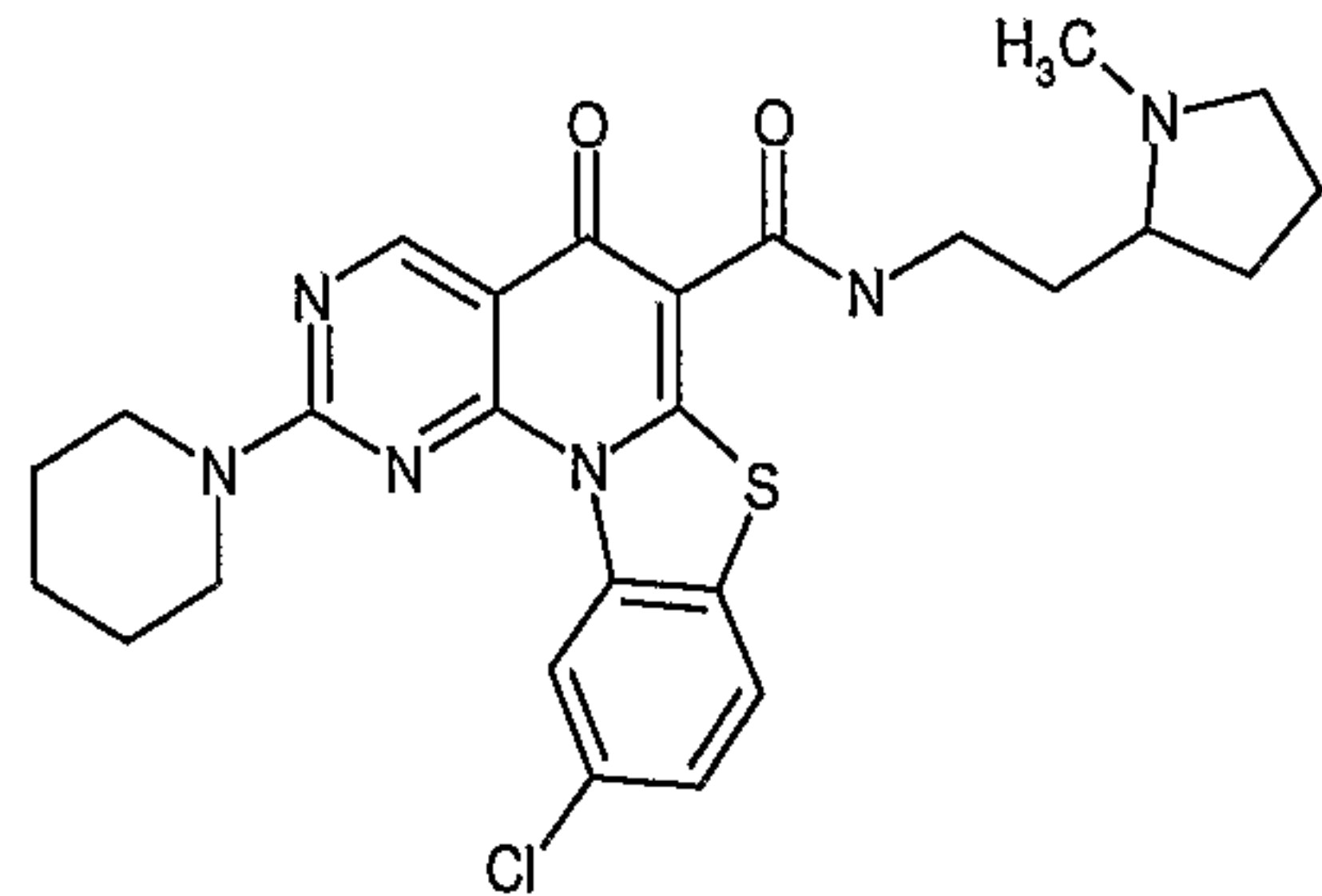
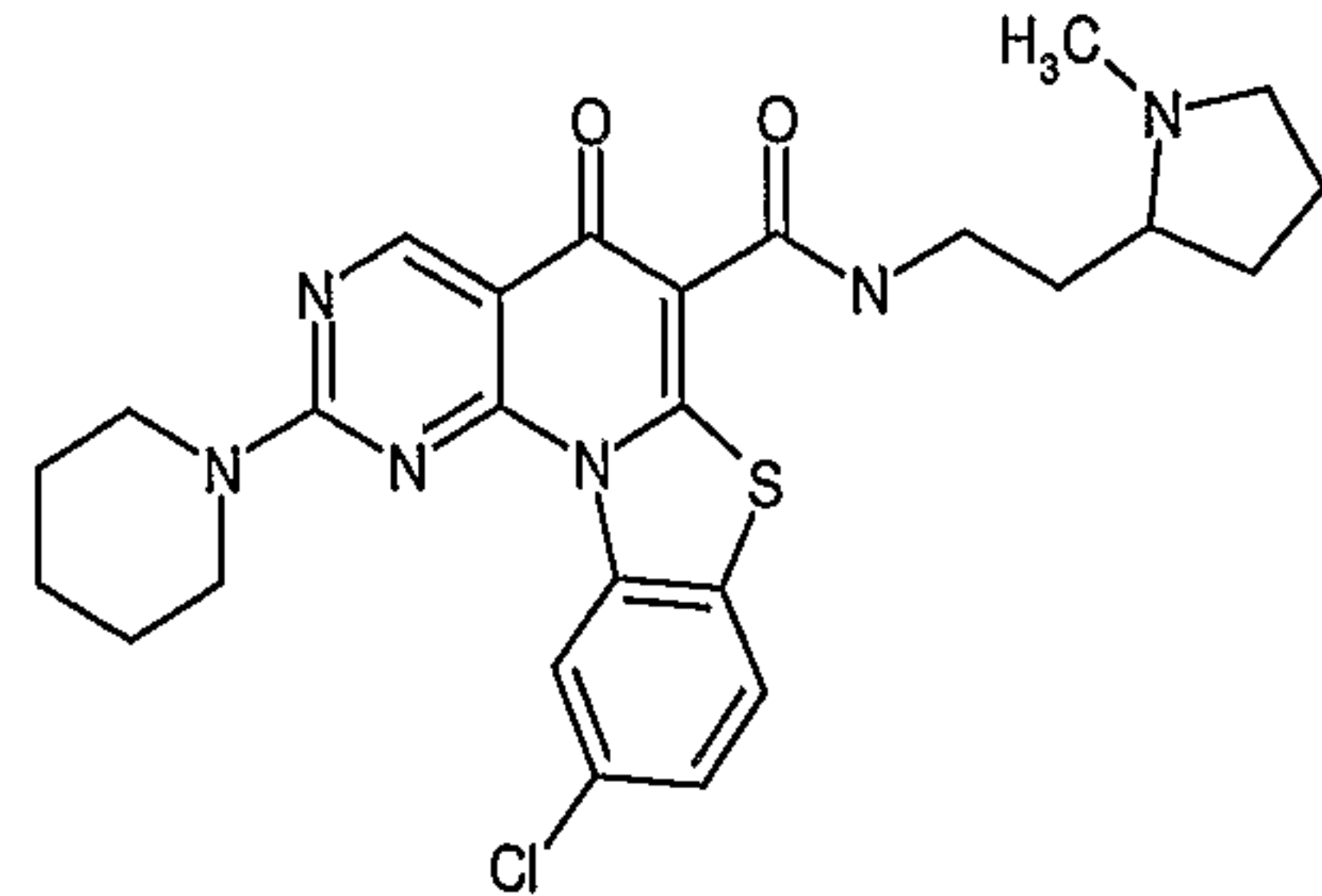
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | 0.37   |
|   | HCT-116 | 0.98   |
|  | Miapaca | 0.04   |
|  | HCT-116 | 0.05   |
|  | Miapaca | 0.04   |
|  | HCT-116 | 0.08   |

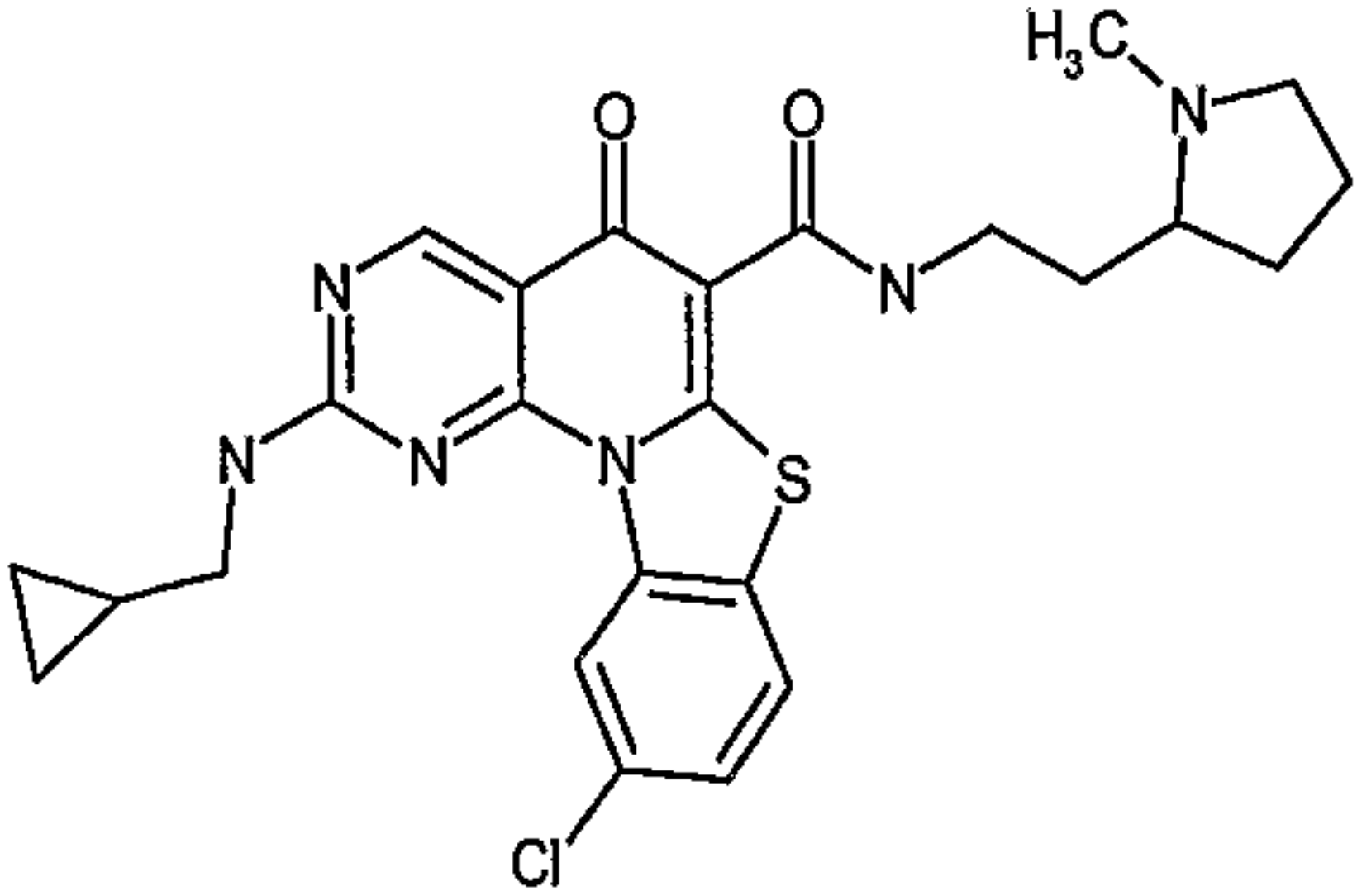
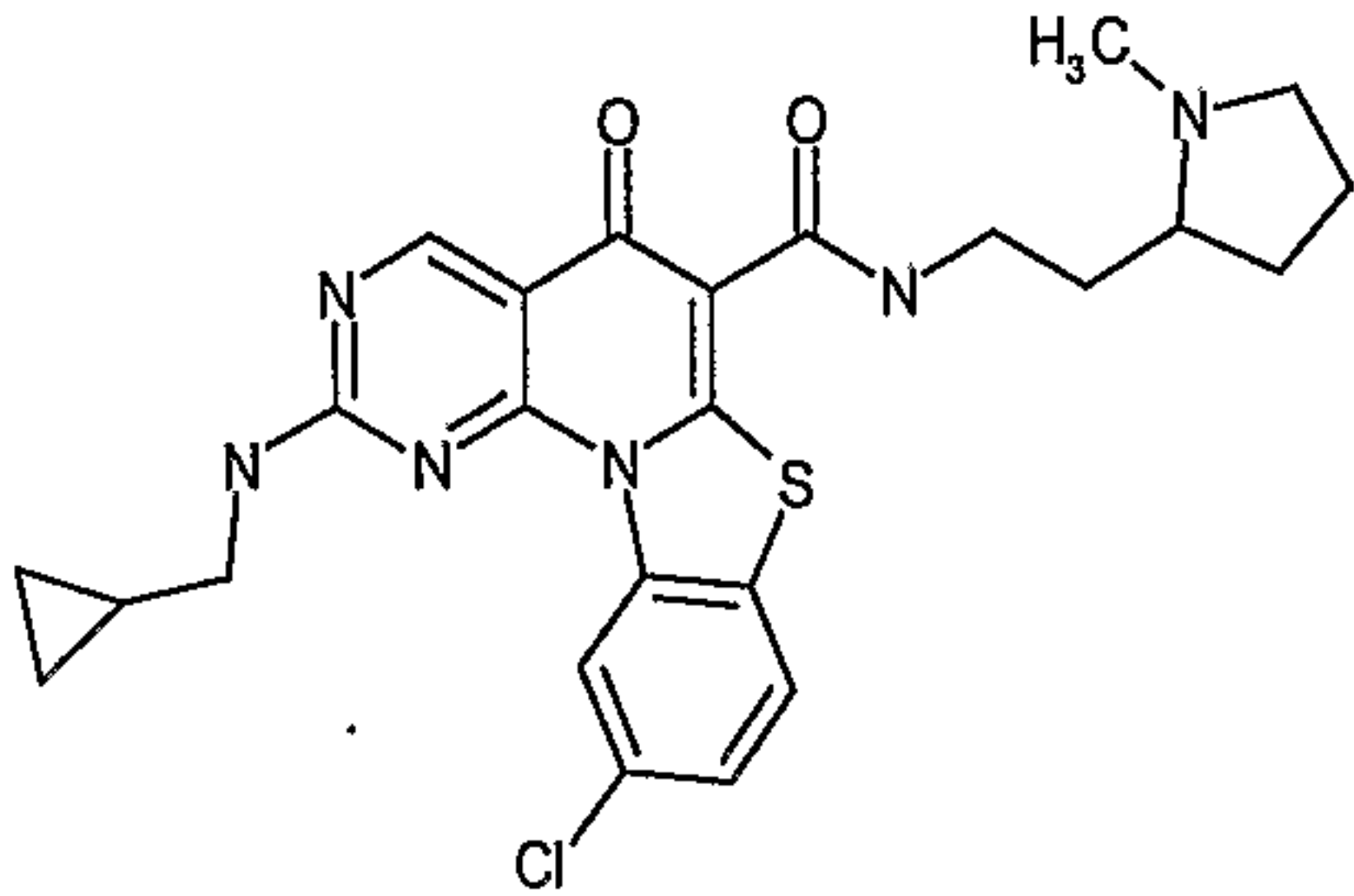
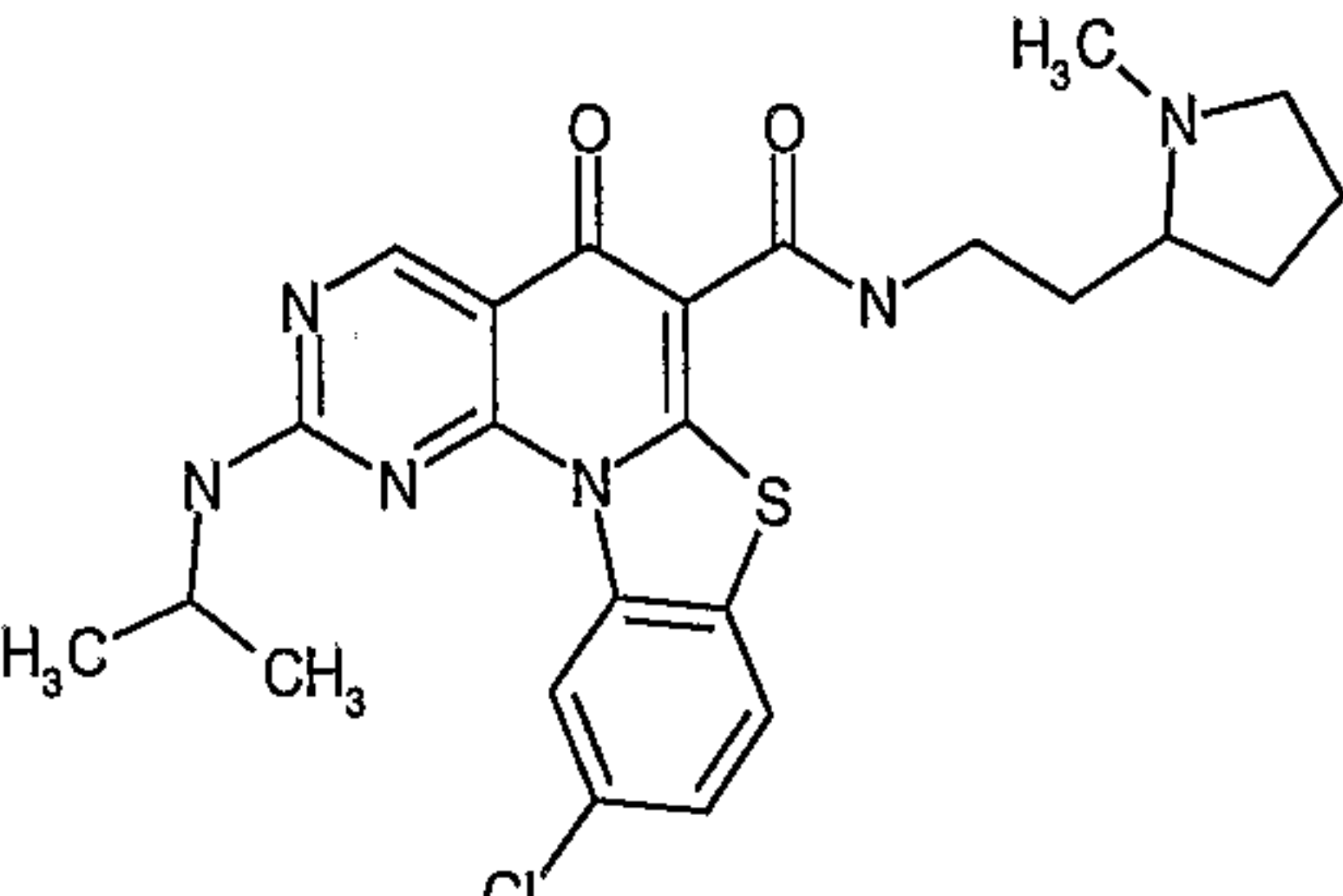
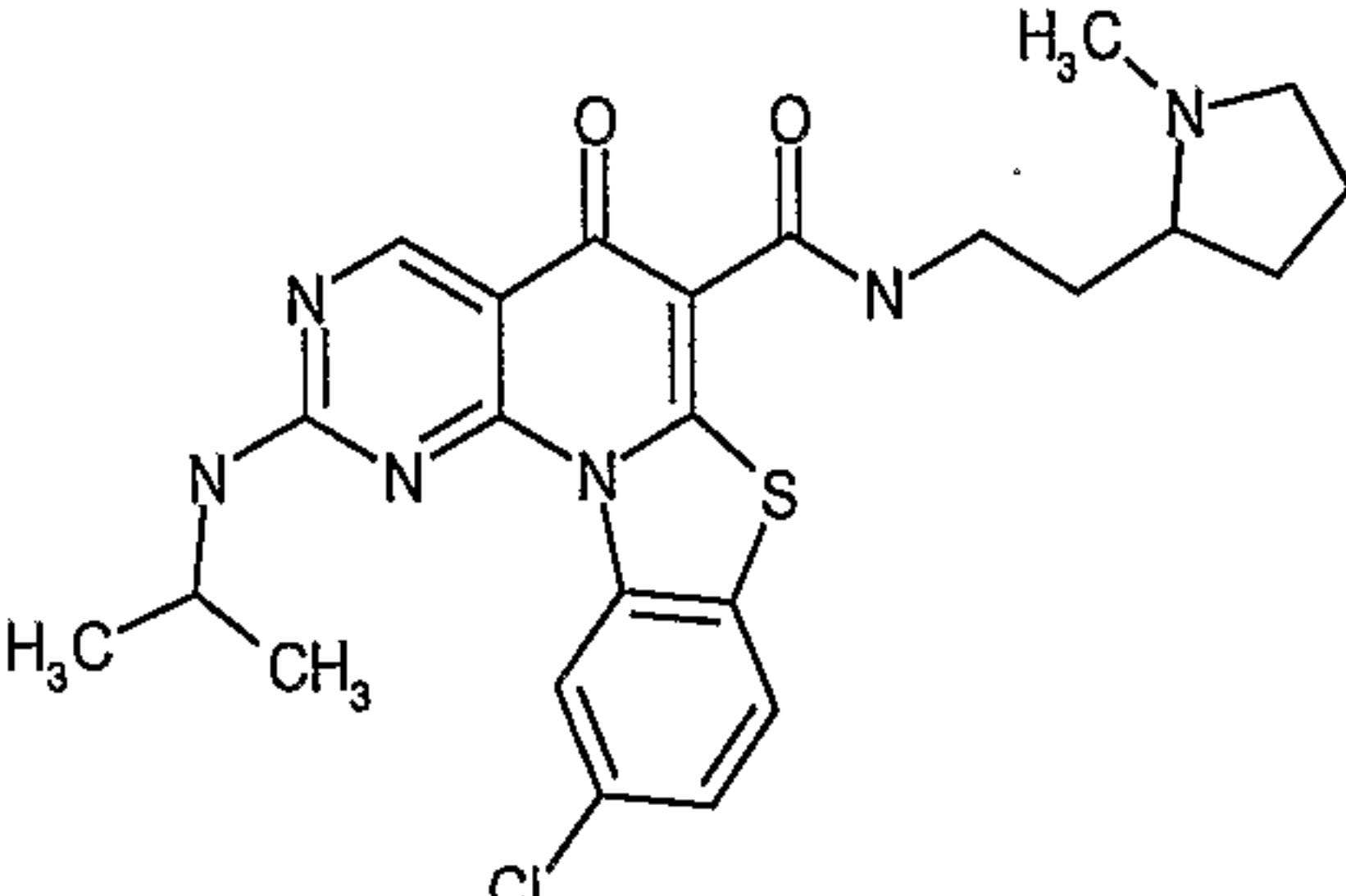
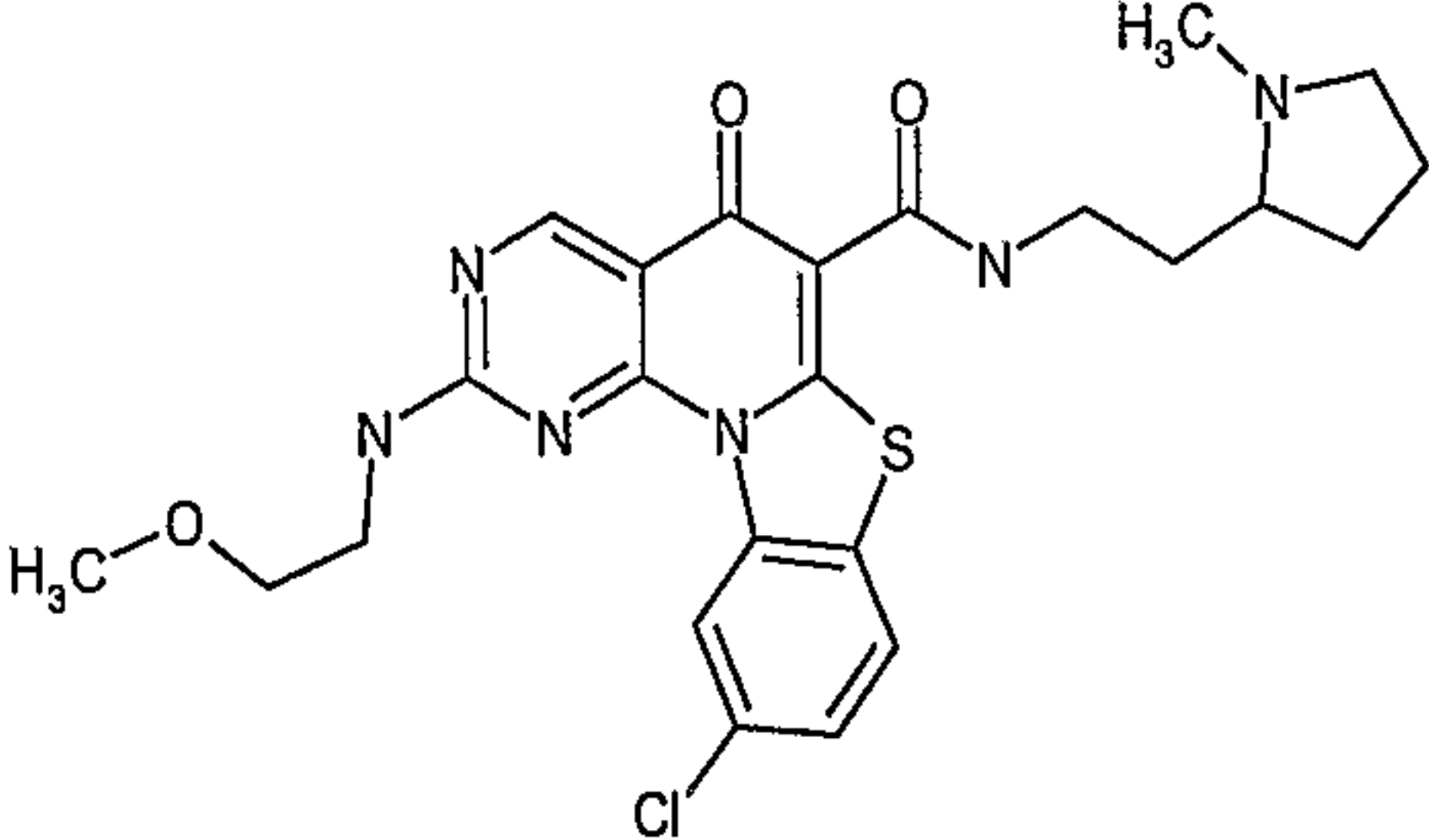
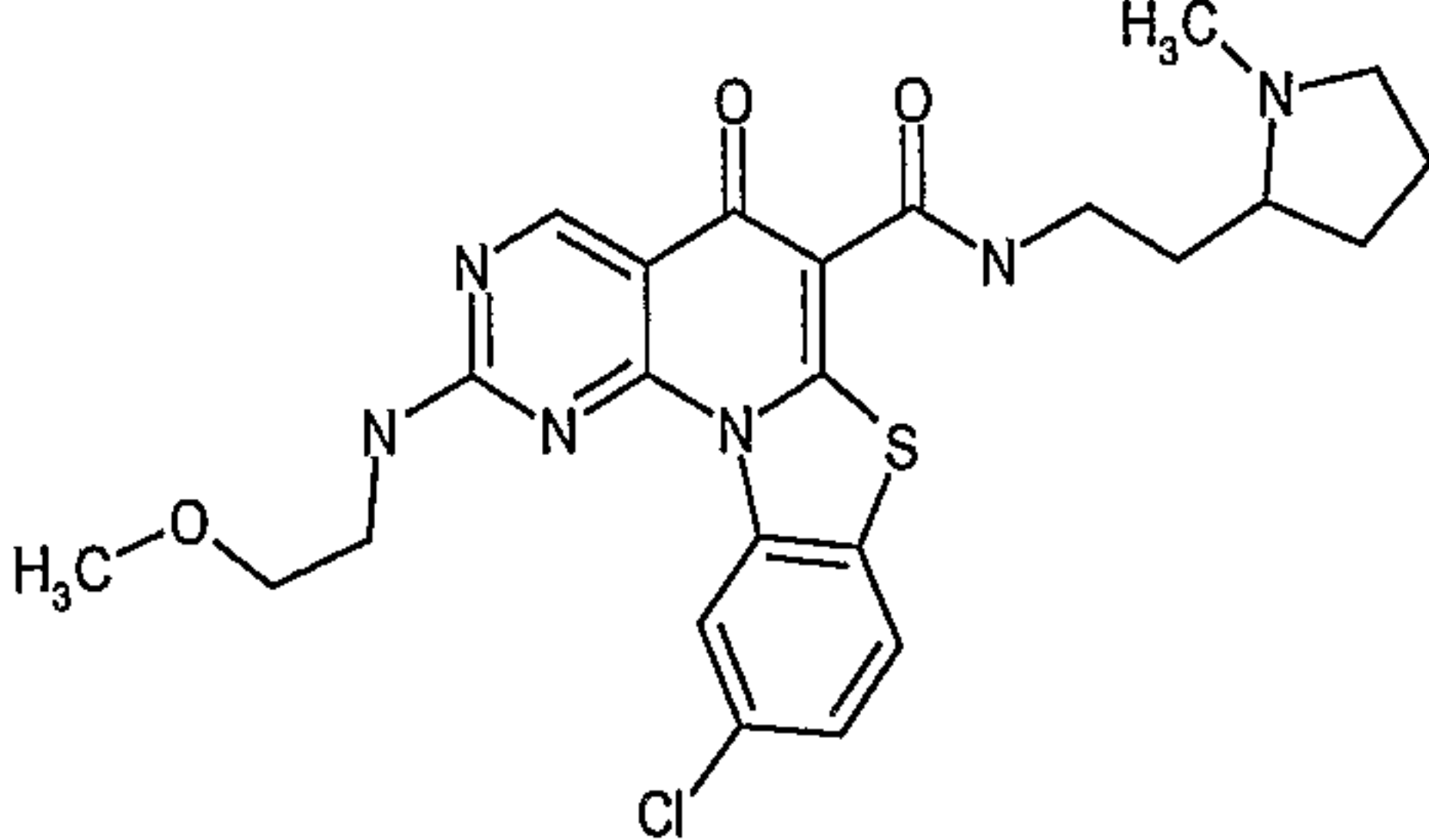
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | 0.15   |
|   | HCT-116 | 0.11   |
|  | Miapaca | 0.01   |
|  | HCT-116 | 0.01   |
|  | Miapaca | <0.01  |
|  | HCT-116 | <0.01  |

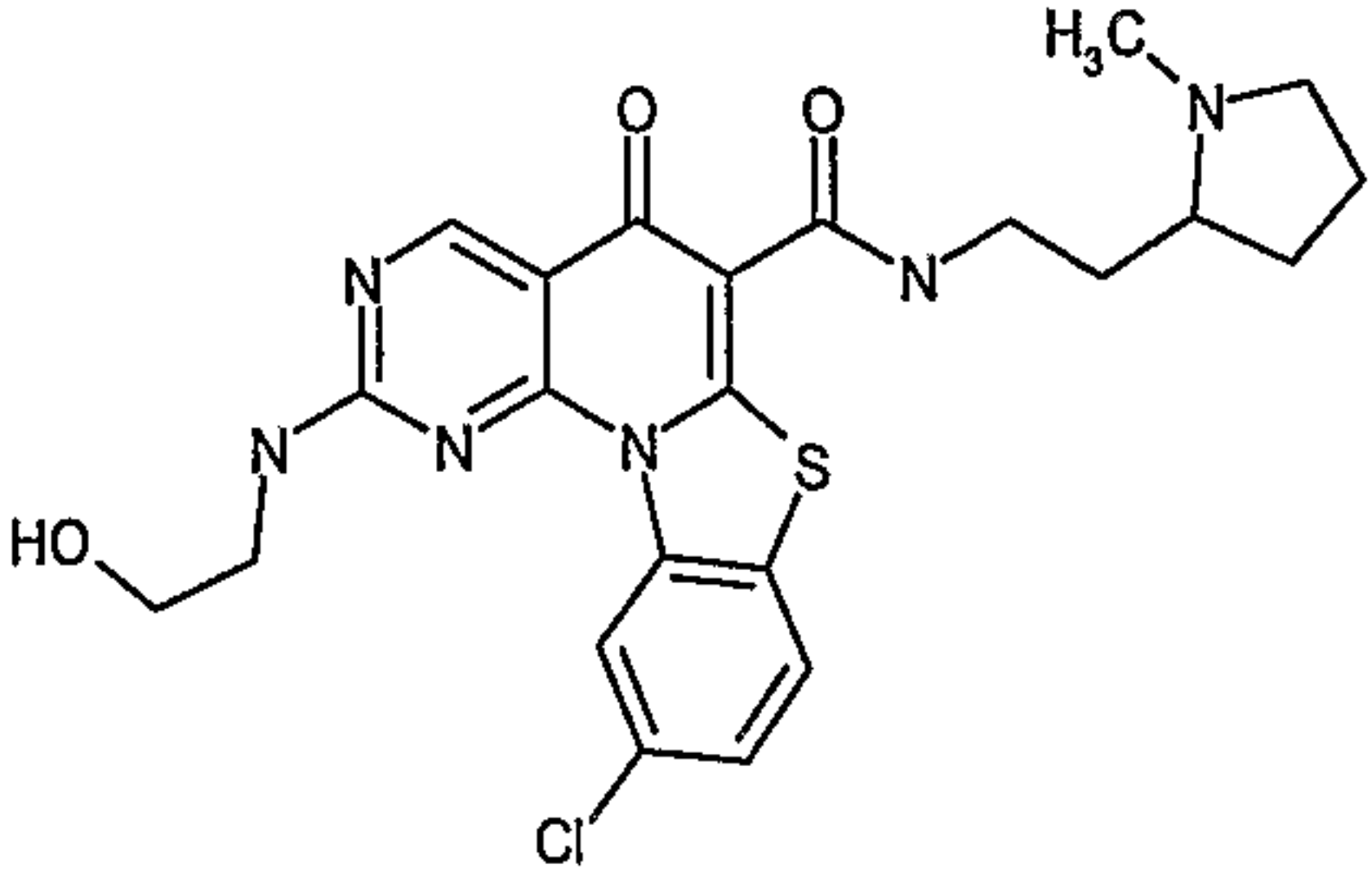
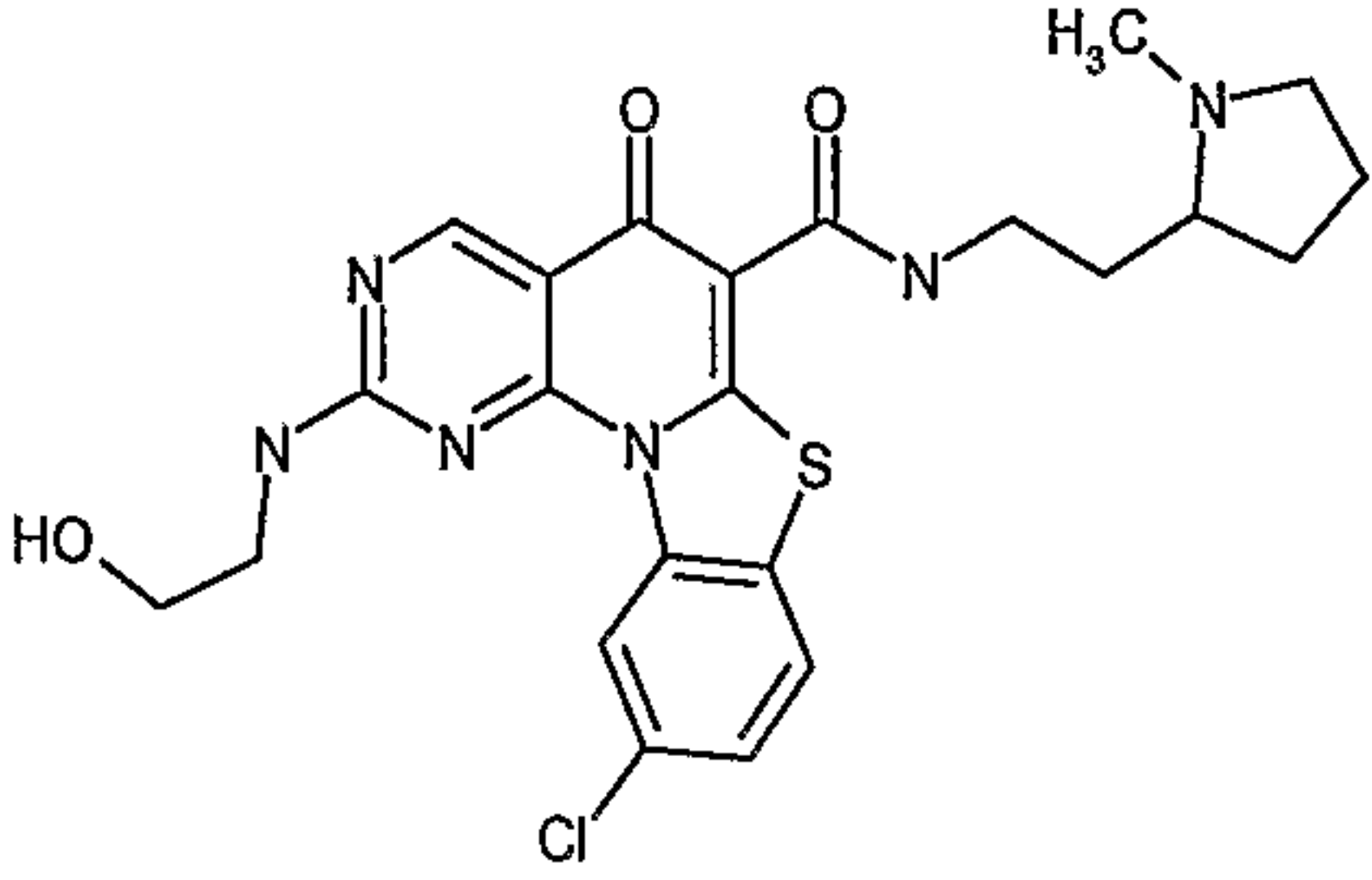
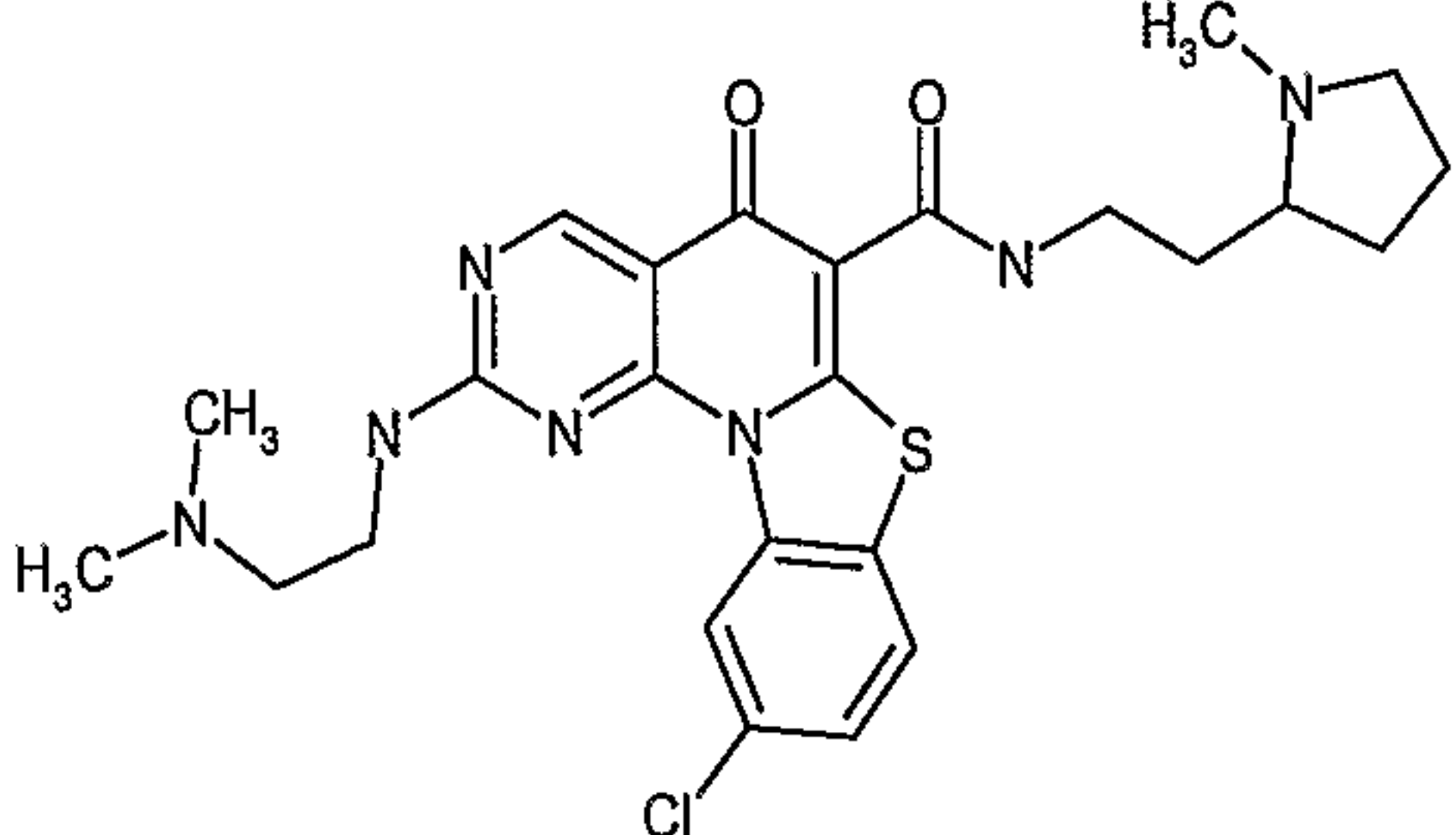
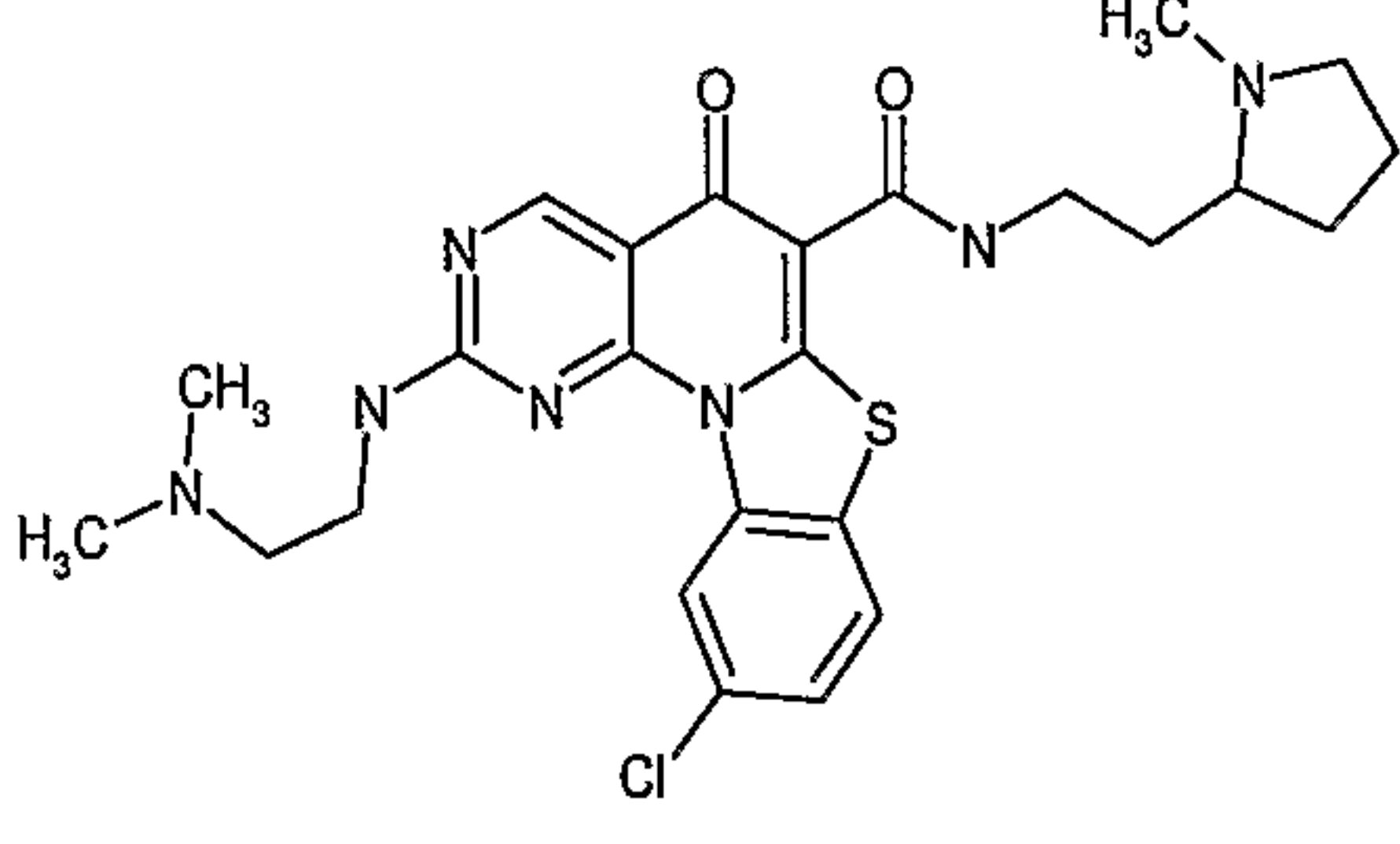
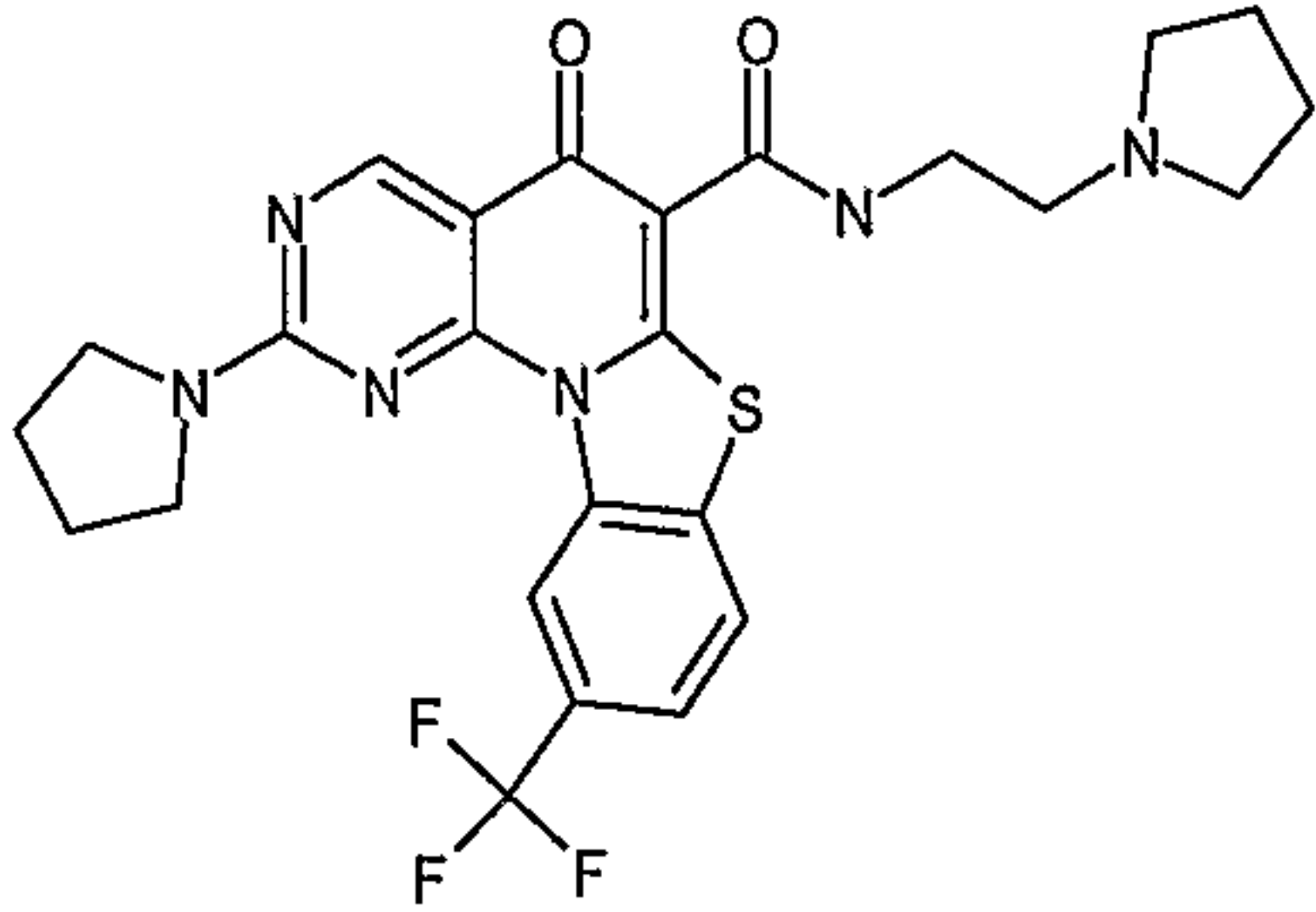
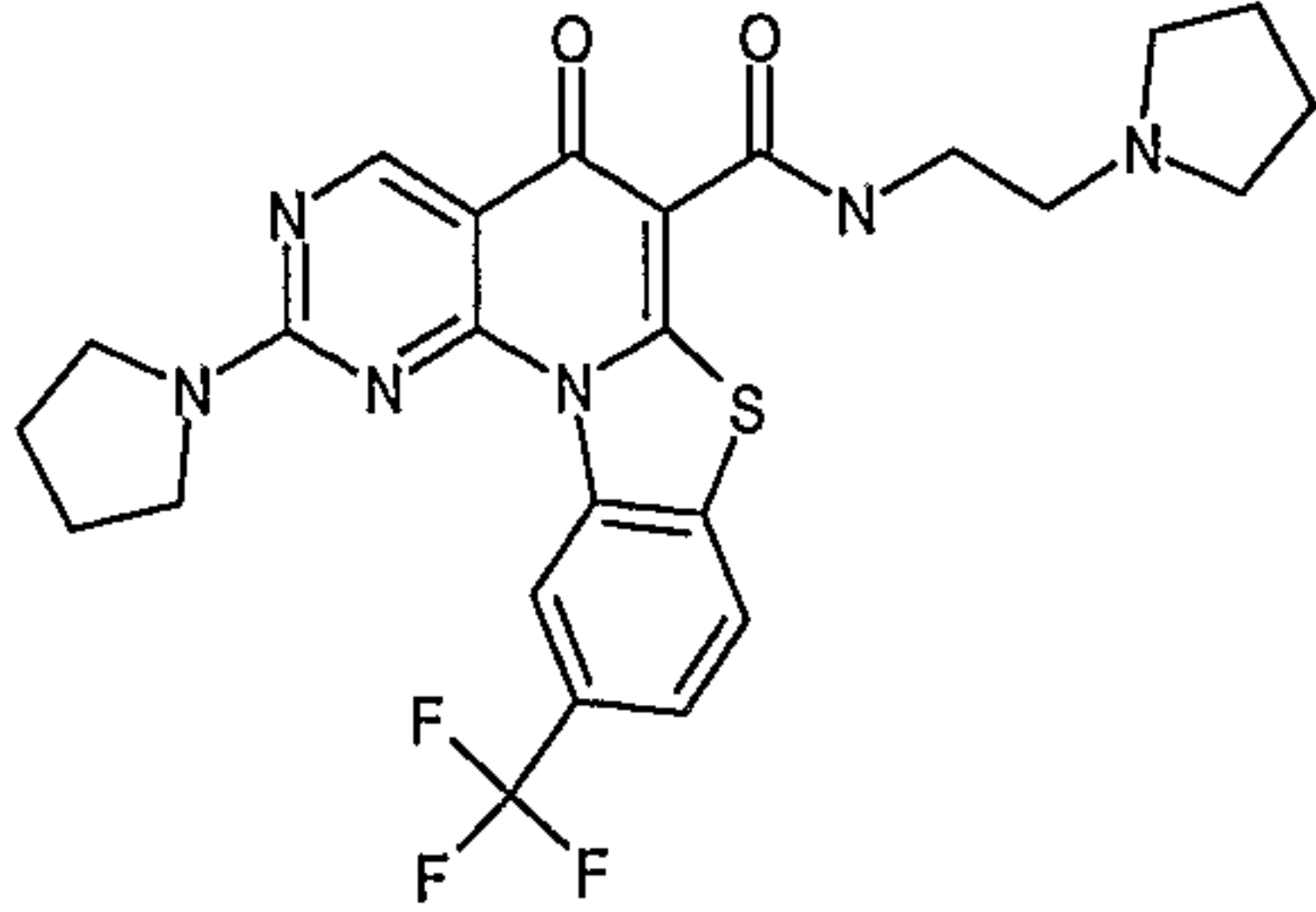
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | 0.34   |
|   | HCT-116 | 0.19   |
|  | Miapaca | 0.05   |
|  | HCT-116 | 0.07   |
|  | Miapaca | 0.02   |
|  | HCT-116 | 0.02   |

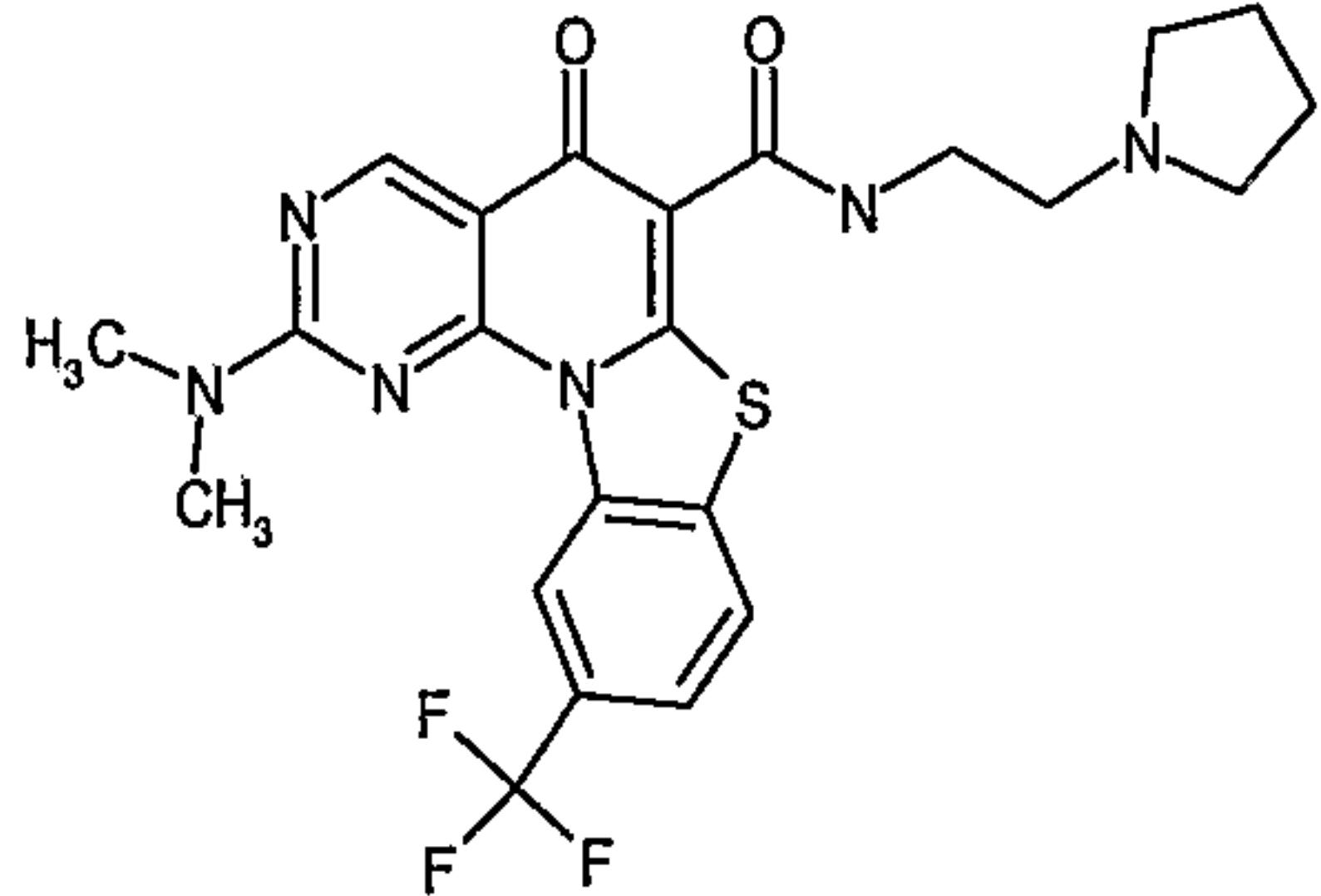
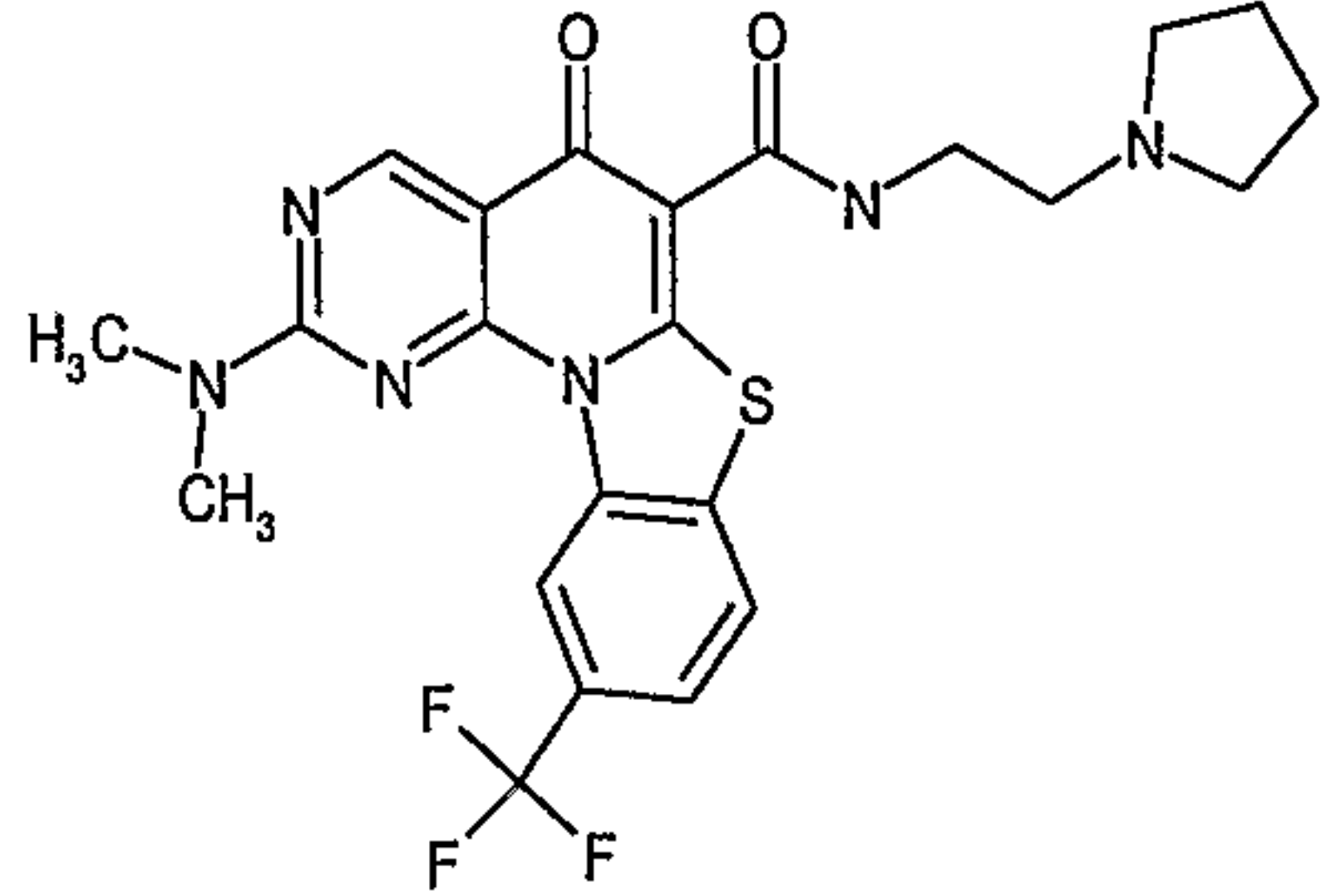
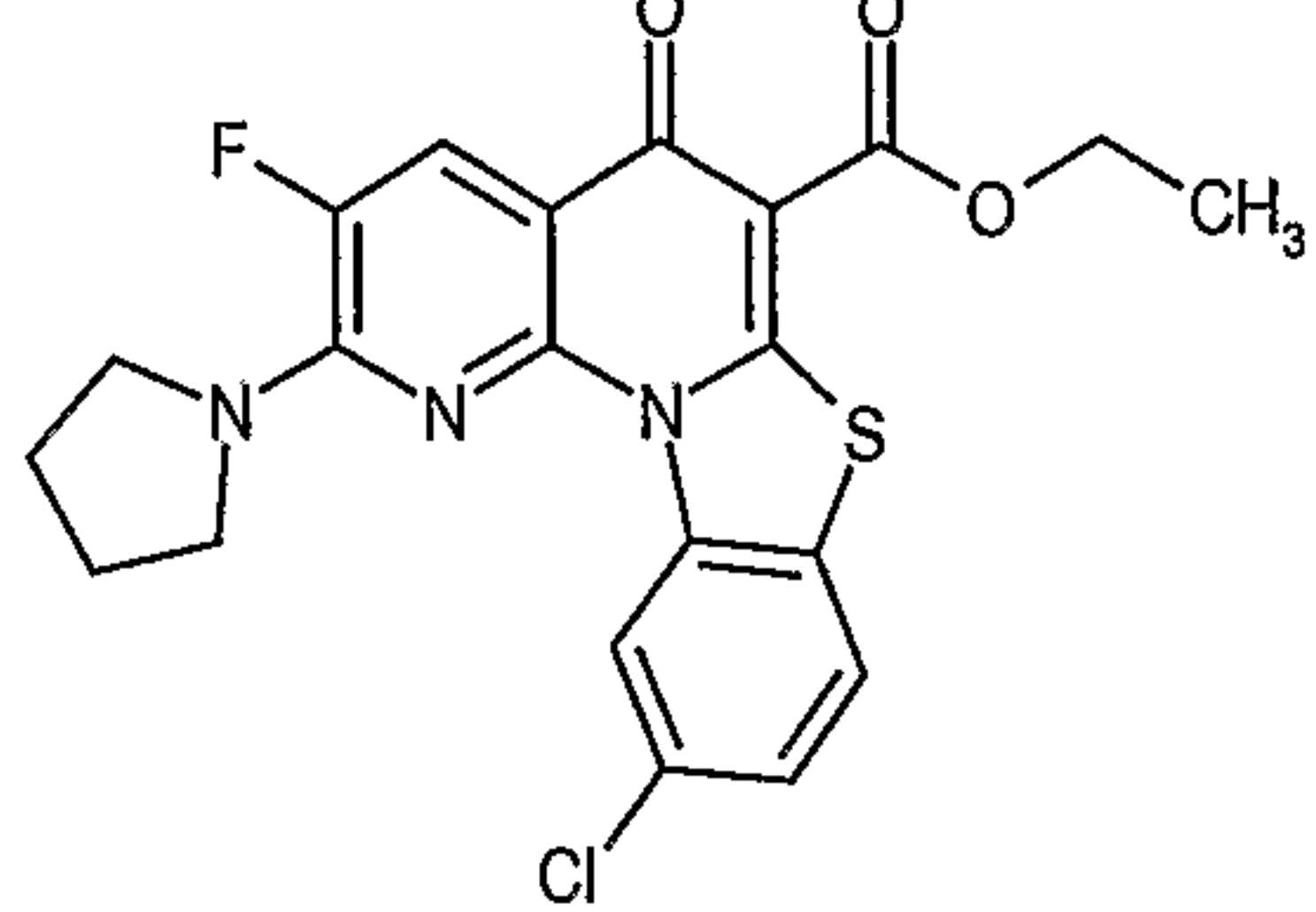
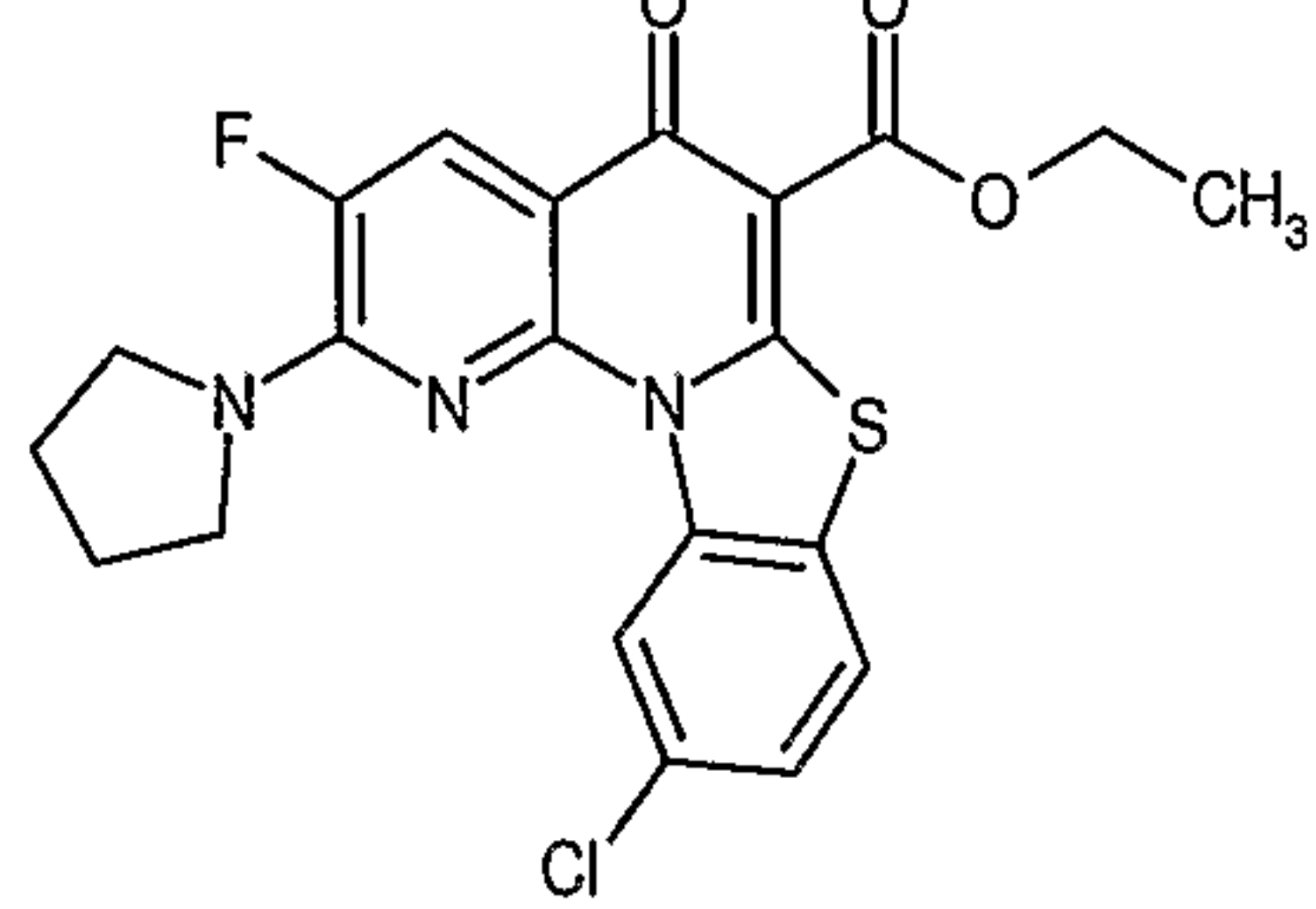
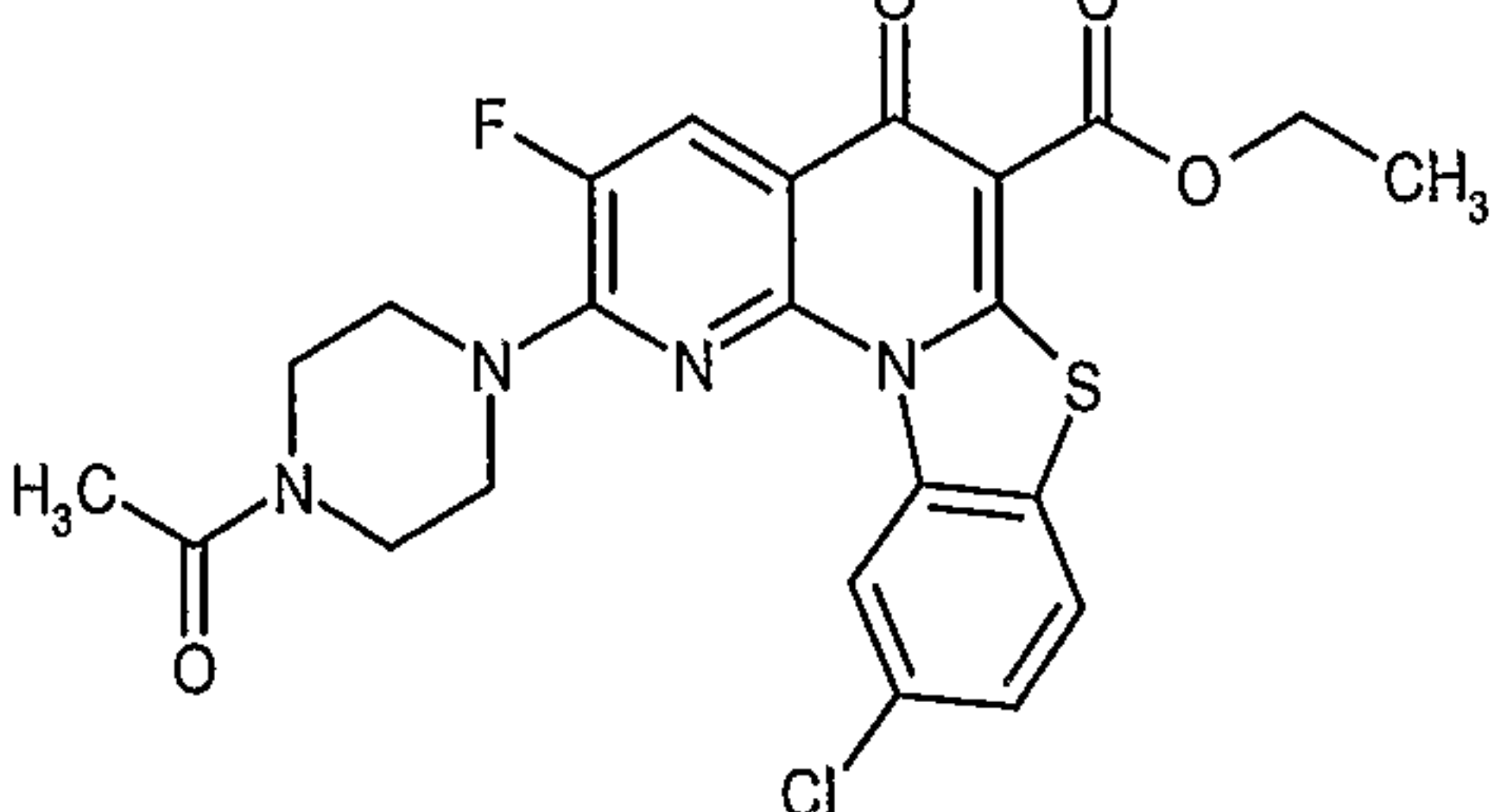
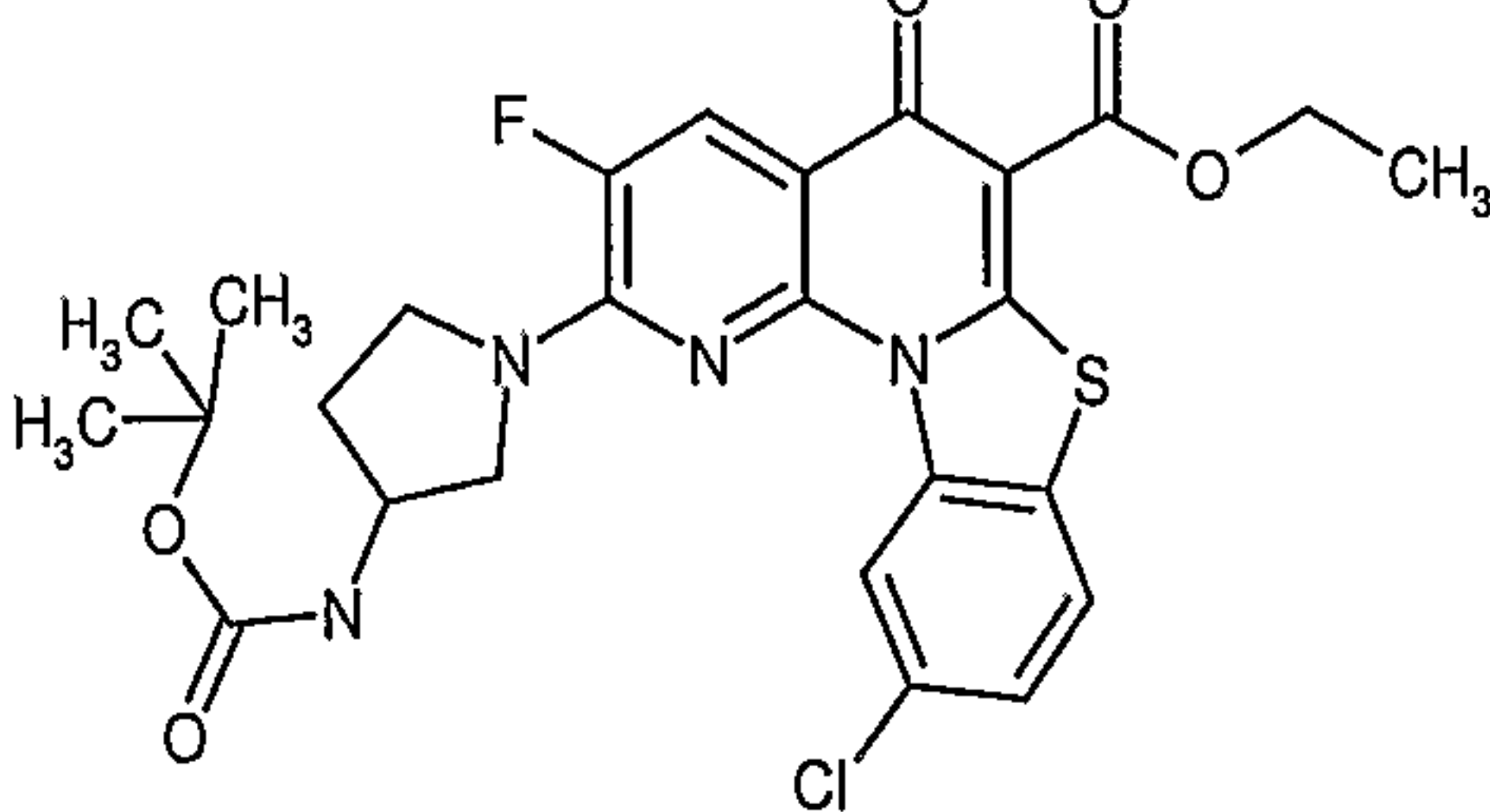
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | 0.02   |
|   | HCT-116 | 0.02   |
|   | Miapaca | <0.01  |
|   | HCT-116 | 0.02   |
|  | Miapaca | 0.03   |
|  | HCT-116 | 0.02   |

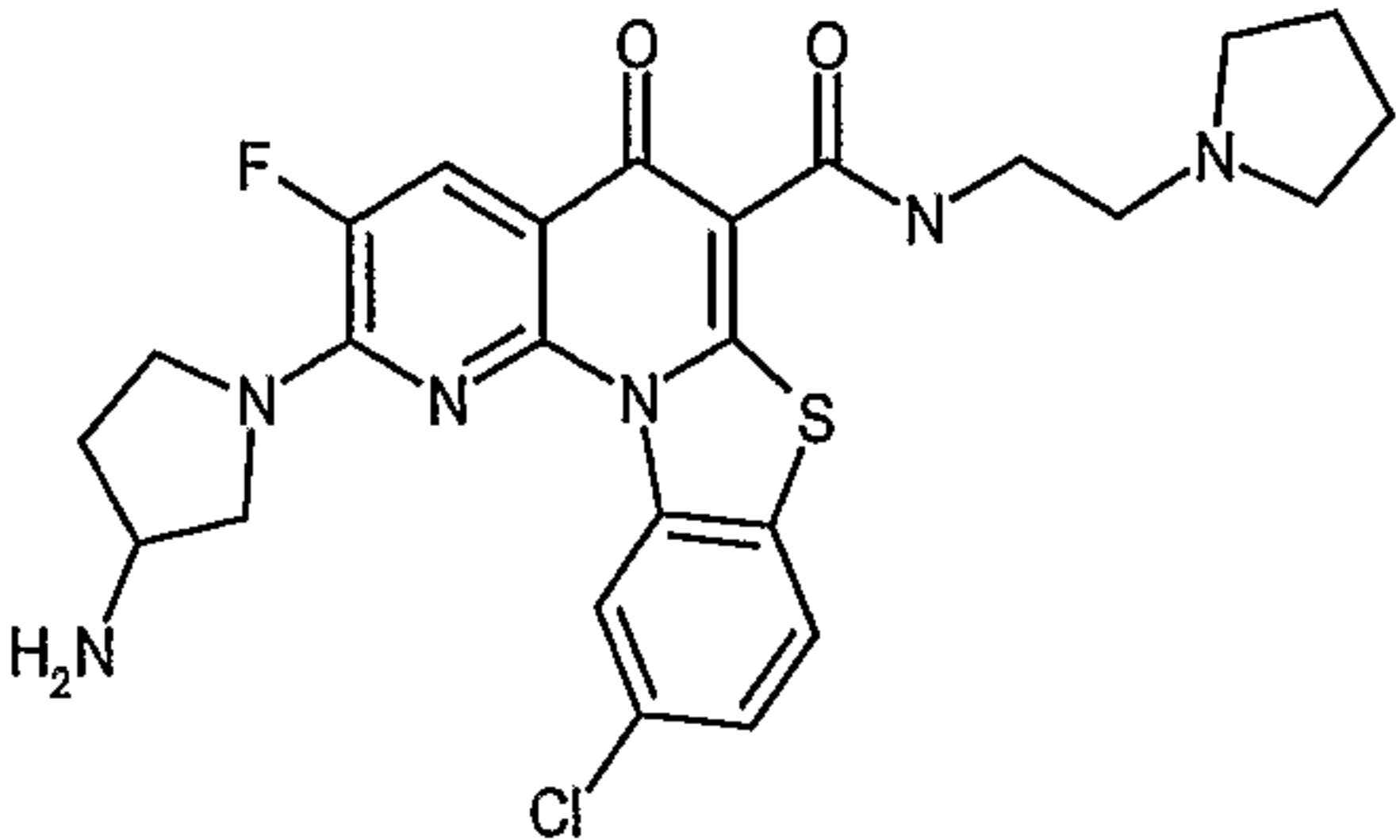
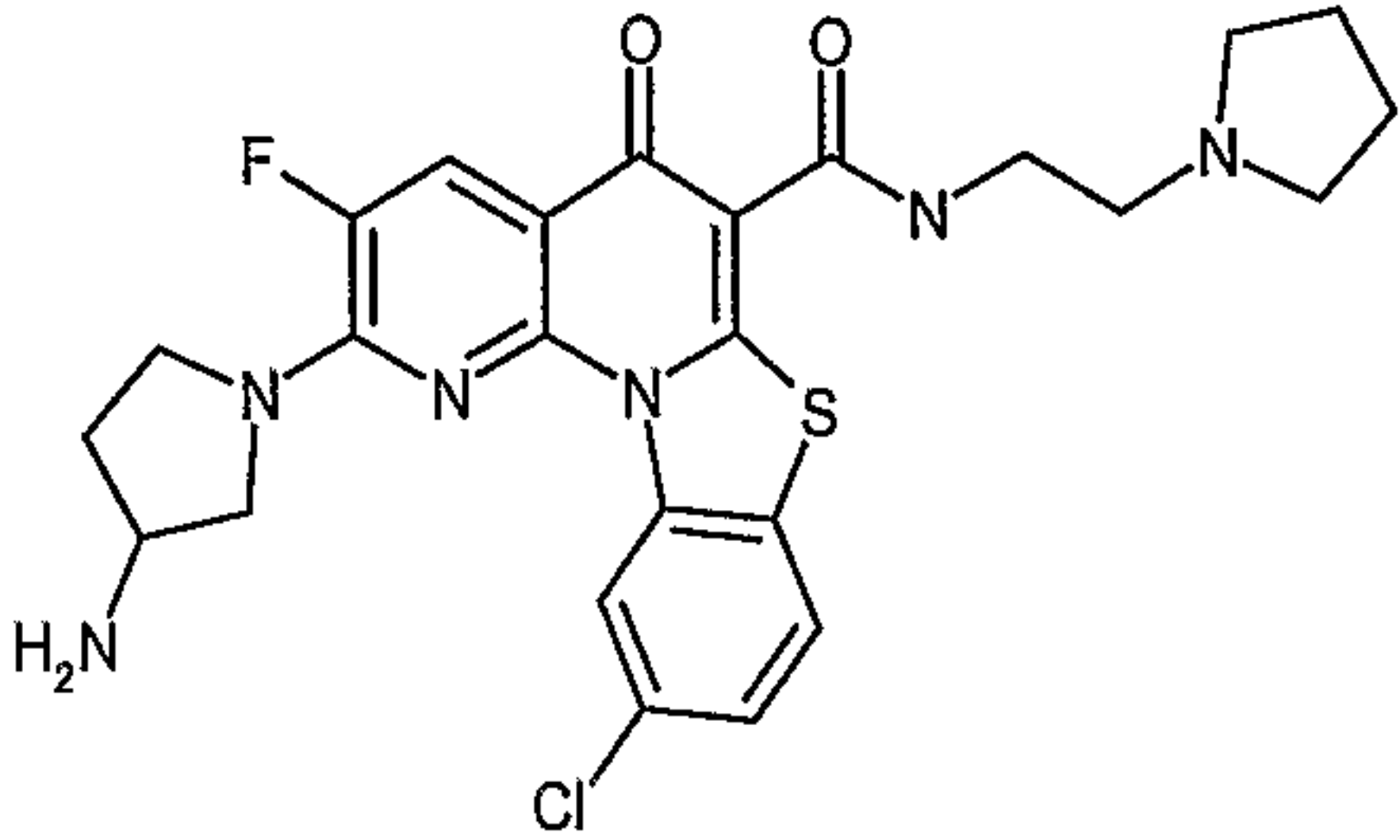
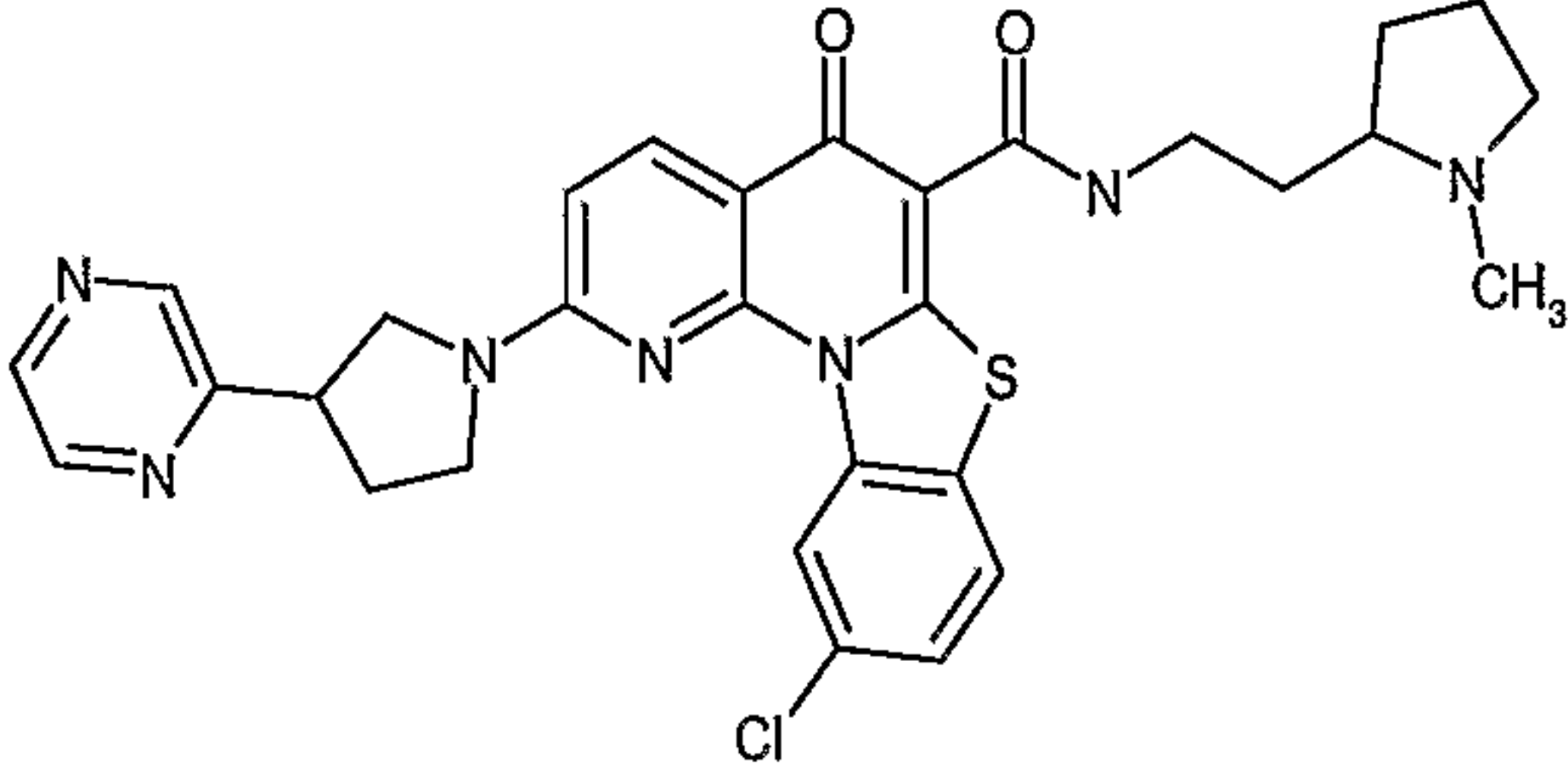
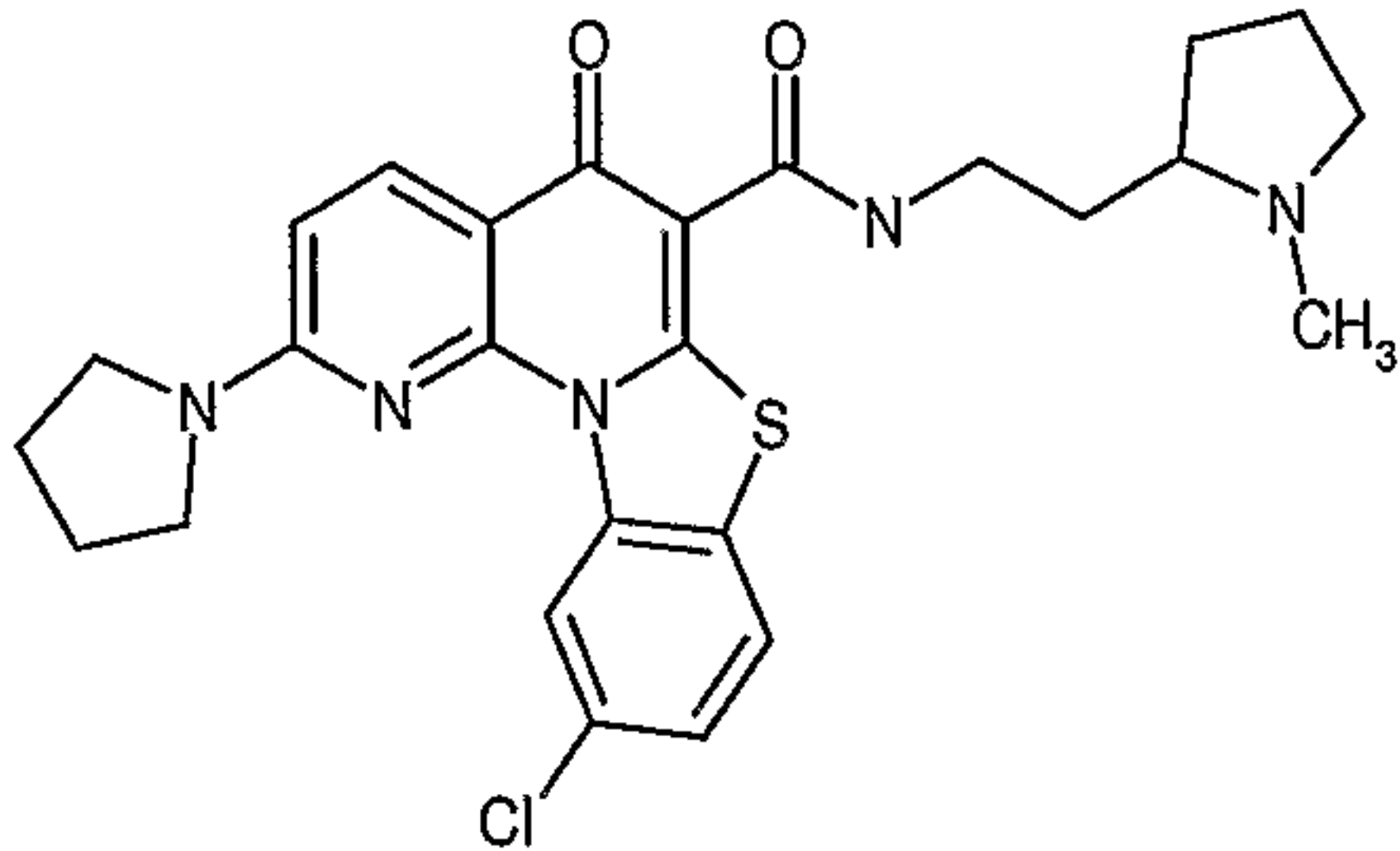
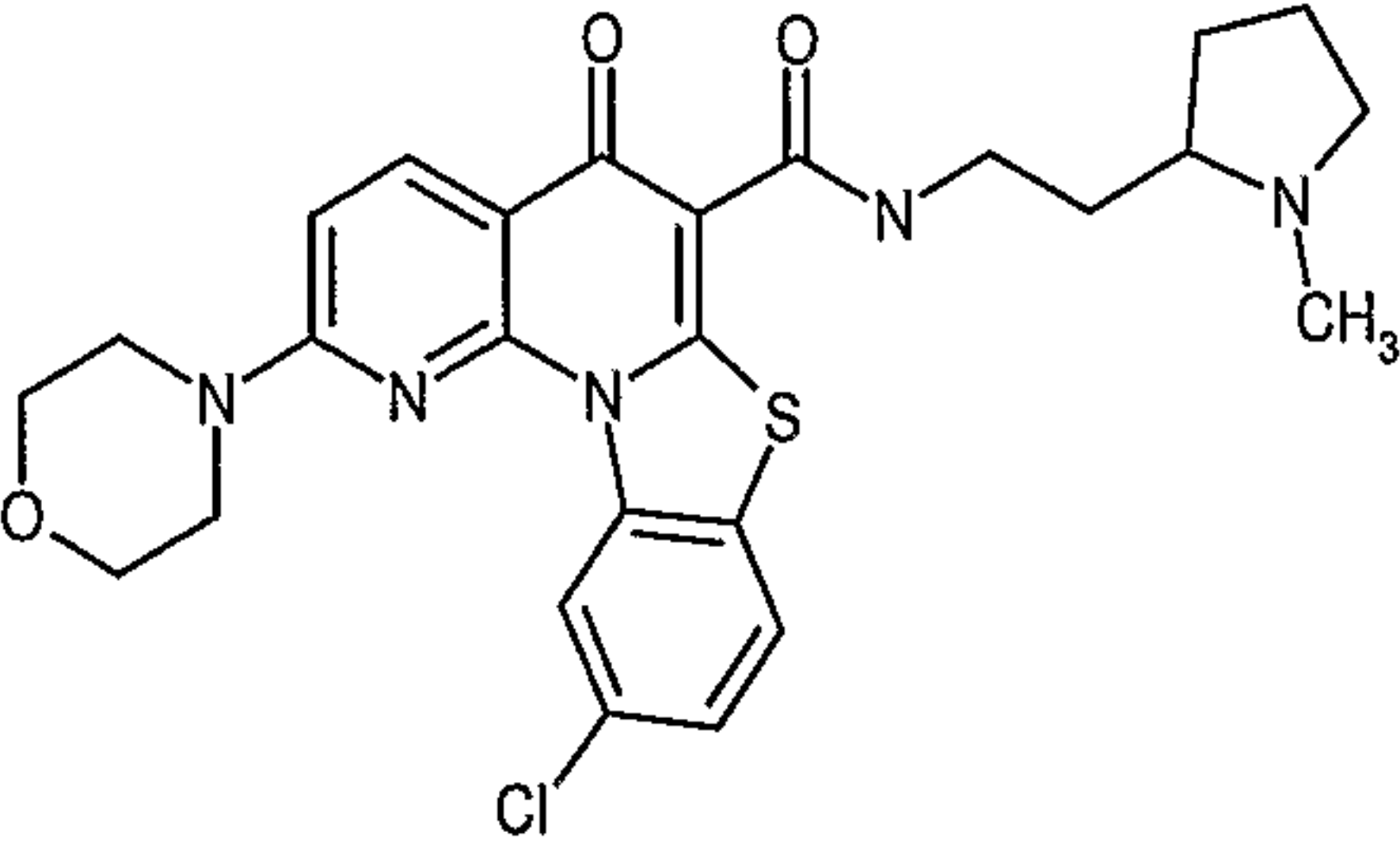
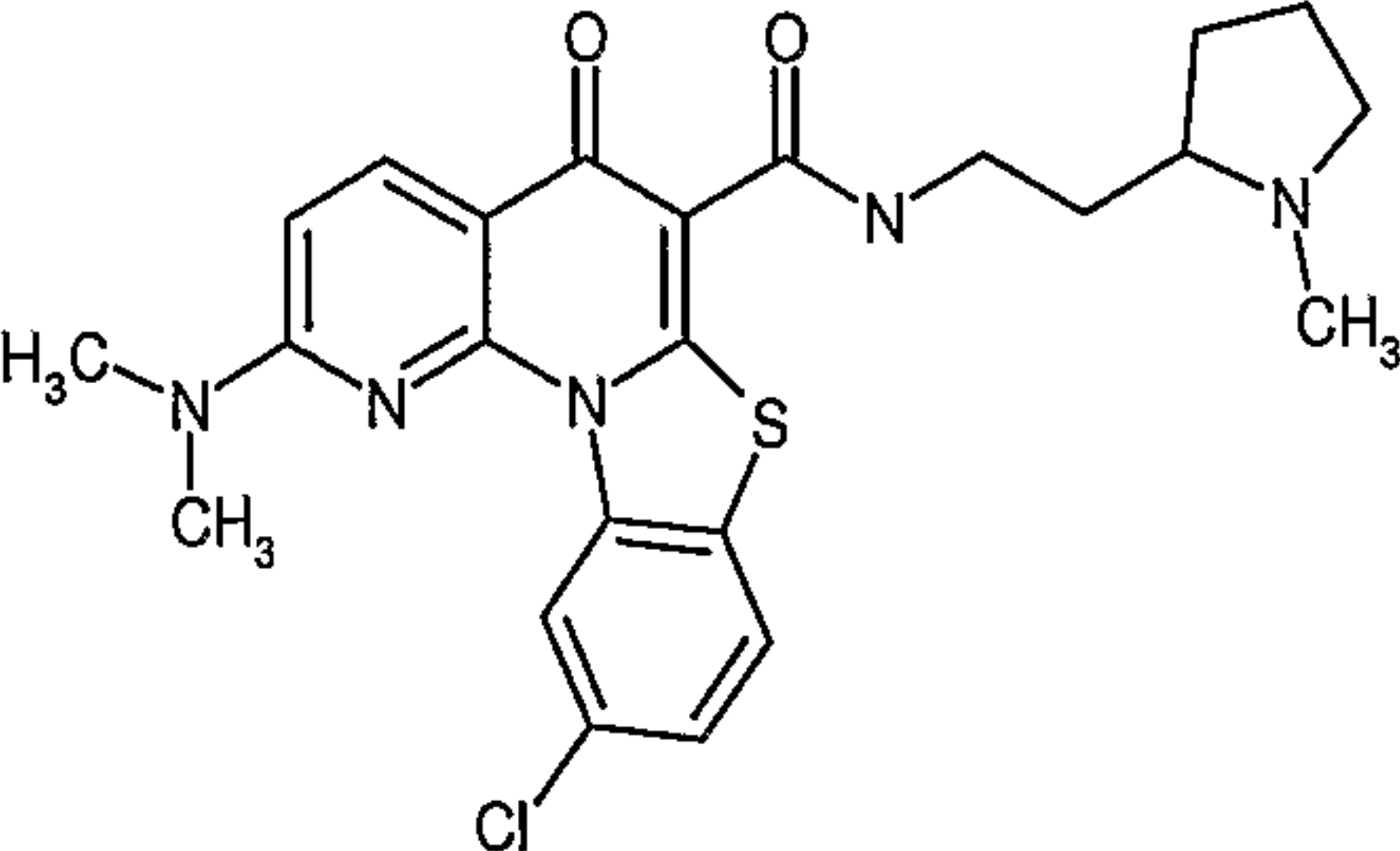
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | <0.01  |
|   | HCT-116 | <0.01  |
|  | Miapaca | 0.73   |
|  | HCT-116 | 2.45   |
|  | Miapaca | 0.02   |
|  | HCT-116 | 0.04   |

| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | 0.04   |
|  | HCT-116 | 0.02   |
|  | Miapaca | 0.16   |
|  | HCT-116 | 1.7    |
|  | Miapaca | 1.50   |
|  | HCT-116 | 0.3    |

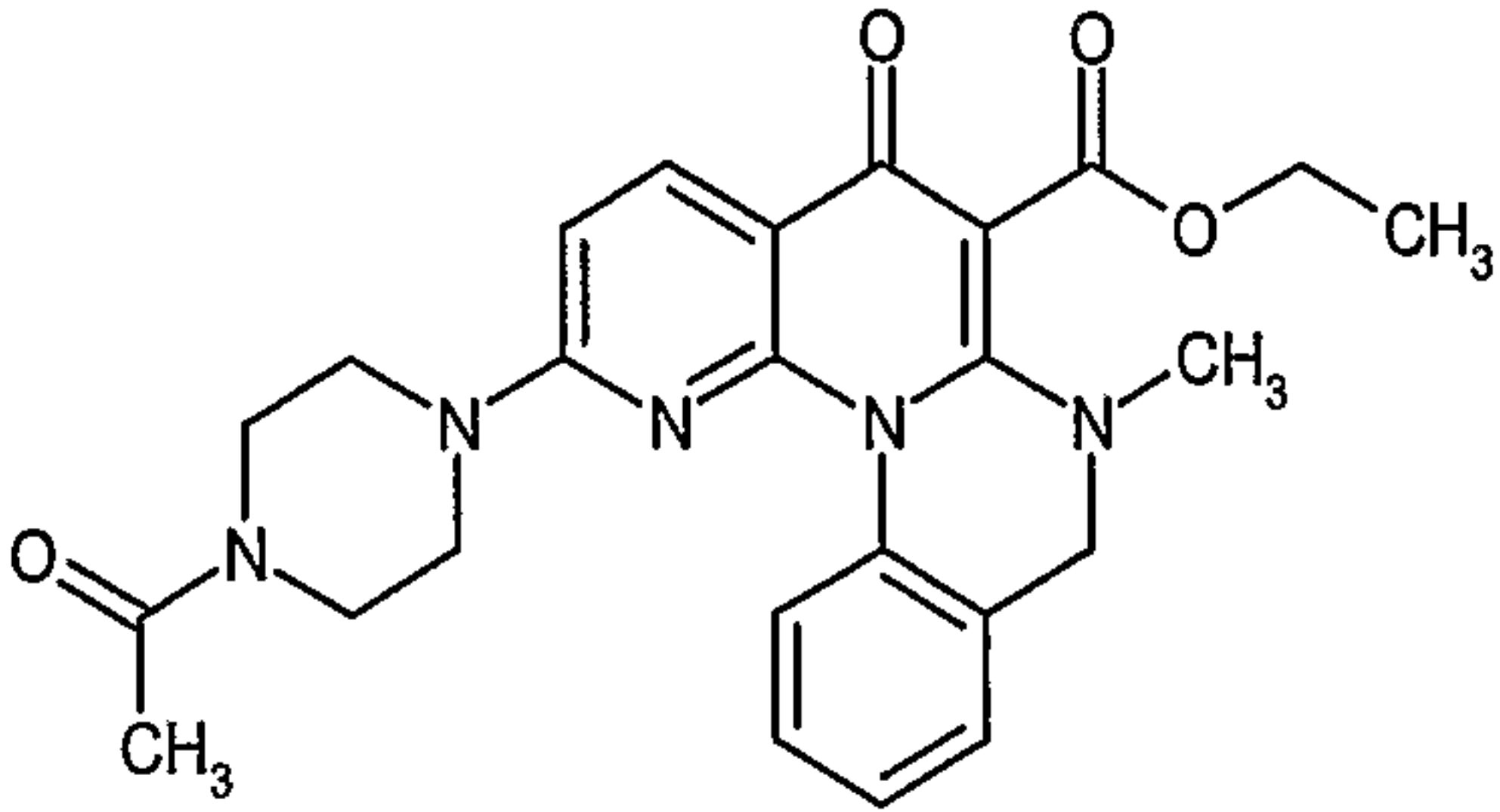
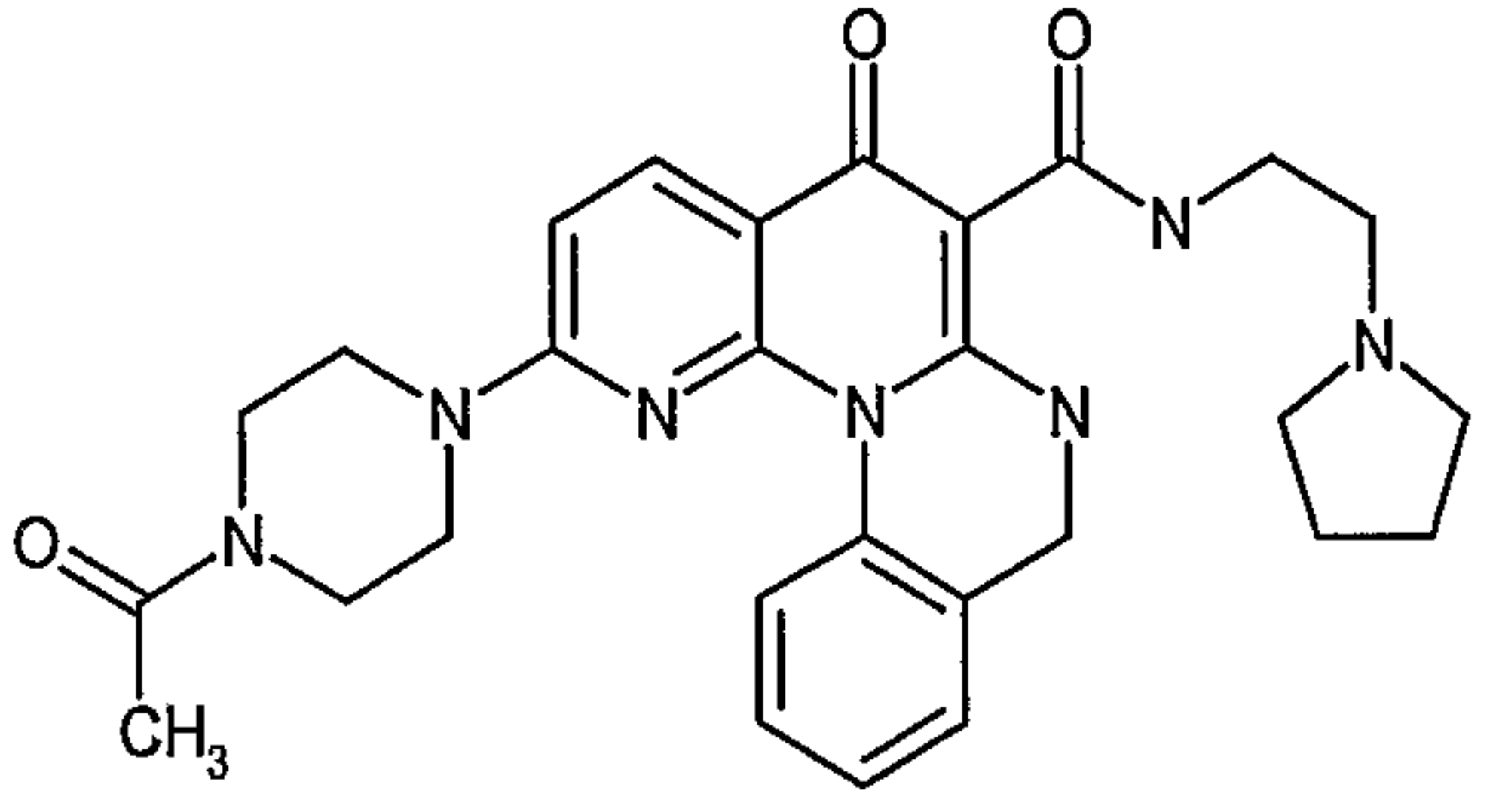
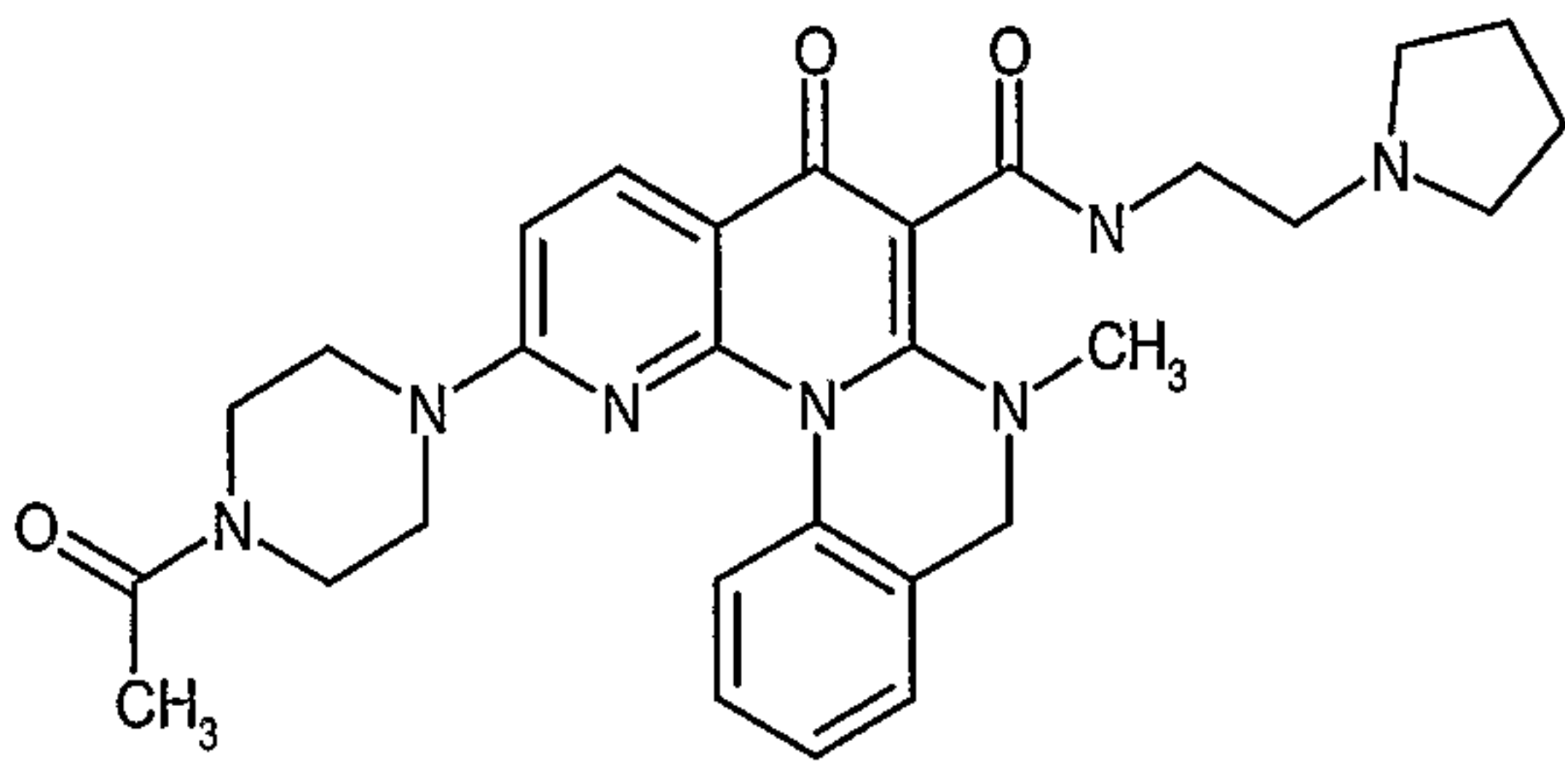
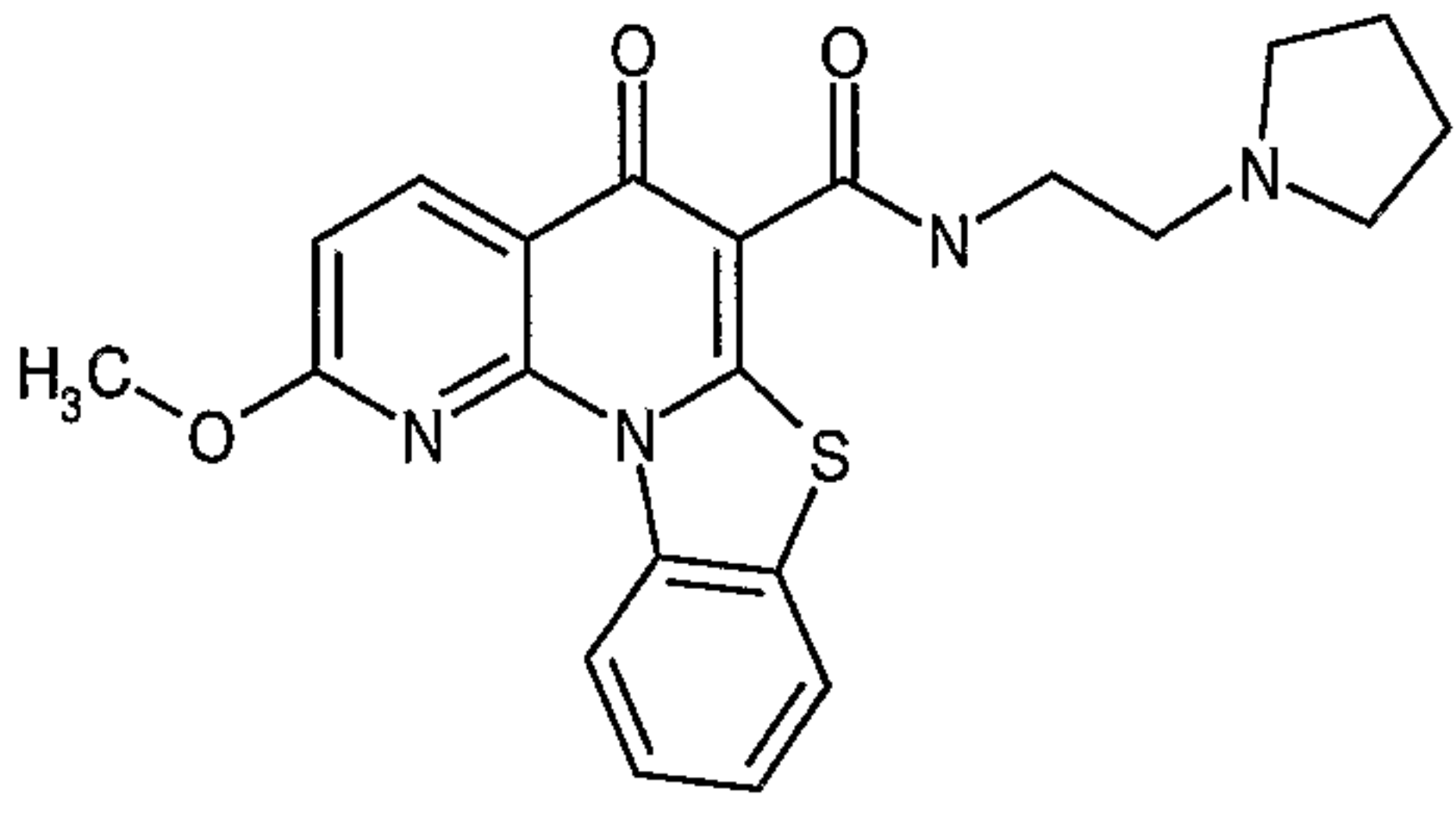
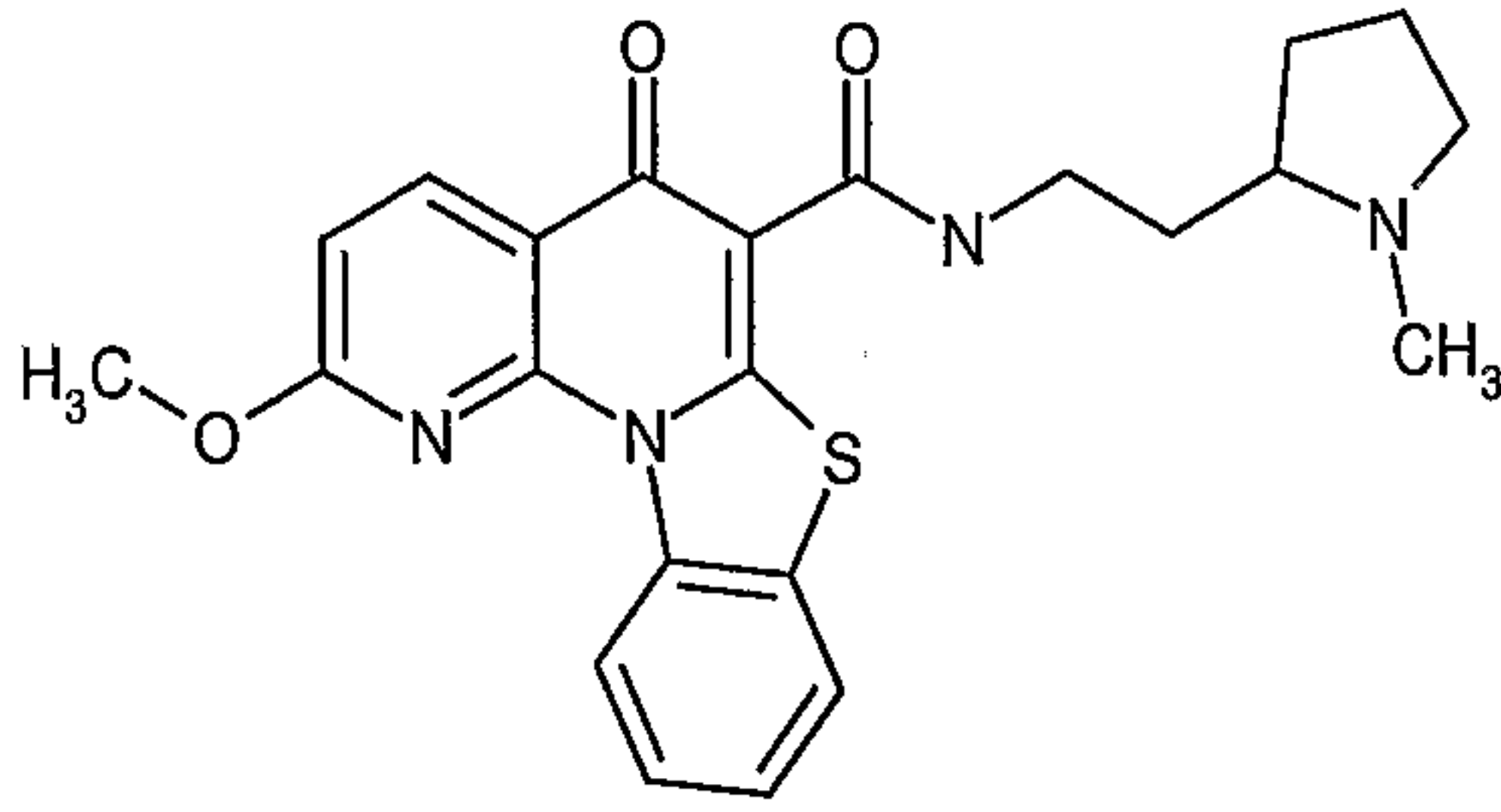
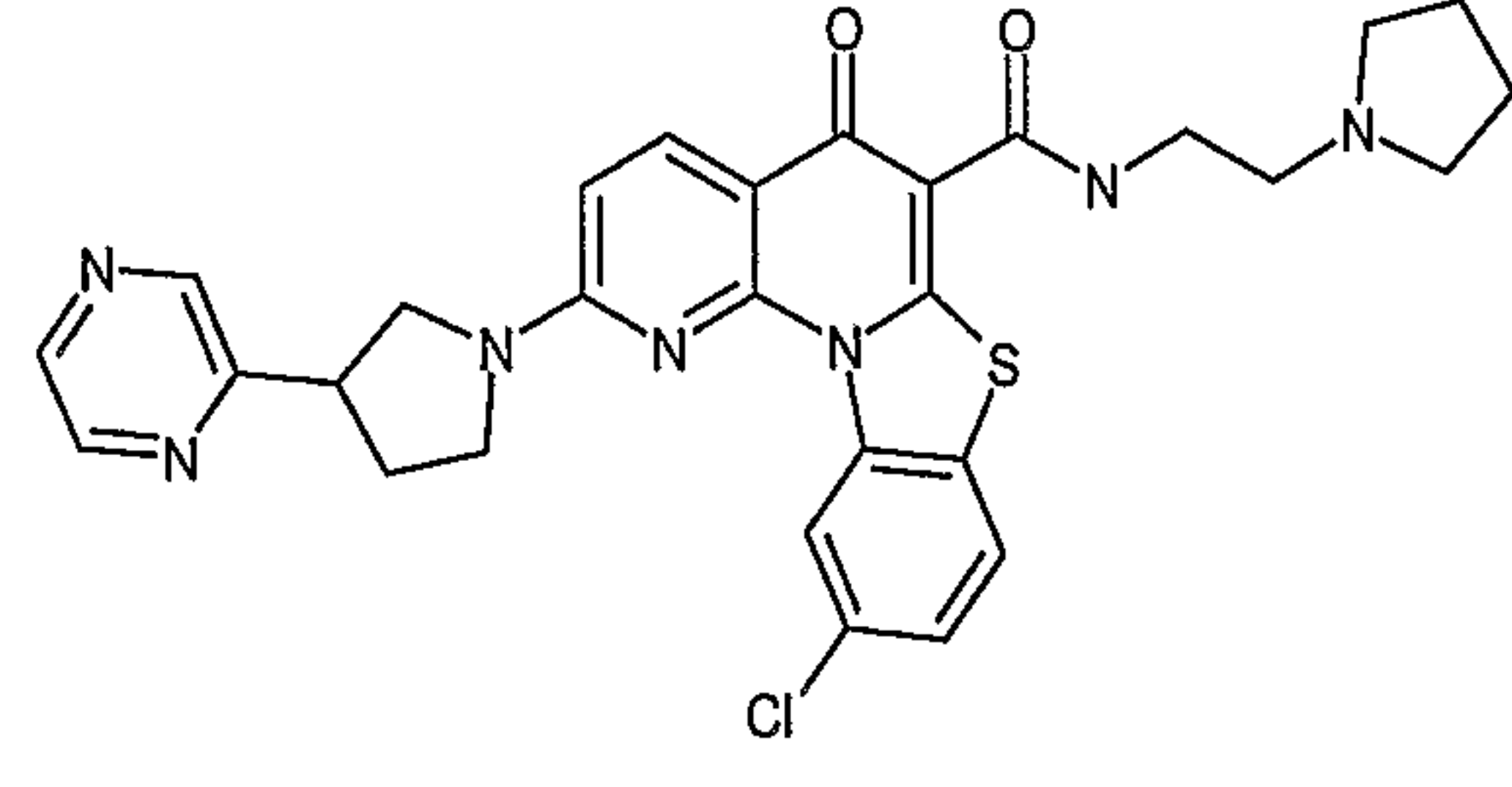
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | 2.00   |
|   | HCT-116 | 0.38   |
|  | Miapaca | 0.43   |
|  | HCT-116 | 0.27   |
|  | Miapaca | 0.04   |
|  | HCT-116 | 0.15   |

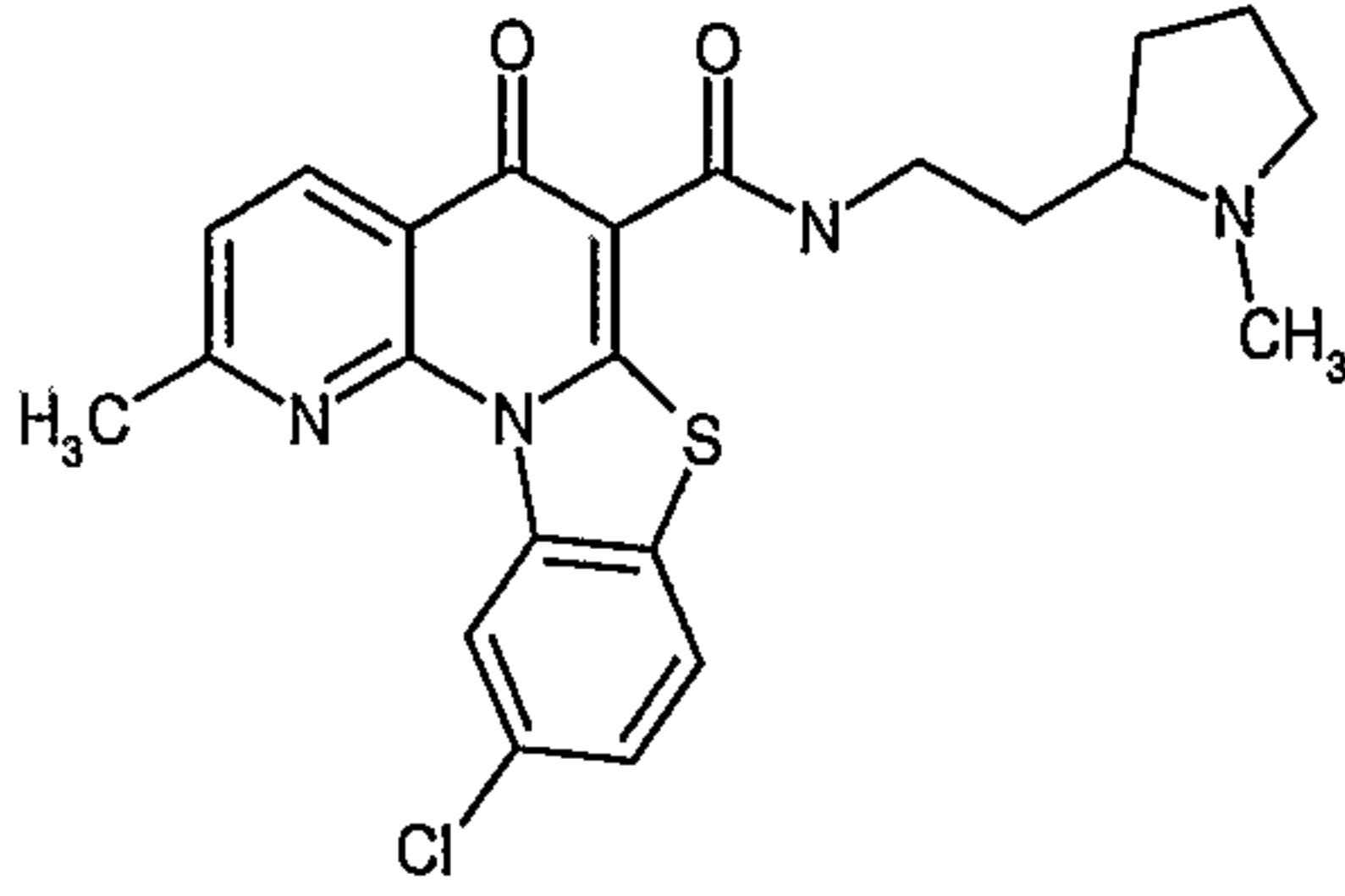
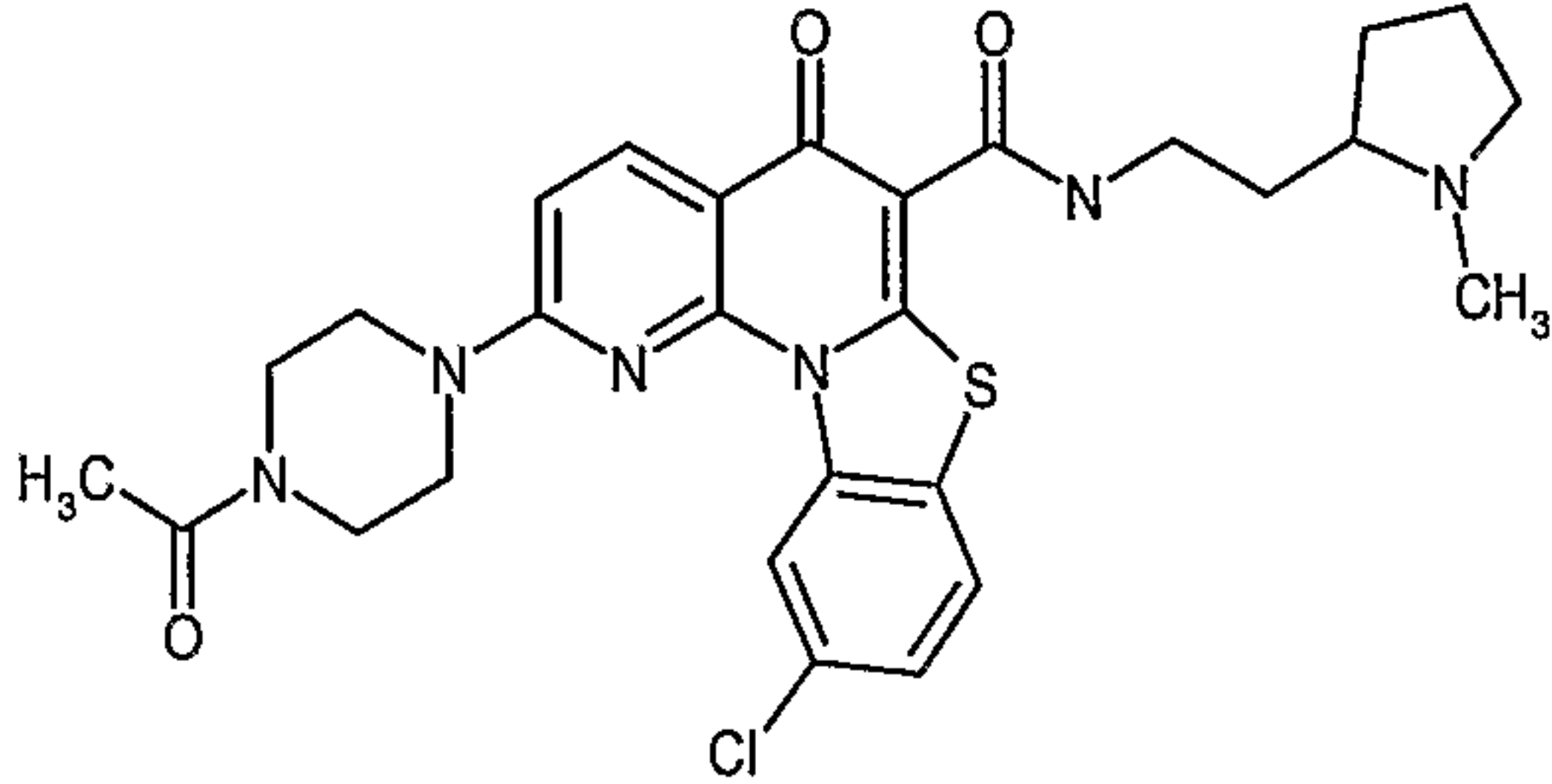
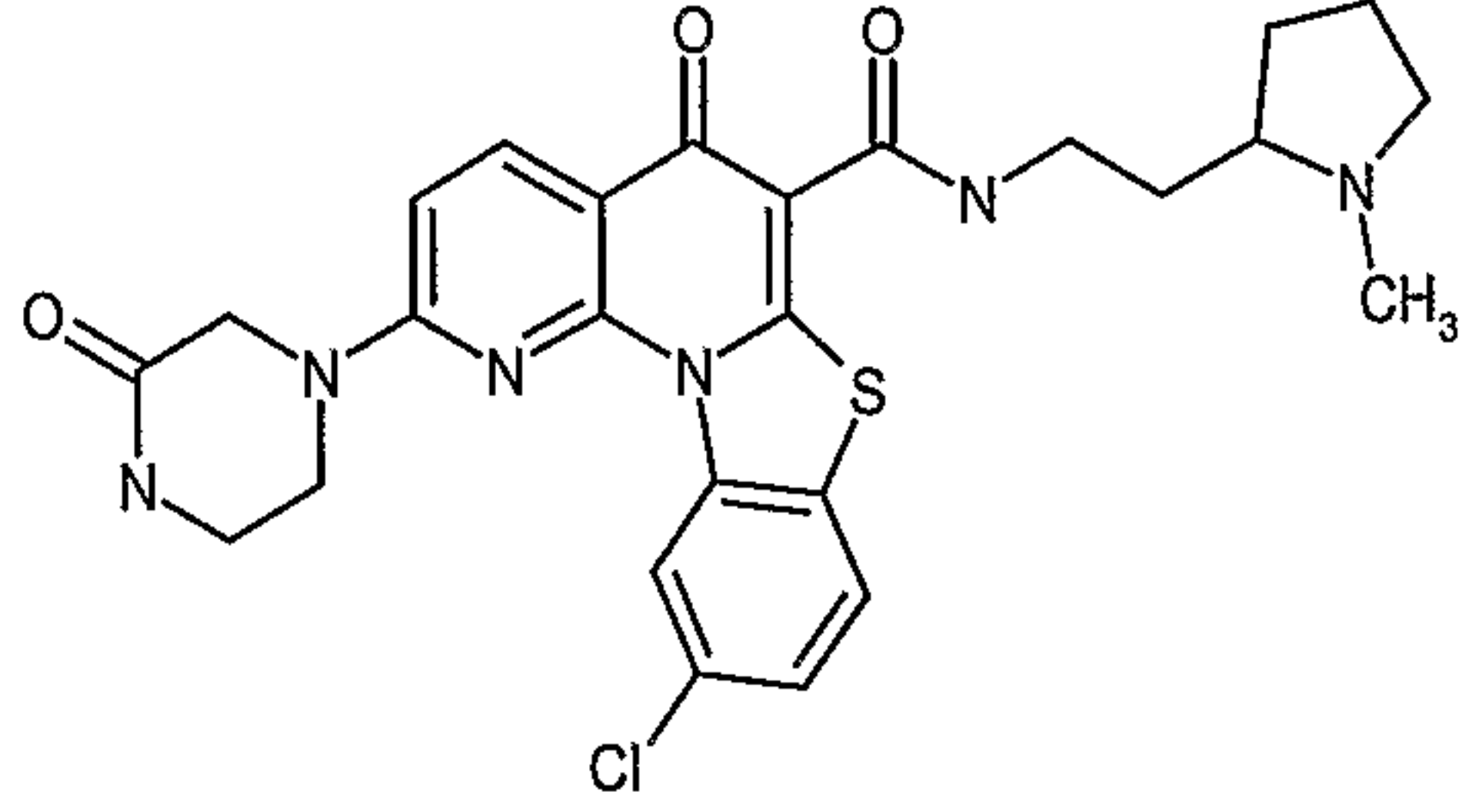
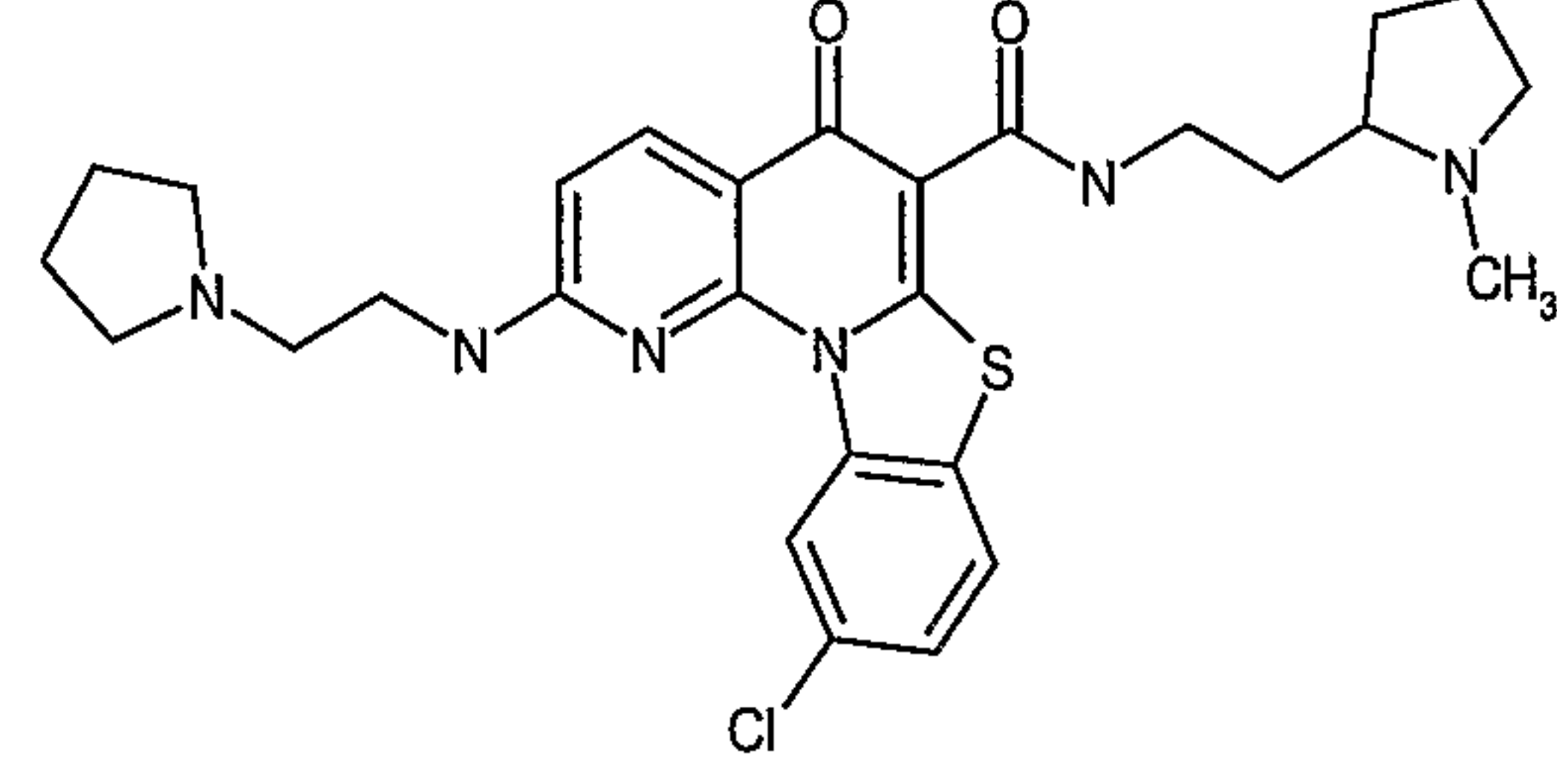
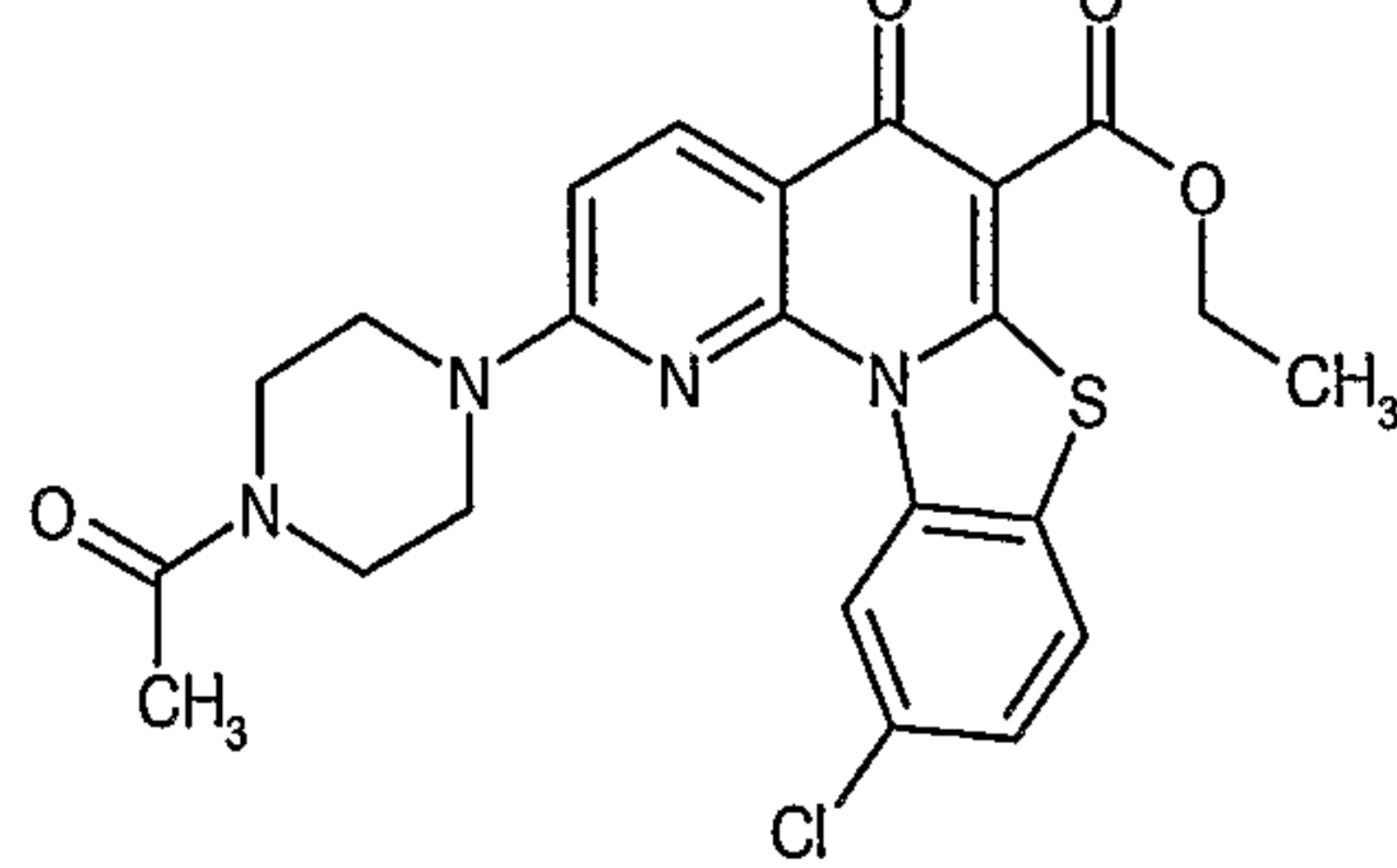
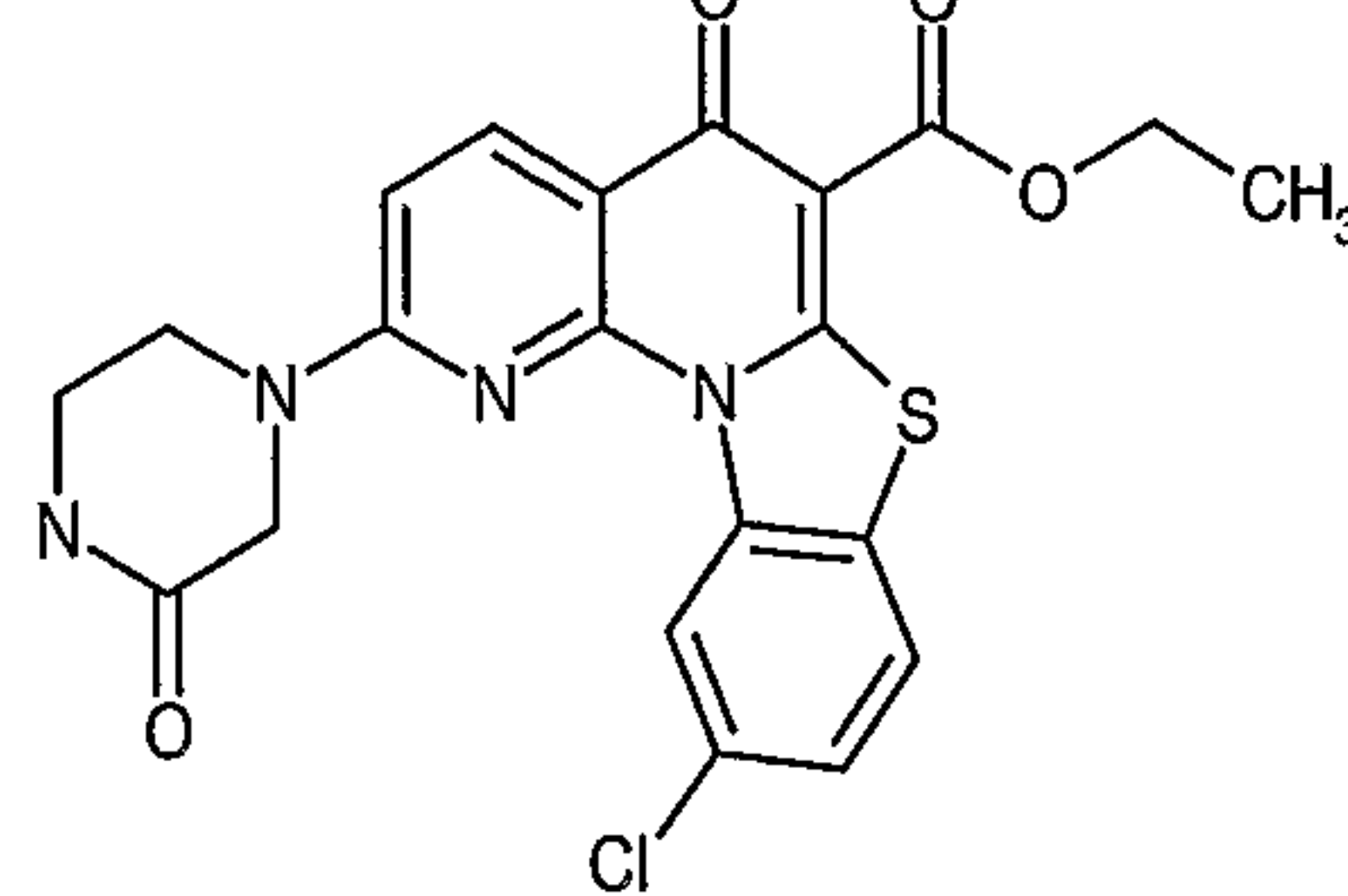
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | Miapaca | 0.07   |
|   | HCT-116 | 0.16   |
|  | Miapaca | 0.01   |
|  | HCT-116 | 0.01   |
|  | HCT-116 | >10    |
|  | Miapaca | 4.00   |

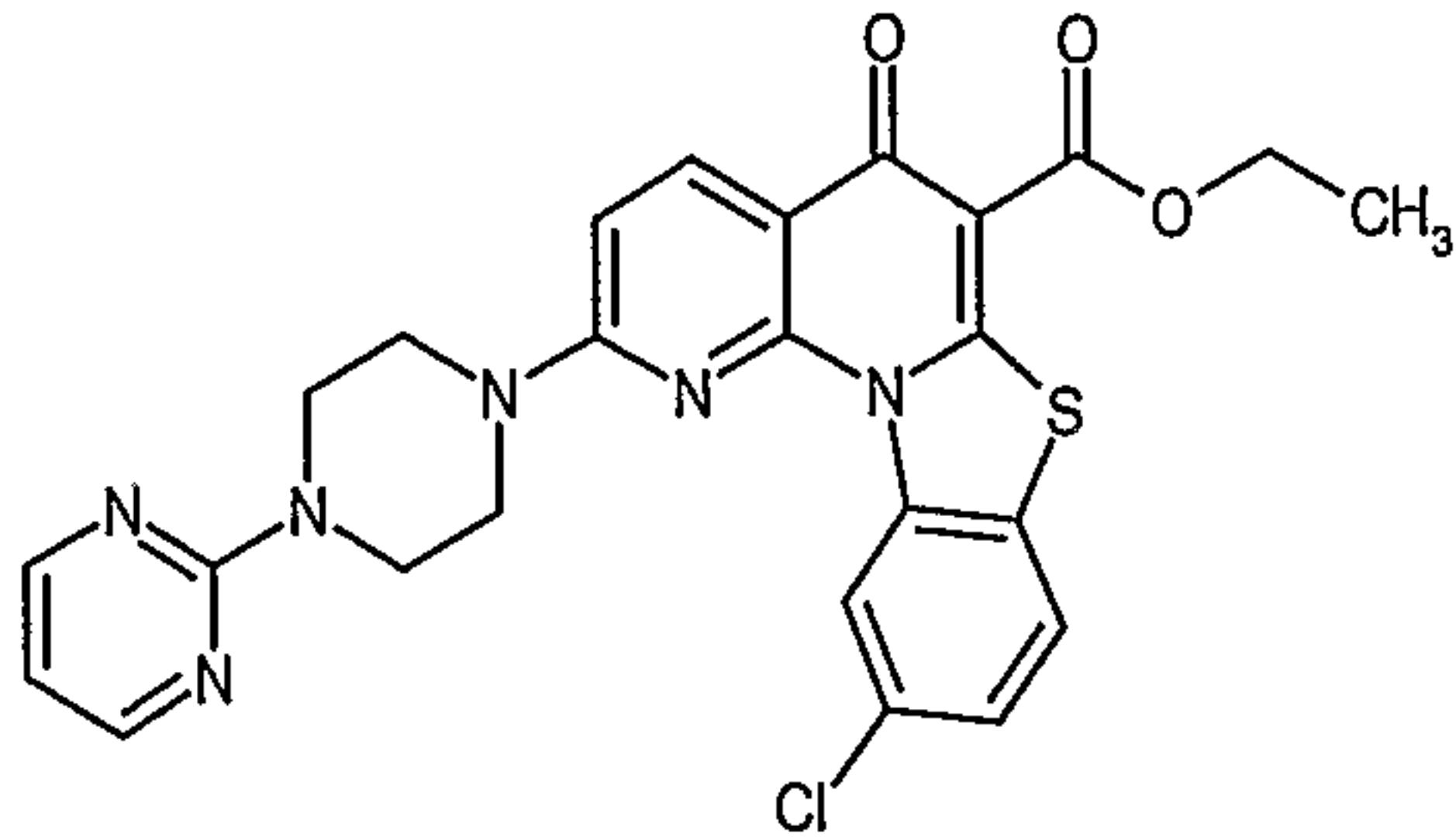
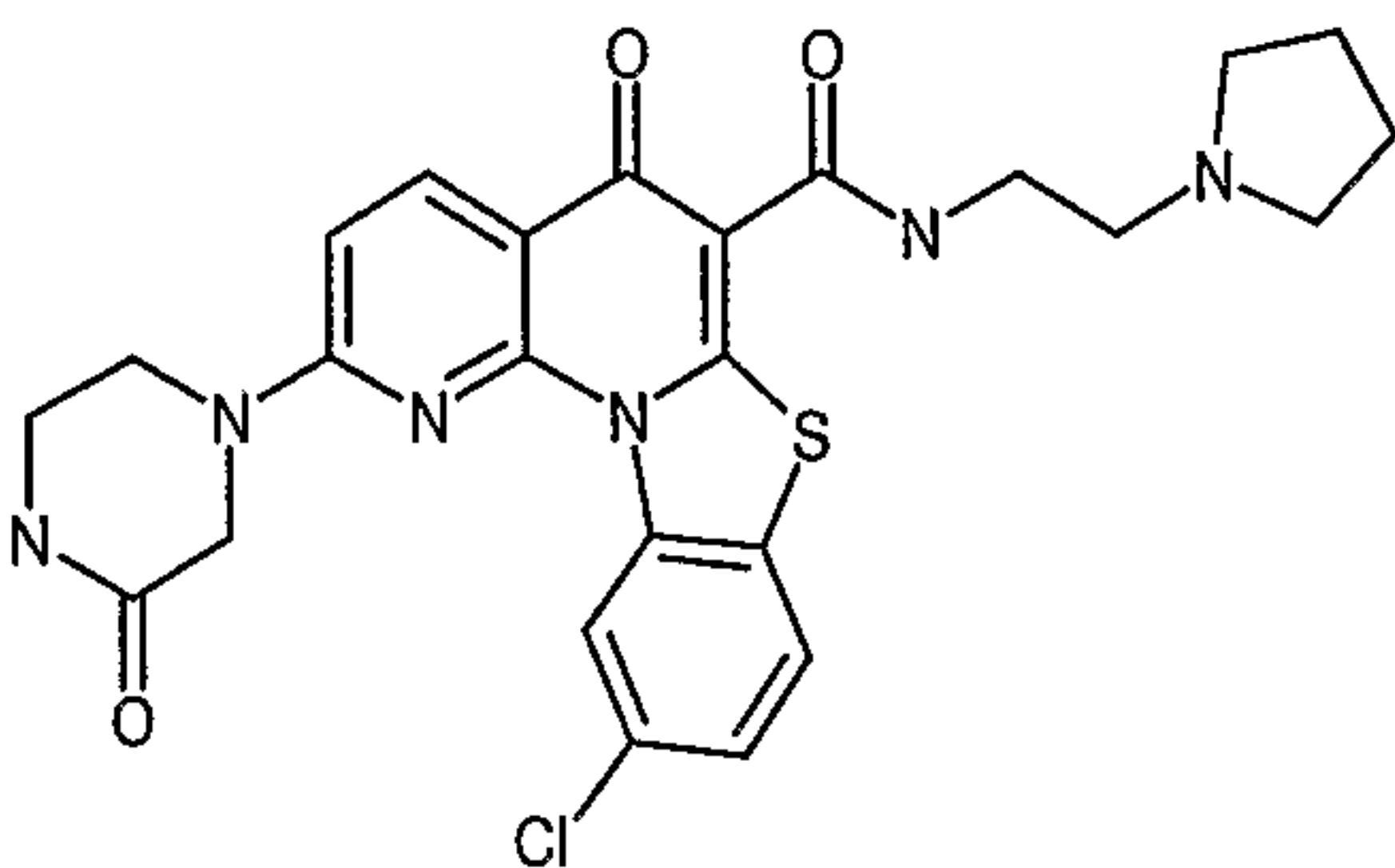
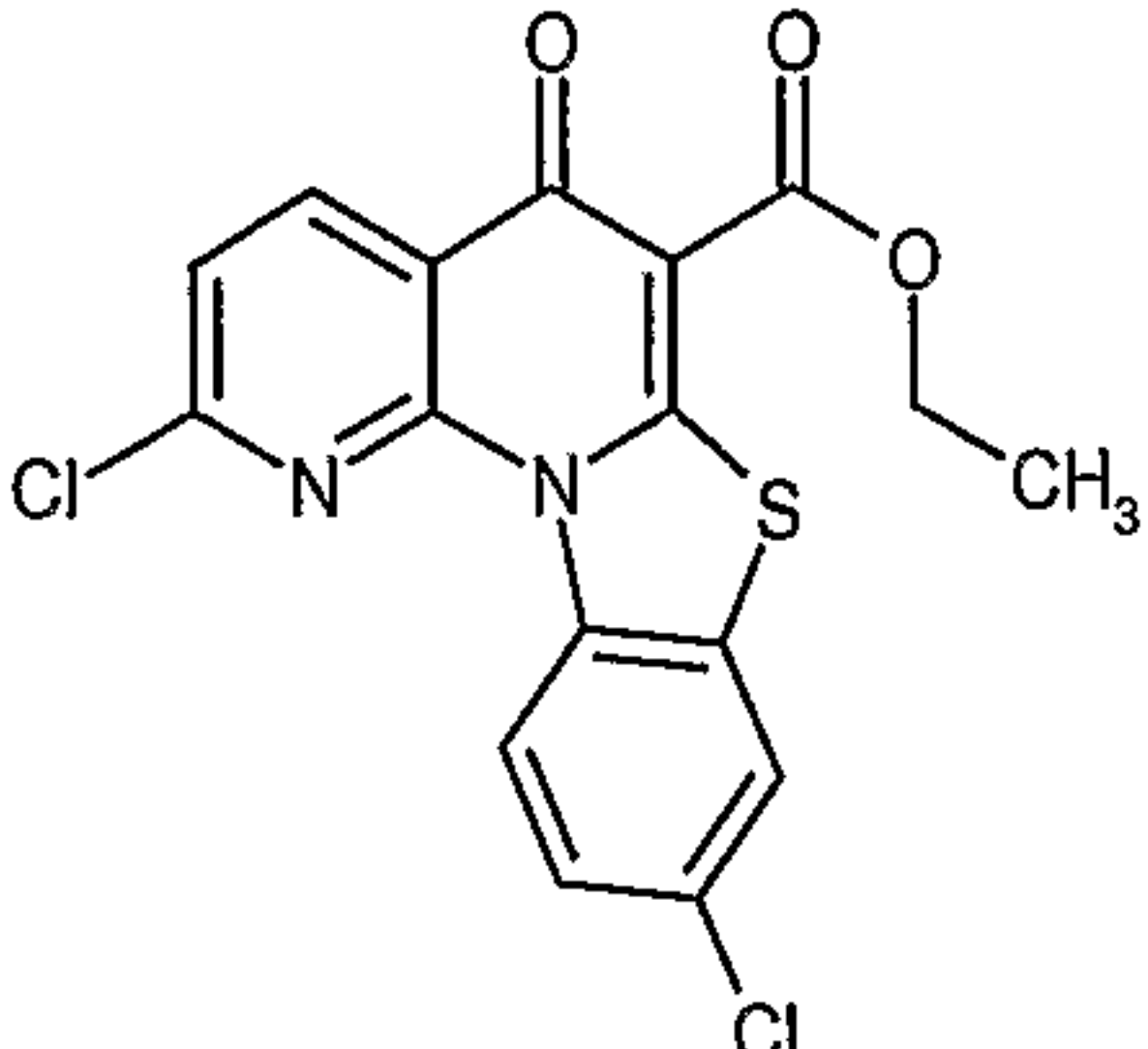
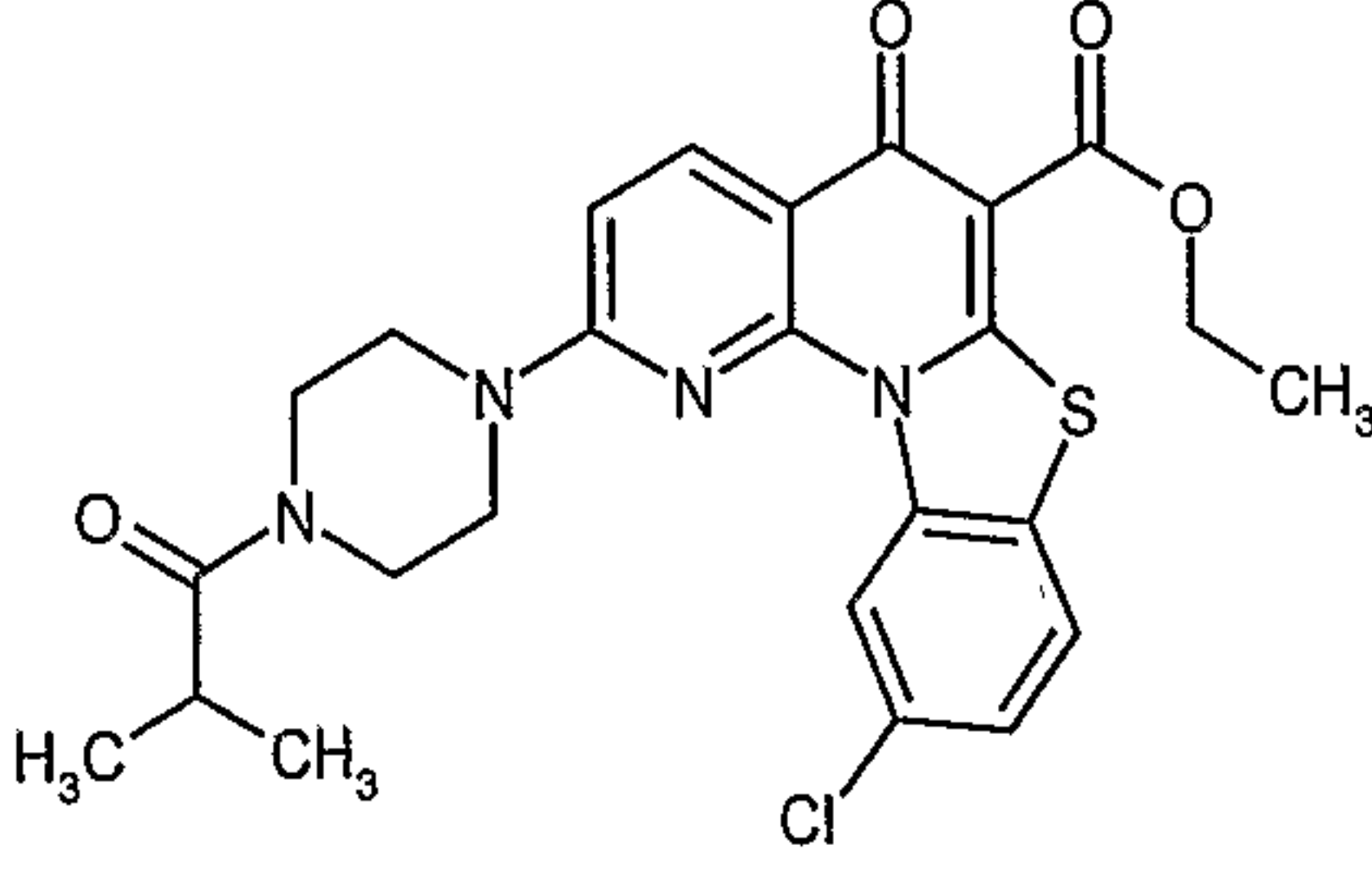
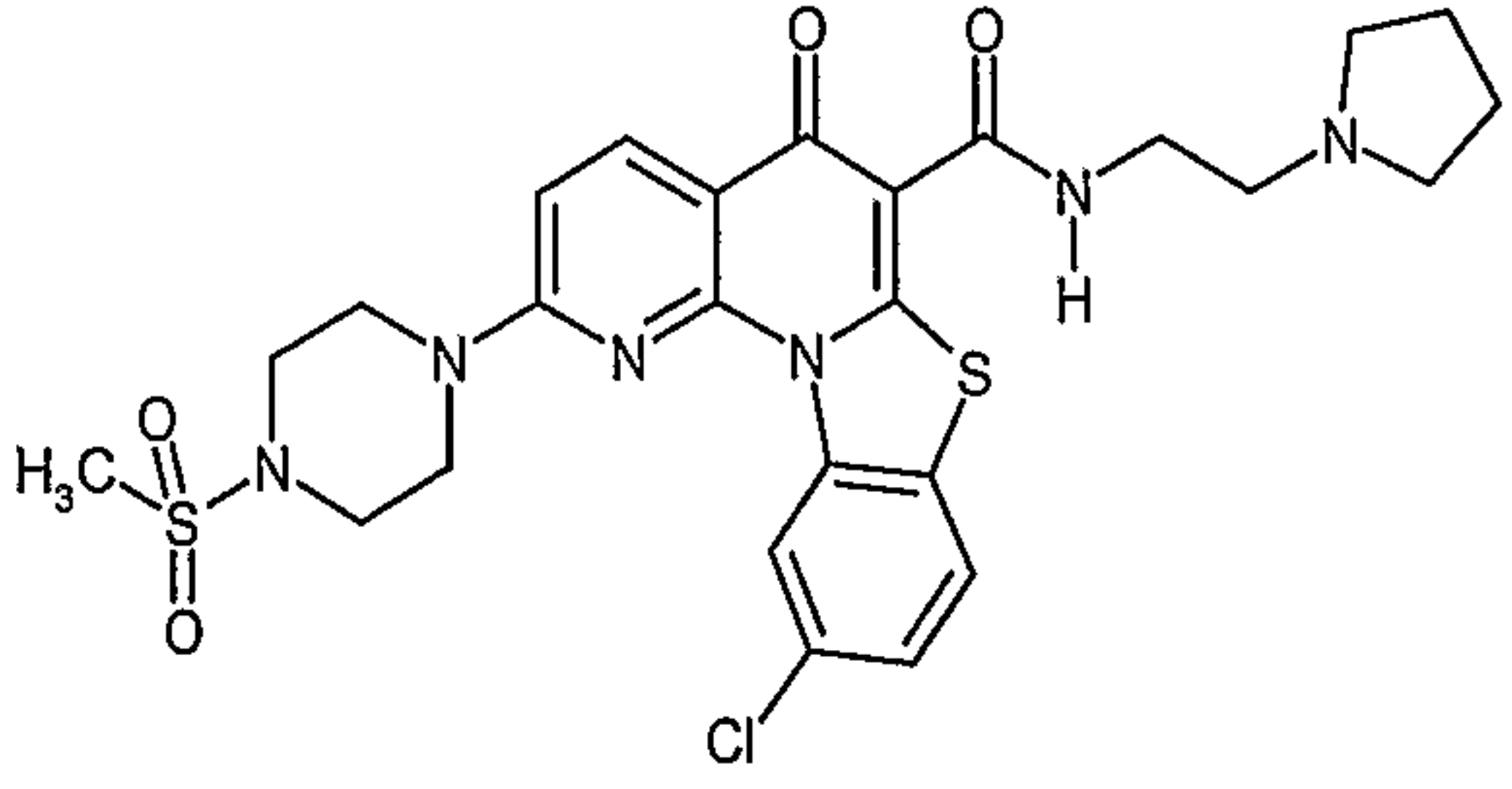
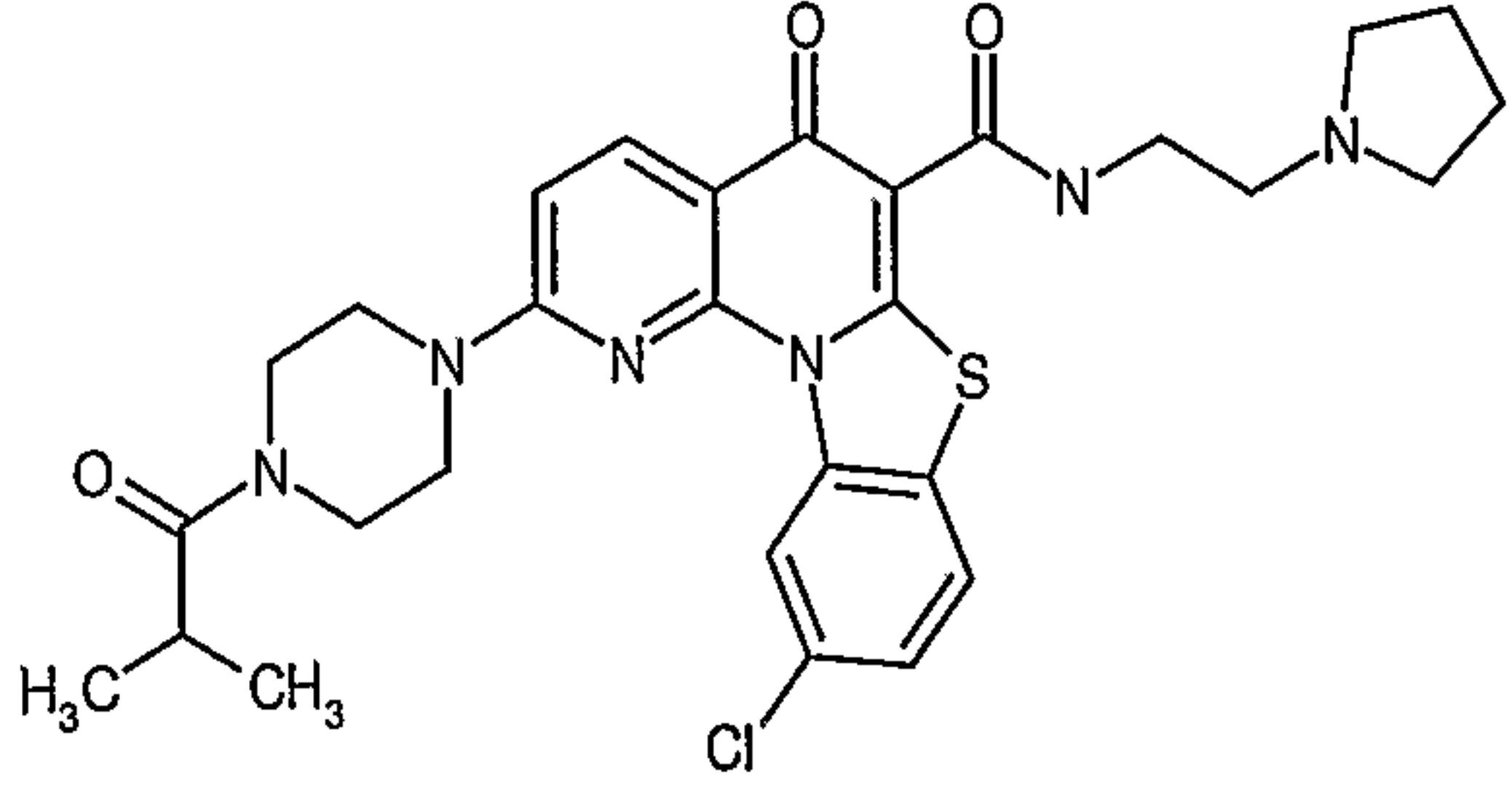
| MOLSTRUCTURE                                                                        | M CELL  | M DATA |
|-------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | 0.82   |
|   | Miapaca | 0.16   |
|  | HCT-116 | >10    |
|  | Miapaca | 6.50   |
|  | HCT-116 | 0.07   |
|  | HCT-116 | >10    |

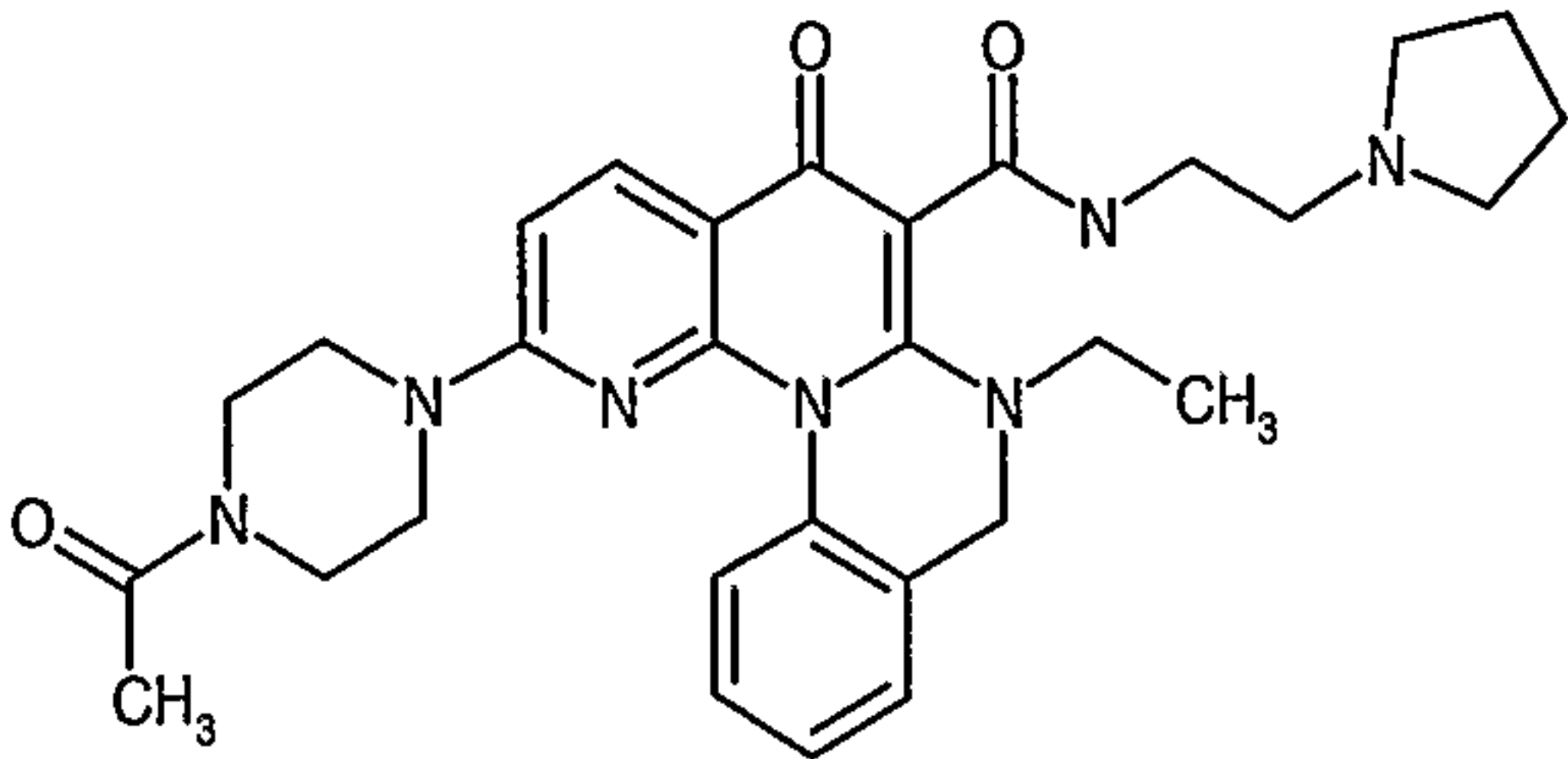
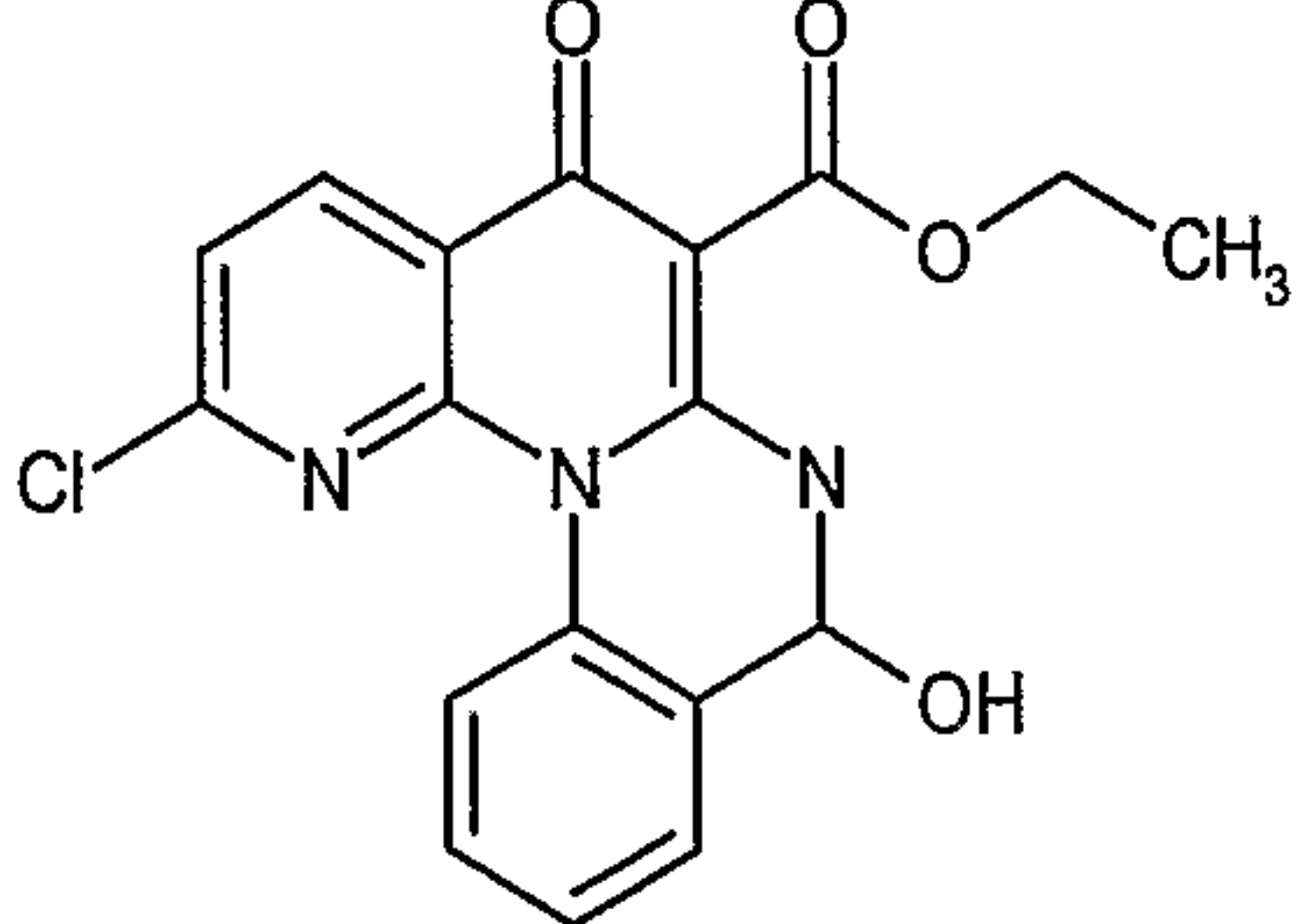
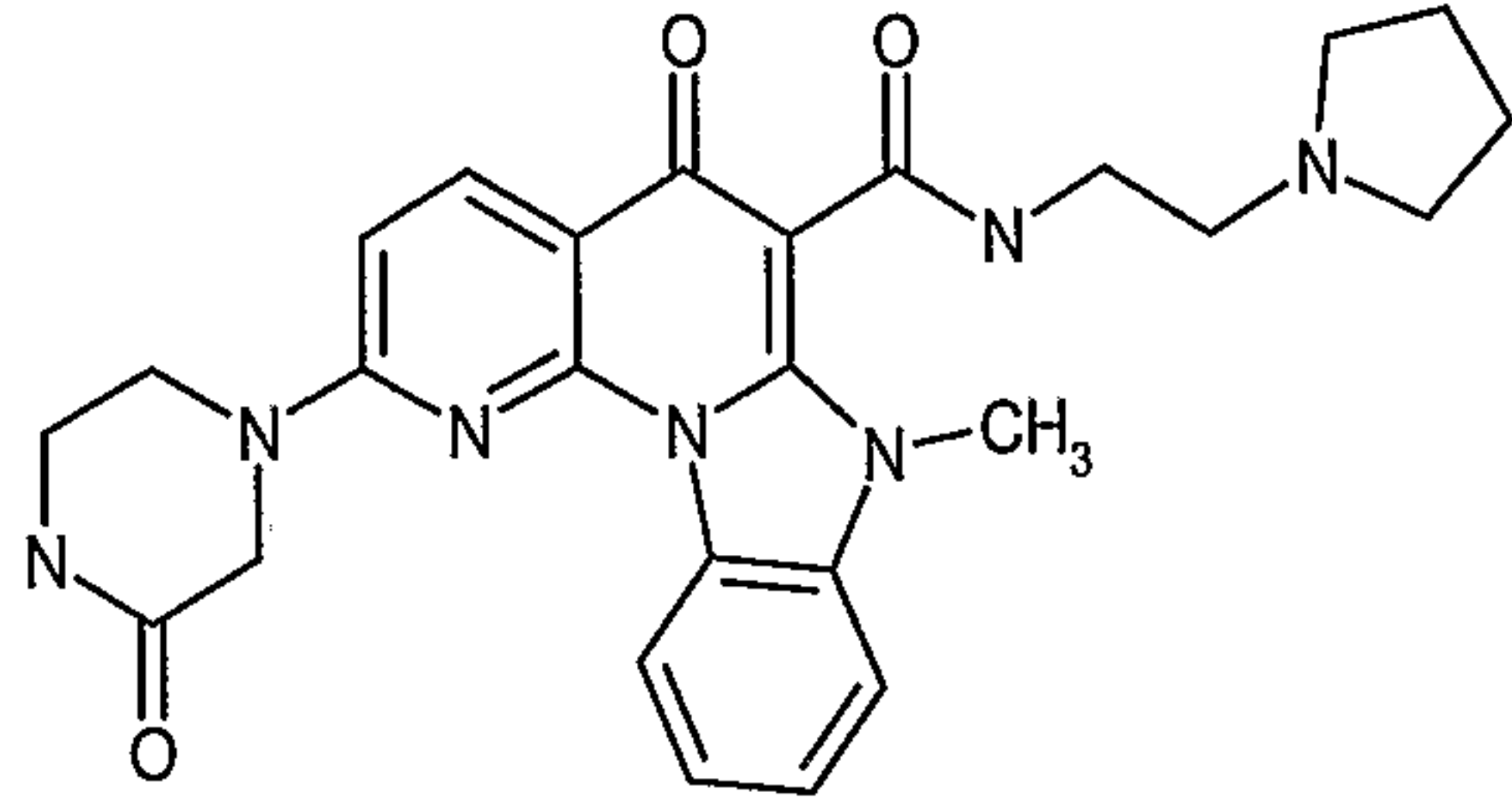
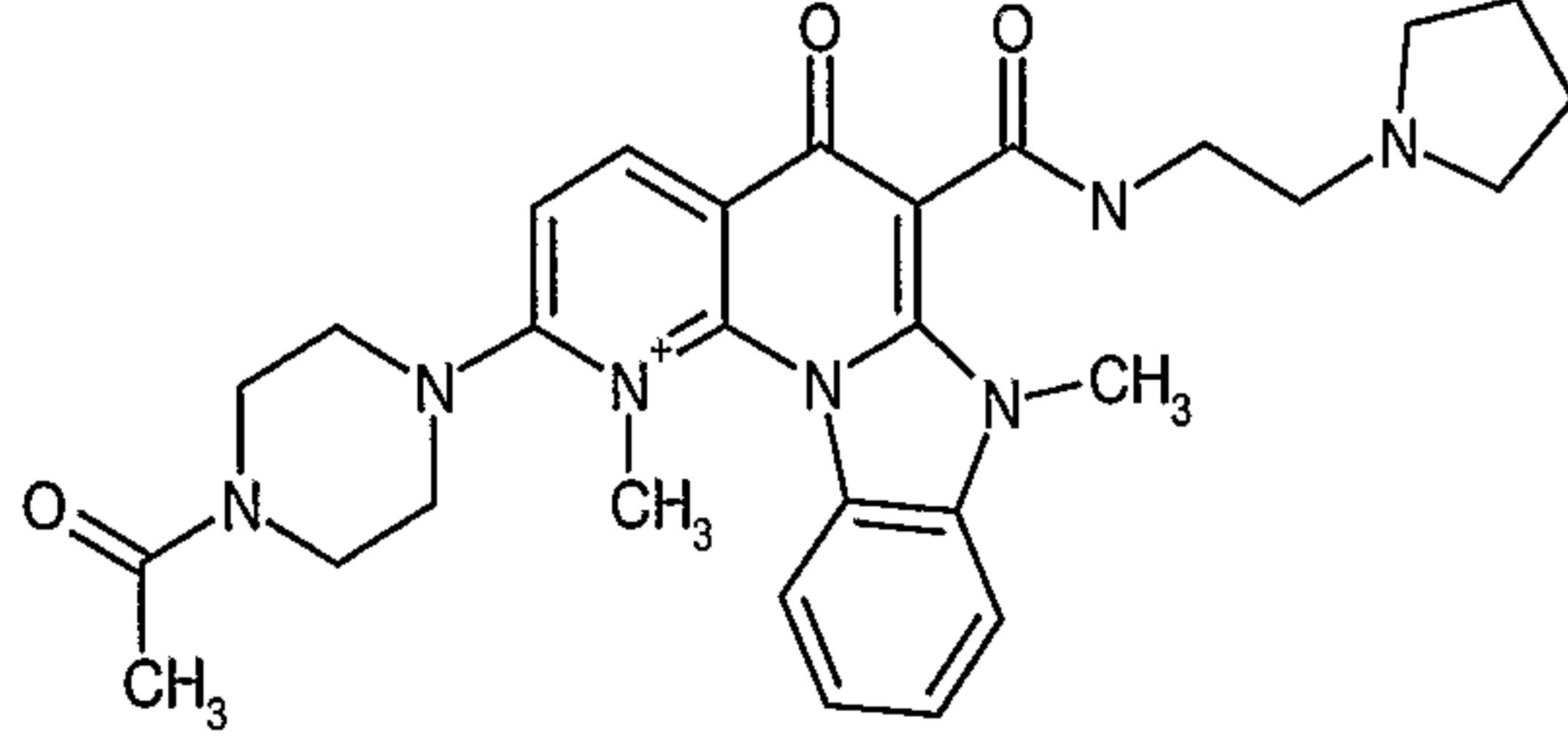
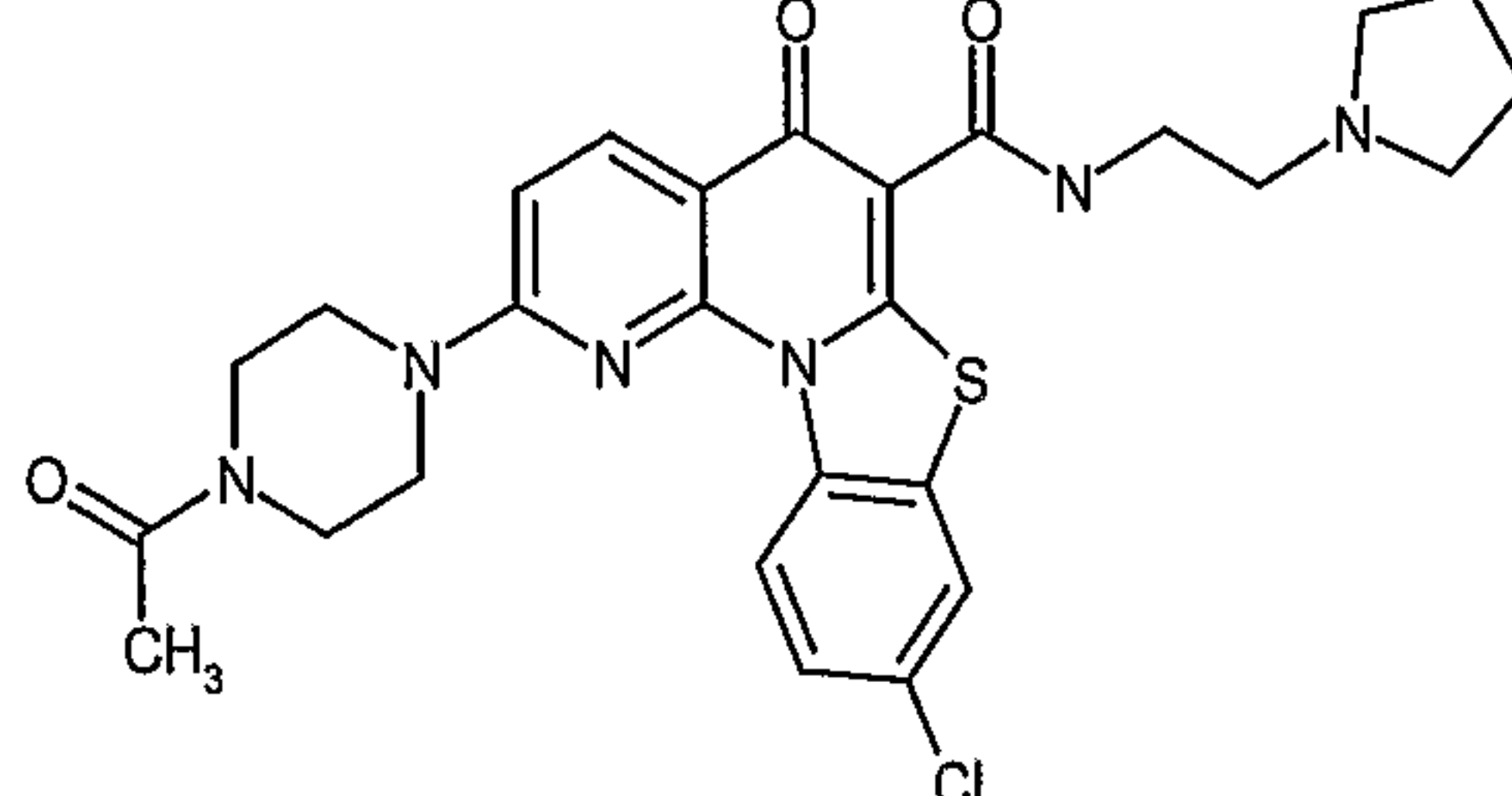
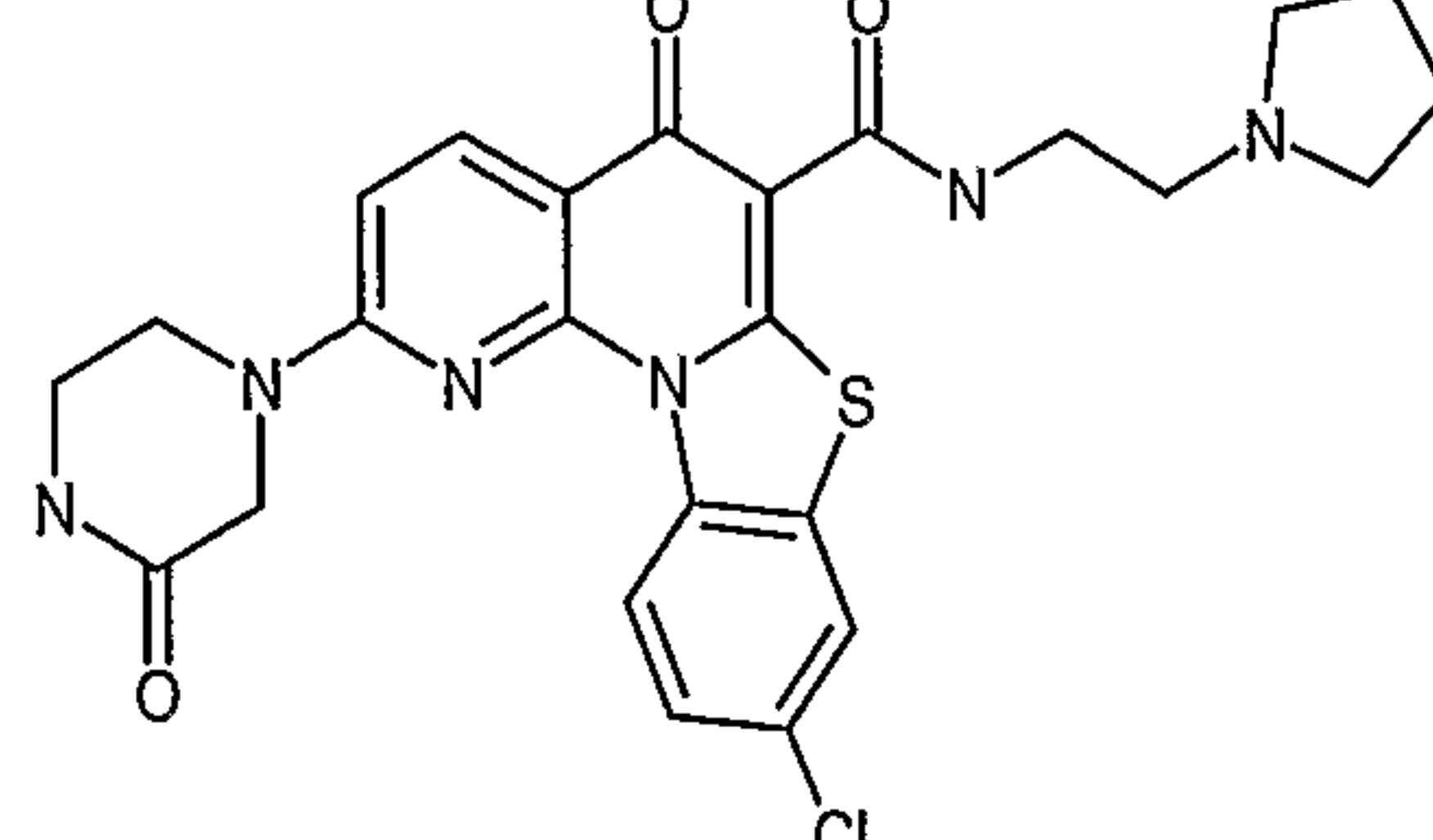
| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | HCT-116 | 0.02   |
|    | Miapaca | 0.01   |
|  | HCT-116 | 1.2    |
|   | HCT-116 | 0.19   |
|   | HCT-116 | 0.57   |
|   | HCT-116 | 0.15   |

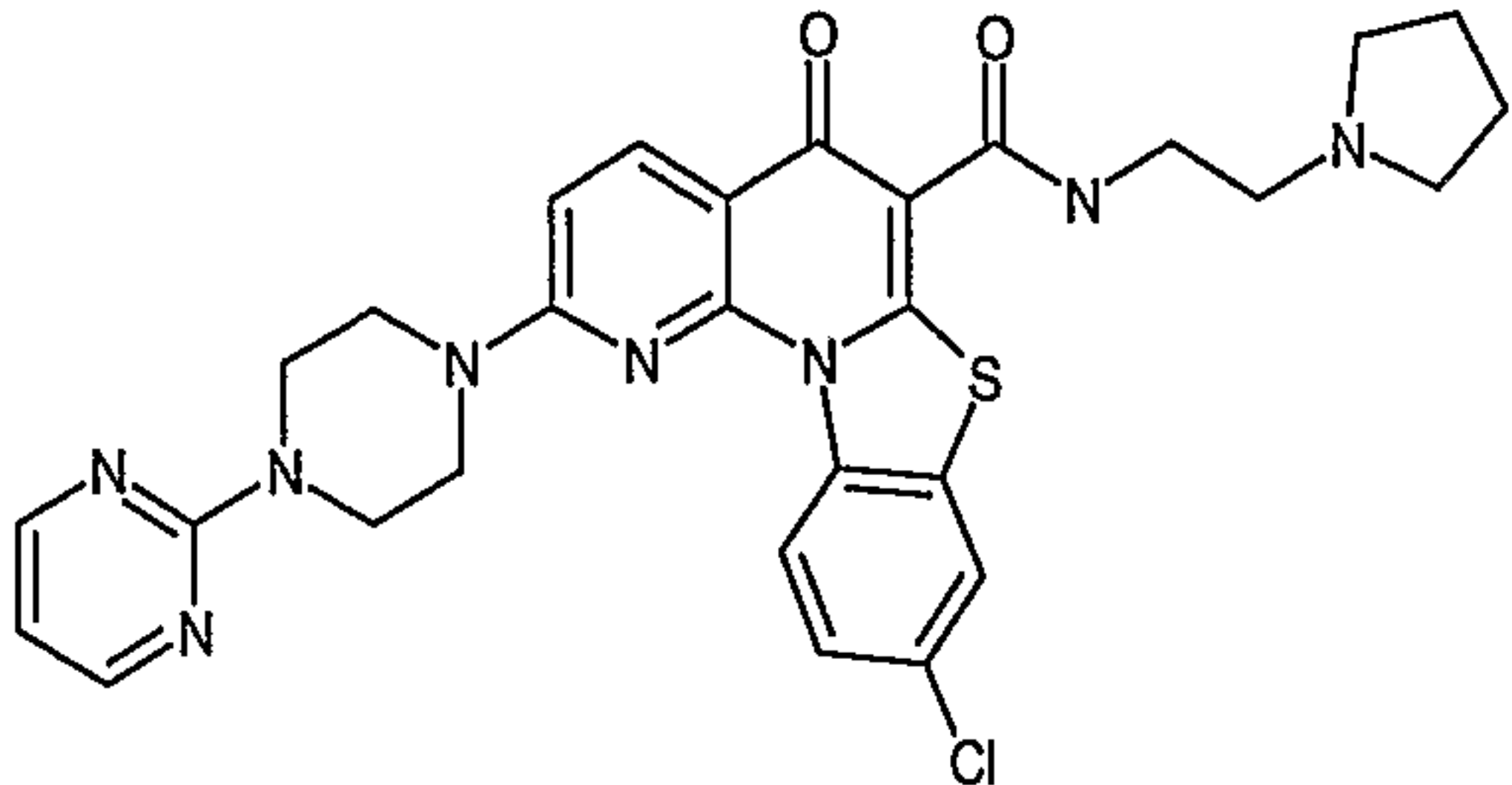
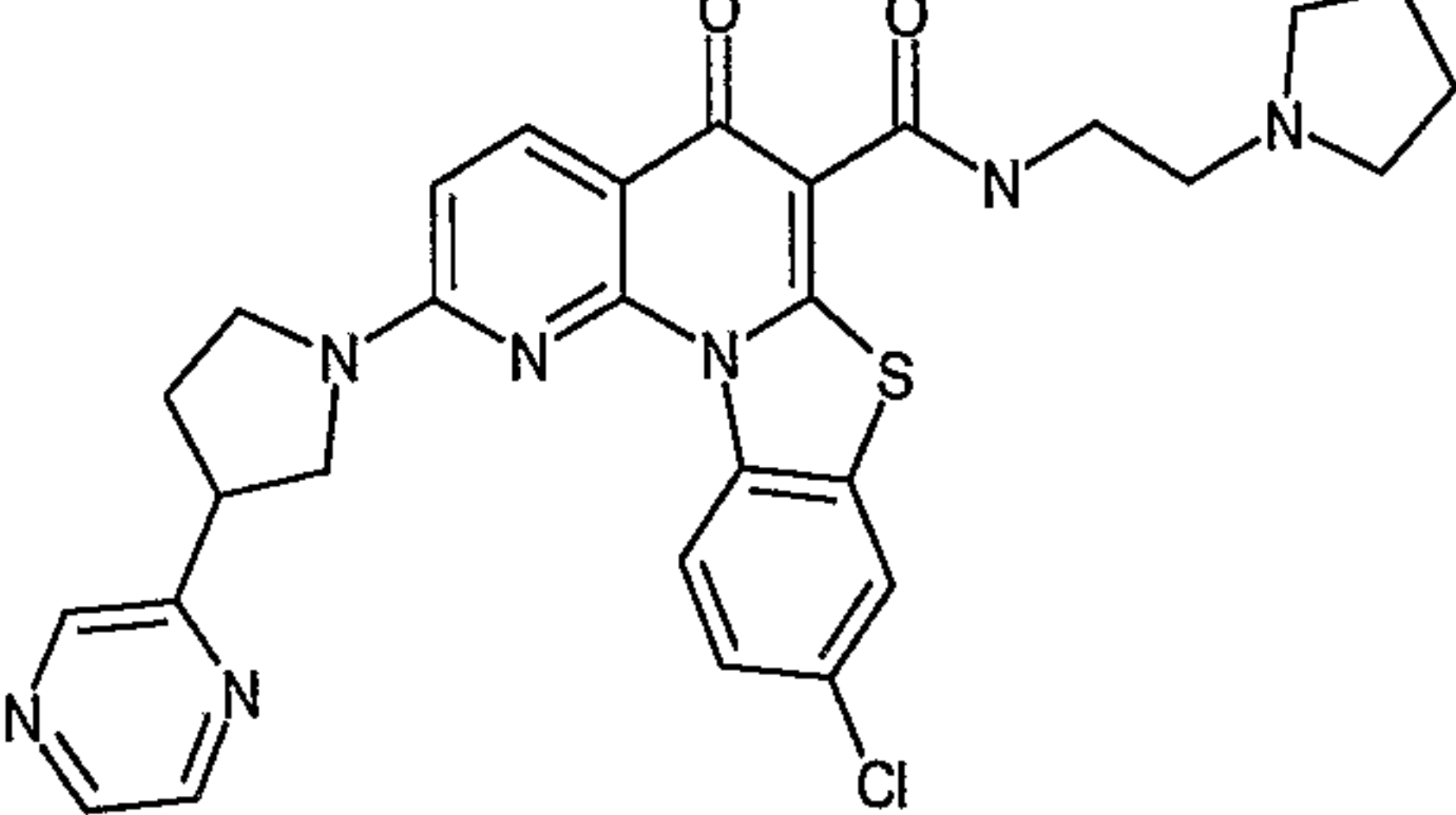
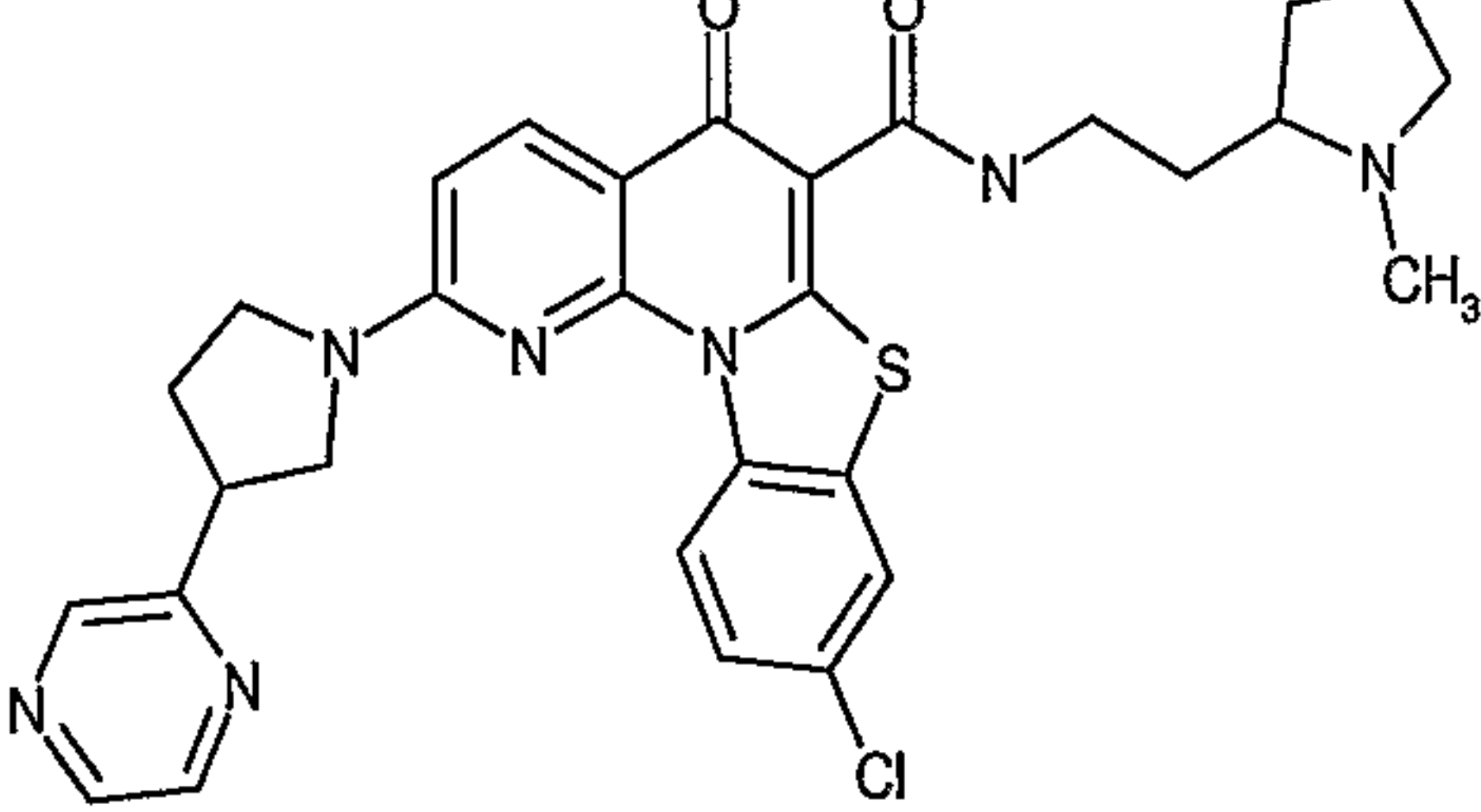
85

| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-116 | >10    |
|   | HCT-116 | >10    |
|  | HCT-116 | >10    |
|   | HCT-116 | 0.04   |
|  | HCT-116 | 0.31   |
|  | HCT-116 | 3.6    |

| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | HCT-116 | 2.9    |
|   | HCT-116 | 0.45   |
|   | HCT-116 | 0.42   |
|  | HCT-116 | 0.16   |
|   | HCT-116 | 0.23   |
|   | HCT-117 | 0.2    |

| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|     | HCT-118 | 0.75   |
|    | HCT-119 | 0.17   |
|   | HCT-121 | >10    |
|   | HCT-122 | 0.07   |
|  | HCT-123 | 0.03   |
|  | HCT-124 | 0.03   |

| MOLSTRUCTURE                                                                         | M CELL  | M DATA |
|--------------------------------------------------------------------------------------|---------|--------|
|    | HCT-117 | >10    |
|    | HCT-118 | 3.5    |
|   |         |        |
|  |         |        |
|   |         |        |
|   |         |        |

| MOLSTRUCTURE                                                                        | M CELL | M DATA |
|-------------------------------------------------------------------------------------|--------|--------|
|   |        |        |
|   |        |        |
|  |        |        |

[0057] The compounds described herein may interact with regions of nucleic acids that can form quadruplexes. Because regions of DNA that can form quadruplexes are regulators of biological processes such as oncogene transcription, modulators of quadruplex biological activity can be utilized as cancer therapeutics. Molecules that interact with regions of DNA that can form quadruplexes can exert a therapeutic effect on certain cell proliferative disorders and related conditions. Particularly, abnormally increased oncogene expression can cause cell proliferative disorders, and quadruplex structures typically down-regulate oncogene expression. Examples of oncogenes include but are not limited to MYC, HIF, VEGF, ABL, TGF, PDGFA, MYB, SPARC, HUMTEL, HER, VAV, RET, H-RAS, EGF, SRC, BCL1, BCL2, DHFR, HMGA, and other oncogenes known to one of skill in the art. Furthermore, the compounds described herein may induce cell death (*e.g.*, apoptosis) and not interact with regions of DNA that can form quadruplexes.

[0058] Molecules that bind to regions of DNA that can form quadruplexes can exert a biological effect according to different mechanisms, which include for example, stabilizing a native quadruplex structure, inhibiting conversion of a native quadruplex to duplex DNA by blocking strand cleavage, and stabilizing a native quadruplex structure having a

quadruplex-destabilizing nucleotide substitution and other sequence specific interactions. Thus, compounds that bind to regions of DNA that can form quadruplexes described herein may be administered to cells, tissues, or organisms for the purpose of down-regulating oncogene transcription and thereby treating cell proliferative disorders.

[0059] Determining whether the biological activity of native DNA that can form quadruplexes is modulated in a cell, tissue, or organism can be accomplished by monitoring quadruplex biological activity. Quadruplex forming regions of DNA biological activity may be monitored in cells, tissues, or organisms, for example, by detecting a decrease or increase of gene transcription in response to contacting the quadruplex forming DNA with a molecule. Transcription can be detected by directly observing RNA transcripts or observing polypeptides translated by transcripts, which are methods well known in the art.

[0060] Molecules that interact with quadruplex forming DNA and quadruplex forming nucleic acids can be utilized to treat many cell proliferative disorders. Cell proliferative disorders include, for example, colorectal cancers and hematopoietic neoplastic disorders (*i.e.*, diseases involving hyperplastic/neoplastic cells of hematopoietic origin such as those arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof). The diseases can arise from poorly differentiated acute leukemias, *e.g.*, erythroblastic leukemia and acute megakaryoblastic leukemia. Additional myeloid disorders include, but are not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (Vaickus, Crit. Rev. in *Oncol./Hematol.* 11:267-297 (1991)). Lymphoid malignancies include, but are not limited to acute lymphoblastic leukemia (ALL), which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas include, but are not limited to non-Hodgkin lymphoma and variants thereof, peripheral T cell lymphomas, adult T cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF), Hodgkin's disease and Reed-Sternberg disease. Cell proliferative disorders also include cancers of the colorectum, breast, lung, liver, pancreas, lymph node, colon, prostate, brain, head and neck, skin, liver, kidney, and heart. Compounds that interact with regions of DNA that may form quadruplexes also can be utilized to target cancer related processes and conditions, such as increased angiogenesis, by inhibiting angiogenesis in a subject.

[0061] The present invention provides a method for reducing cell proliferation or for treating or alleviating cell proliferative disorders, comprising contacting a system having a native DNA capable of forming a quadruplex region with a compound having any one of the above formula. The system may be a group of cells or one or more tissues. In one embodiment, the system is a subject in need of a treatment of a cell proliferative disorder (*e.g.*, a mammal such as a mouse, rat, monkey, or human). The present invention also provides a method for treating colorectal cancer by administering a compound that interacts with a c-MYC quadruplex forming region to a subject in need thereof, thereby reducing the colorectal cancer cell proliferation. Furthermore, the present invention provides a method for inhibiting angiogenesis and optionally treating a cancer associated with angiogenesis, comprising administering a compound that interacts with a vascular endothelial growth factor (VEGF) quadruplex forming region to a subject in need thereof, thereby reducing angiogenesis and optionally treating a cancer associated with angiogenesis.

[0062] Compounds that interact with quadruplex forming regions of DNA can also be used to reduce a microbial infection, such as a viral infection. Retroviruses offer a wealth of potential targets for G-quadruplex targeted therapeutics. G-quadruplex structures have been implicated as functional elements in at least two secondary structures formed by either viral RNA or DNA in HIV, the dimer linker structure (DLS) and the central DNA flap (CDF). Additionally, DNA aptamers which are able to adopt either inter- or intramolecular quadruplex structures are able to inhibit viral replication. In one example, DNA aptamers are able to inhibit viral replication by targeting the envelope glycoprotein (putatively). In another example, DNA aptamers inhibit viral replication by targeting the HIV-integrase respectively, suggesting the involvement of native quadruplex structures in interaction with the integrase enzyme.

[0063] Dimer linker structures, which are common to all retroviruses, serve to bind two copies of the viral genome together by a non-covalent interaction between the two 5' ends of the two viral RNA sequences. The genomic dimer is stably associated with the gag protein in the mature virus particle. In the case of HIV, the origin of this non-covalent binding may be traced to a 98 base-pair sequence containing several runs of at least two consecutive guanines (*e.g.*, the 3' for the formation of RNA dimers *in vitro*). An observed cation (potassium) dependence for the formation and stability of the dimer *in vitro*, in addition to the failure of an antisense sequence to effectively dimerize, has revealed the most likely binding structure to be an intermolecular G-quadruplex.

[0064] Prior to integration into the host genome, reverse transcribed viral DNA forms a pre-integration complex (PIC) with at least two major viral proteins, integrase and reverse transcriptase, which is subsequently transported into the nucleus. The Central DNA Flap (CDF) refers to 99-base length single-stranded tail of the + strand, occurring near the center of the viral duplex DNA, which is known to play a role in the nuclear import of the PIC. Oligonucleotide mimics of the CDF have been shown to form intermolecular G-quadruplex structures in cell-free systems.

[0065] Thus, compounds that recognize quadruplex forming regions can be used to stabilize the dimer linker structure and thus prevent de-coupling of the two RNA strands. Also, by binding to the quadruplex structure formed by the CDF, protein recognition and/or binding events for nuclear transport of the PIC may be disrupted. In either case, a substantial advantage can exist over other anti-viral therapeutics. Current Highly Active Anti-Retroviral Therapeutic (HAART) regimes rely on the use of combinations of drugs targeted towards the HIV protease and HIV integrase. The requirement for multi-drug regimes is to minimize the emergence of resistance, which will usually develop rapidly when agents are used in isolation. The source of such rapid resistance is the infidelity of the reverse transcriptase enzyme which makes a mutation approximately once in every 10,000 base pairs. An advantage of targeting viral quadruplex structures over protein targets, is that the development of resistance is slow or is impossible. A point mutation of the target quadruplex can compromise the integrity of the quadruplex structure and lead to a non-functional copy of the virus. A single therapeutic agent based on this concept may replace the multiple drug regimes currently employed, with the concomitant benefits of reduced costs and the elimination of harmful drug/drug interactions.

[0066] The present invention provides a method for reducing a microbial titer in a system, comprising contacting a system having a native DNA quadruplex forming region with a compound having any one of the above formula. The system may be one or more cells or tissues. Examples of microbial titers include but are not limited to viral, bacterial or fungal titers. In a particular embodiment, the system is a subject in need of a treatment for a viral infection (*e.g.*, a mammal such as a mouse, rat, monkey, or human). Examples of viral infections include infections by a hepatitis virus (*e.g.*, hepatitis B or C), human immunodeficiency virus (HIV), rhinovirus, herpes-zoster virus (VZV), herpes simplex virus (*e.g.*, HSV-1 or HSV-2), cytomegalovirus (CMV), vaccinia virus, influenza virus, encephalitis virus, hantavirus, arbovirus, West Nile virus, human papilloma virus (HPV), Epstein-Barr virus, and respiratory syncytial virus. The present invention also provides a method for

treating HIV infection by administering a compound having any one of the above formula to a subject in need thereof, thereby reducing the HIV infection.

Identifying compounds that can bind to quadruplex forming regions of DNA

[0067] Compounds described herein may bind to quadruplex forming regions of DNA where a biological activity of this region, often expressed as a “signal,” produced in a system containing the compound is different than the signal produced in a system not containing the compound. While background signals may be assessed each time a new molecule is probed by the assay, detecting the background signal is not required each time a new molecule is assayed.

[0068] Examples of quadruplex forming nucleotide sequences are set forth in the following Table 2:

| <u>SEQUENCE</u>                                                                                                                               | <u>SEQ<br/>ID NO</u> | <u>ORIGIN</u>      |
|-----------------------------------------------------------------------------------------------------------------------------------------------|----------------------|--------------------|
| TG <sub>4</sub> AG <sub>3</sub> TG <sub>4</sub> AG <sub>3</sub> TG <sub>4</sub> AAGG                                                          | 1                    | CMYC               |
| GGGGGGGGGGGGGGCGGGGGCGGGGGCGGGGGAGGGGC                                                                                                        | 2                    | PDGFA              |
| G <sub>8</sub> ACGCG <sub>3</sub> AGCTG <sub>5</sub> AG <sub>3</sub> CTTG <sub>4</sub> CCAG <sub>3</sub> CG <sub>4</sub> CGCTTAG <sub>5</sub> | 3                    | PDGFB/c<br>-sis    |
| AGGAAGGGGAGGGCCGGGGGGAGGTGGC                                                                                                                  | 4                    | CABL               |
| AGGGGCGGGGCGGGGCGGGGGC                                                                                                                        | 5                    | RET                |
| AG <sub>4</sub> CG <sub>3</sub> CGCGGGAGGAAGGGGGCGGGAGCGGGGCTG                                                                                | 6                    | BCL-2              |
| GGGGGGCGGGGGCGGGCGCAGGGGGAGGGGGC                                                                                                              | 7                    | Cyclin<br>D1/BCL-1 |
| CGGGGCGGGGCGGGGGCGGGGGC                                                                                                                       | 8                    | H-RAS              |
| AGAGGAGGAGGAGGTCACGGAGGAGGAGGAGAAGGAGGAGGAGGAA<br>or<br>AGAGGAGGAGGAGGACACGGAGGAGGAGGAGAAGGAGGAGGAGGAA                                        | 9                    | CMYB               |
| (GGA) <sub>4</sub>                                                                                                                            | 10                   | VAV                |
| AGAGAAGAGGGGAGGAGGAGGAGGAGAGGAGGAGGAGGCGC                                                                                                     | 11                   | HMGA2              |
| GGAGGGGGAGGGG                                                                                                                                 | 12                   | CPIM               |
| AGGAGAAGGAGGAGGTGGAGGAGGAGG                                                                                                                   | 13                   | HER2/neu           |
| AGGAGGAGGAGAATGCGAGGAGGAGGGAGGAGA                                                                                                             | 14                   | EGFR               |
| GGGGCGGGCCGGGGGGCGGGGTCCCGGCGGGGCGGAG                                                                                                         | 15                   | VEGF               |
| CGGGAGGAGGAGGAAGGAGGAAGCGCG                                                                                                                   | 16                   | CSRC               |

[0069] In addition to determining whether a test molecule or test nucleic acid gives rise to a different signal, the affinity of the interaction between the nucleic acid and the compound

may be quantified.  $IC_{50}$ ,  $K_d$ , or  $K_i$  threshold values may be compared to the measured  $IC_{50}$  or  $K_d$  values for each interaction, and thereby identify a test molecule as a quadruplex interacting molecule or a test nucleic acid as a quadruplex forming nucleic acid. For example,  $IC_{50}$  or  $K_d$  threshold values of 10  $\mu$ M or less, 1  $\mu$ M or less, and 100 nM or less are often utilized. In another example, threshold values of 10 nM or less, 1 nM or less, 100 pM or less, and 10 pM or less may be utilized to identify quadruplex interacting molecules and quadruplex forming nucleic acids.

[0070] Many assays are available for identifying compounds that have affinity for quadruplex forming regions of DNA. In some of these assays, the biological activity is the quadruplex nucleic acid binding to a compound and binding is measured as a signal. In other assays, the biological activity is a polymerase arresting function of a quadruplex and the degree of arrest is measured as a decrease in a signal. In certain assays, the biological activity is transcription and transcription levels can be quantified as a signal. In another assay, the biological activity is cell death and the number of cells undergoing cell death is quantified. Another assay monitors proliferation rates of cancer cells. Examples of assays are fluorescence binding assays, gel mobility shift assays (see, *e.g.*, Jin & Pike, *Mol. Endocrinol.* (1996) 10:196-205), polymerase arrest assays, transcription reporter assays, cancer cell proliferation assays, and apoptosis assays (see, *e.g.*, Amersham Biosciences (Piscataway, New Jersey)), and embodiments of such assays are described hereafter. Also, topoisomerase assays can be utilized to determine whether the quadruplex interacting molecules have a topoisomerase pathway activity (see, *e.g.*, TopoGEN, Inc. (Columbus, Ohio)).

#### Gel Electrophoretic Mobility Shift Assay (EMSA)

[0071] An EMSA is useful for determining whether a nucleic acid forms a quadruplex and whether a nucleotide sequence is quadruplex-destabilizing. EMSA is conducted as described previously (Jin & Pike, *Mol. Endocrinol.* 10: 196-205 (1996)) with minor modifications. Generally, synthetic single-stranded oligonucleotides are labeled in the 5' -terminus with T4-kinase in the presence of [ $\gamma$ - $^{32}$ P] ATP (1,000 mCi/mmol, Amersham Life Science) and purified through a sephadex column.  $^{32}$ P-labeled oligonucleotides (~30,000 cpm) are then incubated with or without various concentrations of a testing compound in 20  $\mu$ l of a buffer containing 10 mM Tris pH 7.5, 100 mM KCl, 5 mM dithiothreitol, 0.1 mM EDTA, 5 mM  $MgCl_2$ , 10% glycerol, 0.05% Nonedit P-40, and 0.1 mg/ml of poly(dI-dC) (Pharmacia). After incubation for 20 minutes at room temperature, binding reactions are loaded on a 5%

polyacrylamide gel in 0.25 x Tris borate-EDTA buffer (0.25 x TBE, 1 x TBE is 89 mM Tris-borate, pH 8.0, 1 mM EDTA). The gel is dried and each band is quantified using a phosphoimager.

#### DMS Methylation Protection Assay

[0072] Chemical footprinting assays are useful for assessing quadruplex structure. Quadruplex structure is assessed by determining which nucleotides in a nucleic acid are protected or unprotected from chemical modification as a result of being inaccessible or accessible, respectively, to the modifying reagent. A DMS methylation assay is an example of a chemical footprinting assay. In such an assay, bands from EMSA are isolated and subjected to DMS-induced strand cleavage. Each band of interest is excised from an electrophoretic mobility shift gel and soaked in 100 mM KCl solution (300  $\mu$ l) for 6 hours at 4°C. The solutions are filtered (microcentrifuge) and 30,000 cpm (per reaction) of DNA solution is diluted further with 100 mM KCl in 0.1X TE to a total volume of 70  $\mu$ l (per reaction). Following the addition of 1  $\mu$ l salmon sperm DNA (0.1  $\mu$ g/ $\mu$ l), the reaction mixture is incubated with 1  $\mu$ l DMS solution (DMS:ethanol; 4:1; v:v) for a period of time. Each reaction is quenched with 18  $\mu$ l of stop buffer (b-mercaptoethanol:water:NaOAc (3 M); 1:6:7; v:v:v). Following ethanol precipitation (twice) and piperidine cleavage, the reactions are separated on a preparative gel (16%) and visualized on a phosphoimager.

#### Polymerase Arrest Assay

[0073] An arrest assay includes a template nucleic acid, which may comprise a quadruplex forming sequence, and a primer nucleic acid which hybridizes to the template nucleic acid 5' of the quadruplex-forming sequence. The primer is extended by a polymerase (*e.g.*, Taq polymerase), which advances from the primer along the template nucleic acid. In this assay, a quadruplex structure can block or arrest the advance of the enzyme, leading to shorter transcription fragments. Also, the arrest assay may be conducted at a variety of temperatures, including 45°C and 60°C, and at a variety of ion concentrations.

[0074] An example of the Taq polymerase stop assay is described in Han, *et al.*, *Nucl. Acids Res.* (1999) 27:537-542, which is a modification of that used by Weitzmann, *et al.*, *J. Biol. Chem.* (1996) 271:20958–20964. Briefly, a reaction mixture of template DNA (50 nM), Tris·HCl (50 mM), MgCl<sub>2</sub> (10 mM), DTT (0.5 mM), EDTA (0.1 mM), BSA (60 ng), and 5'-end-labeled quadruplex nucleic acid (~18 nM) is heated to 90°C for 5 minutes and

allowed to cool to ambient temperature over 30 minutes. Taq Polymerase (1  $\mu$ l) is added to the reaction mixture, and the reaction is maintained at a constant temperature for 30 minutes. Following the addition of 10  $\mu$ l stop buffer (formamide (20 ml), 1 M NaOH (200  $\mu$ l), 0.5 M EDTA (400  $\mu$ l), and 10 mg bromophenol blue), the reactions are separated on a preparative gel (12%) and visualized on a phosphorimager. Adenine sequencing (indicated by "A" at the top of the gel) is performed using double-stranded DNA Cycle Sequencing System from Life Technologies. The general sequence for the template strands is TCCAACCTATGTATAC-INSERT-TTAGCGACACGCAATTGCTATAGTGAGTCGTATTA, where "INSERT" refers to a nucleic acid sequence comprising a quadruplex forming sequence (See *e.g.*, Table 2). Bands on the gel that exhibit slower mobility are indicative of quadruplex formation.

#### High Throughput Polymerase Arrest Assay

[0075] A high throughput polymerase arrest assay has been developed. The assay comprises contacting a template nucleic acid, often DNA, with a primer, which also is often DNA; contacting the primer/template complex with a compound described herein (also referred to as a "test compound"); contacting the primer/template complex with a polymerase; and separating reaction products. The assay often includes the step of denaturing the primer/template complex mixture and then renaturing the complex, which often is carried out before a test molecule is added to the system. Multiple assays often are carried out using varying concentrations of a test compound, such that an IC<sub>50</sub> value can be obtained, for example. The reaction products often include extended primers of different lengths. Where a test compound does not significantly interact with a quadruplex structure in the template, the primer often is extended to the end of the template.

[0076] Where a test compound significantly interacts with a quadruplex structure in the template, the primer often is extended only to the quadruplex structure in the template and no further. Thus, the reaction mixture often includes at least two reaction products when a test compound interacts with a quadruplex structure in the template, one having a completely extended primer and one having an incompletely extended primer, and these two reaction products are separated. The products may be separated using any convenient separation method, such as mass spectrometry and in one embodiment, capillary electrophoresis.

[0077] The reaction products often are identified by detecting a detectable label linked to the primer. The detectable label may be non-covalently linked to the 5' end of the primer (*e.g.*, a biotin molecule covalently linked to the 5' end of the primer which is non-covalently linked

to an avidin molecule joined to a detectable label). The detectable label may be joined to the primer at any stage of the assay, sometimes before the primer is added to the system, after the primer is extended, or after the products are separated. The detectable label often is covalently linked to the primer using a procedure selected based upon the nature of the chemical groups in the detectable label.

**[0078]** Many methods for covalently linking detectable labels to nucleic acids are available, such as chemically coupling an allylamine-derivatized nucleotide to a succinimidyl-ester derivative of a detectable label, and then generating a primer using the labeled nucleotide. (See, *e.g.*, *Nature Biotech* (2000) 18:345-348 and [http address info.med.yale.edu/genetics/ward/tavi/n\\_coupling.html](http://info.med.yale.edu/genetics/ward/tavi/n_coupling.html)). A spacer (often between 5-16 carbon atoms long) sometimes is incorporated between the detectable label and the nucleotide. Any convenient detectable label may be utilized, including but not limited to a radioactive isotope (*e.g.*,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{14}\text{C}$  or  $^3\text{H}$ ); a light scattering label (*e.g.*, a spherical gold or silver label; Genicon Sciences Corporation, San Diego, CA and U.S. Patent No. 6,214,560); an enzymic or protein label (*e.g.*, GFP or peroxidase); or another chromogenic label or dye sometimes is utilized. Often, a fluorescent label is utilized (*e.g.*, amino-methyl coumarin (AMCA); diethyl aminomethyl coumarin (DEAC); cascade blue (CB); fluorescein isothiocyanate (FITC); Oregon green (OG); Alexa 488 (A488); rhodamine green (RGr); lanthanide chelate (*e.g.*, europium), carboxy-rhodamine 6G (R6G); tetramethyl rhodamine (TAMRA); Texas Red (TxR); Cy3; Cy3.5; Cy5, Cy5.5 and carboxynaphthofluorescein (CNF), digoxigenin (DIG); and 2,4-dinitrophenyl (DNP)). Other fluorophores and attendant excitation and emission wavelengths are described in Anantha, *et al.*, *Biochemistry* (1998) 37:2709-2714 and Qu & Chaires, *Methods Enzymol* (2000) 321:353-369).

**[0079]** In an embodiment, a primer oligonucleotide covalently linked to a fluorescent label is contacted with template DNA. The resulting complex is contacted with a test molecule and then contacted with a polymerase capable of extending the primer. The reaction products then are separated and detected by capillary electrophoresis. A longer primer sequence was used for practicing this embodiment as compared to embodiments where the primer includes no covalently-linked fluorophore or where capillary electrophoresis is not utilized for separation. Deoxynucleotides are added at any stage of the assay before the separation, often when the primer is contacted with the template DNA. The template DNA/primer complex often is denatured (*e.g.*, by increasing the temperature of the system) and then renatured (*e.g.*, by cooling the system) before a test compound is added).

### Quadruplex Binding Assay

[0080] Generally, a 5'-fluorescent-labeled (FAM) primer (P45, 15 nM) was mixed with template DNA (15 nM) in a Tris-HCL buffer (15 mM Tris, pH 7.5) containing 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA and 0.1 mM mixed deoxynucleotide triphosphates (dNTP's). In one example, the FAM-P45 primer

(5'-6FAM-AGTCTGACTGACTGTACGTAGCTAATACGACTCACTATAG CAATT-3') (SEQ ID NO. 17) and the c-Myc template DNA (5'-TCCAACCTATGTATACTGGGG AGGGTGGGGAGGGTGGGGAAGGTTAGCGACACGCAATTGCTATAGTGAGTCGTAT TAGCTACGTACAGTCAGTCAGACT-3') (SEQ ID NO. 18) were synthesized and HPLC purified by Applied Biosystems. The mixture was denatured at 95°C for 5 minutes and, after cooling down to room temperature, was incubated at 37°C for 15 minutes.

[0081] After cooling down to room temperature, 1 mM KCl<sub>2</sub> and the test compound (various concentrations) were added and the mixture incubated for 15 minutes at room temperature. The primer extension was performed by adding 10 mM KCl and Taq DNA Polymerase (2.5 U/reaction, Promega) and incubating at 70°C for 30 minutes. The reaction was stopped by adding 1 µl of the reaction mixture to 10 µl Hi-Di Formamide mixed and 0.25 µl LIZ120 size standard. Hi-Di Formamide and LIZ120 size standard were purchased from Applied Biosystems. The partially extended quadruplex arrest product was between 61 or 62 bases long and the full-length extended product was 99 bases long. The products were separated and analyzed using capillary electrophoresis. Capillary electrophoresis was performed using an ABI PRISM™ 3100-Avant Genetic Analyzer. The assay was performed using compounds described above and results are shown in Table 1. µM concentrations reported in Table 1 are concentrations at which 50% of the DNA was arrested in the assay (*i.e.*, the ratio of shorter partially extended DNA (arrested DNA) to full-length extended DNA is 1:1).

### Transcription Reporter Assay

[0082] In a transcription reporter assay, test quadruplex DNA is coupled to a reporter system, such that a formation or stabilization of a quadruplex structure can modulate a reporter signal. An example of such a system is a reporter expression system in which a polypeptide, such as luciferase or green fluorescent protein (GFP), is expressed by a gene operably linked to the potential quadruplex forming nucleic acid and expression of the polypeptide can be detected. As used herein, the term "operably linked" refers to a nucleotide sequence which is

regulated by a sequence comprising the potential quadruplex forming nucleic acid. A sequence may be operably linked when it is on the same nucleic acid as the quadruplex DNA, or on a different nucleic acid. An exemplary luciferase reporter system is described herein.

[0083] A luciferase promoter assay described in He, *et al.*, *Science* (1998) 281:1509-1512 often is utilized for the study of quadruplex formation. Specifically, a vector utilized for the assay is set forth in reference 11 of the He, *et al.*, document. In this assay, HeLa cells are transfected using the lipofectamin 2000-based system (Invitrogen) according to the manufacturer's protocol, using 0.1 µg of pRL-TK (Renilla luciferase reporter plasmid) and 0.9 µg of the quadruplex-forming plasmid. Firefly and Renilla luciferase activities are assayed using the Dual Luciferase Reporter Assay System (Promega) in a 96-well plate format according to the manufacturer's protocol.

#### Circular Dichroism Assay

[0084] Circular dichroism (CD) is utilized to determine whether another molecule interacts with a quadruplex nucleic acid. CD is particularly useful for determining whether a PNA or PNA-peptide conjugate hybridizes with a quadruplex nucleic acid *in vitro*. PNA probes are added to quadruplex DNA (5 µM each) in a buffer containing 10 mM potassium phosphate (pH 7.2) and 10 or 250 mM KCl at 37°C and then allowed to stand for 5 minutes at the same temperature before recording spectra. CD spectra are recorded on a Jasco J- 715 spectropolarimeter equipped with a thermoelectrically controlled single cell holder. CD intensity normally is detected between 220 nm and 320 nm and comparative spectra for quadruplex DNA alone, PNA alone, and quadruplex DNA with PNA are generated to determine the presence or absence of an interaction (see, *e.g.*, Datta, *et al.*, *JACS* (2001) 123:9612-9619). Spectra are arranged to represent the average of eight scans recorded at 100 nm/min.

#### Fluorescence Binding Assay

[0085] An example of a fluorescence binding assay is a system that includes a quadruplex nucleic acid, a signal molecule, and a test molecule. The signal molecule generates a fluorescent signal when bound to the quadruplex nucleic acid (*e.g.*, N-methylmesoporphyrin IX (NMM)), and the signal is altered when a test compound competes with the signal molecule for binding to the quadruplex nucleic acid. An alteration in the signal when test molecule is

present as compared to when test compound is not present identifies the test compound as a quadruplex interacting compound.

[0086] 50  $\mu$ l of quadruplex nucleic acid or a nucleic acid not capable of forming a quadruplex is added in 96-well plate. A test compound also is added in varying concentrations. A typical assay is carried out in 100  $\mu$ l of 20 mM HEPES buffer, pH 7.0, 140 mM NaCl, and 100 mM KCl. 50  $\mu$ l of the signal molecule NMM then is added for a final concentration of 3  $\mu$ M. NMM is obtained from Frontier Scientific Inc, Logan, Utah. Fluorescence is measured at an excitation wavelength of 420 nm and an emission wavelength of 660 nm using a FluroStar 2000™ fluorometer (BMG Labtechnologies, Durham, NC). Fluorescence often is plotted as a function of concentration of the test compound or quadruplex-targeted nucleic acid and maximum fluorescent signals for NMM are assessed in the absence of these molecules.

#### Cell Proliferation Assay

[0087] In a cancer cell proliferation assay, cell proliferation rates are assessed as a function of different concentrations of test compounds added to the cell culture medium. Any cancer cell type can be utilized in the assay. In one embodiment, colon cancer cells are cultured *in vitro* and test compounds are added to the culture medium at varying concentrations. A useful colon cancer cell line is colo320, which is a colon adenocarcinoma cell line deposited with the National Institutes of Health as accession number JCRB0225. Parameters for using such cells are available at the http address [cellbank.nihs.go.jp/cell/data/jcrb0225.htm](http://cellbank.nihs.go.jp/cell/data/jcrb0225.htm).

#### Formulation of Compounds

[0088] As used herein, the term "pharmaceutically acceptable salts, esters and amides" includes but are not limited to carboxylate salts, amino acid addition salts, esters and amides of the compounds, as well as the zwitterionic forms thereof, which are known to those skilled in the art as suitable for use with humans and animals. (See, *e.g.*, Gerge, S.M., *et al.*, "Pharmaceutical Salts," *J. Pharm. Sci.* (1977) 66:1-19).

[0089] Any suitable formulation of the compounds described herein can be prepared. In cases where compounds are sufficiently basic or acidic to form stable nontoxic acid or base salts, administration of the compounds as salts may be appropriate. Examples of pharmaceutically acceptable salts are organic acid addition salts formed with acids that form a

physiological acceptable anion, for example, tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate,  $\alpha$ -ketoglutarate, and  $\alpha$ -glycerophosphate. Suitable inorganic salts may also be formed, including hydrochloride, sulfate, nitrate, bicarbonate, and carbonate salts. Pharmaceutically acceptable salts are obtained using standard procedures well known in the art. For example, pharmaceutically acceptable salts may be obtained by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion. Alkali metal (*e.g.*, sodium, potassium or lithium) or alkaline earth metal (*e.g.*, calcium) salts of carboxylic acids also are made.

[0090] A compound may be formulated as a pharmaceutical composition and administered to a mammalian host in need of such treatment. In one embodiment, the mammalian host is human. Any suitable route of administration may be used, including but not limited to oral, parenteral, intravenous, intramuscular, topical and subcutaneous routes.

[0091] In one embodiment, a compound is administered systemically (*e.g.*, orally) in combination with a pharmaceutically acceptable vehicle such as an inert diluent or an assimilable edible carrier. They may be enclosed in hard or soft shell gelatin capsules, compressed into tablets, or incorporated directly with the food of the patient's diet. For oral therapeutic administration, the active compound may be combined with one or more excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compositions and preparations may be varied and may conveniently be between about 2 to about 60% of the weight of a given unit dosage form. The amount of active compound in such therapeutically useful compositions is such that an effective dosage level will be obtained.

[0092] Tablets, troches, pills, capsules, and the like also may contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring may be added. When the unit dosage form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac or sugar and the like. A syrup or elixir may contain the active compound, sucrose

or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Any material used in preparing any unit dosage form is pharmaceutically acceptable and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and devices.

[0093] The active compound also may be administered intravenously or intraperitoneally by infusion or injection. Solutions of the active compound or its salts may be prepared in a buffered solution, often phosphate buffered saline, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The compound is sometimes prepared as a polymatrix-containing formulation for such administration (*e.g.*, a liposome or microsome). Liposomes are described for example in U.S. Patent No. 5,703,055 (Felgner, *et al.*) and Gregoriadis, Liposome Technology vols. I to III (2nd ed. 1993).

[0094] The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient that are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. In all cases, the ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the particle size in the case of dispersions or by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0095] Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the

preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

[0096] For topical administration, the present compounds may be applied in liquid form. Compounds often are administered as compositions or formulations, in combination with a dermatologically acceptable carrier, which may be a solid or a liquid. Examples of useful dermatological compositions used to deliver compounds to the skin are known (see, *e.g.*, Jacquet, *et al.* (U.S. Pat. No. 4,608,392), Geria (U.S. Pat. No. 4,992,478), Smith, *et al.* (U.S. Pat. No. 4,559,157) and Wortzman (U.S. Pat. No. 4,820,508).

[0097] Compounds may be formulated with a solid carrier, which include finely divided solids such as talc, clay, microcrystalline cellulose, silica, alumina and the like. Useful liquid carriers include water, alcohols or glycols or water-alcohol/glycol blends, in which the present compounds can be dissolved or dispersed at effective levels, optionally with the aid of non-toxic surfactants. Adjuvants such as fragrances and additional antimicrobial agents can be added to optimize the properties for a given use. The resultant liquid compositions can be applied from absorbent pads, used to impregnate bandages and other dressings, or sprayed onto the affected area using pump-type or aerosol sprayers. Thickeners such as synthetic polymers, fatty acids, fatty acid salts and esters, fatty alcohols, modified celluloses or modified mineral materials can also be employed with liquid carriers to form spreadable pastes, gels, ointments, soaps, and the like, for application directly to the skin of the user.

[0098] Generally, the concentration of the compound in a liquid composition often is from about 0.1 wt% to about 25 wt%, sometimes from about 0.5 wt% to about 10 wt%. The concentration in a semi-solid or solid composition such as a gel or a powder often is about 0.1 wt% to about 5 wt%, sometimes about 0.5 wt% to about 2.5 wt%. A compound composition may be prepared as a unit dosage form, which is prepared according to conventional techniques known in the pharmaceutical industry. In general terms, such techniques include bringing a compound into association with pharmaceutical carrier(s) and/or excipient(s) in liquid form or finely divided solid form, or both, and then shaping the product if required.

[0099] Table 3 shows examples of formulations for use with compounds described herein. For example, a compound may be formulated having dosages from 10 mg/mL to 20 mg/mL solution, using the formulations herein. In Table 3, the designation "D5W" refers to deionized water with 5% dextrose. Each component in each formulation may be varied without affecting

the activity of the compound. In one example, the compound is formulated in a solution comprising polyethylene glycol and propylene glycol in a buffer solution such as a phosphate buffer.

**Table 3**

|    | Formulations                        | %<br>(w/w) | Compound (mL)<br>+ Placebo<br>solution (mL) | pH of the<br>Placebo<br>solution | pH of the<br>formulated<br>solution<br>(10mg/mL) |
|----|-------------------------------------|------------|---------------------------------------------|----------------------------------|--------------------------------------------------|
| 1. | Mannitol                            | 4          | 35ml + 35mL                                 | 6.1                              | 6.1                                              |
|    | Sucrose                             | 0.5        |                                             |                                  |                                                  |
|    | 5% D5W solution                     | 95.5       |                                             |                                  |                                                  |
| 2. | Mannitol                            | 4          | 35ml + 35mL                                 | 6                                | 5.8                                              |
|    | 50mM PO <sub>4</sub> buffer, pH=6.0 | 96         |                                             |                                  |                                                  |
| 3. | Mannitol                            | 4          | 35ml + 35mL                                 | 5                                | 5                                                |
|    | 50mM Citrate buffer, pH=5.0         | 96         |                                             |                                  |                                                  |
| 4. | Mannitol                            | 4          | 35ml + 35mL                                 | 6                                | 6                                                |
|    | 5% D5W                              | 96         |                                             |                                  |                                                  |
| 5. | Test compound (20mg/mL)             | 1          | 35ml + 35mL                                 | 6.4                              | 6.1                                              |
|    | 5% D5W                              | 99         |                                             |                                  |                                                  |
| 6. | PEG 300                             | 7          | 5ml + 5mL                                   | N/A                              | 5.80                                             |
|    | Propylene glycol                    | 9          |                                             |                                  |                                                  |
|    | 5% D5W                              | 84         |                                             |                                  |                                                  |
| 7. | PEG 300                             | 7          | 5ml + 5mL                                   | N/A                              | 5.8                                              |
|    | Propylene glycol                    | 9          |                                             |                                  |                                                  |
|    | 50mM PO <sub>4</sub> buffer, pH=6.0 | 84         |                                             |                                  |                                                  |
| 8. | Mannitol                            | 4          | 5ml + 5mL                                   | N/A                              | 5.7                                              |
|    | PEG 300                             | 20         |                                             |                                  |                                                  |
|    | 50mM PO <sub>4</sub> buffer, pH=6.0 | 76         |                                             |                                  |                                                  |
| 9. | Mannitol                            | 4          | 5ml + 5mL                                   | N/A                              | 5.8                                              |
|    | Propylene glycol                    | 10         |                                             |                                  |                                                  |
|    | 50mM PO <sub>4</sub> buffer, pH=6.0 | 86         |                                             |                                  |                                                  |

[0100] The compound composition may be formulated into any dosage form, such as tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The

compositions also may be formulated as suspensions in aqueous, non-aqueous, or mixed media. Aqueous suspensions may further contain substances which increase viscosity, including for example, sodium carboxymethylcellulose, sorbitol, and/or dextran. The suspension may also contain one or more stabilizers. The amount of the compound, or an active salt or derivative thereof, required for use in treatment will vary not only with the particular salt selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient and will be ultimately at the discretion of the attendant physician or clinician.

### Dosages

[0101] A useful compound dosage often is determined by assessing its *in vitro* activity in a cell or tissue system and/or *in vivo* activity in an animal system. For example, methods for extrapolating an effective dosage in mice and other animals to humans are known to the art (see, *e.g.*, U.S. Pat. No. 4,938,949). Such systems can be used for determining the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population) of a compound. The dose ratio between a toxic and therapeutic effect is the therapeutic index and it can be expressed as the ratio ED<sub>50</sub>/LD<sub>50</sub>. The compound dosage often lies within a range of circulating concentrations for which the ED<sub>50</sub> is associated with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compounds used in the methods described herein, the therapeutically effective dose can be estimated initially from cell culture assays. A dose sometimes is formulated to achieve a circulating plasma concentration range covering the IC<sub>50</sub> (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in *in vitro* assays, as such information often is used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

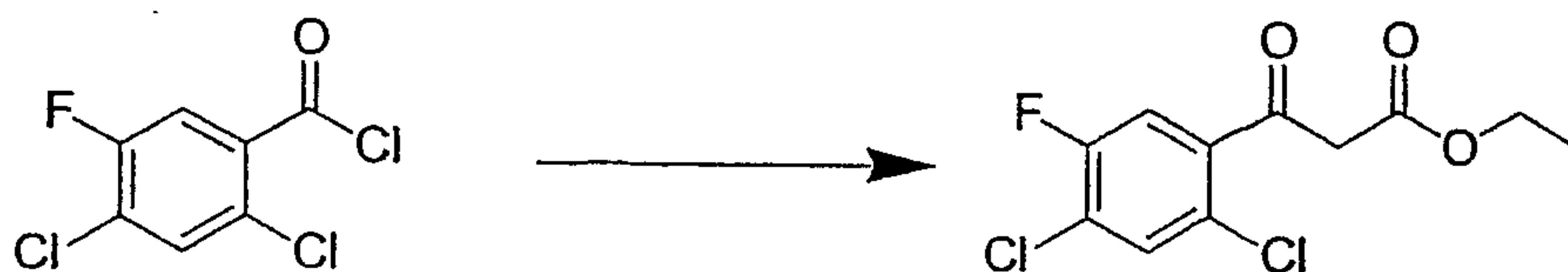
[0102] Another example of effective dose determination for a subject is the ability to directly assay levels of “free” and “bound” compound in the serum of the test subject. Such assays may utilize antibody mimics and/or “biosensors” generated by molecular imprinting techniques. The compound is used as a template, or “imprinting molecule”, to spatially organize polymerizable monomers prior to their polymerization with catalytic reagents. Subsequent removal of the imprinted molecule leaves a polymer matrix which contains a repeated “negative image” of the compound and is able to selectively rebind the molecule

under biological assay conditions (see, *e.g.*, Ansell, *et al.*, *Current Opinion in Biotechnology* (1996) 7:89-94 and in Shea, *Trends in Polymer Science* (1994) 2:166-173).

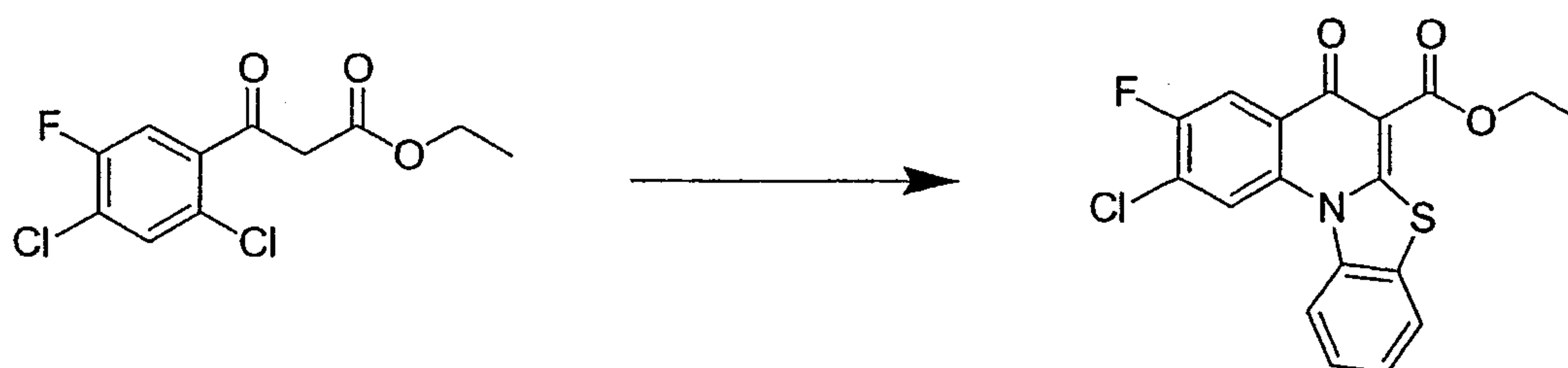
[0103] Such “imprinted” affinity matrixes are amenable to ligand-binding assays, whereby the immobilized monoclonal antibody component is replaced by an appropriately imprinted matrix (see, *e.g.*, Vlatakis, *et al.*, *Nature* (1993) 361:645-647). Through the use of isotope-labeling, “free” concentration of compound can be readily monitored and used in calculations of  $IC_{50}$ . Such “imprinted” affinity matrixes can also be designed to include fluorescent groups whose photon-emitting properties measurably change upon local and selective binding of compound. These changes can be readily assayed in real time using appropriate fiberoptic devices, in turn allowing the dose in a test subject to be quickly optimized based on its individual  $IC_{50}$ . An example of such a “biosensor” is discussed in Kriz, *et al.*, *Analytical Chemistry* (1995) 67:2142-2144.

[0104] Exemplary doses include milligram or microgram amounts of the compound per kilogram of subject or sample weight, for example, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. When one or more of these small molecules is to be administered to an animal (*e.g.*, a human) in order to modulate expression or activity of a polypeptide or nucleic acid described herein, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

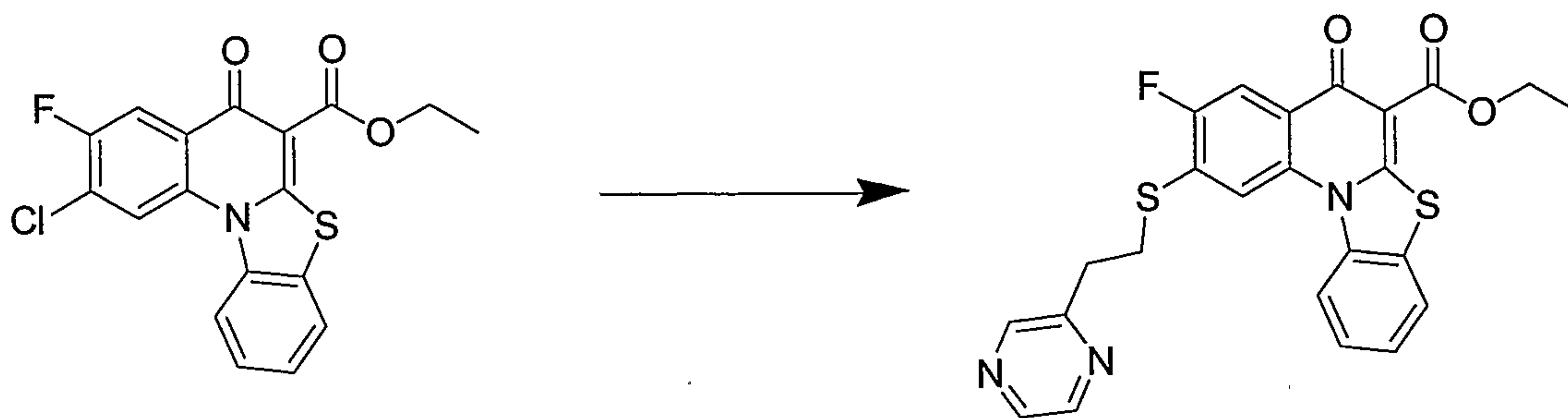
[0105] The following examples are offered to illustrate but not to limit the invention.

Example 1

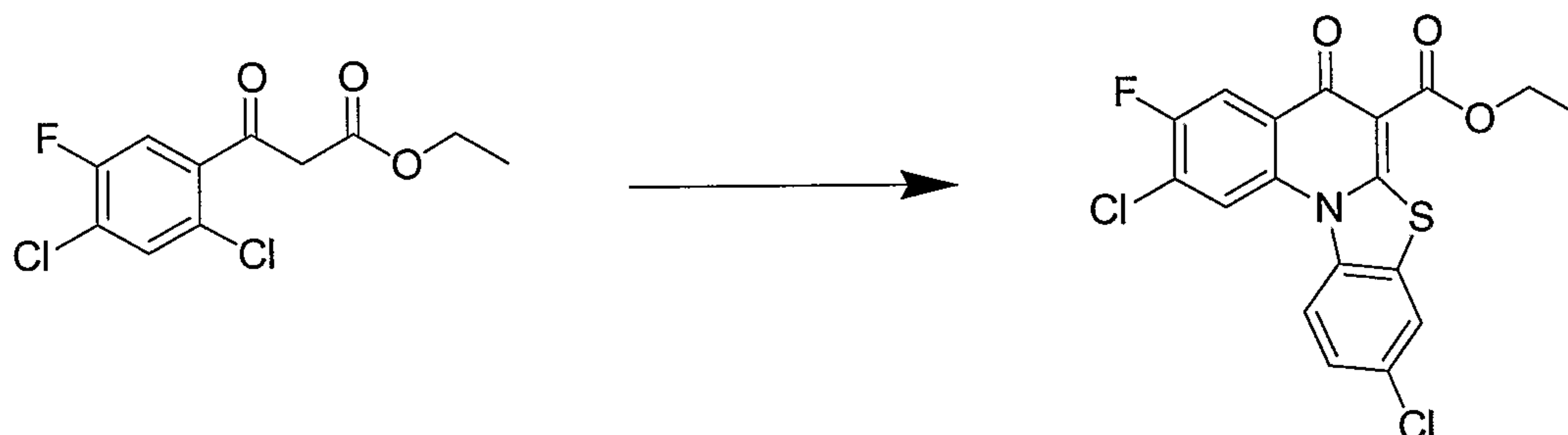
[0106] To a solution of magnesium chloride (6.74 g, 70.8 mmol) and potassium ethyl malonate (6.78 g, 39.8 mmol) in dry acetonitrile (100 mL) at 0 °C was added 2,4-dichloro-5-fluorobenzoyl chloride (5.0 g, 22.1 mmol) dropwise, keeping the internal temperature below 5 °C. The mixture was stirred an additional 30 minutes and triethyl amine (6.1 mL, 44.25 mmol) was added dropwise, again keeping the temperature below 5 °C and the reaction mixture was allowed to stir overnight. The mixture was concentrated *in vacuo*, diluted with toluene (250 mL) and 1N HCl was added (100 mL) and the mixture was allowed to stir for 30 minutes. The layers were separated and the organic layer was washed once more with 1N HCl (100 mL), and brine (200 mL) and dried over sodium sulfate. The organic layer was then filtered, concentrated *in vacuo* and purified over silica gel (1:10 ethyl acetate/hexanes) to afford the ketoester as an oil that solidified on standing (5.02 g, 81%).



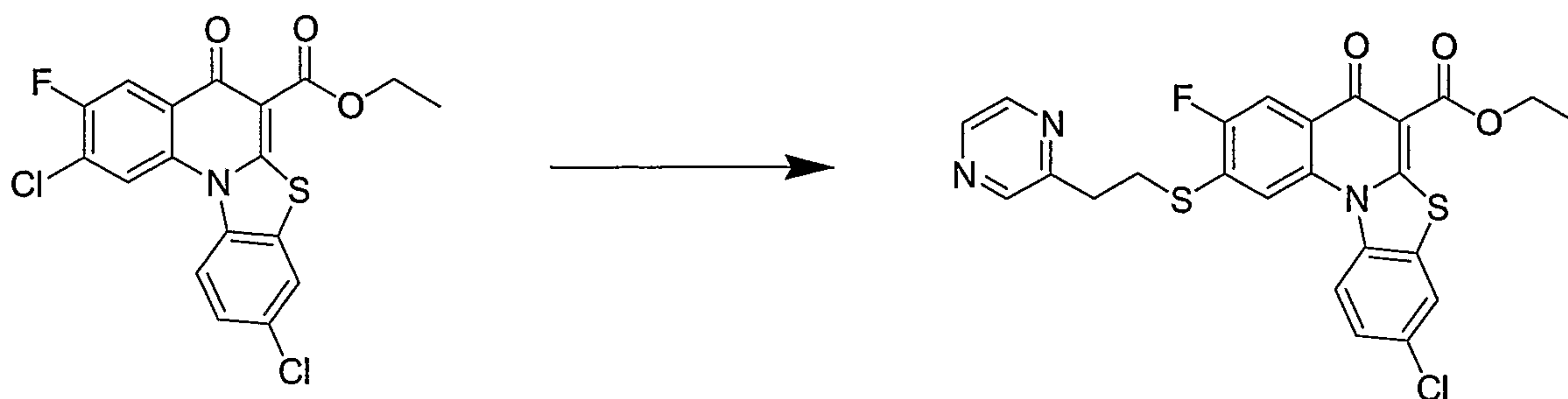
[0107] To a solution of the ketoester (5.0 g, 18 mmol) in diglyme (50 mL) was added 2-chlorobenzothiazole (3.66 g, 21.6 mmol) followed by sodium hydride (1.52 g, 39.6 mmol, 60% in oil) portion-wise over 10 minutes. The reaction was heated to 160 °C for 24 hours and allowed to cool to room temperature. The mixture was quenched by careful addition of water (250 mL) and the resulting brown precipitate was removed by filtration and washed with water. The product was then dissolved in methylene chloride (300 mL), washed with brine and filtered through Celite™. The resulting organic layer was dried over sodium sulfate, filtered and concentrated *in vacuo*. The product was purified over silica gel (7% ethyl acetate in hexanes) to afford the cyclized material (1.76g, 26%) as a solid.

Example 2

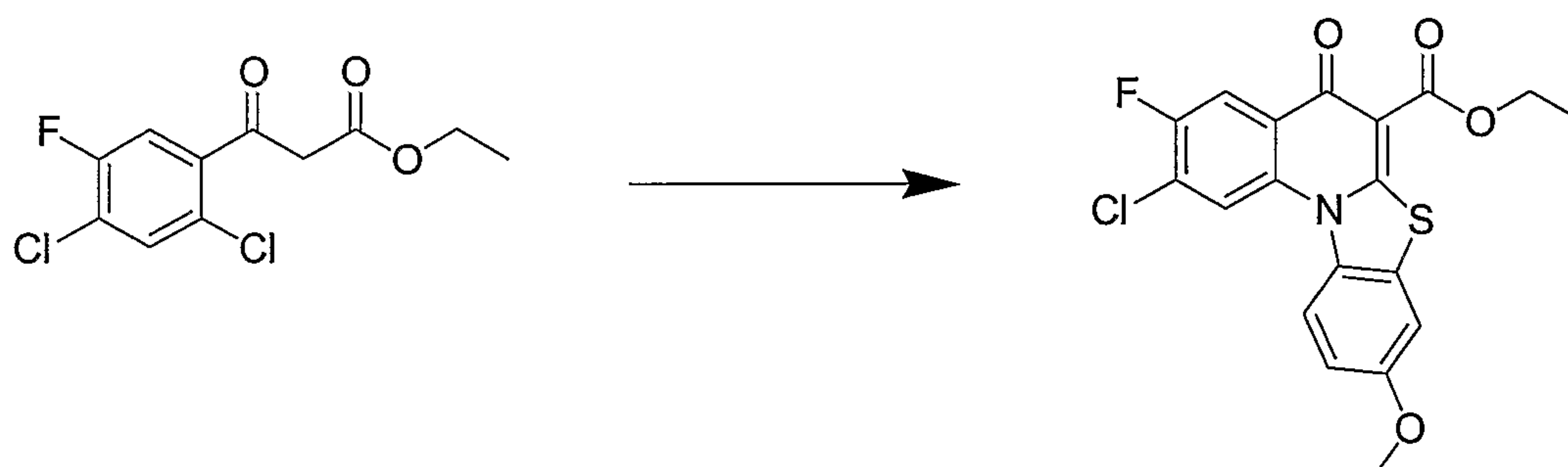
[0108] To a solution of the chloroester (250 mg, 0.66 mmol) in N-methylpyrrolidinone (NMP, 2 mL) was added 2-pyrazineethanethiol (81  $\mu$ L, 0.66 mmol), and potassium carbonate (182 mg, 1.32 mmol) and the mixture was heated to 100 °C for 2 hours. The mixture was allowed to cool to room temperature and water (50 mL) was added and stirring continued overnight. The crude product was collected by filtration and purified by preparative TLC (2% methanol in methylene chloride) to afford the pure product as a tan fluffy solid (122 mg, 38%).

Example 3

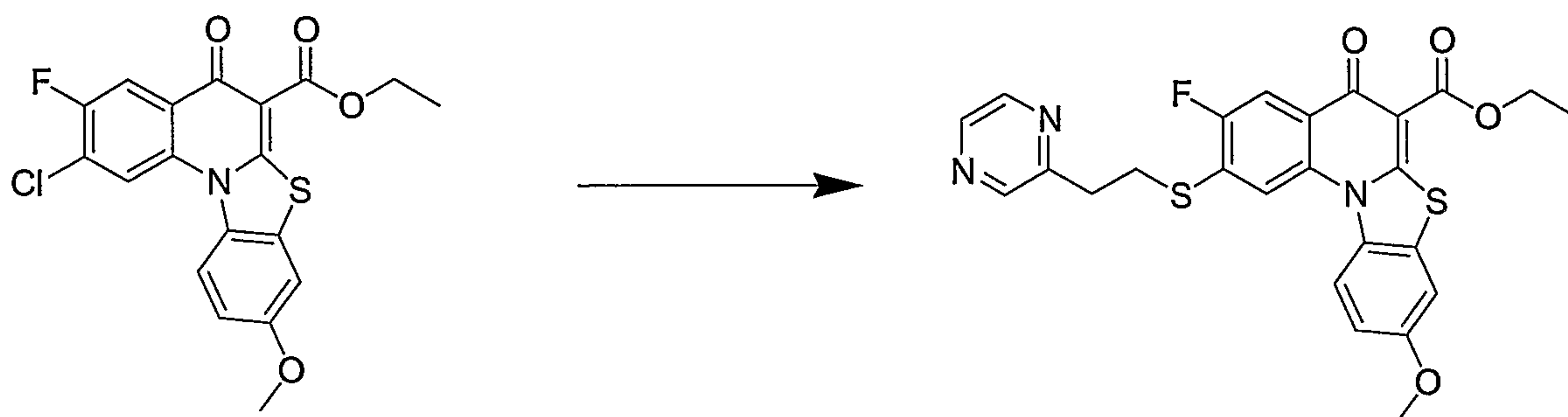
[0109] To a solution of the ketoester (2.0 g, 7.2 mmol) in diglyme (20 mL) was added the 2,6-dichlorobenzothiazole (1.76 g, 8.63 mmol) followed by sodium hydride (0.63 g, 15.8 mmol, 60% in oil) portion-wise over 10 minutes. The reaction was heated to 160 °C for 24 hours and allowed to cool to room temperature. The mixture was quenched by careful addition of water (200 mL) and the resulting brown precipitate was removed by filtration and washed with water. The product was then dissolved in methylene chloride (30 mL), washed with brine and filtered through celite. The resulting organic layer was dried over sodium sulfate, filtered and concentrated *in vacuo*. The product was purified over silica gel (5% ethyl acetate in hexanes) to afford the cyclized material (0.55 g, 18.8%) as a solid.

Example 4

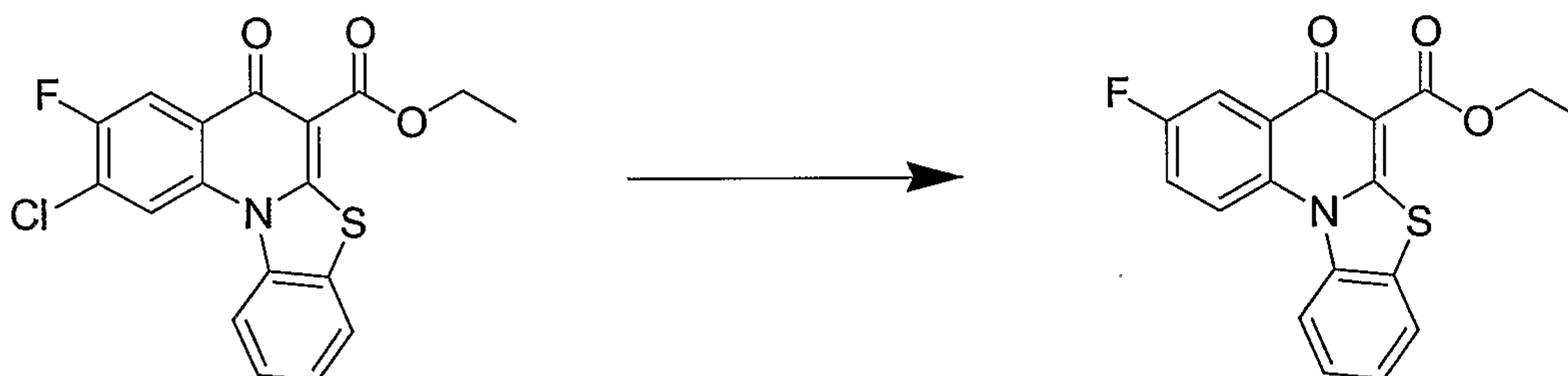
**[0110]** To a solution of the chloroester (250 mg, 0.61 mmol) in N-methylpyrrolidinone (NMP, 2 mL) was added 2-pyrazineethanethiol (75  $\mu$ L, 0.61 mmol), and potassium carbonate (170 mg, 1.2 mmol) and the mixture was heated to 100  $^{\circ}$ C for 2 hours. The mixture was allowed to cool to room temperature and water (50 mL) was added and stirring continued overnight. The crude product was collected by filtration and purified by preparative TLC (2% methanol in methylene chloride) to afford the pure product as a tan fluffy solid (125 mg, 40%).

Example 5

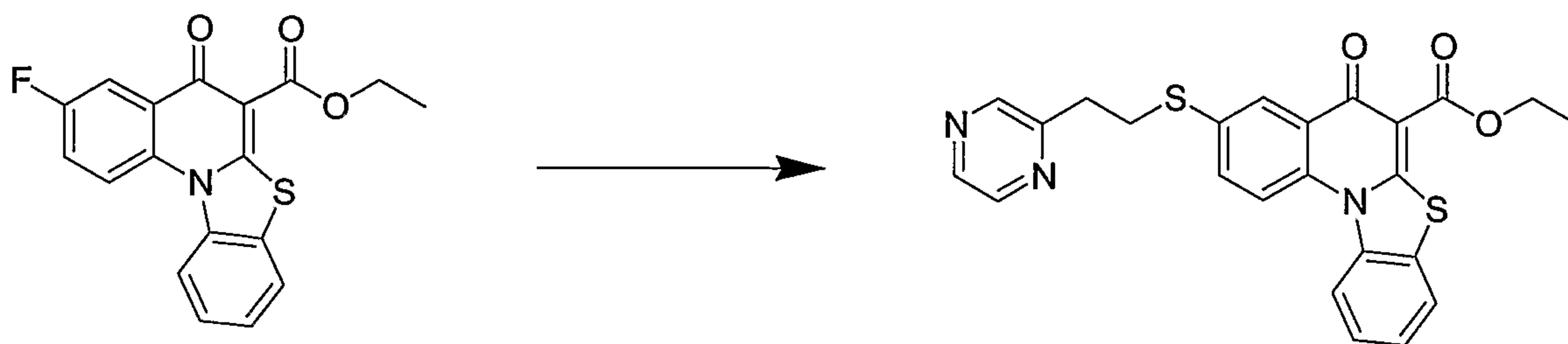
**[0111]** To a solution of the ketoester (2.0 g, 7.2 mmol) in diglyme (20 mL) was added the 2-chloro-6-methoxybenzothiazole (1.73 g, 8.63 mmol) followed by sodium hydride (0.63 g, 15.8 mmol, 60% in oil) portion-wise over 10 minutes. The reaction was heated to 160  $^{\circ}$ C for 24 hours and allowed to cool to room temperature. The mixture was quenched by careful addition of water (200 mL) and the resulting brown precipitate was removed by filtration and washed with water. The product was then dissolved in methylene chloride (30 mL), washed with brine and filtered through celite. The resulting organic layer was dried over sodium sulfate, filtered and concentrated *in vacuo*. The product was purified over silica gel (5% ethyl acetate in hexanes) to afford the cyclized material (0.23 g, 7.6%) as a solid.

Example 6

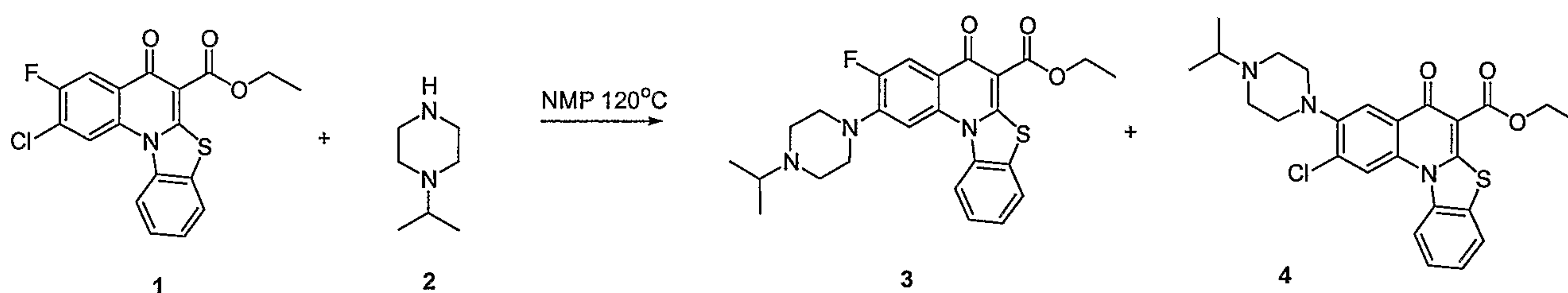
[0112] To a solution of the chloroester (220 mg, 0.54 mmol) in N-methylpyrrolidinone (NMP, 2 mL) was added 2-pyrazineethanethiol (66  $\mu$ L, 0.54 mmol), and potassium carbonate (150 mg, 1.1 mmol) and the mixture was heated to 100 °C for 2 hours. The mixture was allowed to cool to room temperature and water (50 mL) was added and stirring continued overnight. The crude product was collected by filtration and purified by preparative TLC (2% methanol in methylene chloride) to afford the pure product as a tan fluffy solid (75 mg, 28%).

Example 7

[0113] To a suspension of the chloroester (0.25 g, 0.67 mmol) in glacial acetic acid (5 mL) was added ammonium formate (0.6 g, 4.0 mmol) and the mixture was degassed with argon for 2 minutes. Next, palladium on charcoal (10% degussa type, 0.6 g) was added and the mixture was heated to 60 °C for 1 hour. More ammonium formate (0.1 g) and catalyst (0.1 g) were added and heating continued overnight. Finally, more ammonium formate and catalyst (150 mg each) were added and heating continued for 1.5 hr. The mixture was allowed to cool to room temperature and filtered through celite and the solvent was removed *in vacuo* and replaced with methylene chloride (100 mL). The organics were washed with water (100 mL) and brine (100 mL) and dried over sodium sulfate. The solvent was removed *in vacuo* to afford the benzothiazole as a tan solid (153 mg, 67%).

Example 8

[0114] To a solution of the fluoroester (50 mg, 0.15 mmol) in N-methylpyrrolidinone (NMP, 0.5 mL) was added 2-pyrazineethanethiol 126  $\mu$ L, 1.0 mmol), and potassium carbonate (40 mg, 0.3 mmol) and the mixture was heated to 100 °C for 2 hours. The mixture was allowed to cool to room temperature and water (50 mL) was added and stirring continued overnight. The crude product was collected by filtration and purified by preparative TLC (2% methanol in methylene chloride) to afford the pure product as a tan fluffy solid (17 mg, 25%).

Example 9

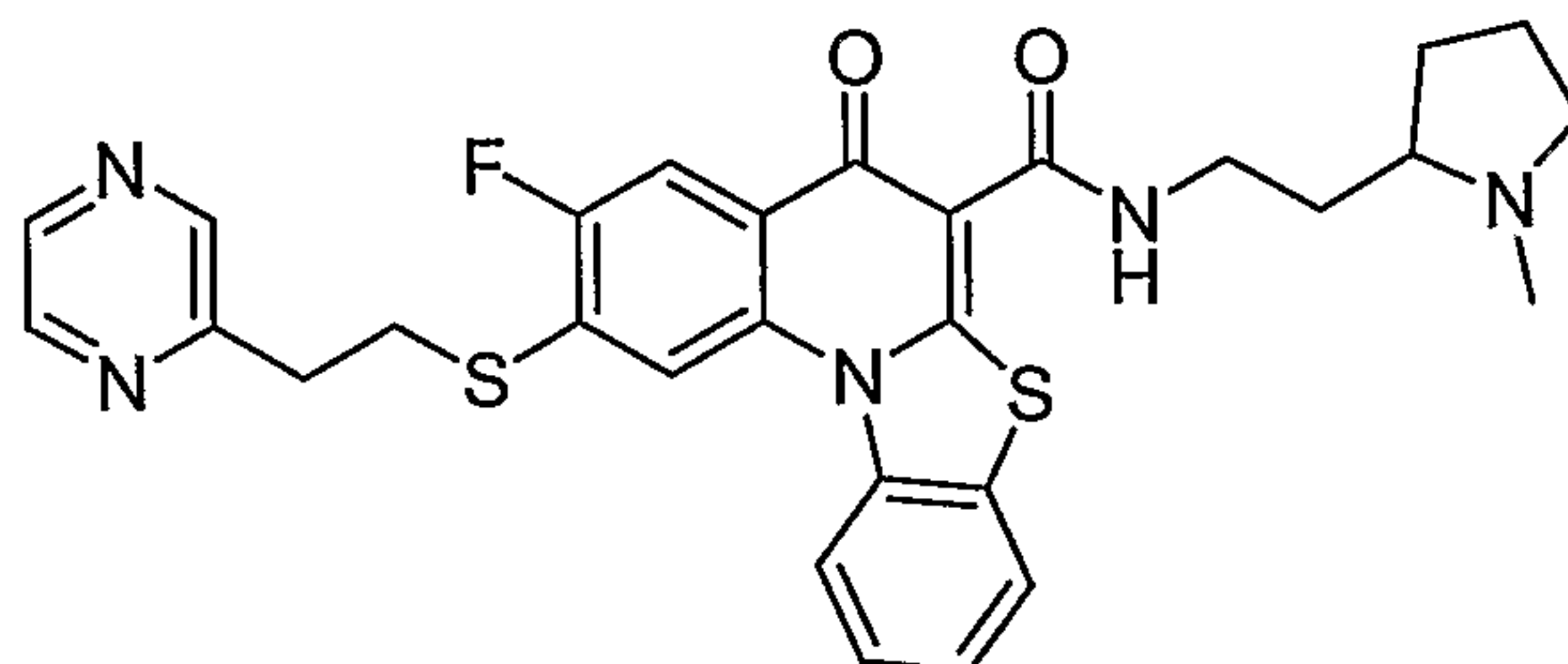
[0115] Compound 1 (1.0 eq, 399 mg, 1.06 mmol) and 1-isopropyl piperazine 2 (5.0 eq., 0.76 ml, 5.31 mmol) were dissolved in N-methylpyrrolidinone (NMP, 5 ml). The resulting mixture was stirred at 120°C for 3 days. LC monitoring revealed formation of two main products 3 and 4 in equal amounts. The solution was poured into water and the resulting precipitate isolated by filtration. The two compounds were separated by flash chromatography on SiO<sub>2</sub> (gradient MeOH 0.5 to 7% in CH<sub>2</sub>Cl<sub>2</sub>) to afford 3 (135 mg, 27% yield) and 4 (135 mg, 26%).

[0116] 3: R<sub>f</sub> = 0.40 (SiO<sub>2</sub>, 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>), LCMS (ES): 95% pure, m/z 468 [M+H]<sup>+</sup>.

[0117] 4: R<sub>f</sub> = 0.26 (SiO<sub>2</sub>, 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>), LCMS (ES): 95% pure, m/z 484 [M]<sup>+</sup>, 486 [M+2]<sup>+</sup>.

Example 10

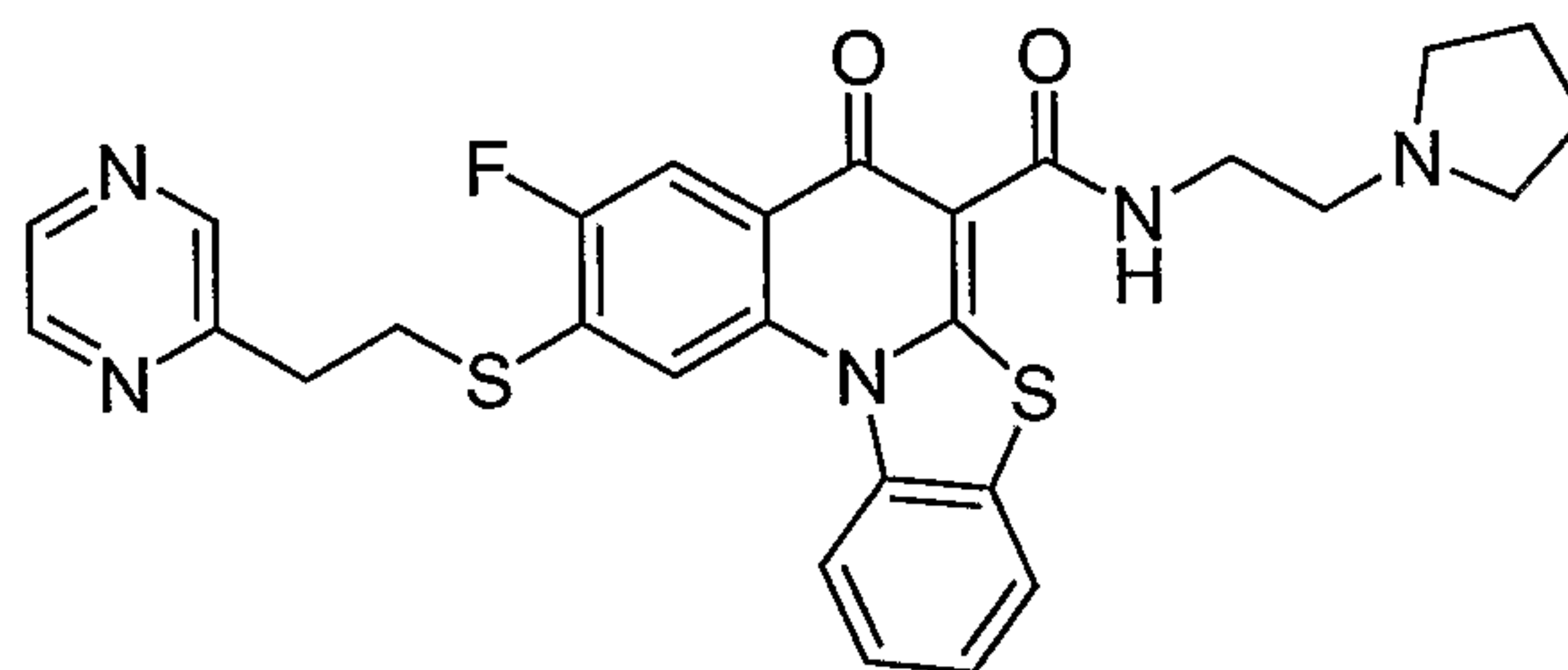
[0118] This example provides activity data for two particular compounds:



Quadruplex Binding Affinity  $K_m$  5.5  $\mu M$

TumorCell MeanTimeSurvival (IC50) (Alamar Blue Stain)

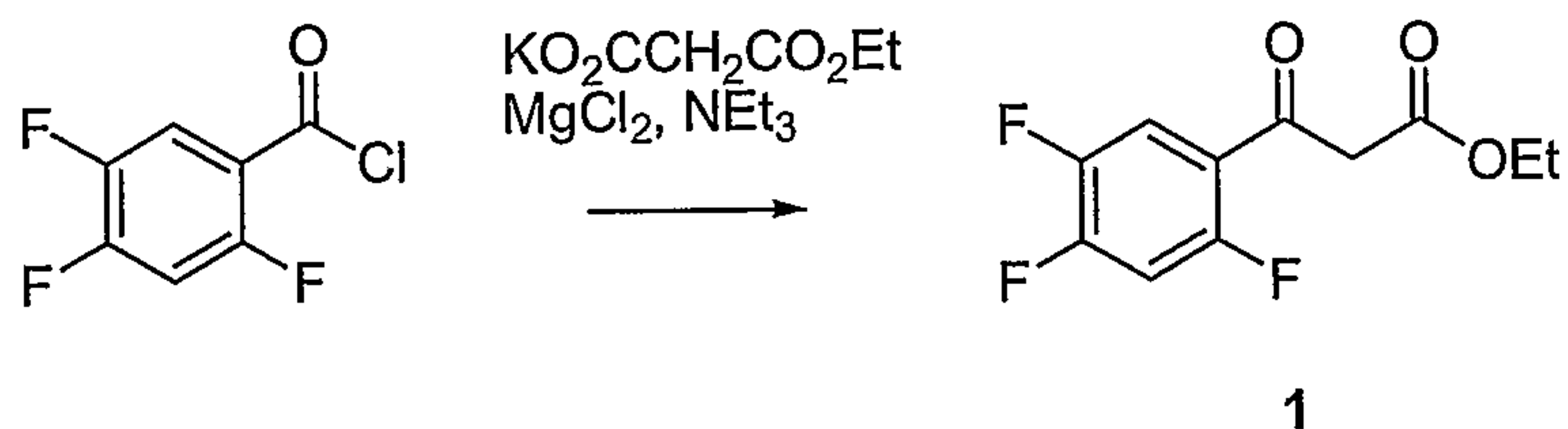
|         |             |
|---------|-------------|
| Hela    | 2.8 $\mu M$ |
| PC3     | 2.7 $\mu M$ |
| HCT-116 | 1.1 $\mu M$ |



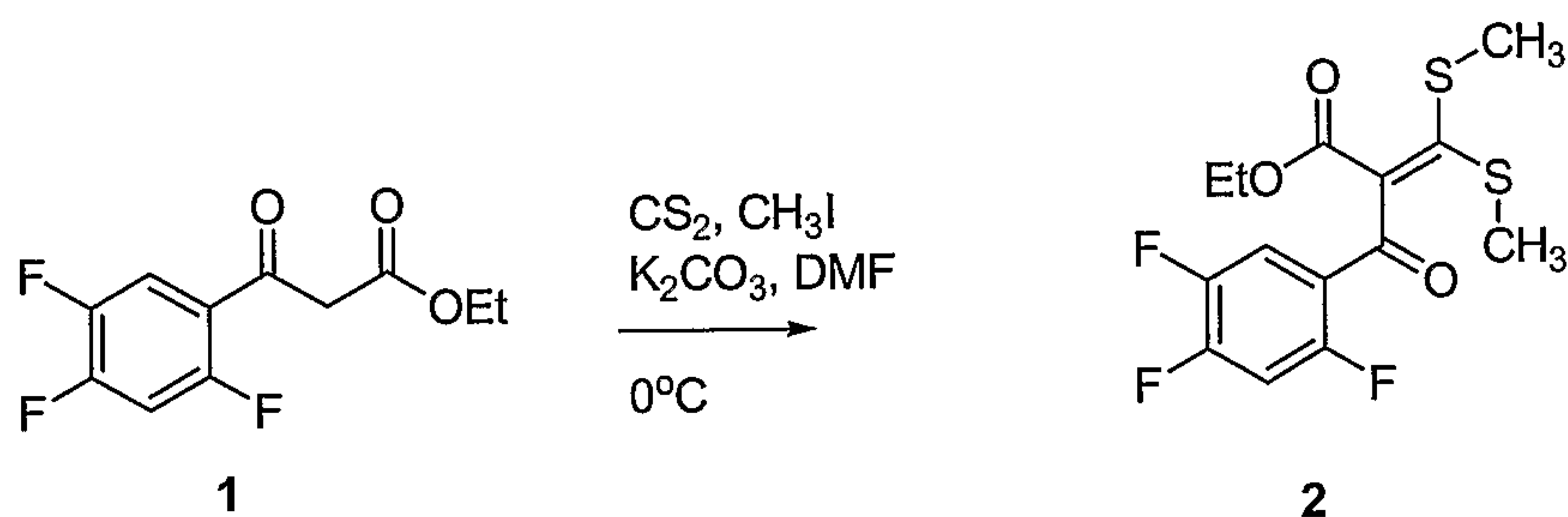
Quadruplex Binding Affinity  $K_m$  4.5  $\mu M$

TumorCell MeanTimeSurvival (IC50) (Alamar Blue Stain)

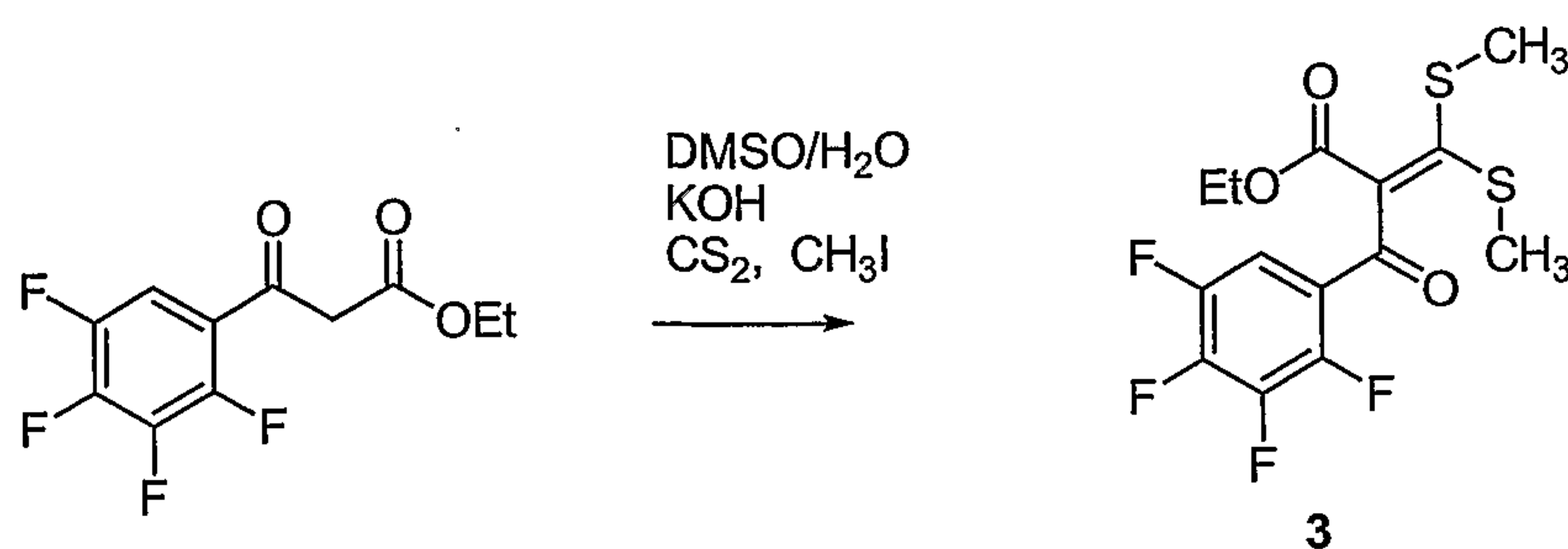
|         |              |
|---------|--------------|
| Hela    | 2.7 $\mu M$  |
| PC3     | 0.52 $\mu M$ |
| HCT-116 | 0.57 $\mu M$ |

Example 11

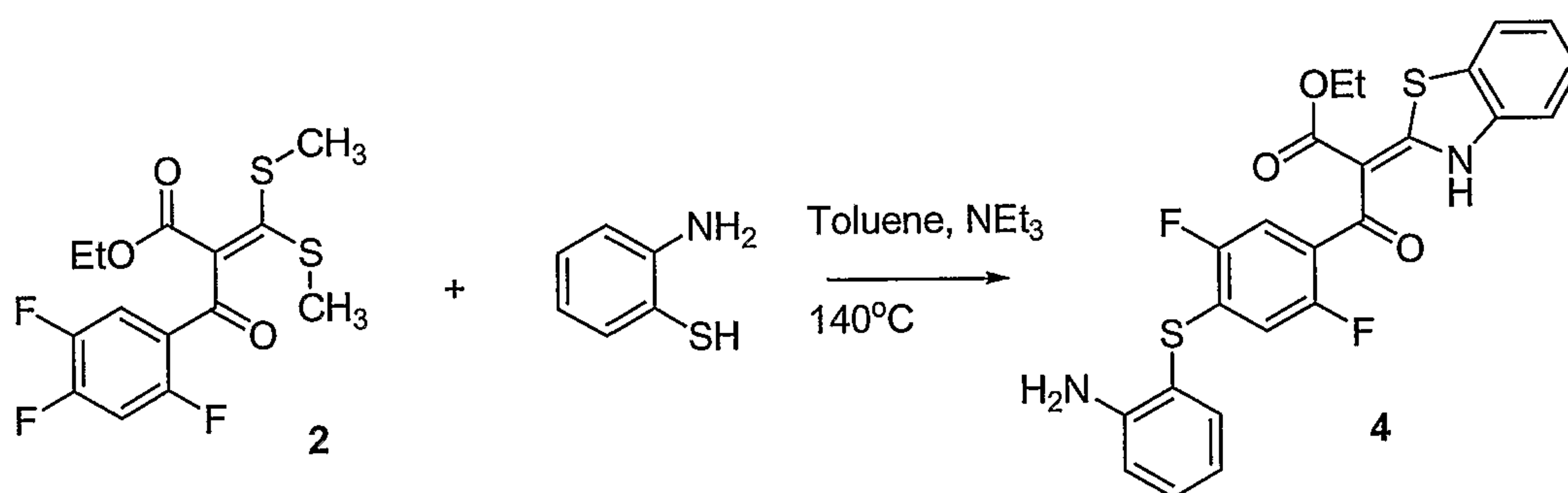
[0119] In a three necked flask equipped with a nitrogen inlet, potassium ethylmalonate (1.5 eq, 32.8 g, 0.192 mol) and  $\text{MgCl}_2$  (1.5 eq., 18.4 g, 0.193 mol) were suspended in acetonitrile (120 ml) under mechanical stirring. The suspension was cooled down with an ice bath. A solution of 2,4,5-trifluorobenzoyl chloride (1.0 eq, 25 g, 0.128 mol) in acetonitrile (60 ml) was added dropwise. A solution of triethylamine (2.0 eq, 36 ml, 0.258 mol) in acetonitrile (50 ml) was added over 30 min while keeping the internal temperature below  $10^\circ\text{C}$  by external cooling with an ice-salt mixture. The very thick slurry that resulted was allowed to warm to room temperature. An extra amount of acetonitrile (150 ml) was added to allow a proper stirring of the slurry. The reaction was stirred for 2 days and the volatiles were removed *in vacuo*. A 10% aqueous solution of HCl and EtOAc were added and the mixture stirred for 3 hours. The material was extracted with EtOAc (3x). The combined extracts were washed with brine and dried over  $\text{Na}_2\text{SO}_4$ . After evaporation of the solvent *in vacuo*, the material was recrystallized in a 10% water/EtOH mixture to provide compound 1 as a white crystalline material (17.8 g, 56% yield). LCMS (ES): 95% pure,  $m/z$  269  $[\text{M}+23]^+$ . Mixture of two tautomeric isomers.



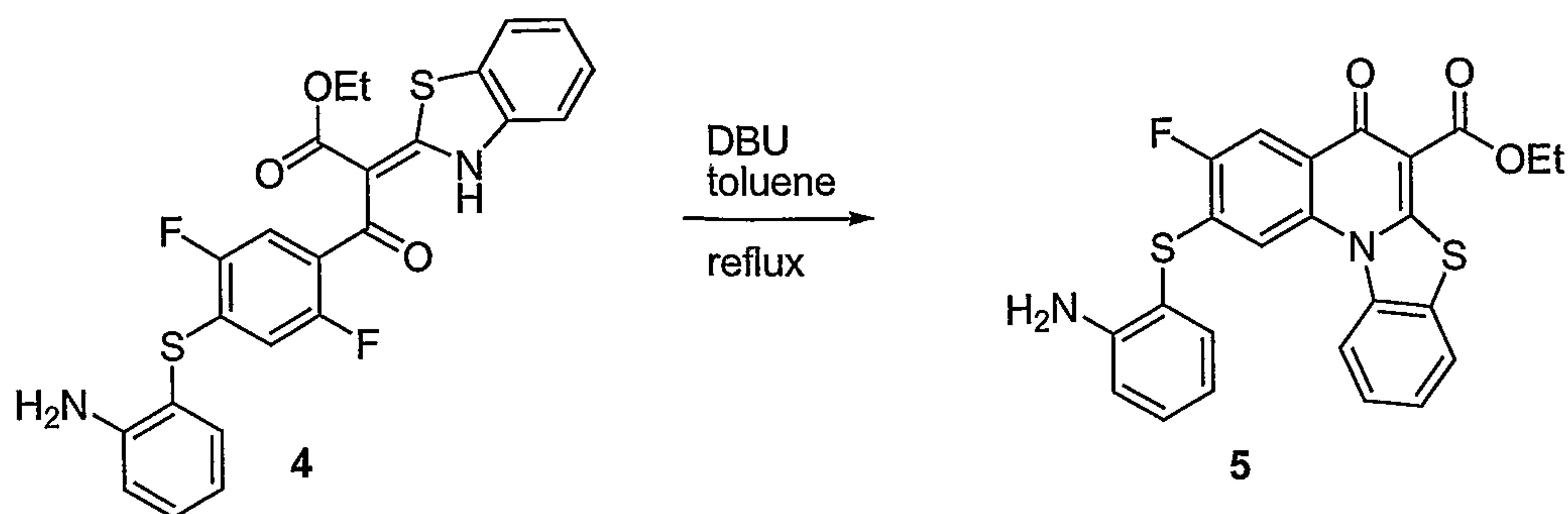
[0120] Compound 1 (1.0 eq, 3.27 g, 13.29 mmol) was dissolved in anhydrous DMF (30 ml). The solution was cooled down with ice,  $\text{K}_2\text{CO}_3$  (3.0 eq, 5.51 g, 39.87 mmol) was added and the solution stirred for 15 min. To the resulting white slurry was quickly added carbon disulfide (1.5 eq, 1.20 ml, 19.86 mmol) and the mixture stirred at  $0^\circ\text{C}$  for 5 min. Methyl Iodide (3.0 eq, 2.5 ml, 40.2 mmol) was added dropwise through syringe and the reaction stirred at  $0^\circ\text{C}$  for 2 hours. After adding iced water, the compound was extracted with EtOAc (3x). The combined extracts were washed with water (1x) and brine (2x), dried over  $\text{Na}_2\text{SO}_4$  and the volatiles removed *in vacuo*. Upon adding hexanes and bit of EtOAc the compound started to crystallize. The crude material was isolated by filtration and recrystallized in hexanes to afford a white crystalline solid (3.27 g, 70% yield). LCMS (ES): 95% pure,  $m/z$  373  $[\text{M}+23]^+$ , 351  $[\text{M}+1]^+$ .



[0121] Ethyl 3-oxo-3-(2,3,4,5-tetrafluorophenyl)propanoate (1.0 eq, 5.77 g, 21.84 mmol) was dissolved in a mixture of DMSO (55 ml) and water (12 ml). A solution of KOH (2.3 eq, 2.82 g, 50.26 mmol) in water (25 ml) was added dropwise while keeping the internal temperature below 15°C using an ice bath. After stirring for 15 min, a mixture of carbon disulfide (3.2 eq, 4.2 ml, 69.50 mmol) and iodomethane (3.8 eq, 5.2 ml, 83.35 mmol) was added quickly and the resulting mixture stirred at room temperature overnight. After adding water, the material was extracted with EtOAc (2x). The combined extracts were washed with water, dried over Na<sub>2</sub>SO<sub>4</sub> and the solvent removed *in vacuo*. The compound was purified by flash chromatography on silica gel (5 to 15% gradient of EtOAc in hexanes) to afford a yellow oil (1.69 g, 21% yield). LCMS (ES): 95% pure, *m/z* 323 [M+1-EtO]<sup>+</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz) δ 1.15 (t, *J* = 7.0, 3H), 2.40 (br s, 6H), 4.18 (q, *J* = 7.2, 2H), 7.54-7.60 (m, 1H) ppm.

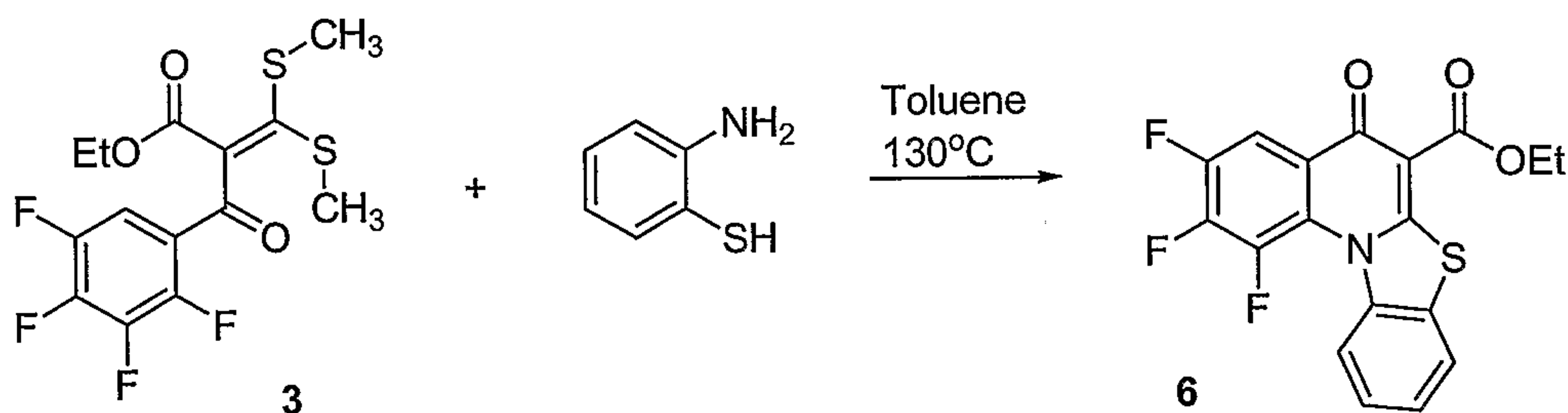


[0122] Compound 2 (1.0 eq, 993 mg, 2.84 mmol), 2-aminothiophenol (5.0 eq, 1.52 ml, 14.2 mmol) and NEt<sub>3</sub> (4.0 eq, 1.59 ml, 11.0 mmol) were mixed in anhydrous toluene (10 ml). The mixture was stirred at reflux for a few hours. After removal of the solvent *in vacuo*, the material was purified by sonication in EtOAc/hexanes to give a solid (724 mg, 53% yield). LCMS (ES): 95% pure, *m/z* 485 [M+1]<sup>+</sup>.

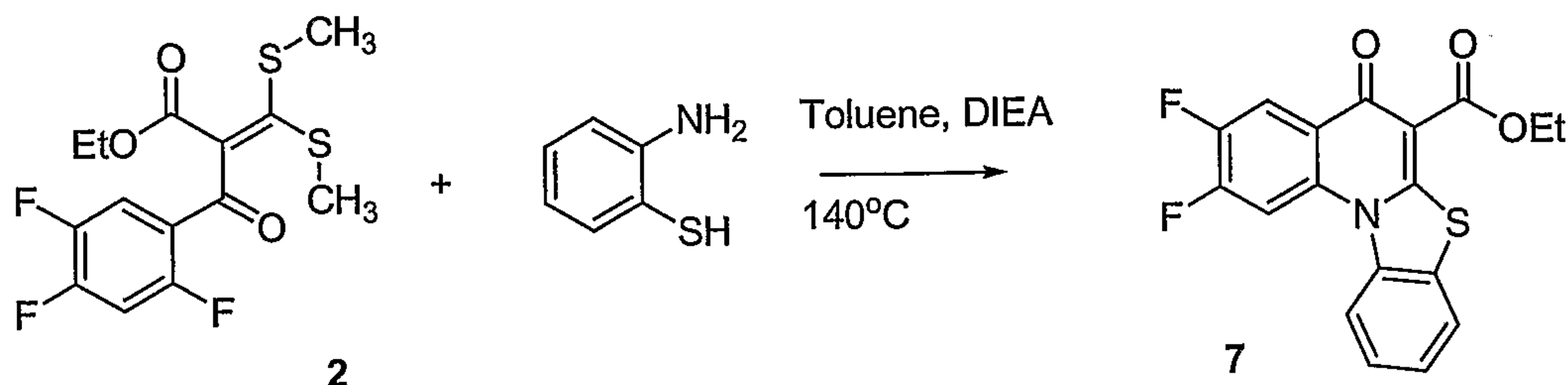


[0123] Compound 4 (1.0 eq, 70 mg, 0.144 mmol) was mixed with DBU (4.0 eq, 65  $\mu$ l, 0.43 mmol) in toluene (1.5 ml). The solution was stirred under reflux for 45 min. The compound was purified by flash chromatography on silica gel (1% MeOH in  $\text{CH}_2\text{Cl}_2$ ). LCMS (ES): 95% pure,  $m/z$  465  $[\text{M}+1]^+$ .

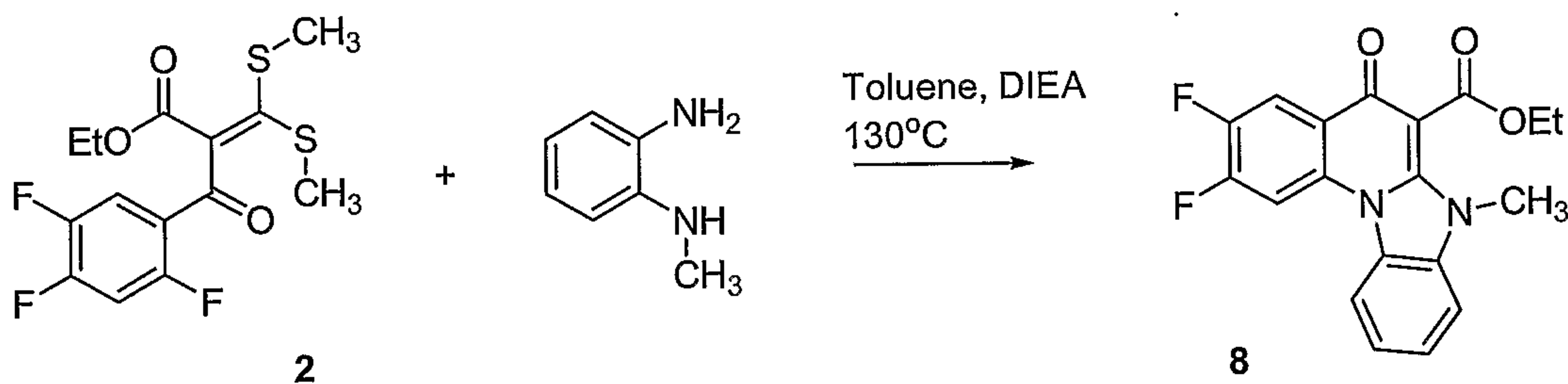
### Example 12



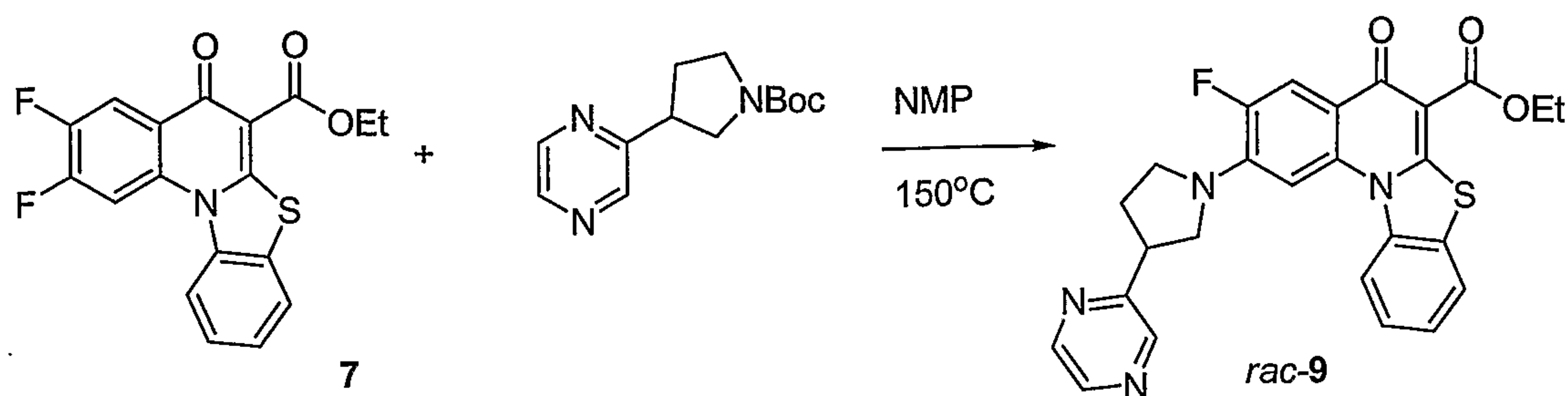
[0124] Compound 3 (1.0 eq, 347 mg, 0.942 mmol) and 2-aminothiophenol (1.0 eq, 0.10 ml, 0.934 mmol) were mixed in toluene (1 ml) and stirred at 130°C for 14 hours. After cooling down some solid impurities were removed by filtration. The toluene solution was poured onto a silica gel column. Toluene was removed first by eluting with hexanes. The column was eluted then with a 10 to 30% gradient of EtOAc in hexanes to provide the expected compound. After evaporation of the volatiles, the solid was further purified by recrystallization using EtOAc/hexanes. Compound 6 was isolated as a gray solid (62 mg, 18% yield). LCMS (ES): 90% pure,  $m/z$  378  $[\text{M}+1]^+$ .

Example 13

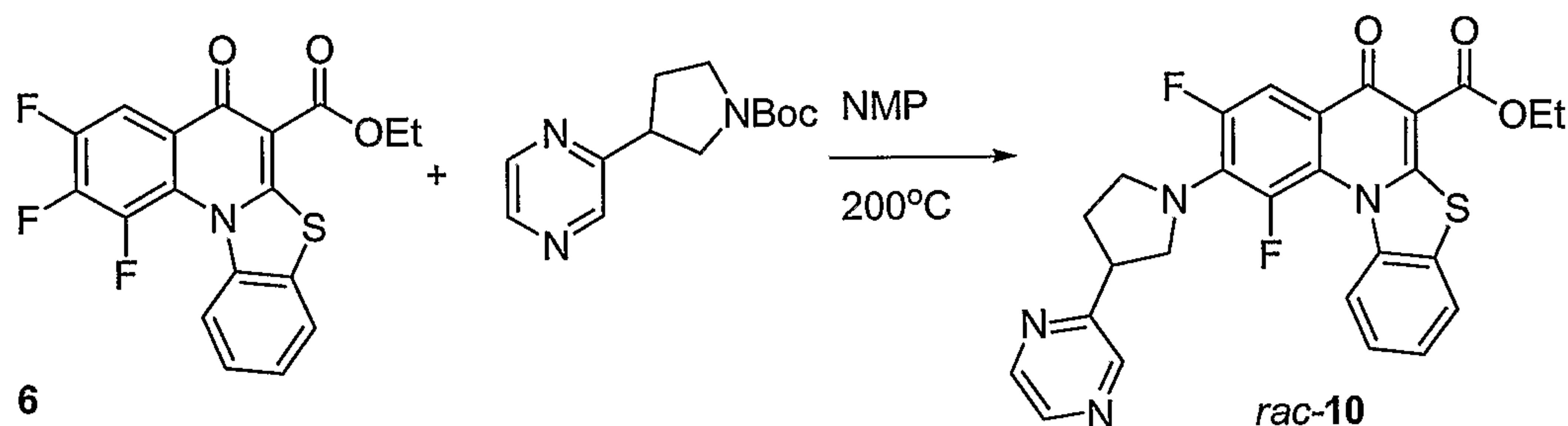
[0125] Compound **2** (1.0 eq, 3.15 g, 8.99 mmol) and 2-aminothiophenol (1.1 eq, 1.06 ml, 9.91 mmol) were mixed in toluene (150 ml). Nitrogen gas was bubbled into the solution for 10 min. The reaction was stirred at reflux (oil bath T = 140°C) for 30 hours. The volatiles were removed *in vacuo* and CH<sub>2</sub>Cl<sub>2</sub> was added. Solid impurities were removed by filtration. The material was purified by flash chromatography on silica gel (10 to 50% gradient of EtOAc in hexanes) to afford compound **7** as an off-white powder (576 mg, 18% yield). LCMS (ES): 90% pure, m/z 360 [M+1]<sup>+</sup>.

Example 14

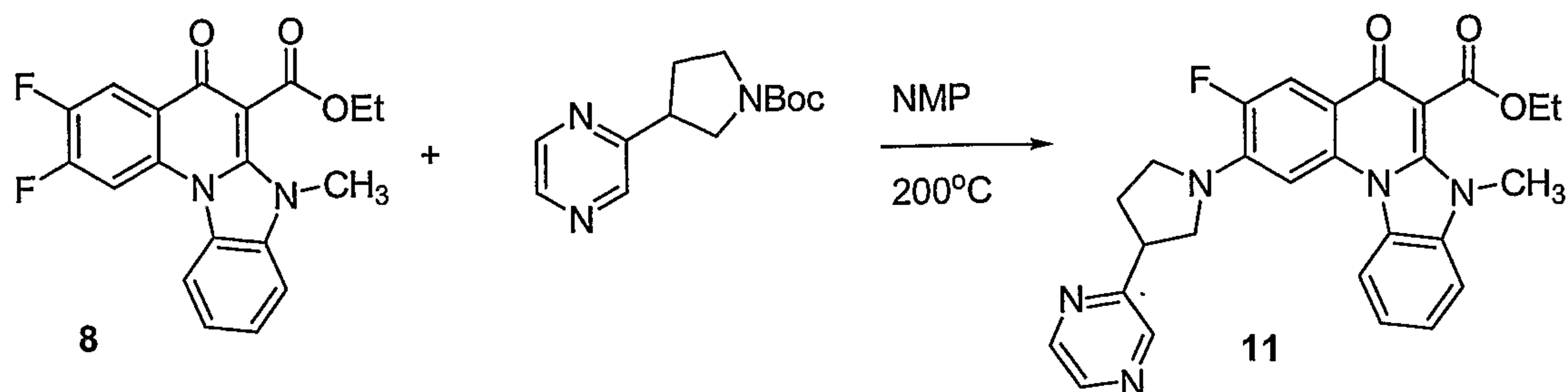
[0126] Compound **2** (1.0 eq, 2.52 g, 7.20 mmol) was mixed with N-methylbenzene-1,2-diamine (1.2 eq, 0.98 ml) and DIEA (1.2 eq, 1.5 ml) in toluene (10 ml) and the reaction stirred at 120°C for one day. Compound **8** was isolated by filtration after cooling the reaction mixture (679 mg, 26% yield). LCMS (ES): 95% pure, m/z 358 [M+1]<sup>+</sup>.

Example 15

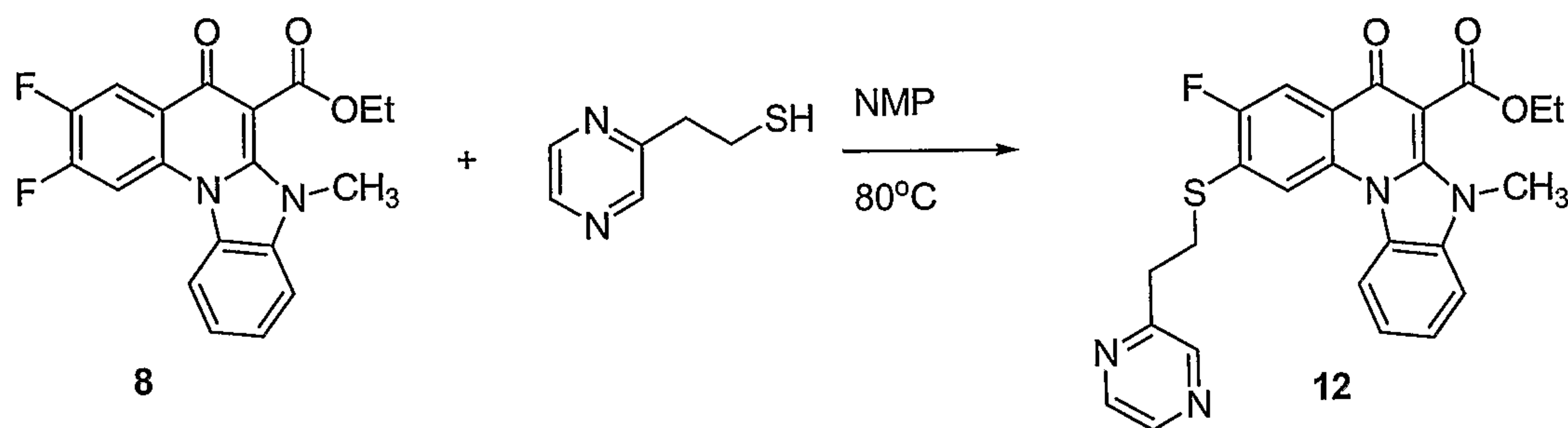
[0127] Compound **7** (1.0 eq, 392 mg, 1.09 mmol) and racemic tert-butyl 3-(pyrazin-2-yl)pyrrolidine-1-carboxylate (1.6 eq, 436 mg, 1.74 mmol) were suspended in 1 ml of NMP. The mixture was heated by microwaves at 150°C for 20 min. Water was added and the resulting solid isolated by filtration. Purification by flash chromatography afforded compound **9** (265 mg, 50% yield) as a solid. LCMS (ES): 95% pure,  $m/z$  489  $[M+1]^+$ .

Example 16

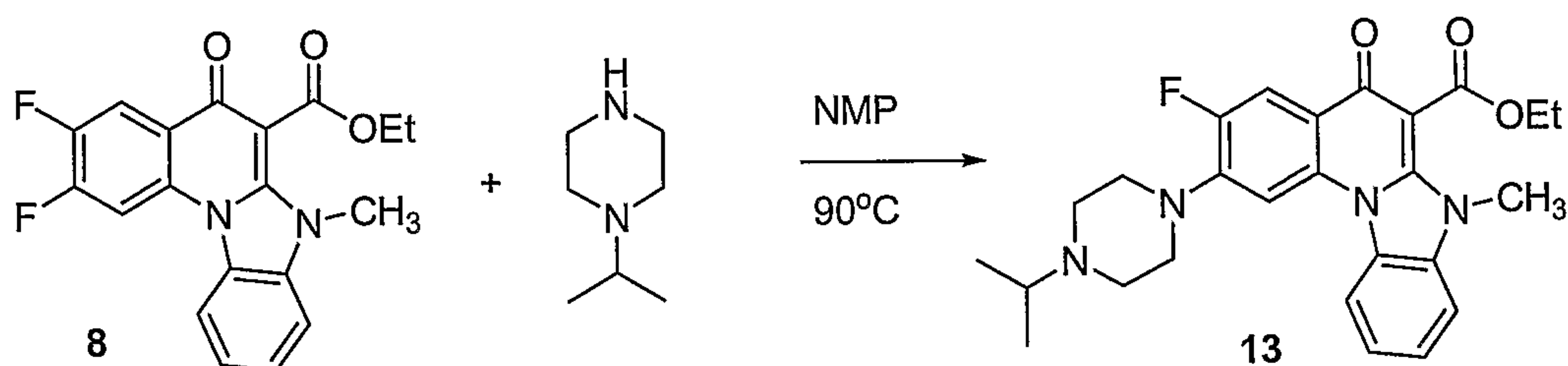
[0128] Compound **6** (1.0 eq, 66 mg, 0.175 mmol) and racemic tert-butyl 3-(pyrazin-2-yl)pyrrolidine-1-carboxylate (3.8 eq, 165 mg, 0.661 mmol) were mixed in NMP (0.5 ml). The mixture was stirred at 200°C for 23 hours. After adding water, the solid that formed was removed by filtration. Purification by flash chromatography on silica gel (1 to 4% gradient of MeOH in  $CH_2Cl_2$ ) afforded compound **10** as a brown solid (70 mg, 79% yield). LCMS (ES): 90% pure,  $m/z$  507  $[M+1]^+$ .

Example 17

[0129] Compound **11** was prepared according to the procedure used for compound **10**. The compound was purified by flash chromatography on silica gel (1 to 10% gradient of MeOH in CH<sub>2</sub>Cl<sub>2</sub>) to afford a solid (220 mg, 47%). LCMS (ES): 95% pure, m/z 486 [M+1]<sup>+</sup>.

Example 18

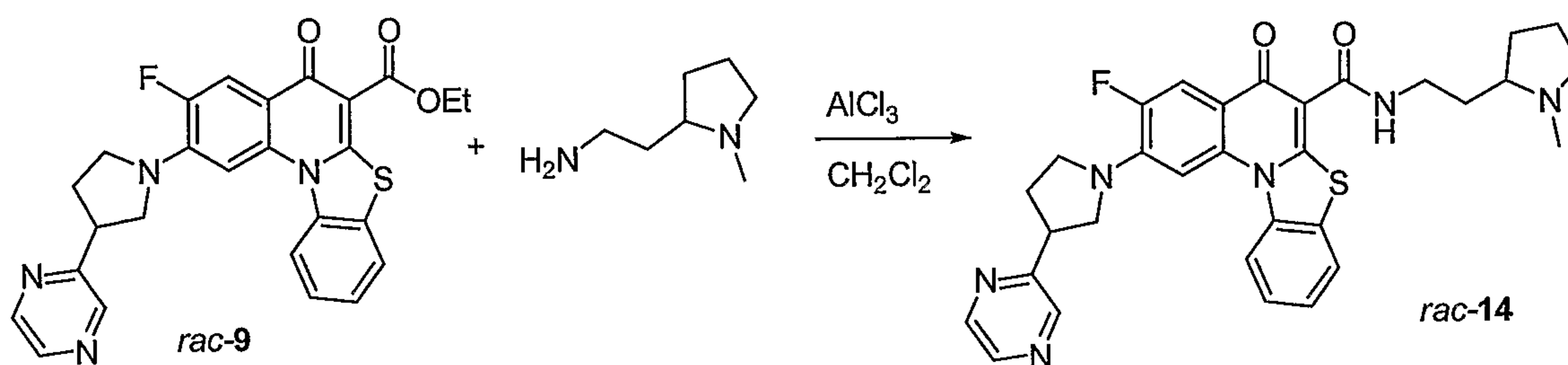
[0130] Compound **8** (1.0 eq, 202 mg, 0.56 mmol) was mixed with 2-(pyrimidin-2-yl)ethanethiol (1.05 eq, 76 µl) and K<sub>2</sub>CO<sub>3</sub> (1.2 eq, 94 mg) in NMP. The mixture was stirred for 3 hours at 80°C. After adding water, the solid was filtered and dried to afford compound **12** (202 mg, 85% yield). LCMS (ES): 95% pure, m/z 477 [M+1]<sup>+</sup>.

Example 19

[0131] Compound **8** (313 mg) was mixed with N-isopropyl piperazine (1.5 eq, 188 µl) in NMP and the mixture was stirred at 90°C for several hours. The compound was isolated by

filtration after adding water. Purification by flash chromatography on silica gel (MeOH/CH<sub>2</sub>Cl<sub>2</sub>) afforded pure compound **13** (222 mg; 54% yield). LCMS (ES): 95% pure, m/z 465 [M+1]<sup>+</sup>.

### Example 20

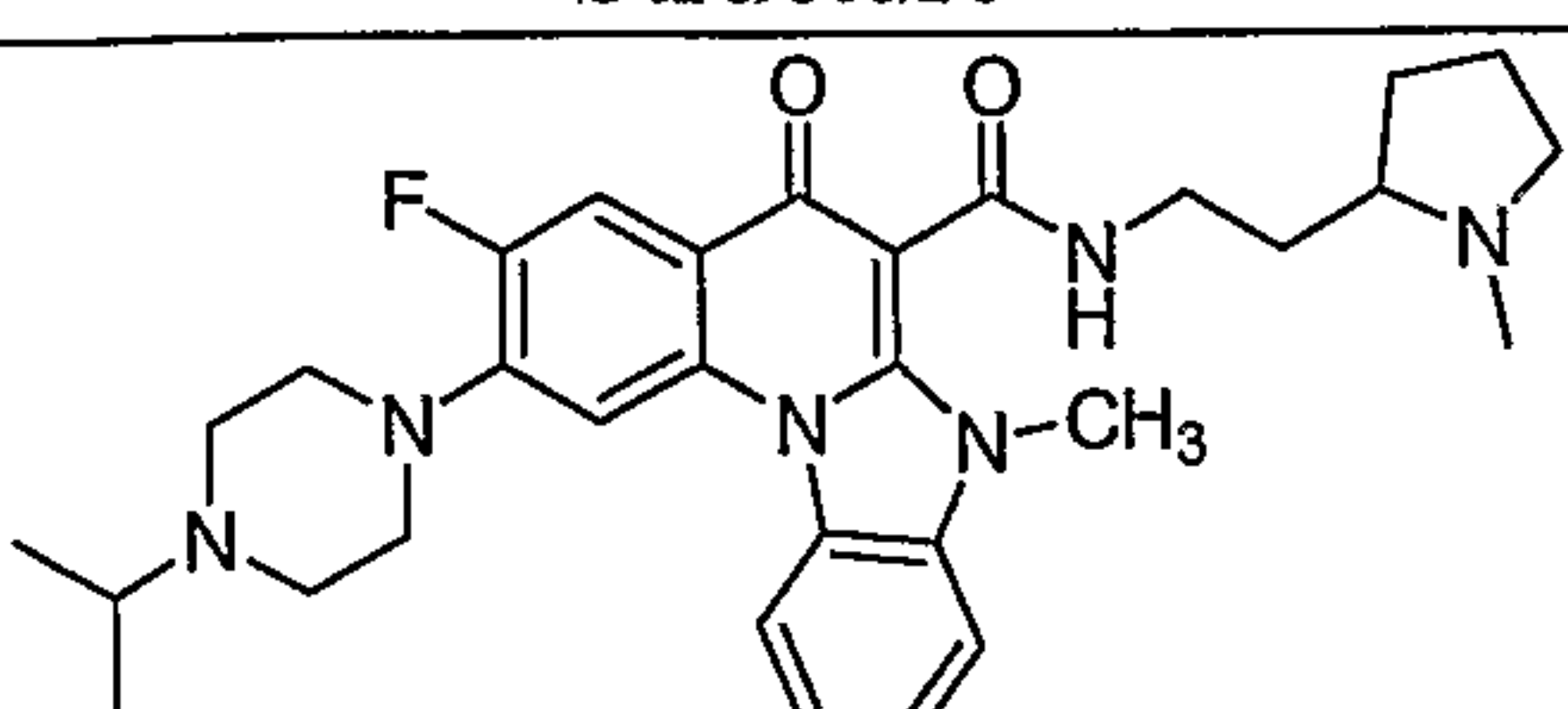


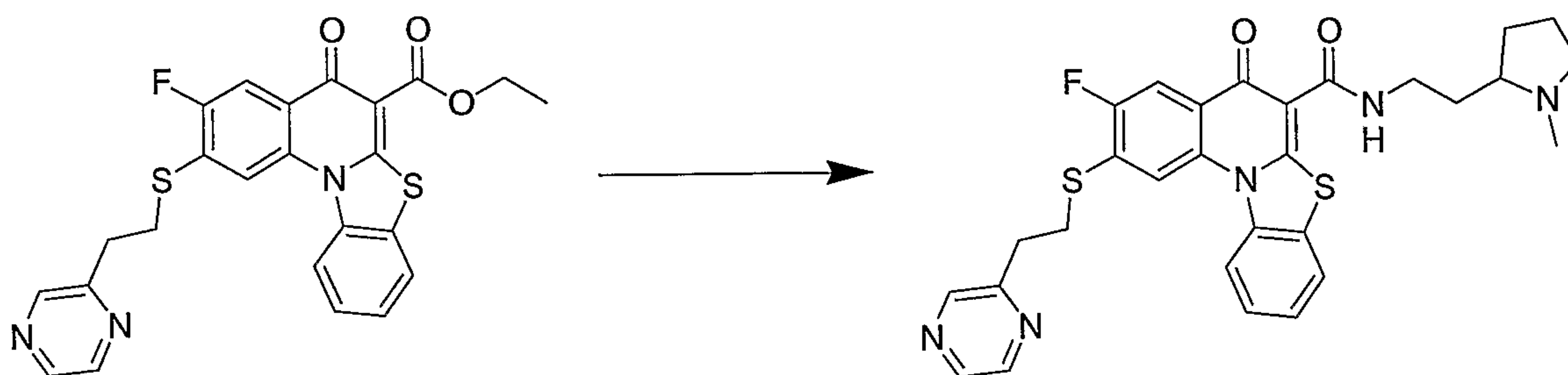
[0132] Compound **9** (1.0 eq, 181 mg, 0.370 mmol) and racemic 2-(1-methylpyrrolidin-2-yl)ethanamine (4.0 eq, 0.21 ml, 1.449 mmol) were mixed in CH<sub>2</sub>Cl<sub>2</sub> (1.5 ml). AlCl<sub>3</sub> (2.9eq, 142 mg, 1.06 mmol) was added and the solution stirred for 22 hours. After removal of CH<sub>2</sub>Cl<sub>2</sub> *in vacuo*, the resulting slurry was treated with a saturated aqueous tartaric acid solution (*ca.* 1 ml) and stirred until all solid disappeared (about 1 hr for completion of the hydrolysis). Water was added and the pH was adjusted to 14 by adding NaOH. The material was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3x) and the combined extracts washed with water (2x). After drying over Na<sub>2</sub>SO<sub>4</sub>, the volatiles were removed *in vacuo*. The material was purified by flash chromatography on alumina (0.1 to 1% gradient of MeOH in CH<sub>2</sub>Cl<sub>2</sub>). The CH<sub>2</sub>Cl<sub>2</sub>/MeOH solution was concentrated *in vacuo*. Addition of EtOAc induced precipitation of **14** as a yellow solid (93 mg, 44% yield). LCMS (ES): 95 pure, m/z 571 [M+1]<sup>+</sup>.

[0133] The following compounds were prepared by the same method, using the appropriate amines and quinolone ethyl esters.

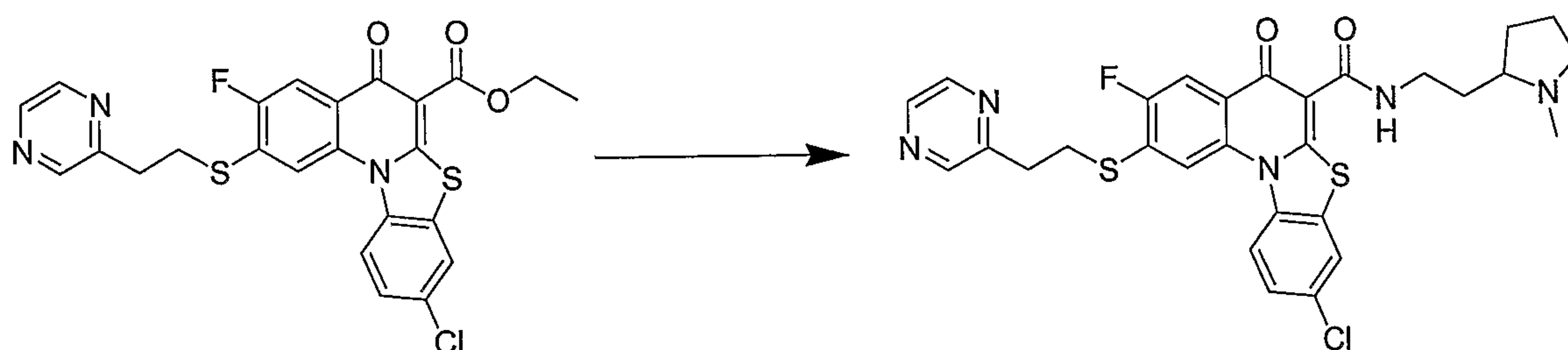
| Structure | M.W.   | LCMS (ES) m/z          |
|-----------|--------|------------------------|
|           | 574.20 | 575 [M+1] <sup>+</sup> |

| Structure | M.W.   | LCMS (ES) m/z          |
|-----------|--------|------------------------|
|           | 532.65 | 533 [M+1] <sup>+</sup> |
|           | 567.66 | 568 [M+1] <sup>+</sup> |
|           | 553.63 | 554 [M+1] <sup>+</sup> |
|           | 424.44 | 425 [M+1] <sup>+</sup> |
|           | 558.67 | 559 [M+1] <sup>+</sup> |
|           | 544.64 | 545 [M+1] <sup>+</sup> |
|           | 532.65 | 533 [M+1] <sup>+</sup> |

| Structure                                                                          | M.W.   | LCMS (ES) m/z          |
|------------------------------------------------------------------------------------|--------|------------------------|
|  | 546.68 | 547 [M+1] <sup>+</sup> |

Example 21

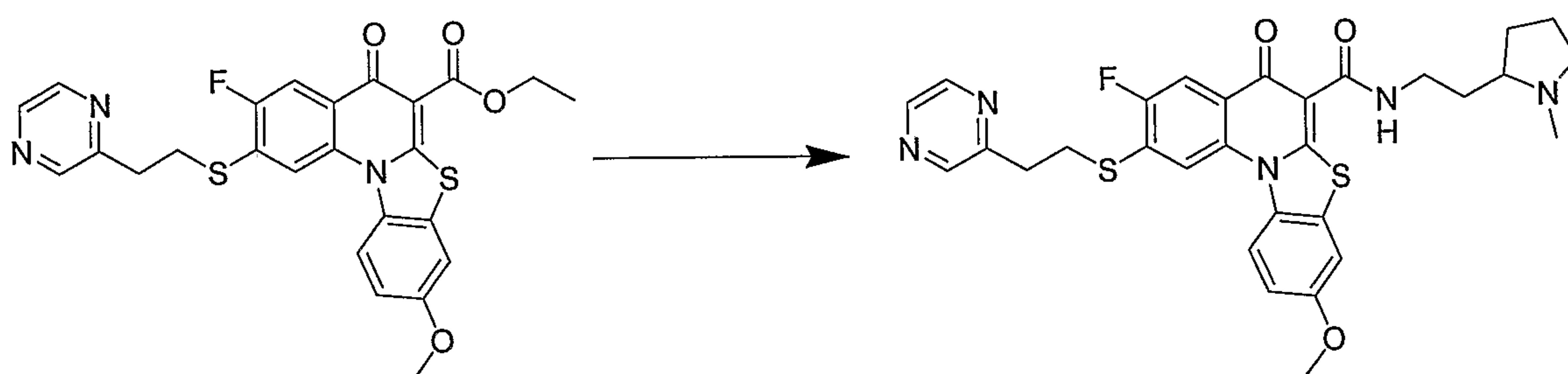
[0134] To a solution of the quinolone ester (60 mg, 0.13 mmol) and 2-(2-aminoethyl)-1-methyl pyrrolidine (30  $\mu$ L, 0.19 mmol) in methylene chloride (1.0 mL) was added aluminum chloride (25 mg, 0.19 mmol) and the reaction mixture was allowed to stir for 30 minutes. The solvent was removed *in vacuo* and saturated L-tartaric acid (1.0 mL) was added, stirring for 45 minutes, until all of the solid dissolved. The aqueous solution was washed with methylene chloride (1.0 mL), basified with 1N NaOH and extracted with methylene chloride. The resulting extract was washed with brine, dried over sodium sulfate, filtered and the solvent was removed *in vacuo*. The resulting yellow material was purified on preparative TLC (Alumina, 2% Methanol in CH<sub>2</sub>Cl<sub>2</sub>) to afford the product as a yellowish solid (30 mg, 43%).

Example 22

[0135] To a solution of the quinolone ester (60 mg, 0.11 mmol) and 2-(2-aminoethyl)-1-methyl pyrrolidine (25  $\mu$ L, 0.17 mmol) in methylene chloride (1.0 mL) was added aluminum

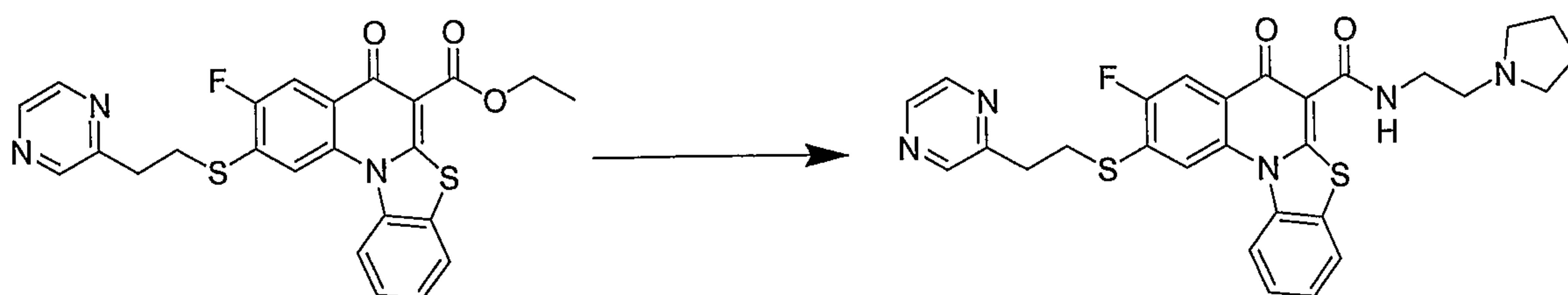
chloride (23 mg, 0.17 mmol) and the reaction mixture was allowed to stir for 30 minutes. The solvent was removed *in vacuo* and saturated L-tartaric acid (1.0 mL) was added, stirring for 45 minutes, until all of the solid dissolved. The aqueous solution was washed with methylene chloride (1.0 mL), basified with 1N NaOH and extracted with methylene chloride. The resulting extract was washed with brine, dried over sodium sulfate, filtered and the solvent was removed *in vacuo*. The resulting yellow material was purified on preparative TLC (Alumina, 2% Methanol in CH<sub>2</sub>Cl<sub>2</sub>) to afford the product as a yellowish solid (30 mg, 46%).

### Example 23



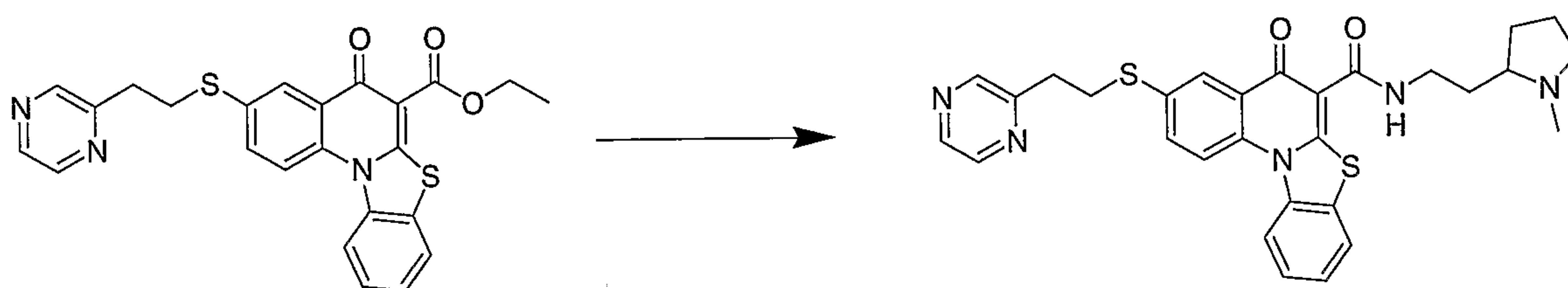
[0136] To a solution of the quinolone ester (75 mg, 0.15 mmol) and 2-(2-aminoethyl)-1-methyl pyrrolidine (32  $\mu$ L, 0.22 mmol) in methylene chloride (1.0 mL) was added aluminum chloride (29 mg, 0.22 mmol) and the reaction mixture was allowed to stir for 30 minutes. The solvent was removed *in vacuo* and saturated L-tartaric acid (1.0 mL) was added, stirring for 45 minutes, until all of the solid dissolved. The aqueous solution was washed with methylene chloride (1.0 mL), basified with 1N NaOH and extracted with methylene chloride. The resulting extract was washed with brine, dried over sodium sulfate, filtered and the solvent was removed *in vacuo*. The resulting yellow material was purified on preparative TLC (Alumina, 2% Methanol in CH<sub>2</sub>Cl<sub>2</sub>) to afford the product as a yellowish solid (30 mg, 34%).

### Example 24



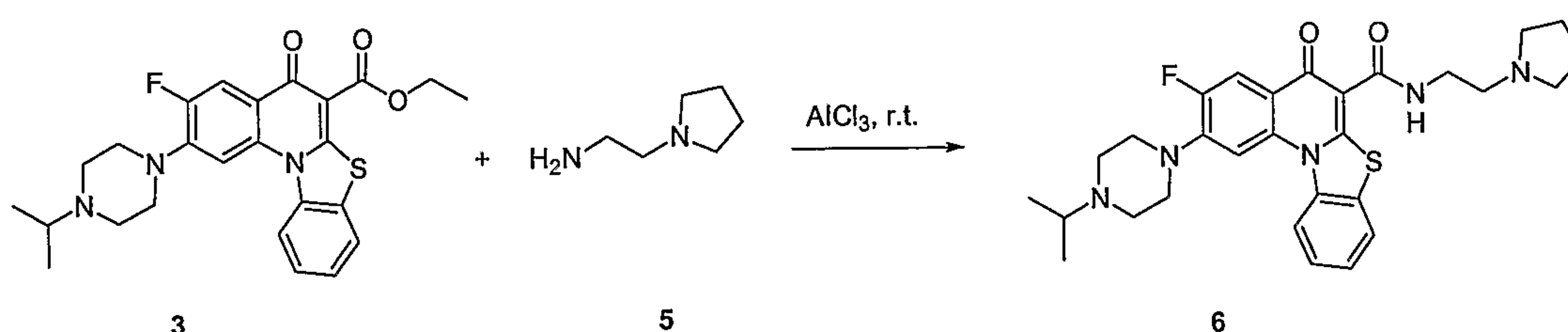
[0137] To a solution of the quinolone ester (34 mg, 0.7 mmol) and 1-(2-aminoethyl)pyrrolidine (15  $\mu$ L, 0.11 mmol) in methylene chloride (1.0 mL) was added aluminum chloride (15 mg, 0.11 mmol) and the reaction mixture was allowed to stir for 30 minutes. The solvent was removed *in vacuo* and saturated L-tartaric acid (1.0 mL) was added, stirring for 45 minutes, until all of the solid dissolved. The aqueous solution was washed with methylene chloride (1.0 mL), basified with 1N NaOH and extracted with methylene chloride. The resulting extract was washed with brine, dried over sodium sulfate, filtered and the solvent was removed *in vacuo*. The resulting yellow material was purified on preparative TLC (Alumina, 2% Methanol in  $\text{CH}_2\text{Cl}_2$ ) to afford the product as a yellowish solid (28 mg, 73%).

#### Example 25



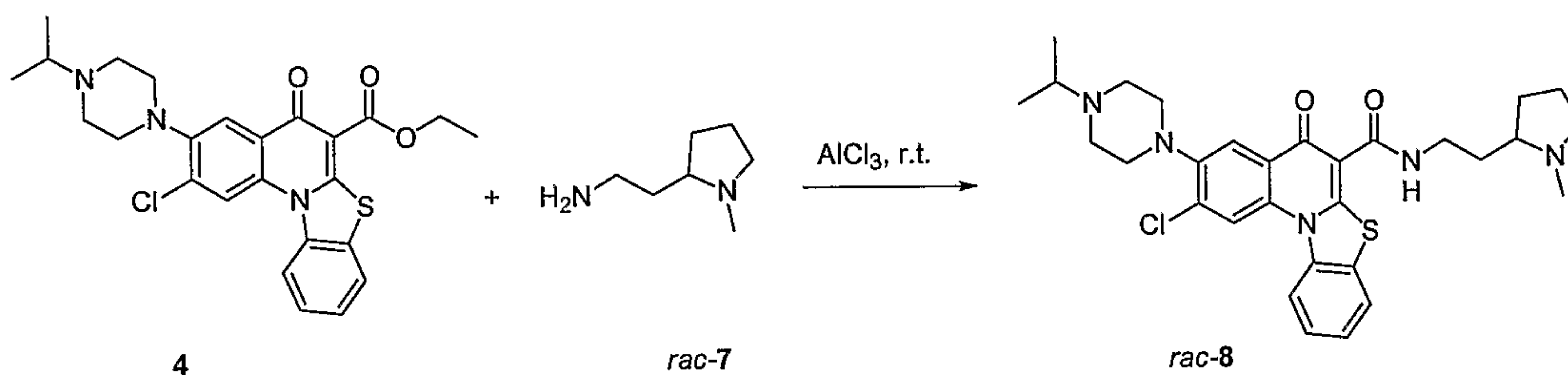
[0138] To a solution of the quinolone ester (146 mg, 0.65 mmol) and 2-(2-aminoethyl)-1-methyl pyrrolidine (1 mmol) in methylene chloride (1.0 mL) was added aluminum chloride (1 mmol) and the reaction mixture was allowed to stir for 30 minutes. The solvent was removed *in vacuo* and saturated L-tartaric acid (1.0 mL) was added, stirring for 45 minutes, until the entire solid dissolved. The aqueous solution was washed with methylene chloride (1.0 mL), basified with 1N NaOH and extracted with methylene chloride. The resulting extract was washed with brine, dried over sodium sulfate, filtered and the solvent was removed *in vacuo*. The resulting yellow material was purified on preparative TLC (Alumina, 2% Methanol in  $\text{CH}_2\text{Cl}_2$ ) to afford the product as a yellowish solid (1.7 mg, 5%).

#### Example 26



[0139] Compound **3** (1.0 eq, 126 mg, 0.27 mmol) and amine **5** (2.0 eq, 68  $\mu$ L, 0.54 mmol) were dissolved in anhydrous  $\text{CH}_2\text{Cl}_2$  (1 ml).  $\text{AlCl}_3$  (2.0 eq, 72 mg, 0.54 mmol) was added and the mixture was stirred at room temperature for 3 hours. The volatiles were removed *in vacuo*. The resulting slurry was treated with a saturated aqueous tartaric acid solution (10 ml) and stirred until all solid disappeared (about 1 hr for completion of the hydrolysis). The solution was neutralized by 1N NaOH (to reach pH = 14) and the compound extracted with  $\text{CH}_2\text{Cl}_2$  (4x). The organic phase was washed with a concentrated aqueous Sodium Potassium tartrate solution, water (2x) and dried over  $\text{Na}_2\text{SO}_4$ . The  $\text{CH}_2\text{Cl}_2$  solution was concentrated. Addition of AcOEt induced crystallization of the expected compound. After filtration, compound **6** was isolated as a pale yellow fluffy solid (76 mg, 53% yield). LCMS (ES): 95% pure,  $m/z$  536  $[\text{M}+\text{H}]^+$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  1.12 (d,  $J = 6.6$ , 6H), 1.80 (br s, 4H), 2.62 (br s, 4H), 2.79 (m, 7H), 3.36 (m, 4H), 3.67 (q,  $J = 6.0$ , 2H), 7.45 (t,  $J = 7.2$ , 1H), 7.53 (td,  $J = 7.3$ ,  $J = 1.3$ , 1H), 7.84 (dd,  $J = 7.8$ ,  $J = 1.2$ , 1H), 7.89 (d,  $J = 6.9$ , 1H), 8.16 (d,  $J = 13.1$ , 1H), 8.23 (d,  $J = 8.5$ , 1H), 10.46 (br t, 1H) ppm.

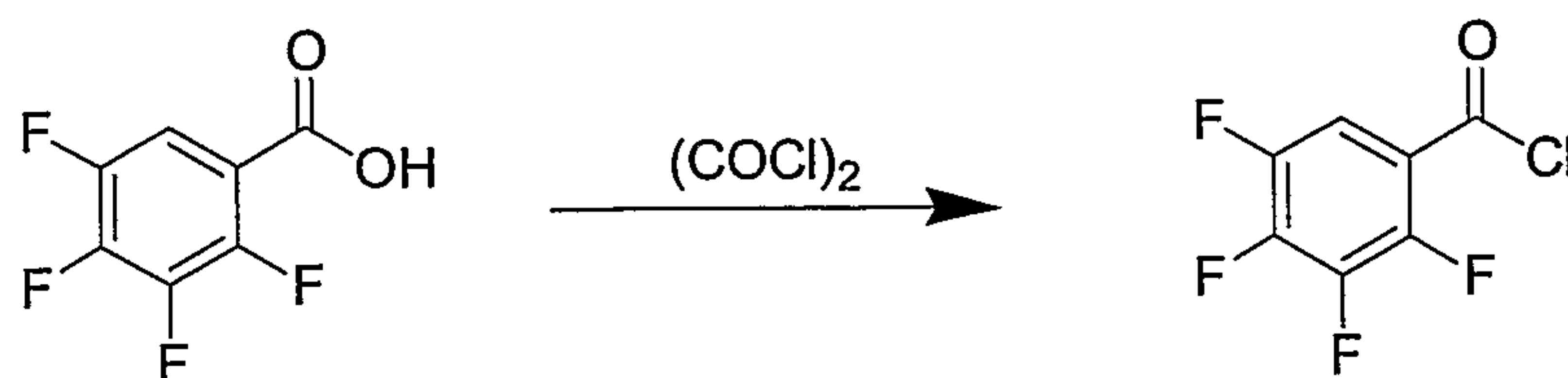
#### Example 27



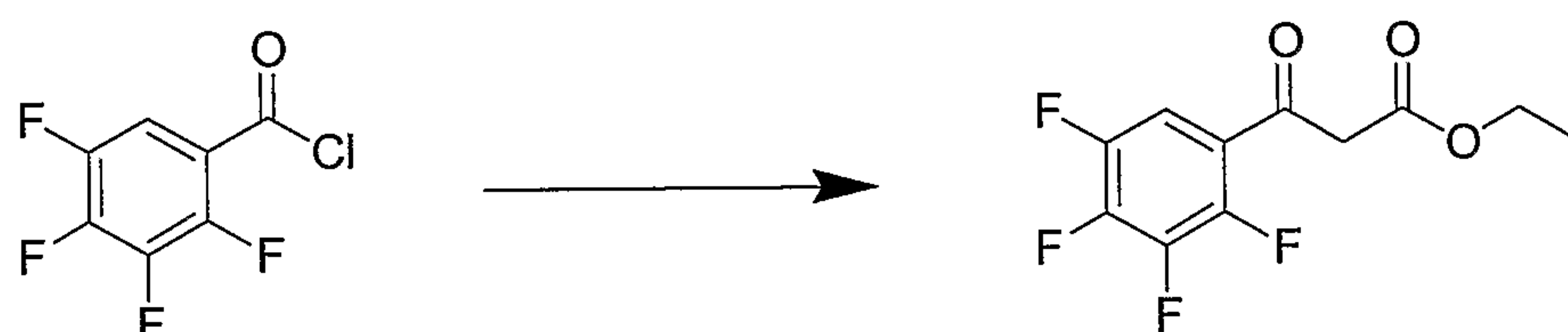
[0140] The compound was prepared according to the procedure used in Example 26, starting from **4** (101 mg, 0.21 mmol) and **7**, providing compound **8** as a white fluffy solid (37 mg, 31% yield). LCMS (ES): 95% pure,  $m/z$  566  $[\text{M}]^+$ , 568  $[\text{M}+2]^+$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  1.13 (d,  $J = 6.5$ , 6H), 1.57 (m overlapped with water signal, 2H), 1.71 (m, 1H), 1.81 (m, 1H), 2.04-2.18 (m, 4H), 2.34 (s, 3H), 2.78 (m, 5H), 3.06 (br t,  $J = 8.6$ , 1H), 3.27 (br s, 4H), 3.52-3.59 (m, 2H), 7.47 (t,  $J = 7.3$ , 1H), 7.57 (td,  $J = 8.4$ ,  $J = 1.1$ , 1H), 7.84 (d,  $J = 7.8$ , 1H), 8.19 (s, 1H), 8.27 (d,  $J = 8.4$ , 1H), 8.57 (s, 1H), 10.38 (br t,  $J = 5.6$ , 1H) ppm.

#### Example 28

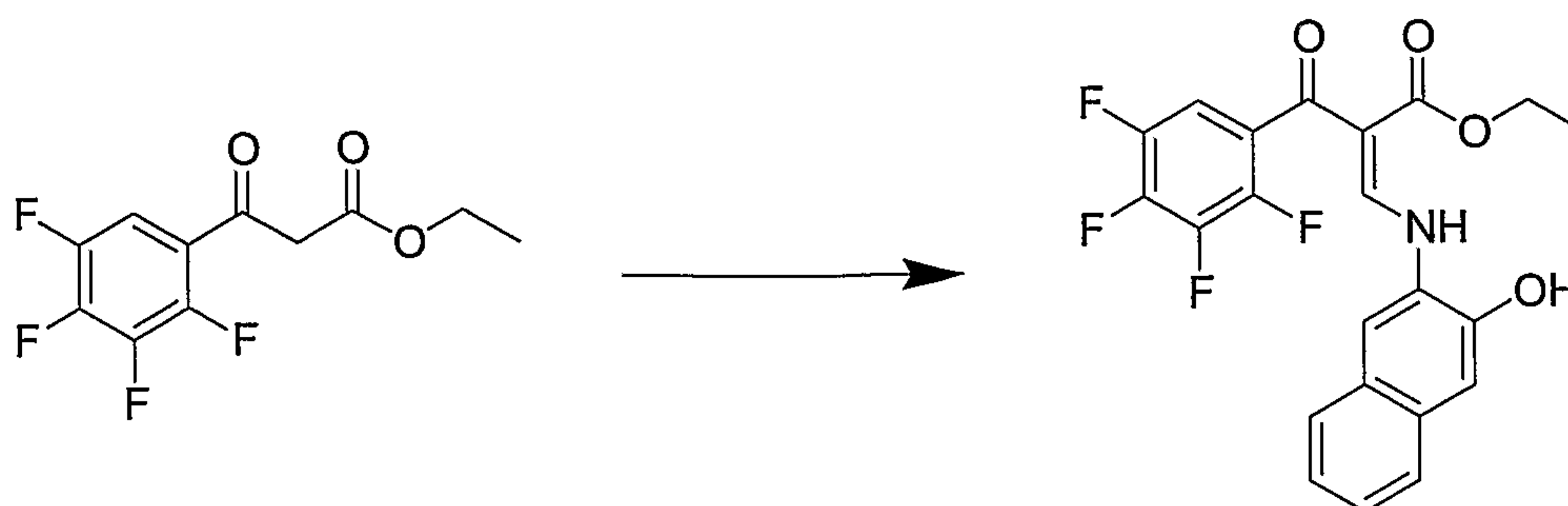
[0141] Example 28 describes a method for preparing a substituted benzoxazine analog from reaction of the corresponding ester with an amine, and aluminum chloride.



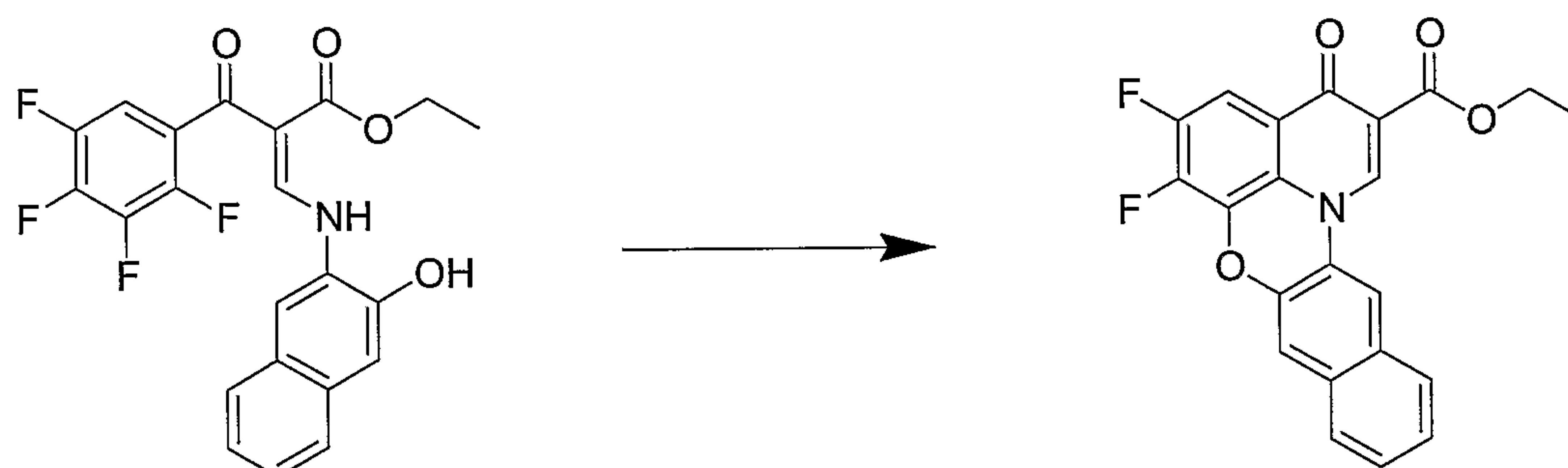
[0142] To a solution of 2,3,4,5-tetrafluorobenzoic acid (100 g, 510 mmol), in methylene chloride (0.5 L) was added oxalyl chloride (68 g, 540 mmol) and DMF (*ca* 3 drops) and the reaction mixture was allowed to stir at room temperature overnight allowing for the produced gasses to escape. The solvent was removed *in vacuo* and the vessel was placed on high vacuum (*ca* 0.5 mm Hg) for 2 hours to afford the acid chloride as a viscous oil (105 g) and was used in the subsequent reaction without further purification.



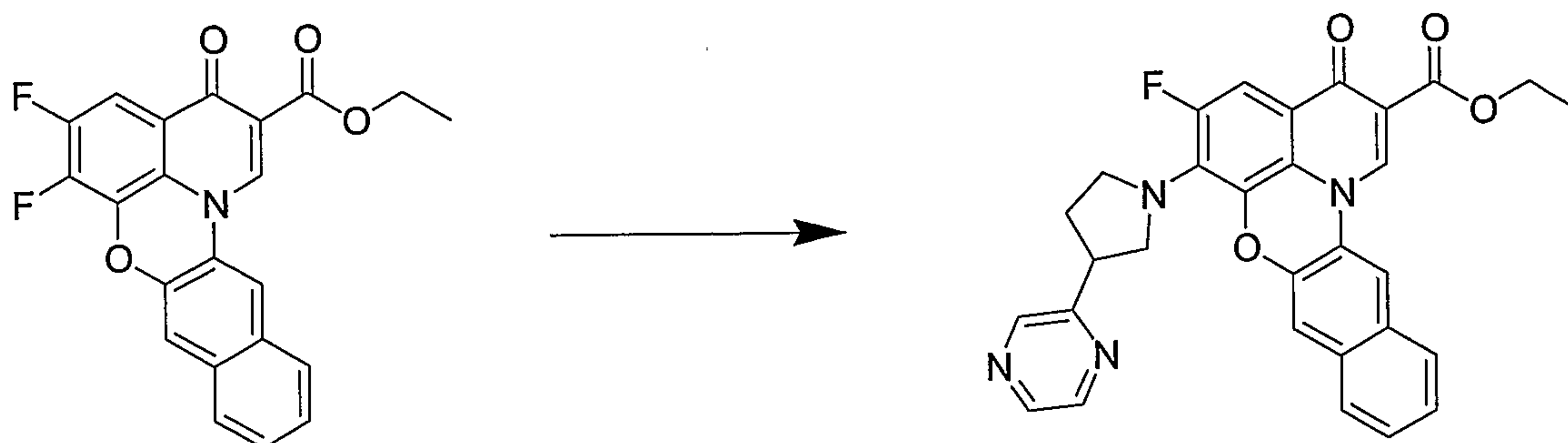
[0143] To a suspension of potassium ethyl malonate (97 g, 570 mmol) and magnesium chloride (55 g, 570 mmol) in acetonitrile and the suspension was chilled to 0 °C. To this suspension was added the crude 2,3,4,5-benzoyl chloride (105 g, 520 mmol) over 5 minutes. Triethylamine was slowly added at a rate sufficient to keep the reaction temperature below 10 °C and the mixture was allowed to warm to room temperature and was stirred overnight. The solvent was removed *in vacuo* and replaced with toluene (300 mL) and 1N HCl (500 mL) was added and the mixture was allowed to stir for 1 hour. The organic layer was separated and washed with 1N HCl (100 mL) and brine (100 mL) and dried over sodium sulfate, filtering over a pad of silica gel (50 X 100 mm), eluting with ethyl acetate. The solvent was removed *in vacuo* and the resulting oil was dissolved in ethanol/water (9:1) and was allowed to crystallize overnight. The resulting crystals were Isolated by filtration, washing with ethanol/water (8:2) to afford the ketoester (43.75 g, 166 mmol) as a white crystalline solid.



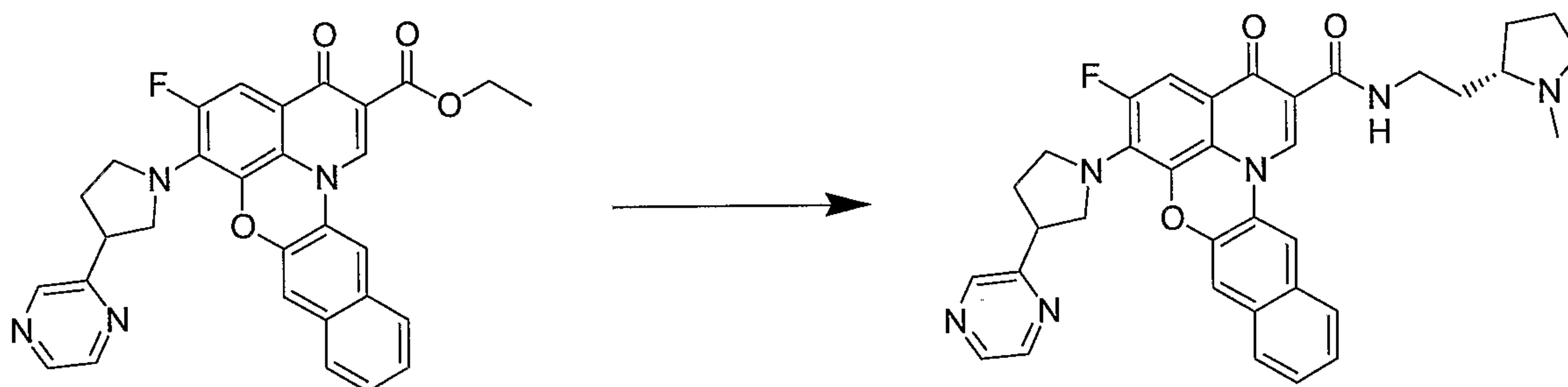
[0144] To a 250 mL round bottom flask was added the tetrafluoroketoester (10.0 g, 37.9 mmol), triethylorthoformate (8.6 mL, 56.8 mmol) and acetic anhydride (7.15 mL, 75.8 mmol) and the reaction mixture was heated to 145 °C for 2 hours. The reaction was allowed to cool to room temperature and placed on high vacuum (*ca* 0.5 mm Hg) for 1 hour. The resulting oil was dissolved in ethanol (100 mL) and 2-amino-1-naphthol (6.02 g, 37.9 mmol) was added at room temperature and the solution became briefly clear and then product began to precipitate. The reaction was allowed to stir for 2 hours and was then filtered and washed with ethanol (100 mL) to afford the enamine as a yellow solid (12.5 g, 28.9 mmol).



[0145] To a solution of the enamine (12.13 g, 27.95 mmol) in dry DMF (50 mL) was added potassium carbonate (4.24g, 1.1 eq.) and the mixture was heated to 90 °C, with constant stirring, for 2 hours. The mixture was allowed to cool to room temperature without stirring and was allowed to remain at room temperature for an additional hour. The crystalline solid was collected by filtration, washing with water. Recrystallization from THF afforded the difluoroester as a white crystalline solid (9.3 g, 23.6 mmol).



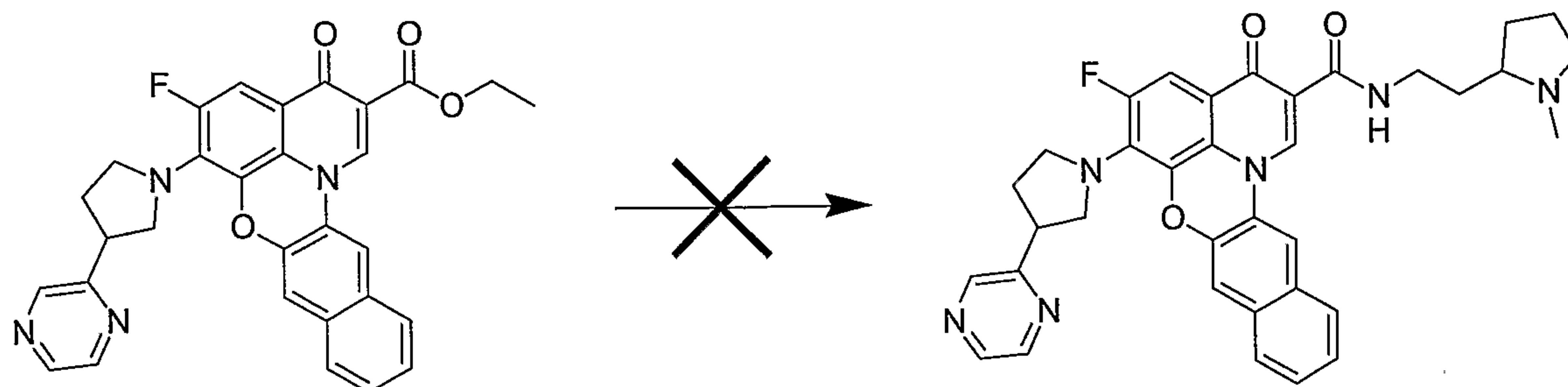
[0146] To a solution of the difluoroester (1.0g, 2.5 mmol) in NMP (10 mL) was added N-Boc-3-(2-pyrazino)pyrrolidine (870 mg, 3.5 mmol) and the mixture was heated to reflux for 3 hours. The reaction mixture was then allowed to cool to room temperature and the product was collected by filtration. Crystallization from THF afforded the pyrazine ester as a yellow solid (910 mg, 1.74 mmol).



[0147] To a solution of the pyrazine ester (250 mg, 0.48 mmol) and 2-(2-aminoethyl)-1-methylpyrrolidine (80 mg, 0.63 mmol) in methylene chloride at room temperature was added aluminum chloride (83 mg, 0.63 mmol) and the reaction mixture was allowed to stir for 2 hours. The solvent was removed *in vacuo* and saturated L-tartaric acid was added (5 mL) and the mixture was allowed to stir for 1 hour. Methylene chloride (10 mL) was then added and the mixture was basified with 1N NaOH. The organic layer was separated and washed with a saturated solution of Rochelle's salt, brine and dried over sodium sulfate. The solvent was removed *in vacuo* and the resulting solid was dissolved in THF and filtered and the solvent was removed again. The crude solid was recrystallized in ethyl acetate to afford the amide as a yellow solid (225 mg, 0.37 mmol, 98.5% pure).

### Example 29

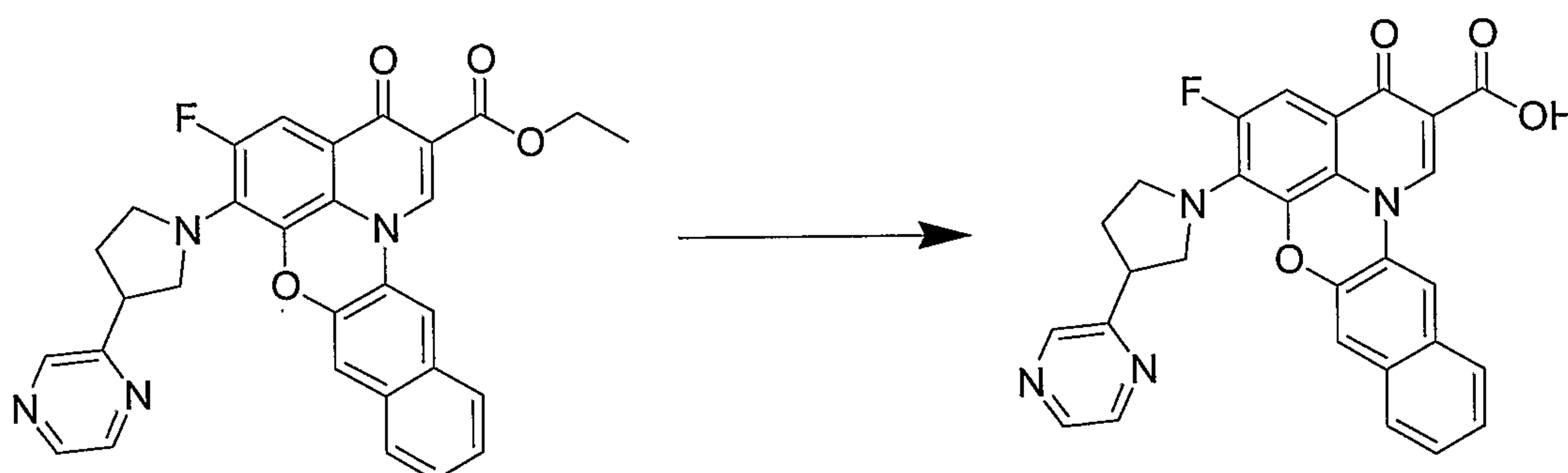
[0148] As shown in Example 29, amide coupling from the corresponding ester resulted in slight or no reaction, where zinc chloride was used as the Lewis acid.



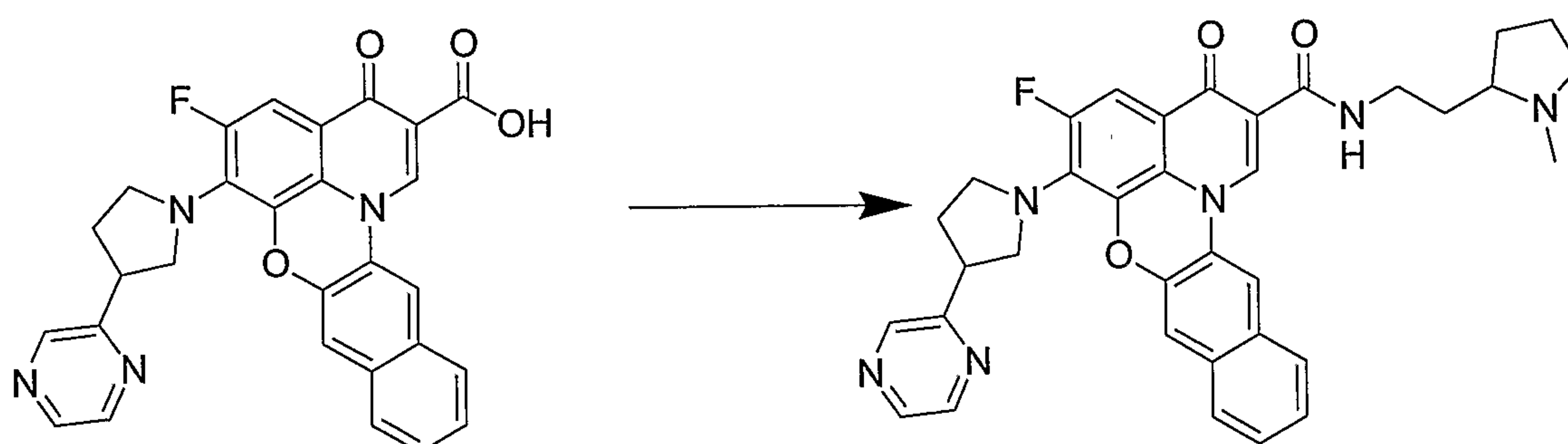
[0149] To a solution of the ester (100 mg, 0.19 mmol) and 2-(2-aminoethyl)-1-methylpyrrolidine (80 mg, 0.63 mmol) in methylene chloride at room temperature was added zinc chloride (86 mg, 0.63 mmol) and the reaction mixture was allowed to stir overnight. LCMS indicated that there was no reaction progress, and the reaction was discontinued.

### Example 30

[0150] Example 30 describes a method for preparing a substituted benzoxazine analog from reaction of the corresponding carboxylic acid with an amine.



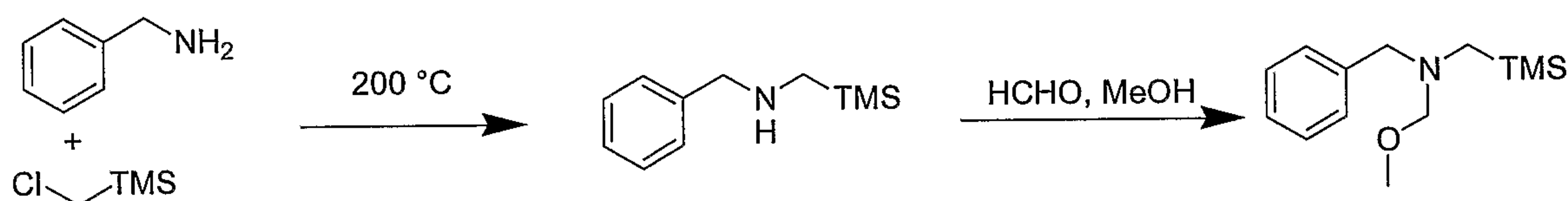
[0151] The pyrazinoester (2.0g, 3.8 mmol) was dissolved in ethanol (100 mL) and *conc* HCl was added (20 mL) and the mixture was refluxed overnight. The mixture was allowed to cool to room temperature and the solid was collected by vacuum filtration, washing with ethanol to afford the pyrazinoacid as a light tan powder (1.6 g, 3.2mmol).



[0152] To a mixture of the fluoroaminoacid (1.6 g, 3.2 mmol) and HBTU (2.0g, 5.3 mmol) in NMP (20 mL) was added N,N-diisopropyl-N-ethylamine (1.0 mL, 6 mmol) and the mixture was allowed to stir at room temperature, under argon, for 1 hour (the solution became clear). (S)-2-(2-aminoethyl)-1-methylpyrrolidine (Mizuno, A.; Hamada, Y.; Shioiri, T., *Synthesis*, **1980**, 12 1007)(1.0 mL, 6.9 mmol) was added and the mixture was allowed to stir for 30 minutes. Water (200 mL) was added and the resulting solid was collected by vacuum filtration, washing with water, and dried to afford the pyrazine as a yellow solid. The yellow solid was purified on silica gel (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub> first eluting off impurities followed by eluting with 5% NH<sub>4</sub>OH/15% MeOH/CH<sub>2</sub>Cl<sub>2</sub>. The combined fractions were evaporated to afford the compound as a yellow solid. (1.2g, 2.0 mmol, 85% pure).

### Example 31

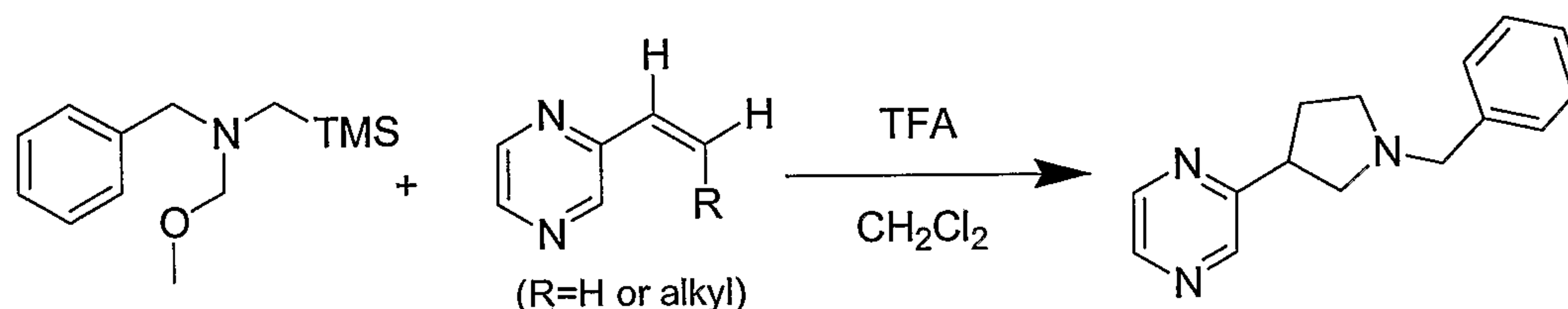
[0153] Example 31 describes the preparation of a Boc-protected pyrrolidine reagent, used as an intermediate in the preparation of benzoxazine and benzothiazole compounds.



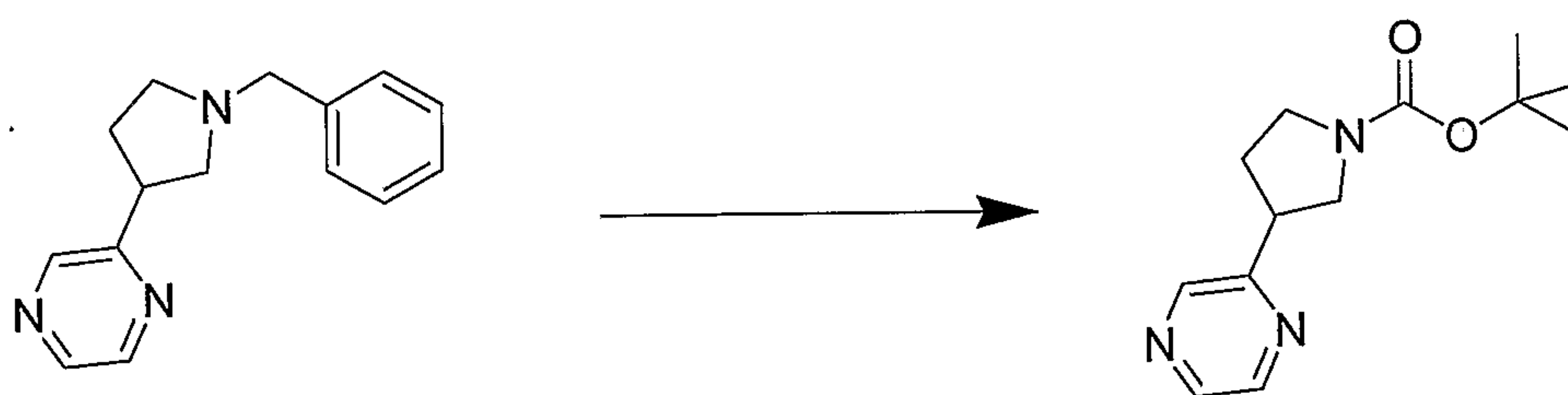
[0154] A mixture of benzyl amine (90g, 841 mmol) and chloromethyltrimethylsilane (30g, 246 mmol) was heated at 200 °C for 2.5 hours. In general, the trimethylsilyl group may be replaced with a -SiR<sup>1</sup>R<sup>2</sup>R<sup>3</sup> moiety, wherein R<sup>1</sup>, R<sup>2</sup> and R<sup>3</sup> are independently alkyl or substituted alkyl. The benzyl groups may also be replaced with other suitable protecting groups.

[0155] The mixture was allowed to cool to room temperature and treated with 1N sodium hydroxide (250 mL) and ether (200 mL) with stirring. The aqueous layer was extracted with ether (3 X 100 mL) and the combined organic extracts were washed with brine, dried over magnesium sulfate and filtered over a pad of silica gel (70 X 50 mm), eluting with ether. The solvent was removed *in vacuo* and the resulting oil was vacuum distilled (bp = 70 °C *ca* 1 mm Hg) to afford the amine as a colorless oil (60.8g) that contained a significant amount of benzyl amine. The resulting oil was then chromatographed on a single biotage column (90g, silica gel, ANALOGIX) eluting with ethyl acetate. The solvent was removed *in vacuo* to afford the pure amine as a colorless oil (43.55g, 225 mmol). The resulting amine was then added to 37%

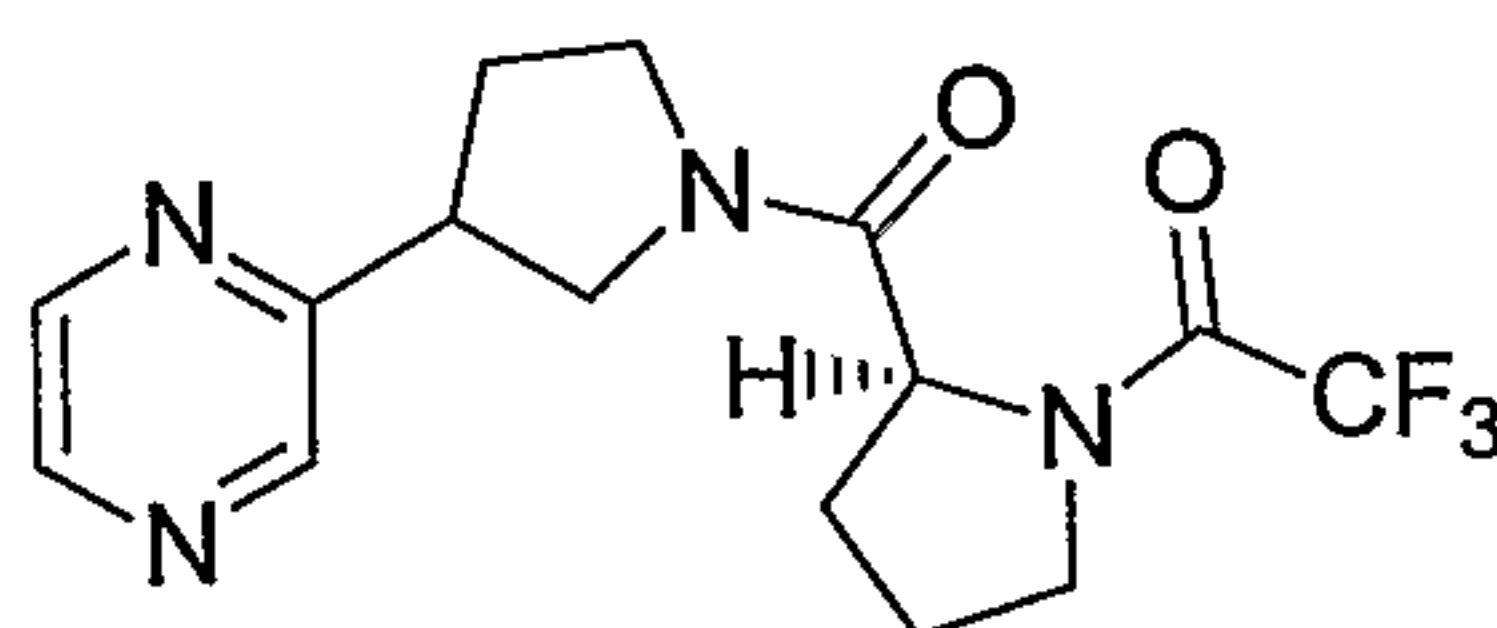
formalin (25 mL) and the mixture was stirred at room temperature for 10 minutes, followed by the addition of methanol (25 mL) and potassium carbonate (20g). The resulting mixture was allowed to stir overnight and then extracted with methylene chloride (3 X 100 mL) and the combined organic extracts were dried with sodium sulfate. The solvent was removed *in vacuo* and the resulting oil was vacuum distilled (bp = 80 °C *ca* 1 mm Hg) to afford the amine as a colorless liquid (39.9g, 168 mmol).



**[0156]** To a solution of vinylpyrazine (10g, 94.3 mmol) in methylene chloride (200 mL) and trifluoroacetic acid (2 mL) was added dropwise a solution of the silylated amine ether (24.33g, 102.7 mmol) dissolved in methylene chloride (100 mL) over 4 hours. The volume was then reduced to 100 mL and extracted with 1N HCl (3 X 75 mL). The aqueous layer was then basified with NaOH and extracted with methylene chloride (3 X 100 mL), dried over magnesium sulfate and filtered over a pad of silica gel (30 X 150 mm) eluting with ethyl acetate. The solvent was evaporated to afford the benzylated pyrazinopyrrolidine (26.19g) as a brownish clear liquid. In general, the pyrazine heterocycle may be replaced with other suitable heterocyclic groups.



**[0157]** To a solution of the benzyl pyrrolidine (7.0 g, 29.3 mmol) and di-*tert*-butyldicarbonate (44.7 g, 205 mmol) in methanol (35 mL) was added 10% Pd/C (degussa type, wet) and the vessel was pressurized with Hydrogen (50 PSI) with shaking. The vessel was vented 3 times to control pressure. After 5 hours the reaction was complete and the mixture was filtered and the solvent was removed *in vacuo*. The resulting material was chromatographed on silica gel (1:1 hexanes/ethyl acetate) to afford the Boc protected pyrrolidine as a light yellow oil (2.3 g, 9.2 mmol).

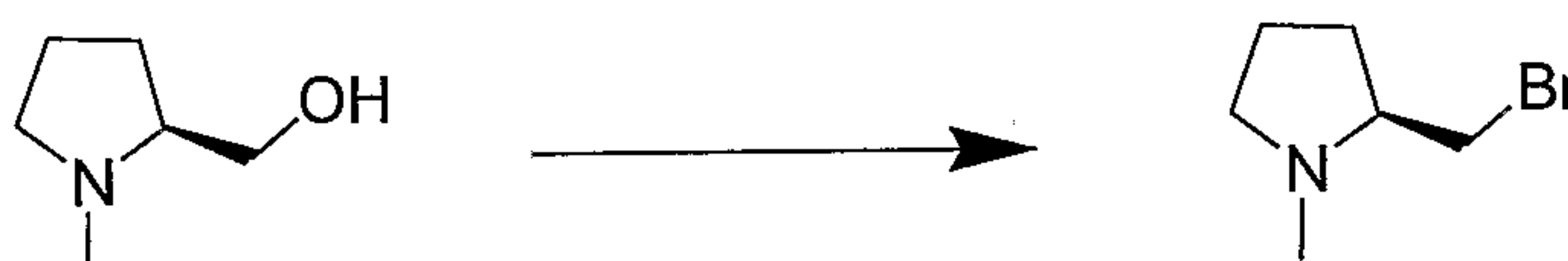


TPC Amide

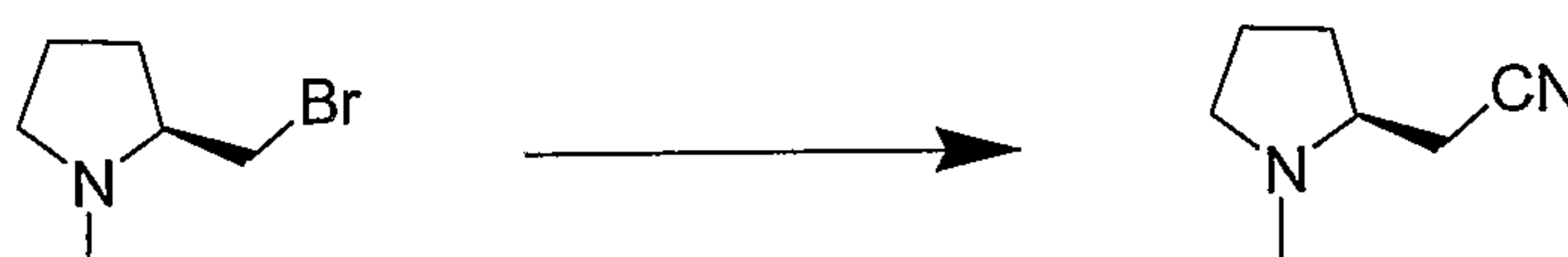
[0158] Enantiomeric ratios can be determined by making a TPC (N-trifluoroacetyl-L-prolyl chloride, Regis #440001) and using GCMS (HP 6890N/5973 MSD) on a Phenomenex Zebron capillary column (ZB-50, 50% Phenyl, 50% Dimethylpolysiloxane, 30M X 0.25 mm, 0.25  $\mu$ M film thickness). Chromatography conditions: 1  $\mu$ L injection split 50:1. Constant Flow He = 1.0 mL/min. Oven; 100 °C for 5 min, 5 °C/min to 300 °C and hold for 8 minutes. The compound comes at 39.08 and 39.31 min but the resolution is very good.

### Example 32

[0159] Example 32 describes the preparation of a chiral amine reagent used in amide coupling.



[0160] To a solution of the hydroxymethyl pyrrolidine (50 g, 434 mmol) in methylene chloride (1 L) was added triphenylphosphine (148 g, 564 mmol) followed by careful addition of carbon tetrabromide (187 g, 564 mmol) at room temperature. The reaction mixture was allowed to stir for 1 hour at room temperature. Water was added and the organic layer was washed with brine, dried over sodium sulfate and the solvent was removed *in vacuo*. The resulting oil was purified by silica gel chromatography (1:1 hexanes/ethyl acetate) to afford the bromide as a clear oil (35 g, 197 mmol).

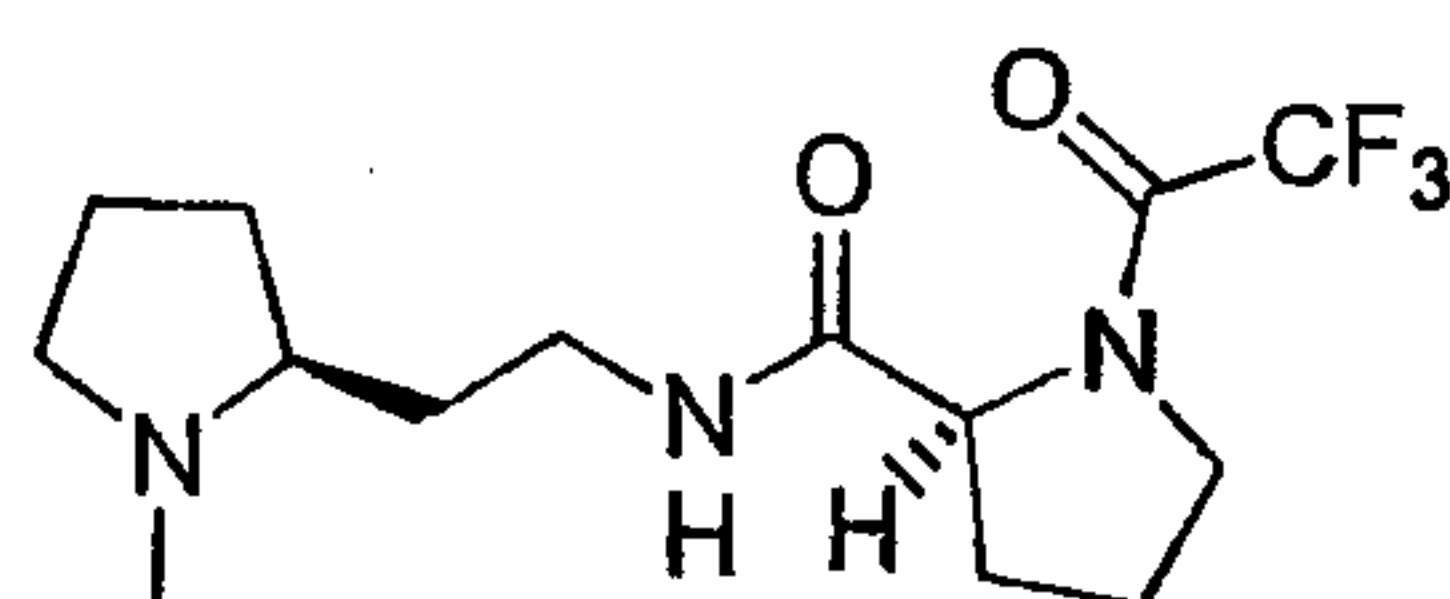


[0161] To a solution of the bromide (23.0 g, 129 mmol) in a solution of acetonitrile and water (75:15, 200 mL) was added potassium cyanide (12.6 g, 194 mmol) and 18-crown-6 (340 mg, 1.3 mmol) and the reaction was allowed to stir overnight at room temperature. The volume was then reduced to 50 mL, under vacuum, and was extracted twice with methylene

chloride (2 X 200 mL). The resulting extracts were combined and washed with brine, dried over sodium sulfate and the solvent was carefully removed *in vacuo* to afford the cyanide as a clear oil (17 g).



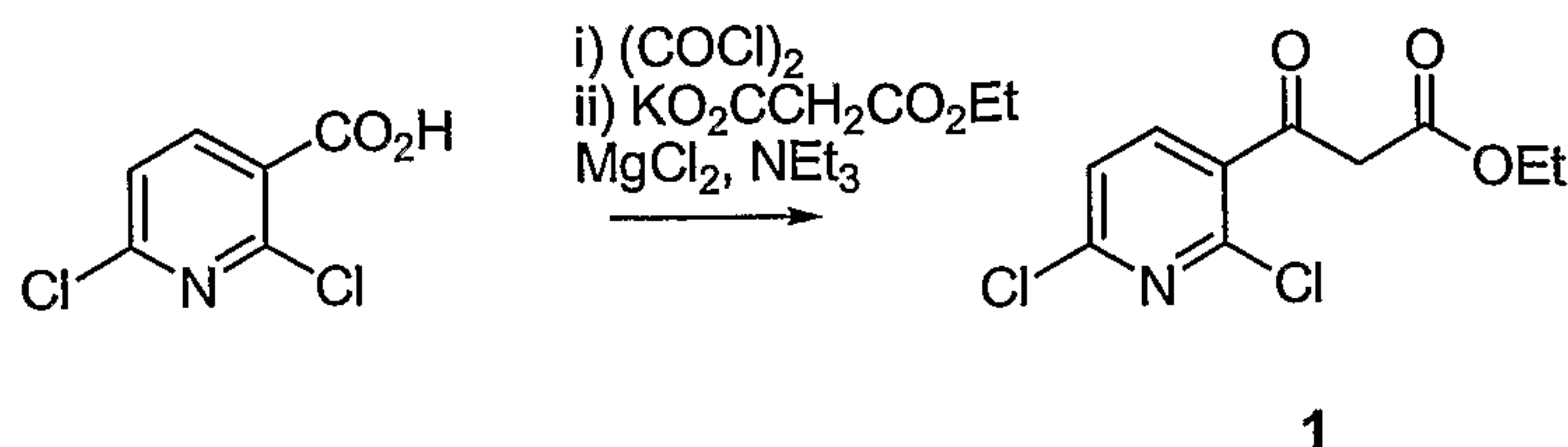
[0162] To a solution of the cyanide (17 g, 137 mmol) in methanol (90 mL) was added Raney Nickel™ (2.0 g, water solution) and the mixture was pressurized with hydrogen (60 PSI) with shaking for 24 hours. The solution was filtered and the solvent was removed *in vacuo*. The pure amine was isolated by distillation (BP = 50 °C, *ca* 10 mm Hg) as a clear oil (7.54 g, 58.9 mmol).



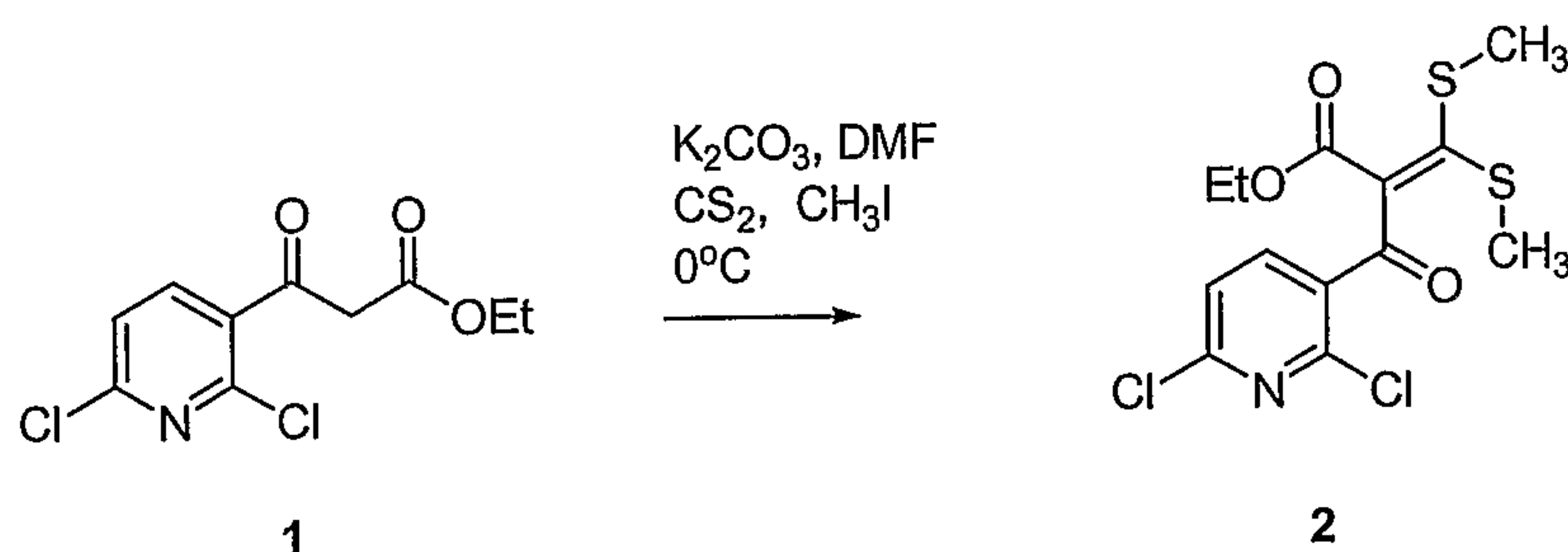
TPC Amide

[0163] Enantiomeric ratios can be determined by making a TPC (N-trifluoromethylacetyl-L-prolyl chloride, Regis #440001) and using GCMS (HP 6890N/5973 MSD) on a Phenomenex Zebron™ capillary column (ZB-50, 50% Phenyl, 50% Dimethylpolysiloxane, 30M X 0.25 mm, 0.25 µm film thickness). Chromatography conditions: 1 µL injection split 50:1. Constant Flow He = 1.0 mL/min. Oven; 100 °C for 5 min, 5 °C/min to 300 °C and hold for 8 minutes. The compound comes at 28.51 and 28.68 min but the resolution is very good.

[0164] It is understood that the foregoing detailed description and accompanying examples are merely illustrative, and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art. Such changes and modifications, including without limitation those relating to the chemical structures, substituents, derivatives, intermediates, syntheses, formulations and/or methods of use of the invention, may be made without departing from the spirit and scope thereof.

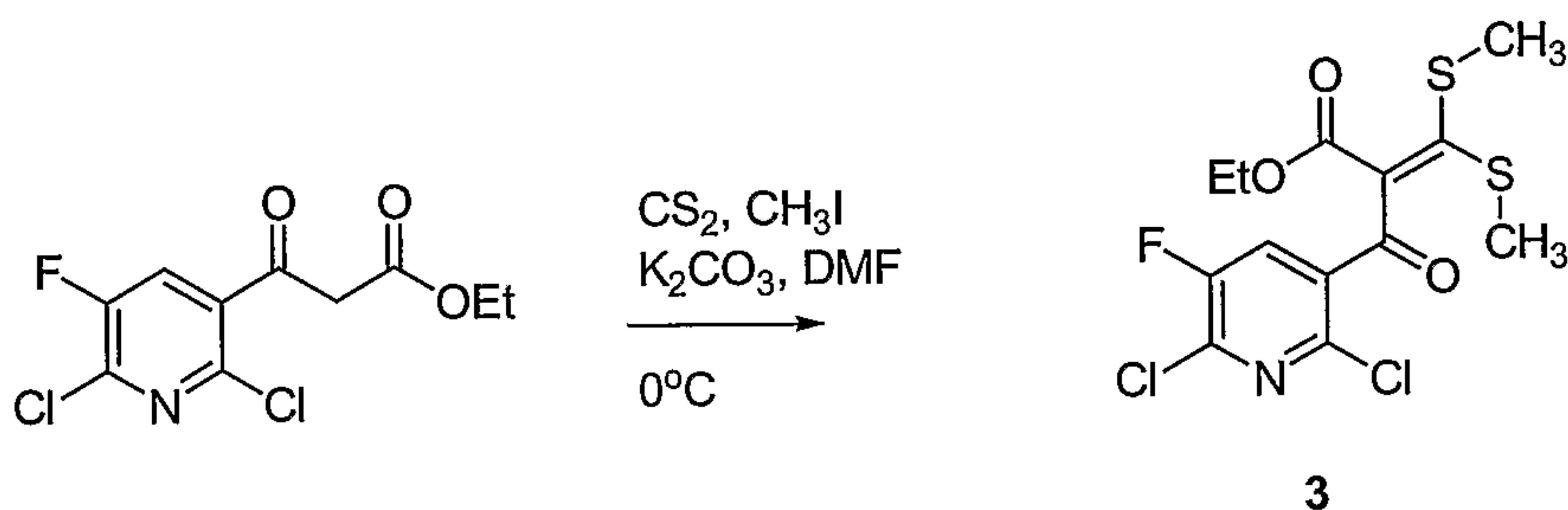
Example 33

[0165] To a solution of the 2,6-dichloronicotinic acid (1.0 eq, 31.24 g, 162.7 mmol) in methylene chloride (500 mL) was added oxalyl chloride (1.2 eq, 23.7g, 187.5 mmol) followed by 3 drops of DMF and the mixture was allowed to stir overnight at room temperature. The solvent was then removed *in vacuo* to afford the crude acid chloride as oil. In a separate flask was dissolved potassium ethyl malonate (1.5 eq., 41.5g, 244 mmol) in acetonitrile (500 mL) and the mixture was cooled to 5°C. Magnesium chloride (1.5 eq, 23.4 g, 245.8 mmol) was then added over 5 minutes, keeping the temperature below 25 °C. The crude acid chloride was then dissolved in acetonitrile (50 mL) and was added *via* dropping funnel keeping the temperature below 5 °C over 30 minutes. Triethylamine (2.0 eq, 42.8 ml, 325 mmol) was then added as quickly as possible while still keeping the temperature below 10 °C. Upon complete addition the reaction was allowed to warm to room temperature overnight with constant stirring. The solvent was removed *in vacuo* and replaced with ethyl acetate. 1N HCl was added (500 mL) and the mixture was stirred for an additional 30 minutes. The organic layer was separated, washed with brine and dried over sodium sulfate and the solvent was removed *in vacuo* to afford the ketoester as orange oil (35.04g). The product was purified by recrystallization from 10% water/methanol to afford the pure ketoester **1** as a white crystalline solid (31.21 g, 74%). LCMS (ES): 95% pure, elutes as 2 peaks,  $m/z$  216, 262.

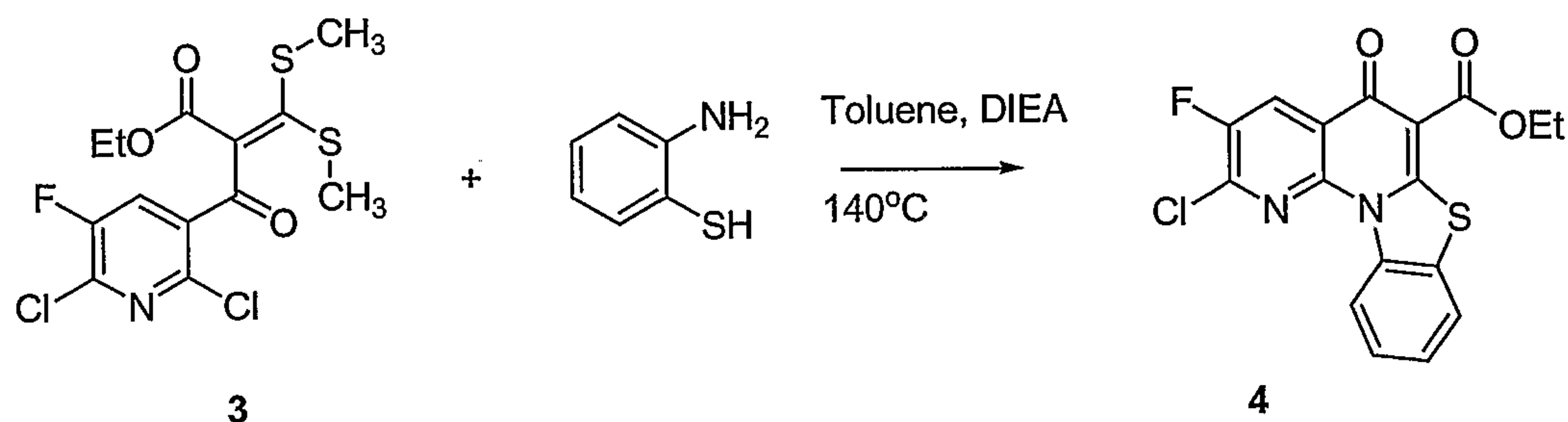
Example 34

[0166] Ketoester **1** (1.0 eq, 11.45g, 43.87 mmol) was dissolved in DMF (60 ml) and the mixture was chilled to 0 °C with an ice bath. Methyl iodide (3.0 eq, 8.2 ml, 132.mmol) was then added and the mixture was cooled to -5 °C. Carbon disulfide was then added (1.5 eq, 4.0 mL, 65.8 mmol) followed by potassium carbonate (2.0 eq, 12.1 g, 88 mmol) keeping the temperature below 5 °C. The mixture was allowed to warm to room temperature with stirring over 2 hours then was extracted with ethyl acetate (5 X 100 mL) and dried over sodium sulfate. The solvent was removed *in vacuo* and the resulting oil was purified by silica gel chromatography (10% ethyl acetate/hexanes) to afford the bisthioether **2** as a yellow oil (70% yield). LCMS (ES): 90% pure,  $m/z$  388  $[M+22]^+$ , 320  $[M+1 -OEt]^+$ .

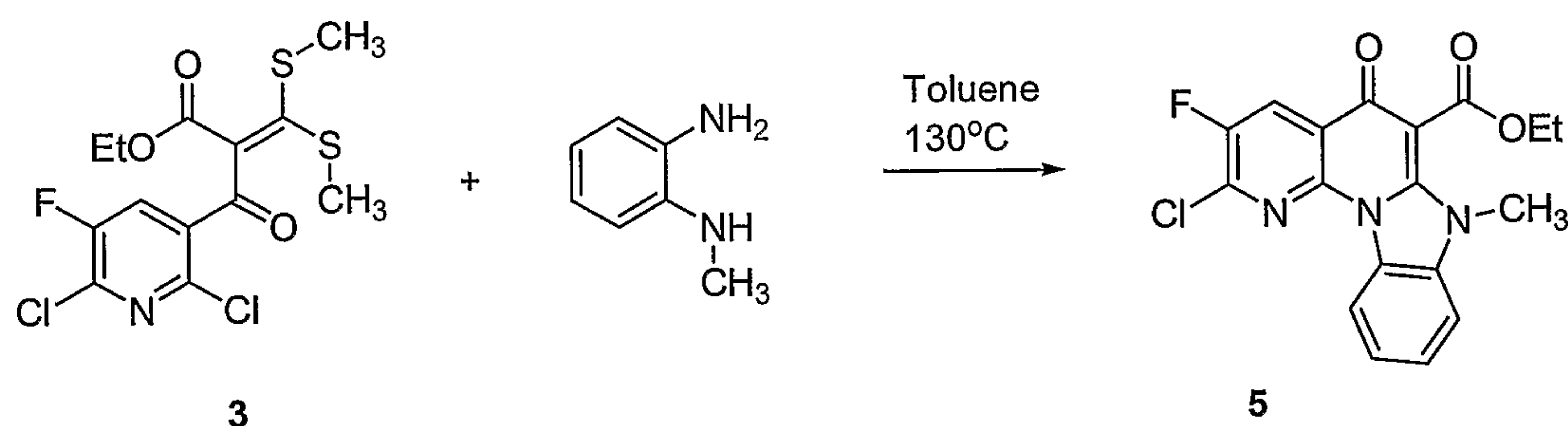
### Example 35



[0167] Ethyl 2,6-dichloro-5-fluoro-3-pyridine-β-keto propionate (1.0 eq., 31.79 g, 0.113 mol) was dissolved in DMF (180 ml). The solution was cooled down using an ice-salt mixture. Iodomethane (3.0 eq, 21 ml, 0.887 mol) and carbon disulfide (1.5 eq, 10.3 ml, 0.170 mol) were added and the mixture stirred until internal temperature reached  $T = -2^{\circ}\text{C}$ .  $\text{K}_2\text{CO}_3$  (2.0 eq, 31.4 g, 0.227 mmol) was added fairly quickly (over 2-3 min) which induced the internal temperature to reach  $T = 12^{\circ}\text{C}$ . The mixture was stirred in an ice bath for 5 hours. After adding water and brine, the compound was extracted with EtOAc (3x). The combined extracts were washed with brine (2x), dried over  $\text{Na}_2\text{SO}_4$  and the volatiles removed *in vacuo*. Purification by flash chromatography on silica gel (5 to 30% gradient of EtOAc in hexanes) afforded compound **3** as a thick yellow oil (22.86 g, 52% yield).  $R_f = 0.14$  (10% EtOAc in hexanes); LCMS (ES): 95% pure,  $m/z$  384  $[M]^+$ , 338  $[M-\text{EtOH}]^+$ , 340  $[M+2-\text{EtOH}]^+$ , 342  $[M+4-\text{EtOH}]^+$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  1.16 (t,  $J = 7.4$ , 3H), 2.43 (s, 6H), 4.18 (q,  $J = 7.4$ , 2H), 7.86 (dd,  $J = 0.6$ ,  $J = 7.4$ , 1H) ppm.

Example 36

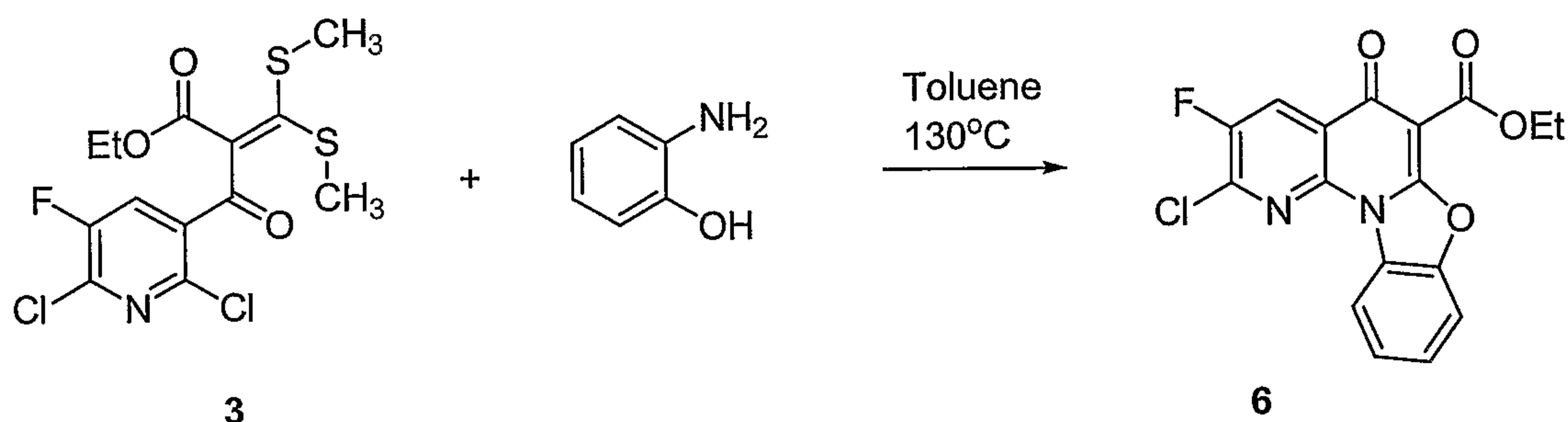
[0168] Compound 3 (1.0 eq, 18.93 g, 49.18 mmol) was dissolved in toluene (400 ml). The solution was carefully degassed by bubbling nitrogen for 10 min. After adding of 2-aminothiophenol (0.9 eq, 4.7 ml, 43.92 mmol) the mixture was stirred at 130-140°C (oil bath temperature) for 6 hours with continuous bubbling of nitrogen inside the reaction. DIEA (1.0 eq, 8.6 ml, 49.37 mmol) was added and the mixture was stirred at 140°C overnight. Upon cooling, compound 4 started to precipitate. The solid was filtered and washed with a bit of toluene. The material was suspended in MeOH and sonicated for a few minutes. After filtration and drying, compound 4 was isolated as a tan solid (9.19 g, 56% yield). LCMS (ES): 95% pure,  $m/z$  377  $[M+1]^+$ , 379  $[M+3]^+$ , 331  $[M+1-\text{EtOH}]^+$ , 333  $[M+3-\text{EtOH}]^+$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  1.50 (t,  $J = 7.0$ , 3H), 4.53 (d,  $J = 7.0$ , 2H), 7.52 (td,  $J = 0.9$ ,  $J = 7.1$ , 1H), 7.63 (td,  $J = 1.4$ ,  $J = 6.0$ , 1H), 7.84 (dd,  $J = 1.2$ ,  $J = 7.9$ , 1H), 8.62 (d,  $J = 7.4$ , 1H), 9.49 (d,  $J = 8.6$ , 1H) ppm.

Example 37

[0169] In a vial, compound 3 (1.0 eq., 202 mg, 0.526 mmol) and N-methyl-benzene-1,2-diamine (1.0 eq., 60  $\mu\text{l}$ , 0.528 mmol) were stirred at 130°C in anhydrous toluene (5 ml) for 16 hours. After removal of the volatiles *in vacuo* the crude mixture was purified by flash chromatography on silica gel (gradient 0.5 to 3% MeOH in  $\text{CH}_2\text{Cl}_2$ ). Upon addition of MeOH

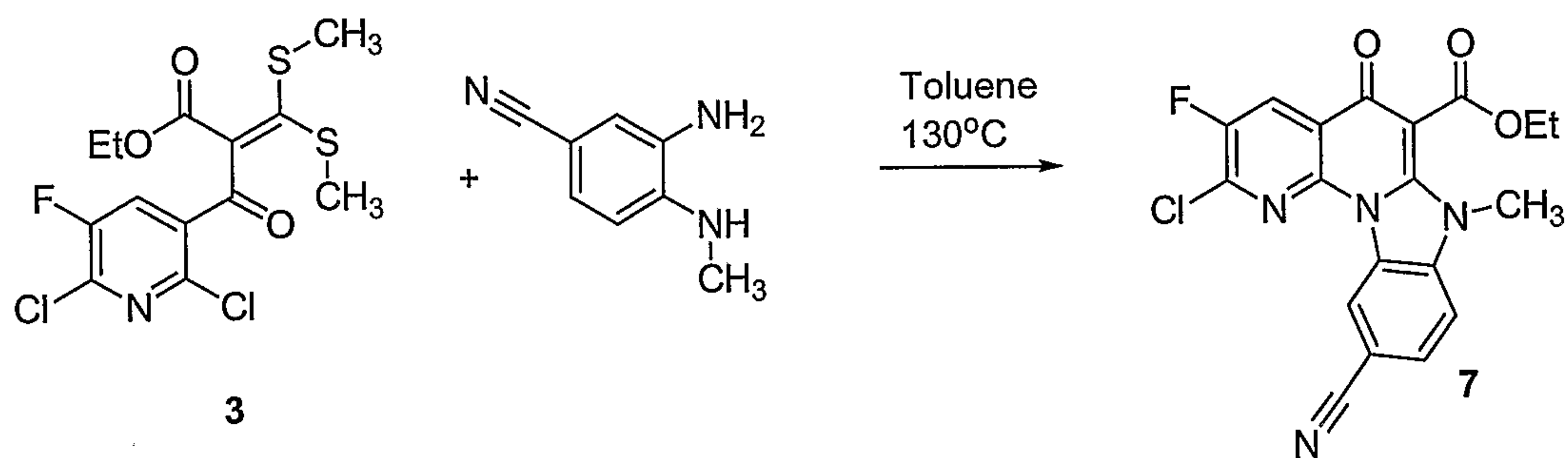
to the resulting oil, pure **5** precipitated out. The material was filtered and dried to afford **5** as a yellow solid (29 mg, 15% yield).  $R_f = 0.26$  (5% MeOH in  $\text{CH}_2\text{Cl}_2$ ); LCMS (ES): 95% pure,  $m/z$  374  $[\text{M}+1]^+$ , 376  $[\text{M}+3]^+$ , 328  $[\text{M}+1-\text{EtOH}]^+$ , 330  $[\text{M}+3-\text{EtOH}]^+$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  1.47 (t,  $J = 7.0$ , 3H), 3.70 (s, 3H), 4.50 (q,  $J = 7.0$ , 2H), 7.37 (dd,  $J = 8.1$ ,  $J = 1.0$ , 1H), 7.44-7.51 (m, 2H), 8.55 (d,  $J = 7.6$ , 1H), 8.94 (dd,  $J = 1.1$ ,  $J = 8.1$ , 1H) ppm.

### Example 38



[0170] In a vial, compound **3** (1.0 eq., 221 mg, 0.575 mmol) and 2-aminophenol (1.1 eq., 70 mg, 0.641 mmol) were stirred at 130°C in anhydrous toluene (5 ml) for 6 hrs. After cooling down the mixture, a brown precipitate that formed was filtered. This material was dissolved in  $\text{CH}_2\text{Cl}_2$  and the solution filtered through a pad of celite. Recrystallization in toluene afforded **6** (48 mg, 24% yield) as a beige solid. LCMS (ES): 95% pure,  $m/z$  361  $[\text{M}+1]^+$ , 363  $[\text{M}+3]^+$ , 315  $[\text{M}+1-\text{EtOH}]^+$ , 317  $[\text{M}+3-\text{EtOH}]^+$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz) shows purity around 80%,  $\delta$  1.46 (t,  $J = 7.0$ , 3H), 4.50 (q,  $J = 7.3$ , 2H), 7.48-7.54 (m, 2H), 7.65 (dd,  $J = 1.4$ ,  $J = 7.9$ , 1H), 8.57 (d,  $J = 7.4$ , 1H), 8.68 (dd,  $J = 1.4$ ,  $J = 7.9$ , 1H) ppm.

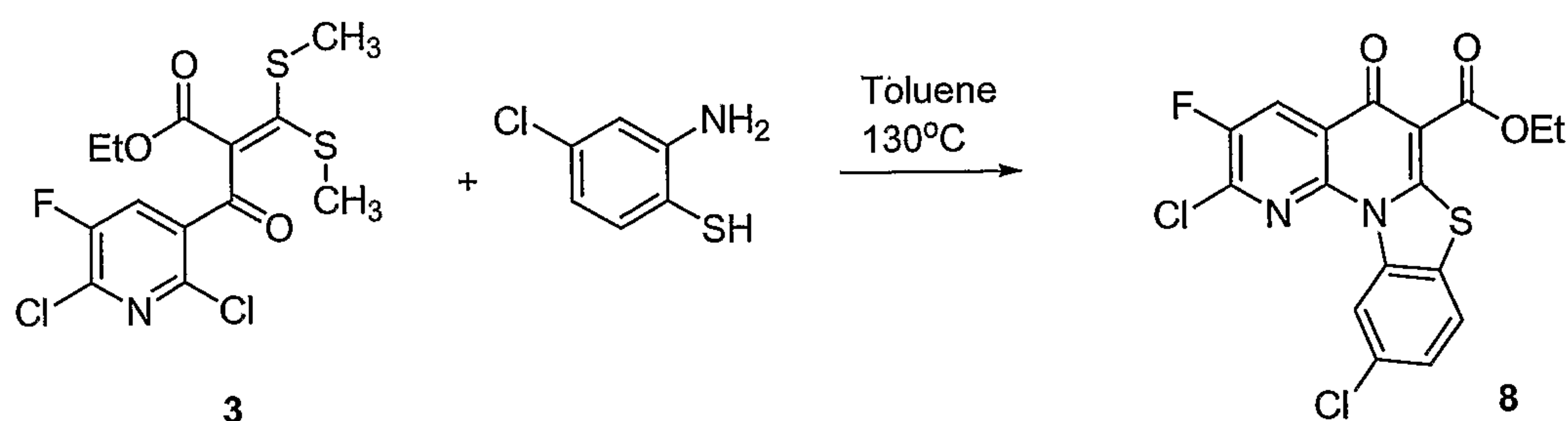
### Example 39



[0171] In a vial, compound **3** (1.0 eq., 266 mg, 0.692 mmol) and 3-amino-4-(methylamino)benzonitrile (1.0 eq., 100 mg, 0.689 mmol) were stirred at 130°C in anhydrous

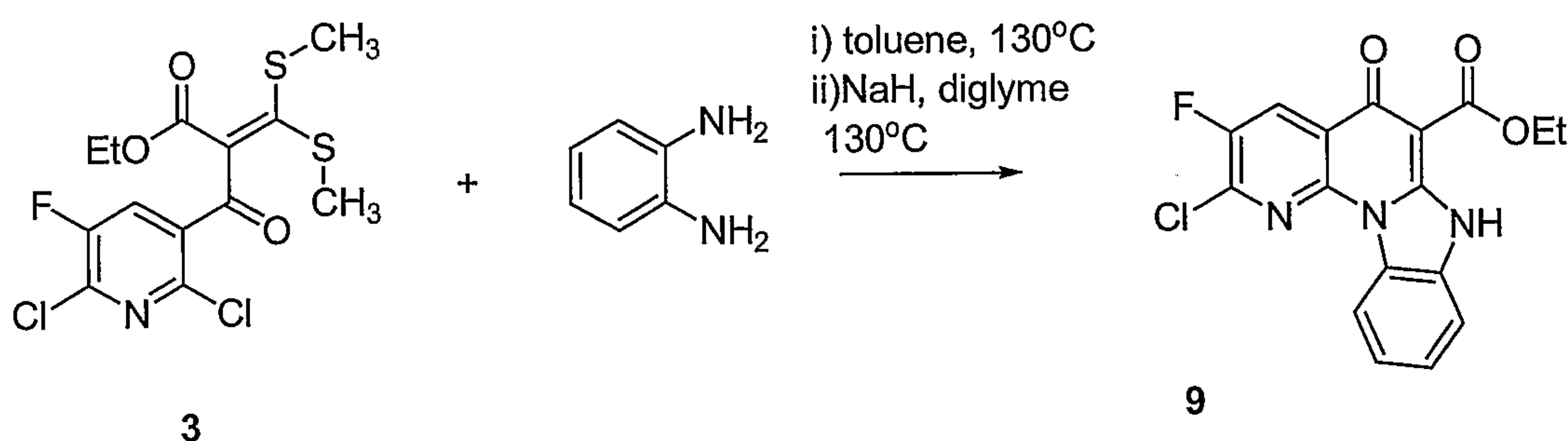
toluene (5 ml) for 2 days. After evaporation of the solvents, the crude mixture was passed through a silica gel column (0.5 to 3% gradient of MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Purification by preparative TLC on silica gel (1 mm, two plates, 4% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) afforded compound 7 as a yellow solid (32 mg, 12% yield). LCMS (ES): 95% pure, m/z 399 [M+1]<sup>+</sup>, 401 [M+3]<sup>+</sup>, 353 [M+1-EtOH]<sup>+</sup>, 355 [M+3-EtOH]<sup>+</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz) δ 1.47 (t, *J* = 7.1, 3H), 3.72 (s, 3H), 4.51 (q, *J* = 7.3, 2H), 7.42 (d, *J* = 8.4, 1H), 7.79 (dd, *J* = 1.5, *J* = 8.4, 1H), 8.55 (d, *J* = 7.3, 1H), 9.22 (d, *J* = 1.4, 1H) ppm.

#### Example 40



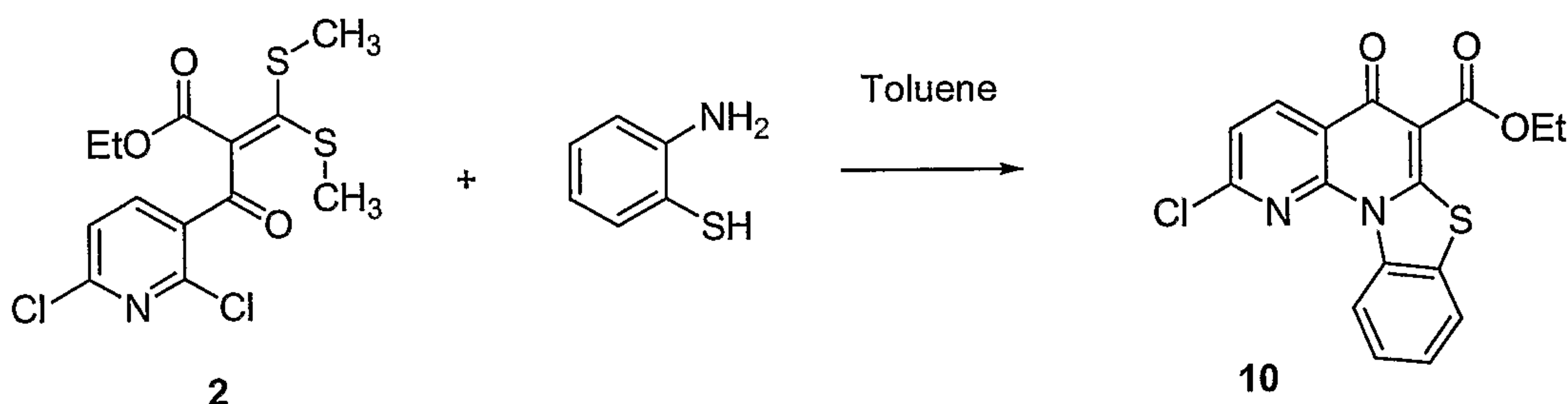
[0172] In a vial, compound 3 (1.0 eq., 288 mg, 0.749 mmol) and 2-amino-4-chlorobenzenethiol (1.0 eq., 120 mg, 0.752 mmol) were stirred at 130°C in anhydrous toluene (5 ml) for 2 days. The solid formed during the reaction was filtered and dissolved in a mixture of MeOH and CH<sub>2</sub>Cl<sub>2</sub> (200 ml). The resulting cloudy solution was filtered through a pad of celite. After evaporation of the volatiles, the material was purified by flash chromatography on silica gel (CH<sub>2</sub>Cl<sub>2</sub> then 0.5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Compound 8 was isolated as a yellow solid (20 mg, 6% yield). LCMS (ES): >85% pure, m/z 411 [M]<sup>+</sup>, 413 [M+2]<sup>+</sup>, 415 [M+4]<sup>+</sup>, 365 [M-EtOH]<sup>+</sup>, 367 [M+2-EtOH]<sup>+</sup>, 369 [M+4-EtOH]<sup>+</sup>.

#### Example 41



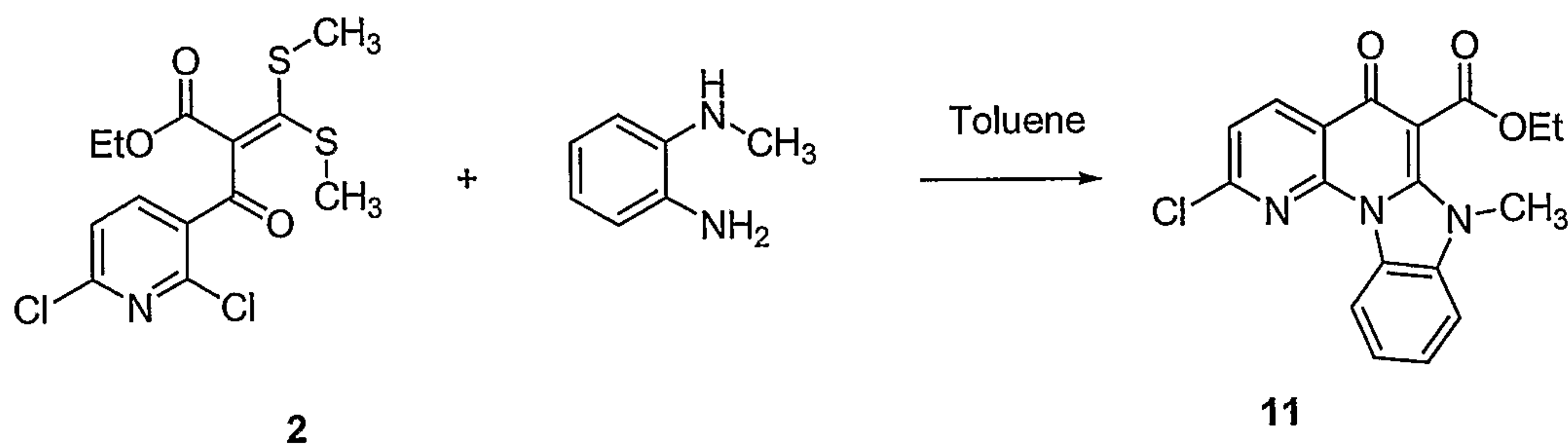
[0173] In a vial, compound **3** (1.0 eq, 255 mg, 0.664 mmol) and benzene-1,2-diamine (1.0 eq, 72 mg, 0.666 mmol) were stirred at 130°C in anhydrous toluene (5 ml) for 5 hours. The volatiles were removed *in vacuo* and the material dissolved in diglyme (2 ml). A 60% suspension of NaH in oil (1.0 eq, 26 mg, 0.63 mmol) was added and the mixture stirred at 130°C for 3 hours. The resulting precipitate was filtered and washed with water. After trituration in MeOH and filtration, compound **9** was isolated as a brown solid (91 mg, 38% yield). LCMS (ES): 95% pure,  $m/z$  360  $[M+1]^+$ , 362  $[M+3]^+$ ;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 500 MHz)  $\delta$  1.44 (t,  $J = 7.1$ , 3H), 4.44 (q,  $J = 7.1$ , 2H), 7.40-7.46 (m, 2H), 7.49 (m, 1H), 8.50 (d,  $J = 7.7$ , 1H), 8.81 (d,  $J = 6.8$ , 1H) ppm.

#### Example 42



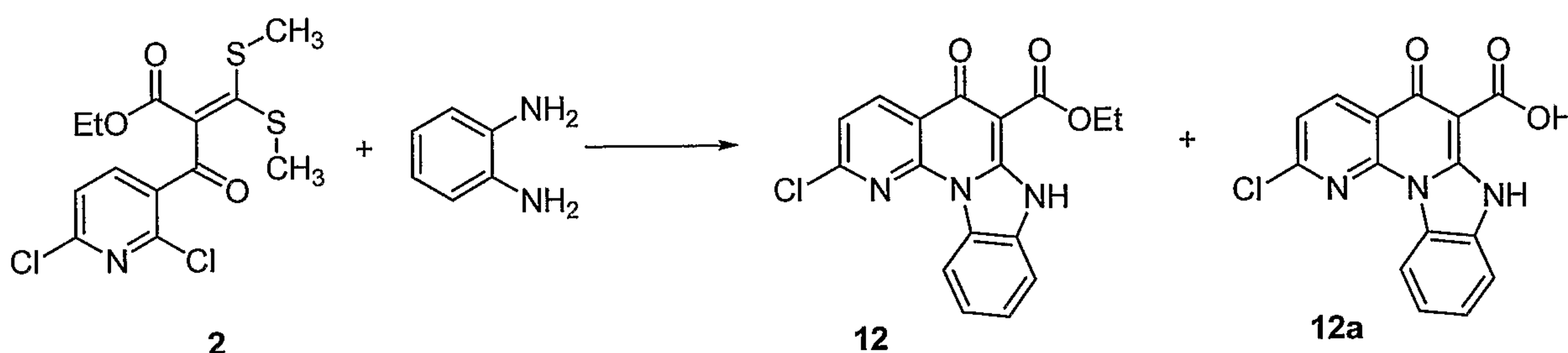
[0174] To a solution of the bisthioether **2** (1.0 eq, 10.14 g, 27.7 mmol) in toluene (300 mL) was added 2-aminothiophenol (1.1 eq, 3.81 g, 30.5 mmol) and the mixture was refluxed overnight with constant nitrogen degassing. The mixture was then allowed to cool to room temperature and diisopropyl ethylamine (1.5 eq, 7.0 mL, 41.55 mmol) was added and the mixture was heated to reflux for 3 hours. The mixture was allowed to cool to room temperature and the product was collected by filtration to afford the cyclized ester **10** as a tan solid (6.6 g, 66% yield). LCMS (ES): 95% pure,  $m/z$  359  $[M+1]^+$ .

#### Example 43

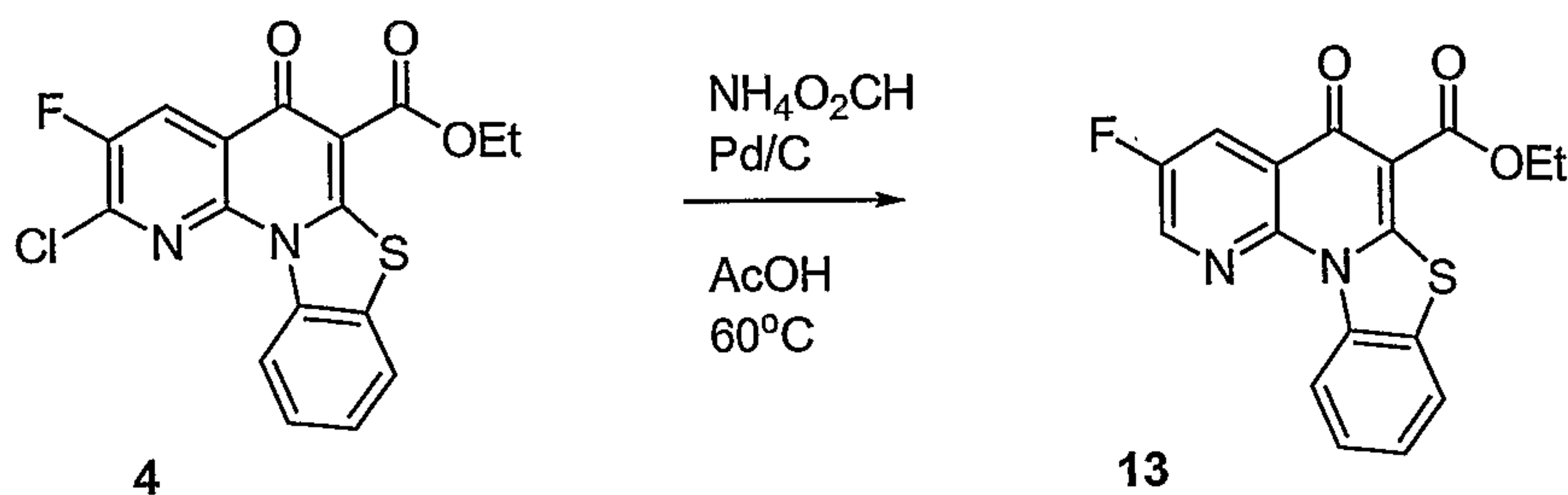


**[0175]** To a solution of the bisthioether **2** (1.0 eq, 5.0 g, 13.66 mmol) in toluene (150 ml) was added N-methyl-1,2-phenylenediamine (1.2 eq, 2.0 g, 16.4 mmol) and the mixture was refluxed overnight with constant nitrogen degassing. The mixture was then allowed to cool to room temperature and diisopropyl ethylamine (1.5 eq, 3.5 mL, 20.75 mmol) was added and the mixture was heated to reflux for 3 hours. The mixture was allowed to cool to room temperature and methanol was added. The volume was reduced to about 100 ml by rotary evaporation and allowed to sit for 2 days. The product was collected by filtration and recrystallized from ethanol to afford the cyclized ester **11** as a tan solid (2.6 g, 53% yield). LCMS (ES): 95% pure, m/z 356 [M+1]<sup>+</sup>.

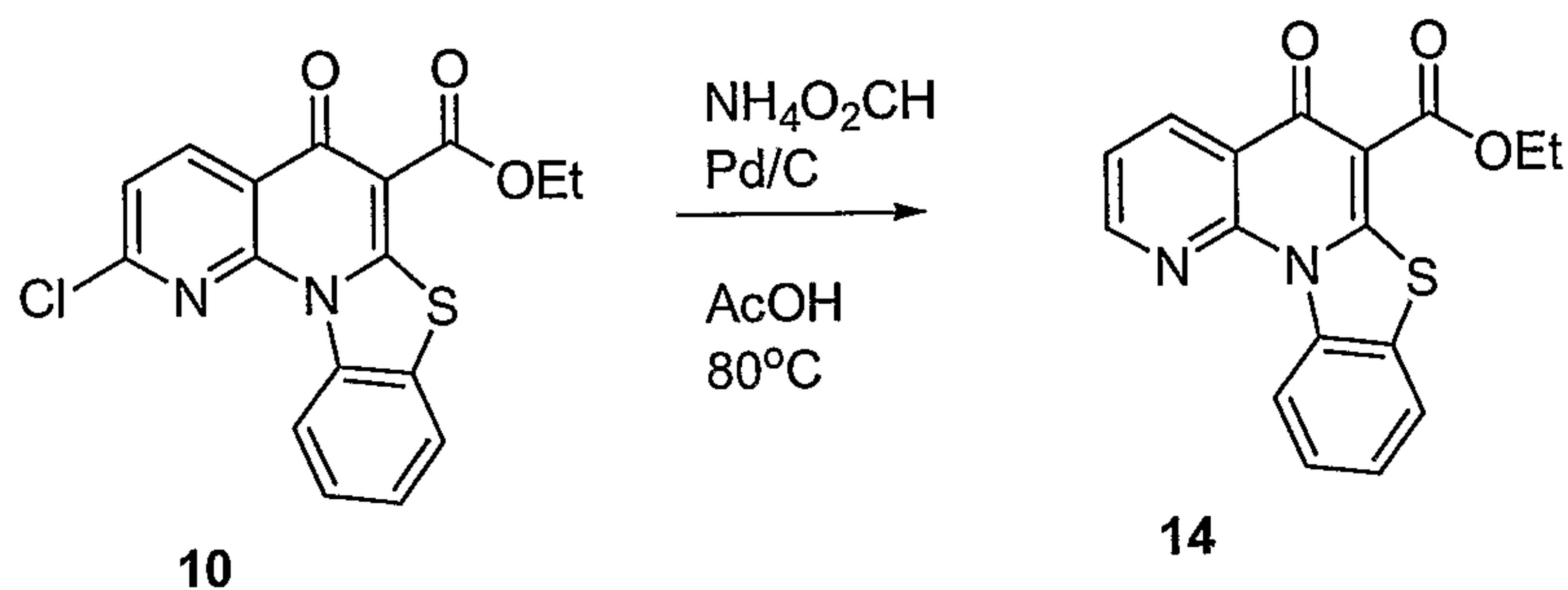
### Example 44



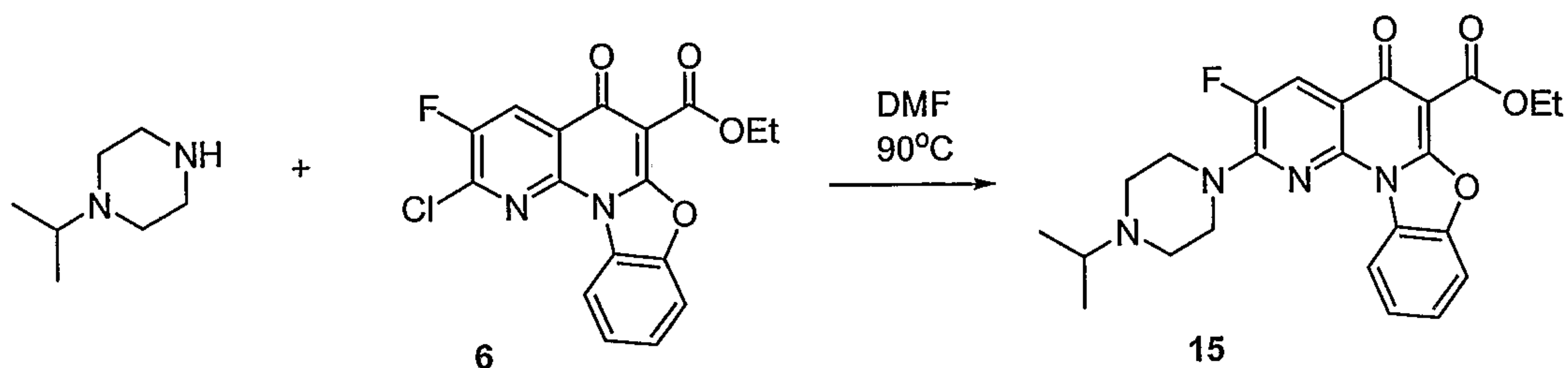
[0176] To a solution of the bithioether **2** (1.0 eq, 10.14 g, 27.7 mmol) in toluene (300 mL) was added 1,2-phenylenediamine (1.1 eq, 3.3 g, 30.5 mmol) and the mixture was refluxed overnight with constant nitrogen degassing. The mixture was then allowed to cool to room temperature and diisopropyl ethylamine (1.5 eq, 7.0 mL, 41.55 mmol) was added and the mixture was heated to reflux for 3 hours. The mixture was allowed to cool to room temperature and the product was collected by filtration to afford the cyclized acid **12a** as a tan solid (3.5 g, 11.2 mmol, 40% yield). LCMS (ES): 95% pure, m/z 314 [M+1]<sup>+</sup>. The resulting filtrate was concentrated *in vacuo* and triturated with ether (100 mL). The product was collected by filtration to afford the cyclized ester **12** as a tan solid (3.5 g, 10.3 mmol, 37% yield). LCMS (ES): 95% pure, m/z 342 [M+1]<sup>+</sup>.

Example 45

[0177] Compound **4** (1.0 eq, 1.57 g, 4.17 mmol) was suspended in acetic acid (15 ml). The suspension was degassed by bubbling nitrogen for 10 min. Ammonium Formate (10 eq, 2.63 g, 41.6 mmol) and Pd/C (10% wet degussa type, 2.5 g) were added and the mixture vigorously stirred at 60°C for 3 hours. The mixture was filtered through a pad of celite. The carbon was treated several times with a hot mixture of CH<sub>2</sub>Cl<sub>2</sub>, MeOH and acetic acid to achieve a complete recovery of the expected material. The combined filtrates were evaporated and CH<sub>2</sub>Cl<sub>2</sub> was added. The organic phase was washed with water (2x), dried over Na<sub>2</sub>SO<sub>4</sub> and the solvents removed *in vacuo*. The resulting solid was sonicated in a mixture of EtOAc and hexanes, filtered and dried to afford pure **13** as an off-white solid (1.11 g, 78% yield). LCMS (ES): 95% pure, m/z 343 [M+1]<sup>+</sup>.

Example 46

[0178] Compound **14** was prepared according to the procedure used for product **13** using a higher temperature (80°C) and longer reaction times. A larger excess of reagents was added several times in order to complete the transformation. The compound was isolated as an off white solid (41 mg, 15% yield). LCMS (ES): 95% pure, m/z 325 [M+1]<sup>+</sup>.

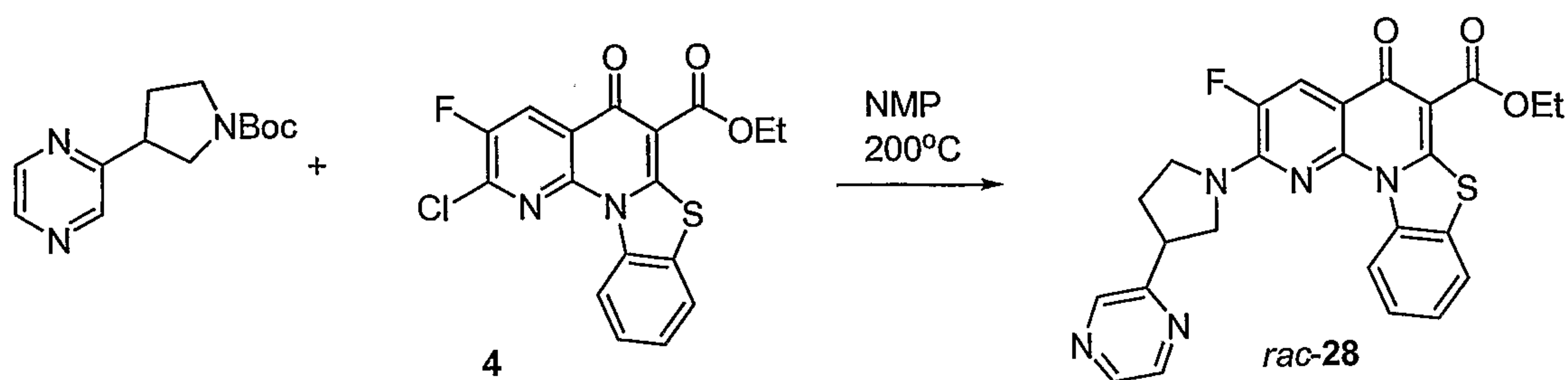
Example 47

**[0179]** In a vial, compound **6** (1.0 eq., 13 mg, 0.0360 mmol) and N-isopropyl piperazine (4.0 eq., 21  $\mu$ l, 0.147 mmol) were stirred in DMF (0.1 ml) at 90°C for 4.5 hrs. After adding water, the precipitate was filtered and dried. The product **15** was purified by precipitation with AcOEt/hexanes to afford a beige solid (7 mg, 43% yield). LCMS (ES): >90% pure, m/z 453 [M+1]<sup>+</sup>.

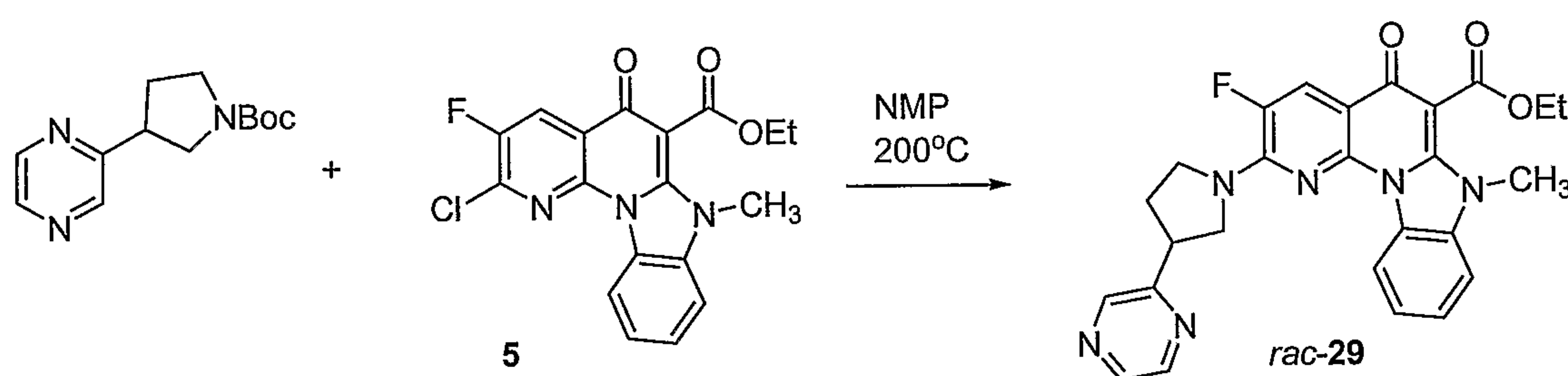
**[0180]** The following analogues were prepared by the same method, using the appropriate amines and chloro-azabenzofluorenones.

| Structure | M.W.   | LCMS (ES) m/z          |
|-----------|--------|------------------------|
|           | 468.54 | 469 [M+1] <sup>+</sup> |
|           | 465.52 | 466 [M+1] <sup>+</sup> |
|           | 468.50 | 469 [M+1] <sup>+</sup> |
|           | 504.54 | 505 [M+1] <sup>+</sup> |

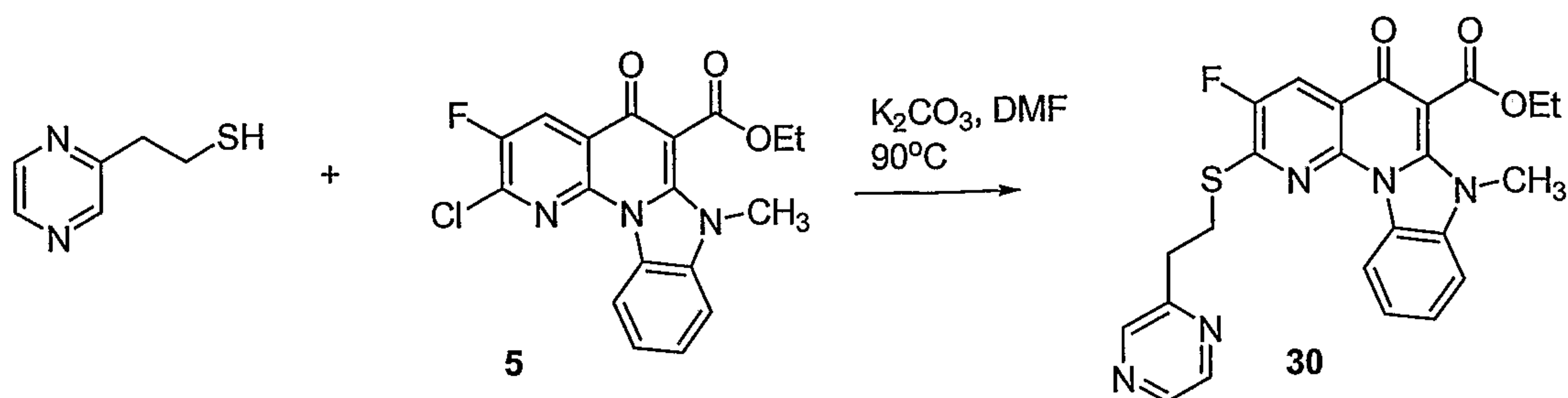
| Structure | M.W.   | LCMS (ES) m/z          |
|-----------|--------|------------------------|
|           | 526.58 | 527 [M+1] <sup>+</sup> |
|           | 490.53 | 491 [M+1] <sup>+</sup> |
|           | 523.56 | 524 [M+1] <sup>+</sup> |
|           | 502.99 | 503 [M+1] <sup>+</sup> |
|           | 451.49 | 452 [M+1] <sup>+</sup> |
|           | 450.51 | 451 [M+1] <sup>+</sup> |
|           | 433.46 | 434 [M+1] <sup>+</sup> |
|           | 505.57 | 506 [M+1] <sup>+</sup> |

Example 48

[0181] In a vial, compound **4** (1.0 eq., 14 mg, 0.0372 mmol) and racemic tert-butyl 3-(pyrazin-2-yl)pyrrolidine-1-carboxylate (4.0 eq., 50 mg, 0.160 mmol) were stirred in anhydrous NMP (0.2 ml) at 200°C for 1 hr. After adding water, the precipitate was filtered and dried. This material was purified by preparative TLC on silica gel (1mm plate, eluted twice with 4% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Compound **28** was isolated as a brown solid (9 mg, 50% yield). LCMS (ES): 90% pure, m/z 490 [M+1]<sup>+</sup>.

Example 49

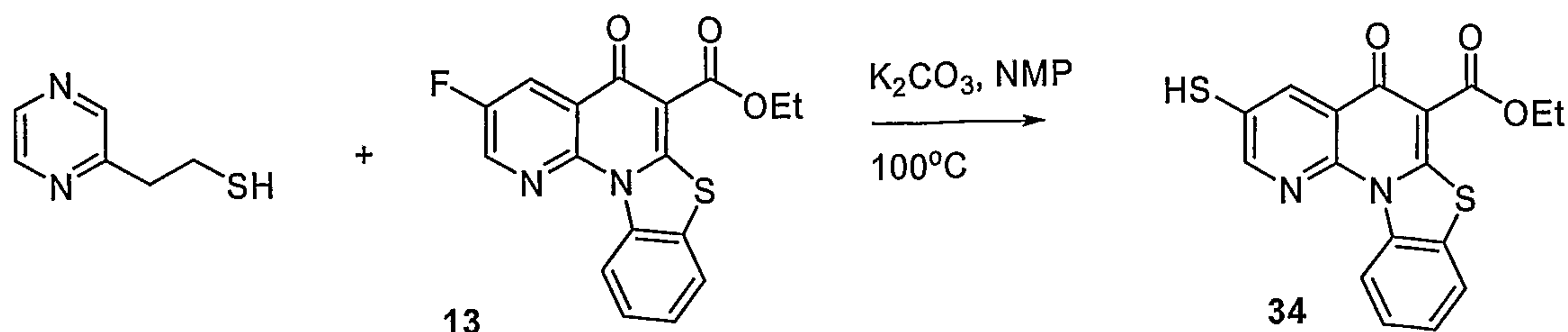
[0182] In a vial, compound **5** (1.0 eq., 21 mg, 0.0562 mmol) and racemic tert-butyl 3-(pyrazin-2-yl)pyrrolidine-1-carboxylate (4.0 eq., 56 mg, 0.225 mmol) were stirred in anhydrous NMP (0.2 ml) at 200°C for 3 hrs. After adding water, the precipitate was filtered and dried. This material was purified by preparative TLC on silica gel (1mm plate, eluted twice with 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>), and by precipitation using CH<sub>2</sub>Cl<sub>2</sub>/hexanes. Compound **29** was isolated as beige solid (15 mg, 55% yield). LCMS (ES): 95% pure, m/z 487 [M+1]<sup>+</sup>.

Example 50

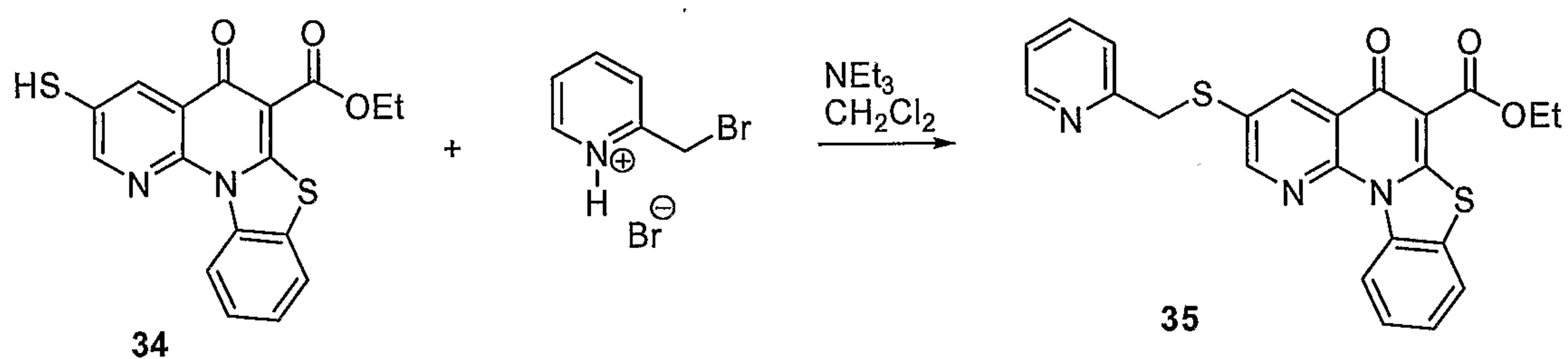
[0183] In a vial, compound **5** (1.0 eq., 21 mg, 0.0562 mmol), 2-(pyrazin-2-yl)ethanethiol (1.1 eq., 8  $\mu$ l, 0.0652 mmol) and  $K_2CO_3$  (1.2 eq., 9 mg, 0.0651 mmol) were mixed in anhydrous DMF (0.2 ml). The mixture was stirred at  $90^\circ C$  for 3 hrs. After adding water, the precipitate was filtered and dried. This material was triturated in MeOH and filtered. Compound **30** was isolated as a yellow solid (19 mg, 71% yield). LCMS (ES): 95% pure,  $m/z$  478  $[M+1]^+$ .

[0184] The following analogues were prepared by the same method, using the appropriate chloro-azabenzofluorenones:

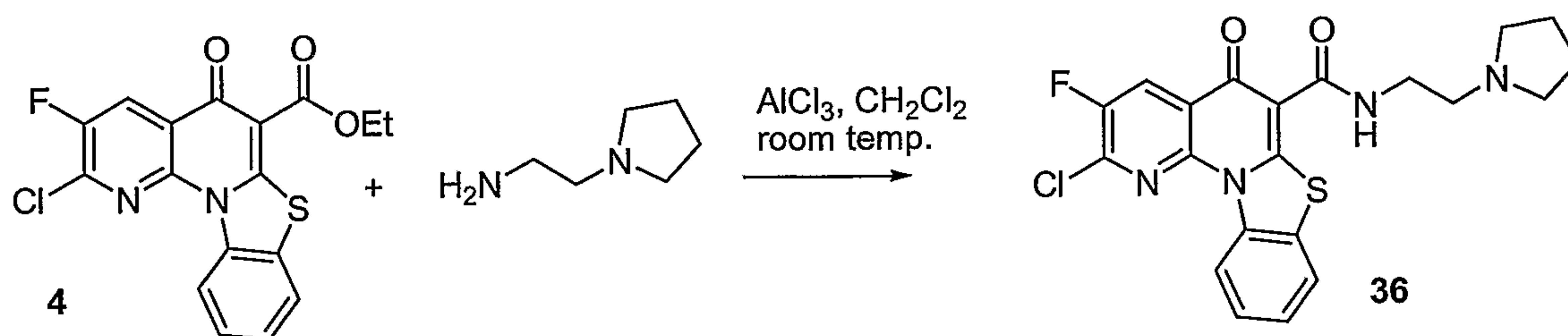
| Structure | M.W.   | LCMS (ES) $m/z$ |
|-----------|--------|-----------------|
|           | 480.53 | 481 $[M+1]^+$   |
|           | 463.58 | 464 $[M+1]^+$   |
|           | 462.54 | 463 $[M+1]^+$   |

Example 51

[0185] Compound 13 (1.0 eq, 266 mg, 0.777 mmol) was suspended in anhydrous NMP (3 ml). Nitrogen was bubbled into the mixture for a few minutes. 2-(pyrazin-2-yl)ethanethiol (5.0 eq, 0.48 ml, 3.91 mmol) and  $K_2CO_3$  (10.0 eq, 1.0 g, 7.23 mmol) were added and the reaction mixture vigorously stirred at  $100^\circ C$  for 1 hour. Water was added to dissolve all the materials. The pH was adjusted to 1-2 by adding a 3N aqueous HCl solution. The resulting orange precipitate was filtered, suspended in a small amount of MeOH and filtered a second time. Crude compound 34 was isolated as an orange-brown solid and was used for next step without any further purification (188 mg, 68% yield). LCMS (ES): 80-90% pure,  $m/z$  357  $[M+1]^+$ .

Example 52

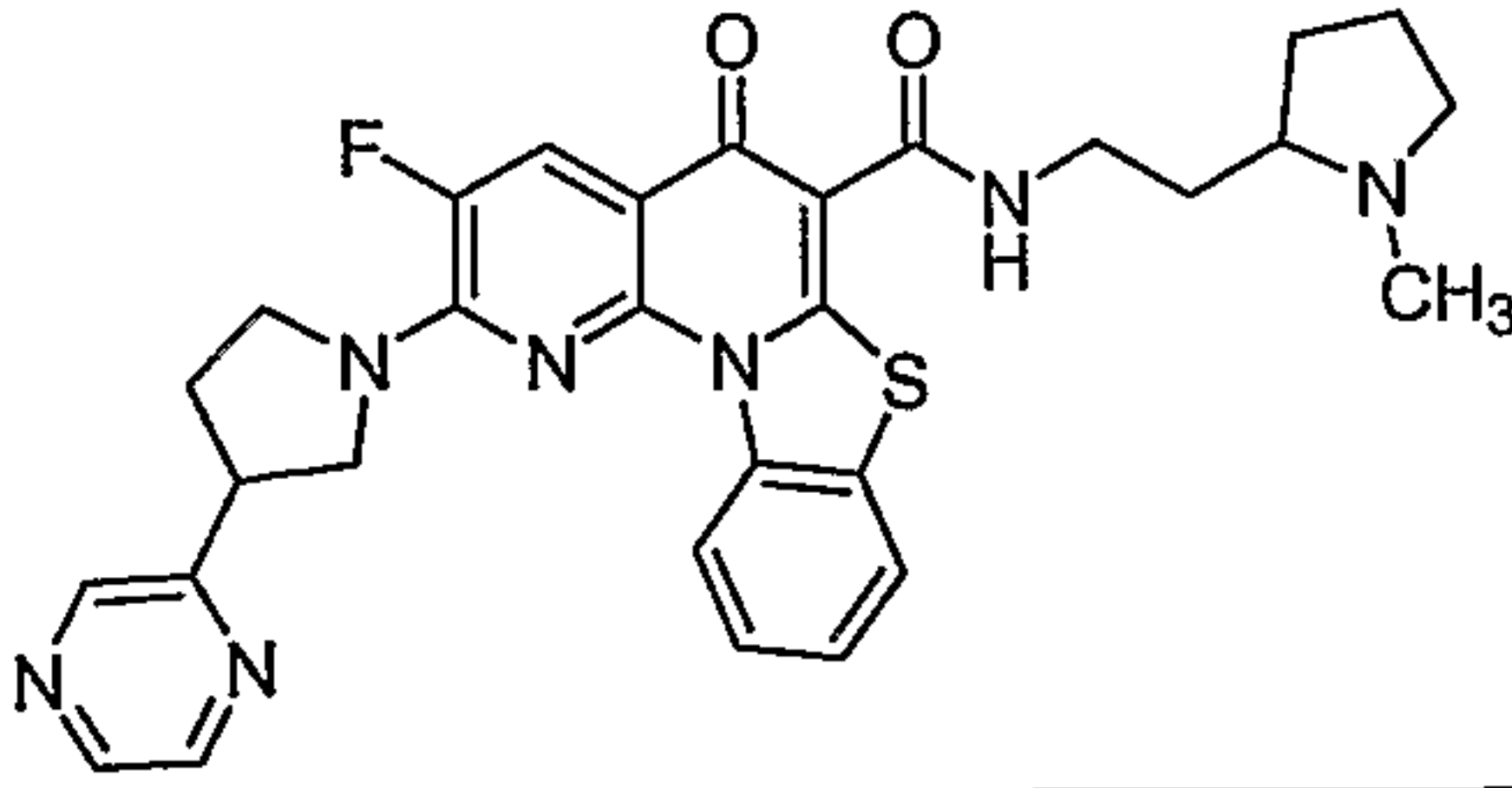
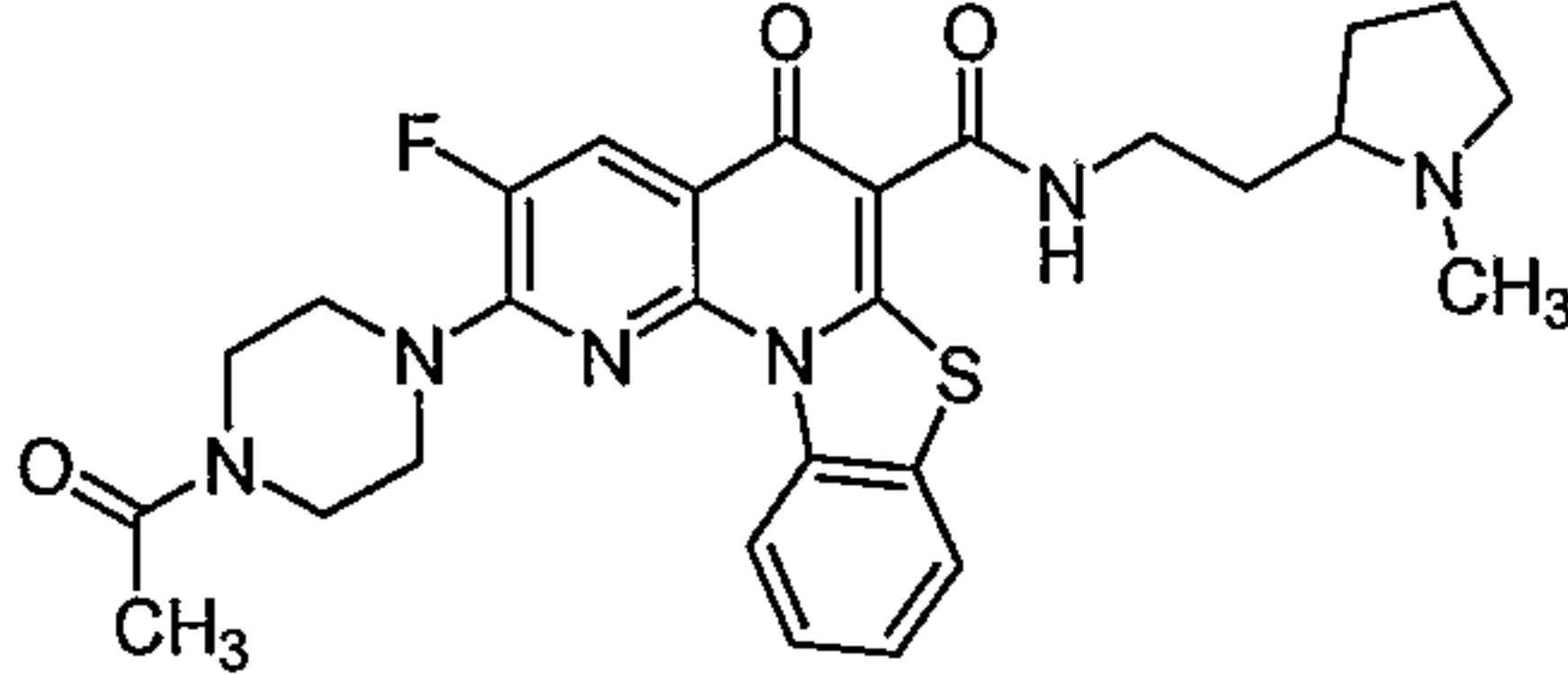
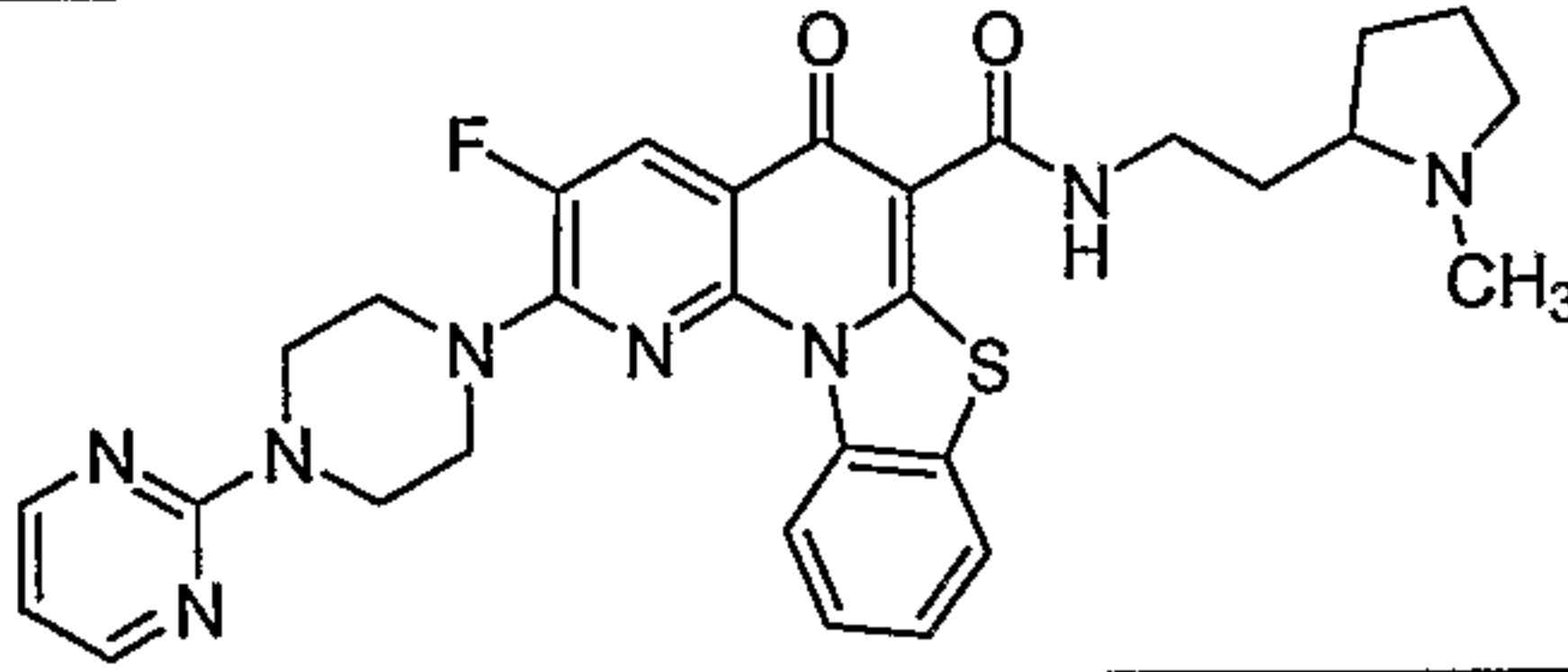
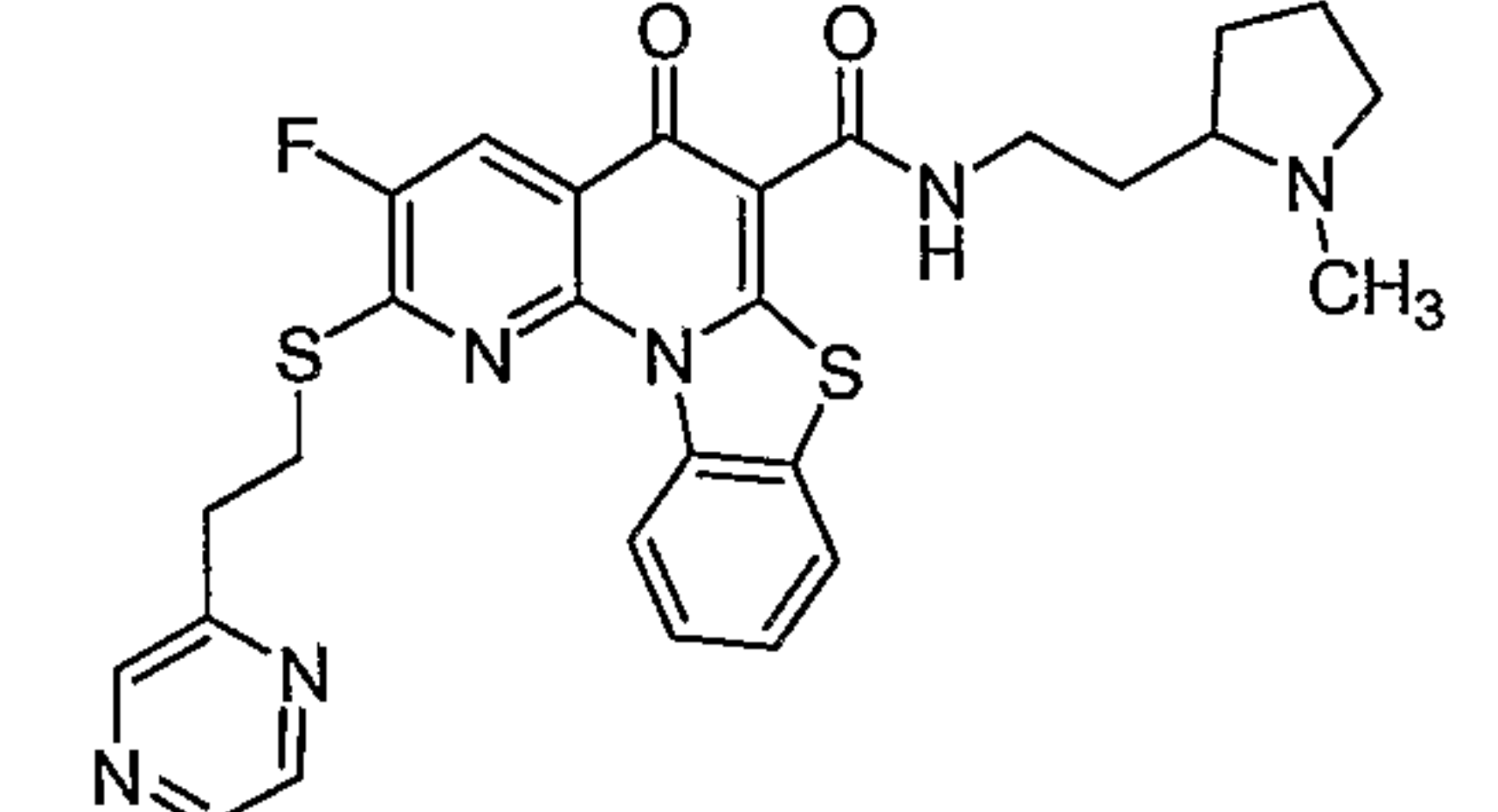
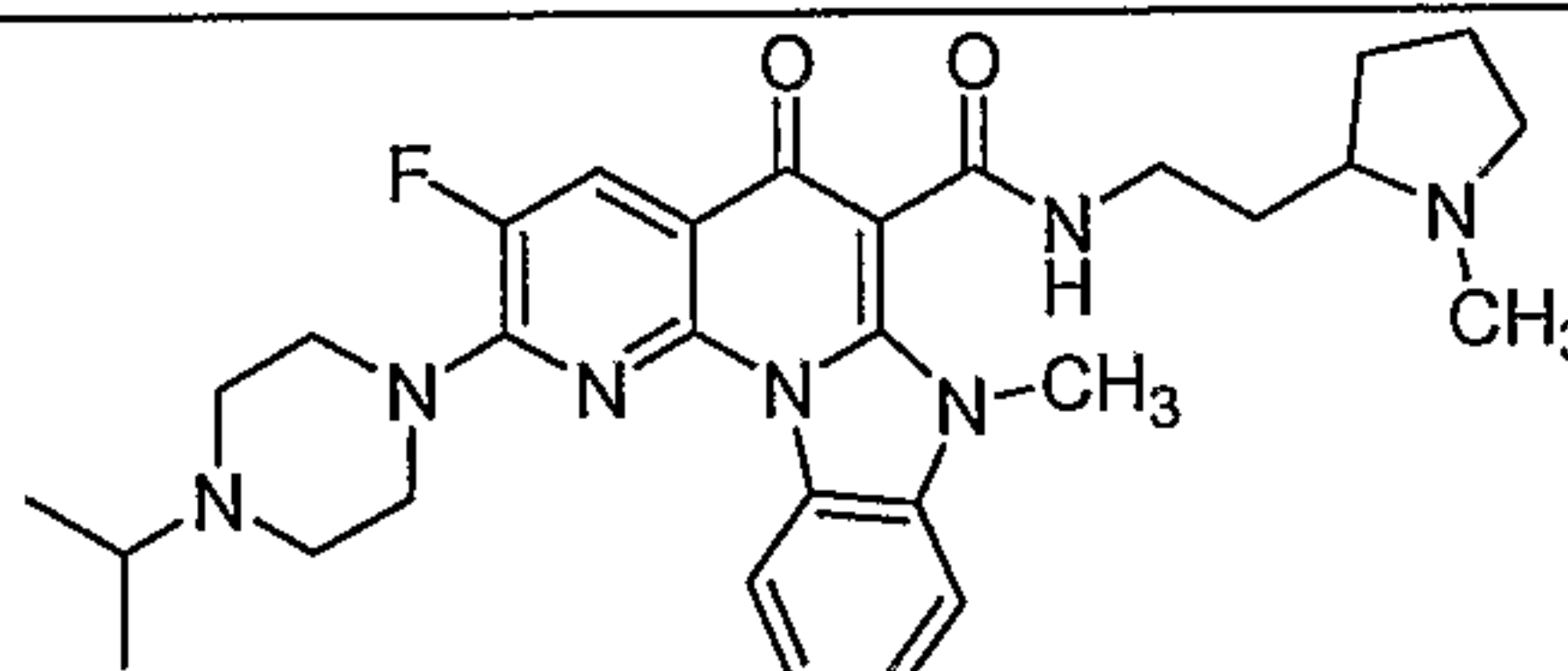
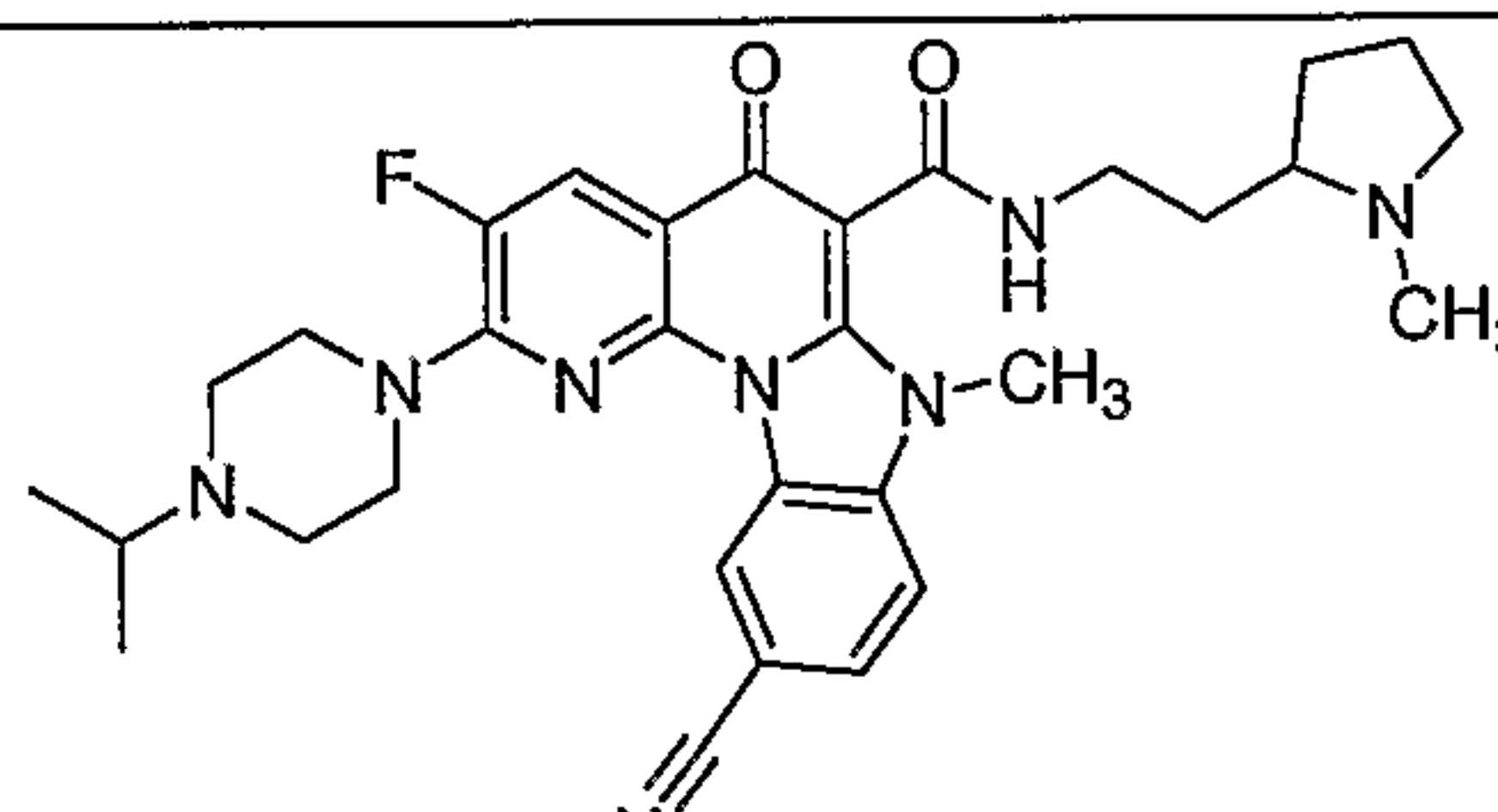
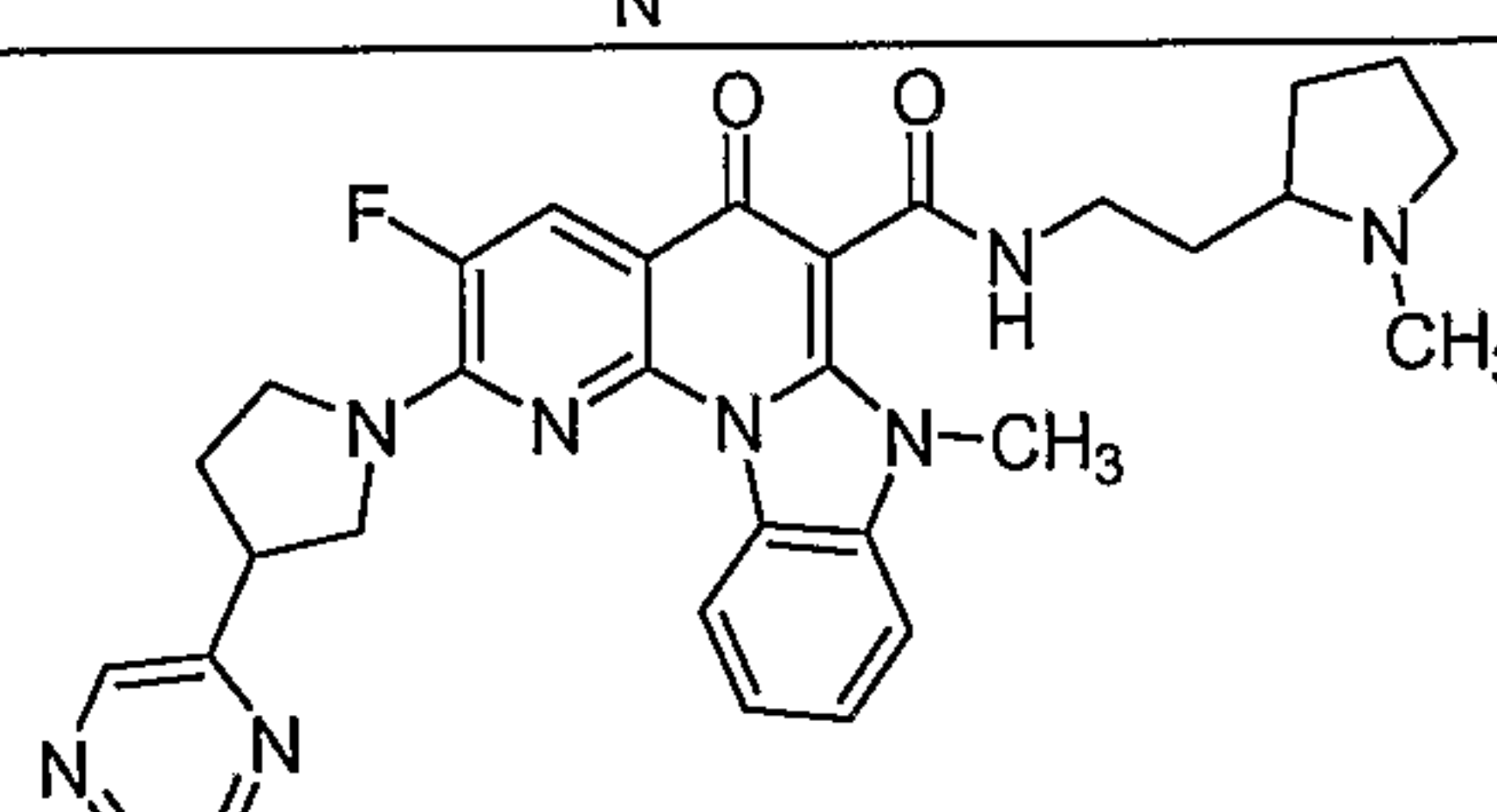
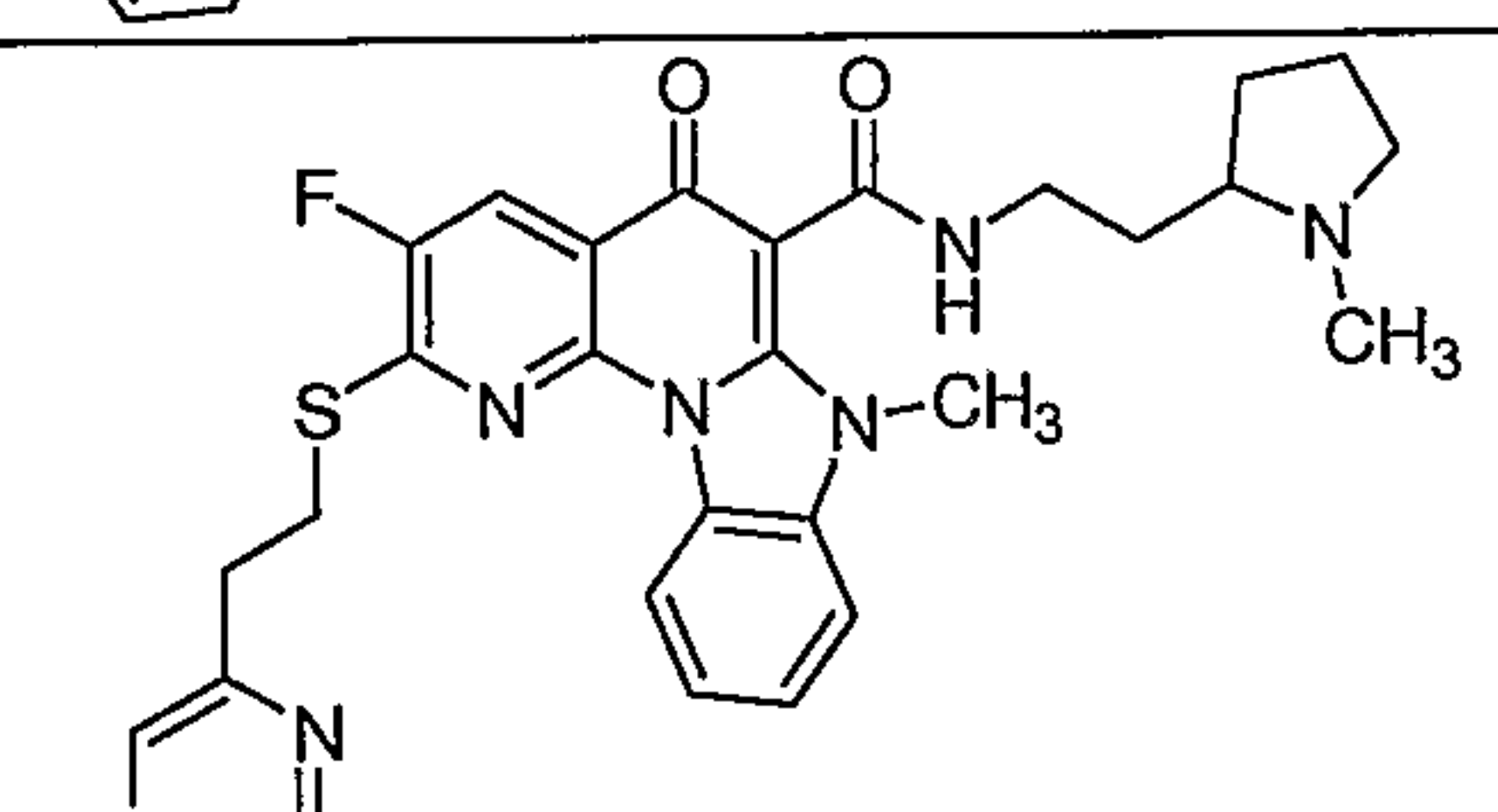
[0186] Compound 34 (1.0 eq, 188 mg, 0.527 mmol) was suspended in  $CH_2Cl_2$  (4 ml). Triethylamine (2.2 eq, 0.16 ml, 1.148 mmol) and 2-(bromomethyl)pyridinium bromide (1.2 eq, 160 mg, 0.6325 mmol) were added and the mixture stirred at room temperature for 15 min. After addition of a saturated aqueous  $NaHCO_3$ , the material was extracted by  $CH_2Cl_2$  (3x). The combined extracts were dried over  $Na_2SO_4$  and the solvents removed *in vacuo*. The compound was purified by preparative HPLC. The resulting solution was concentrated and the pH adjusted to 10 by adding aqueous NaOH. Compound 35 was filtered and triturated in a  $CH_2Cl_2$ /hexanes mixture and filtered to give a pale brown solid (59 mg, 25% yield). LCMS (ES): 95% pure,  $m/z$  448  $[M+1]^+$ .

Example 53

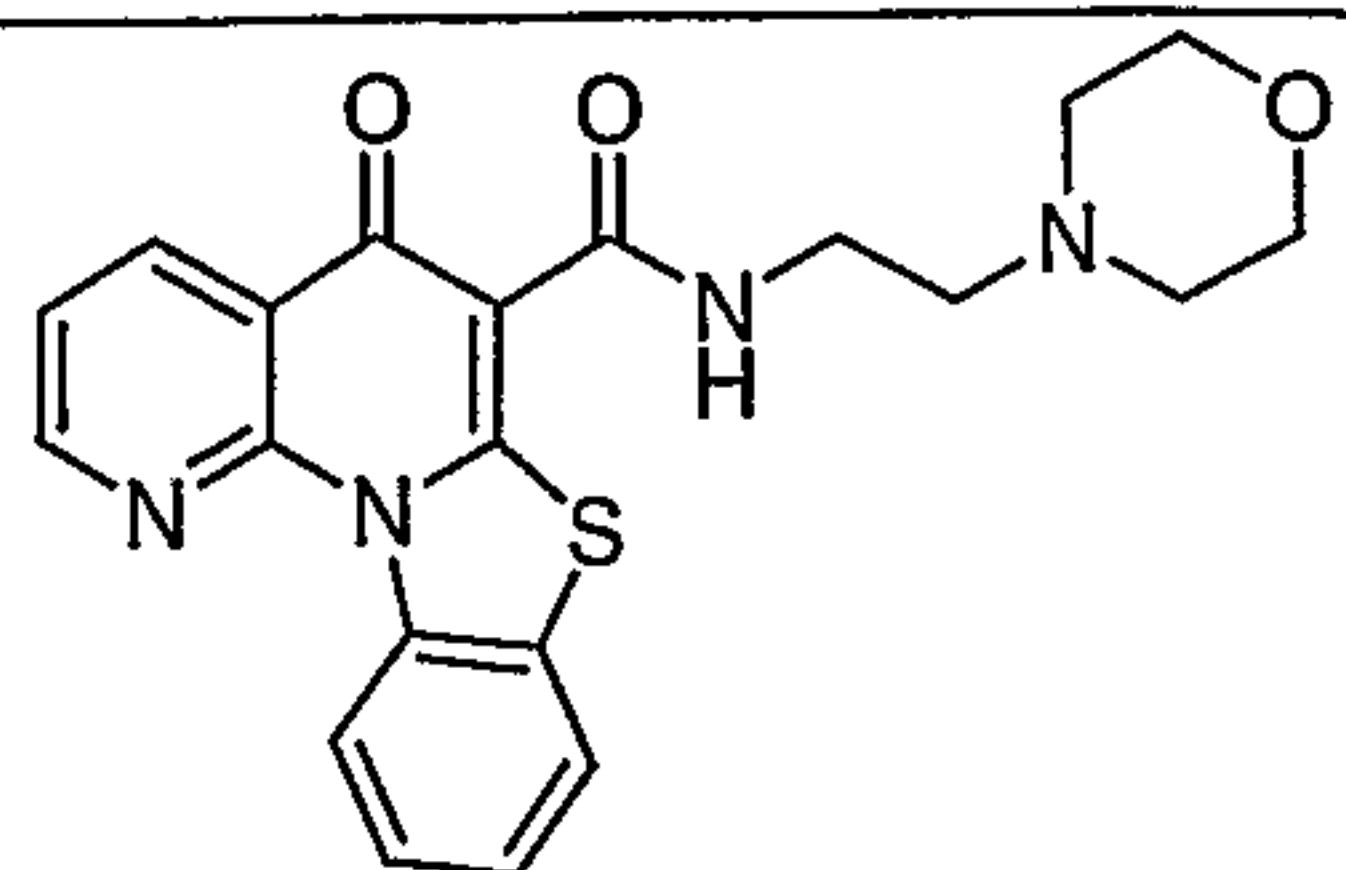
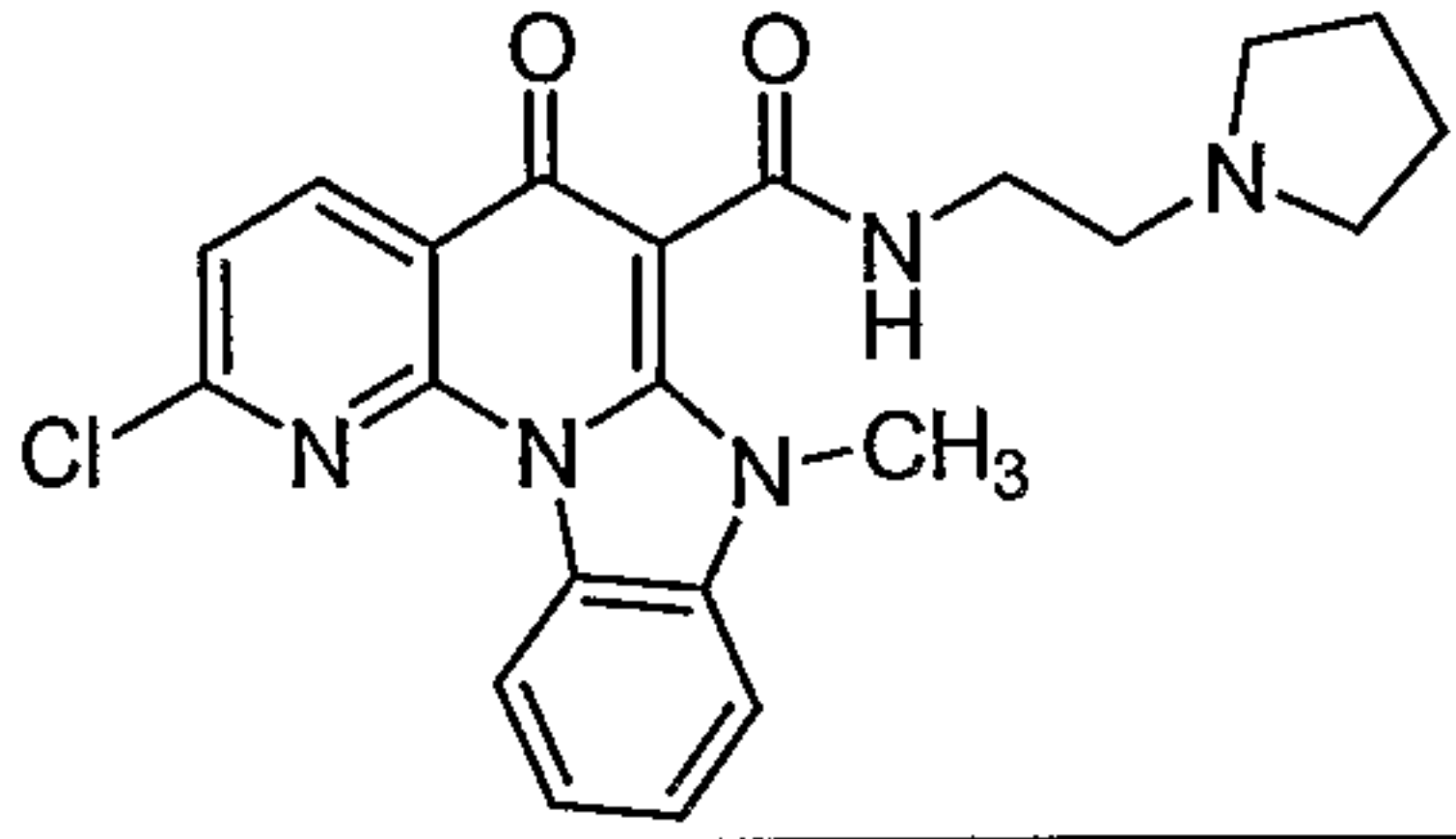
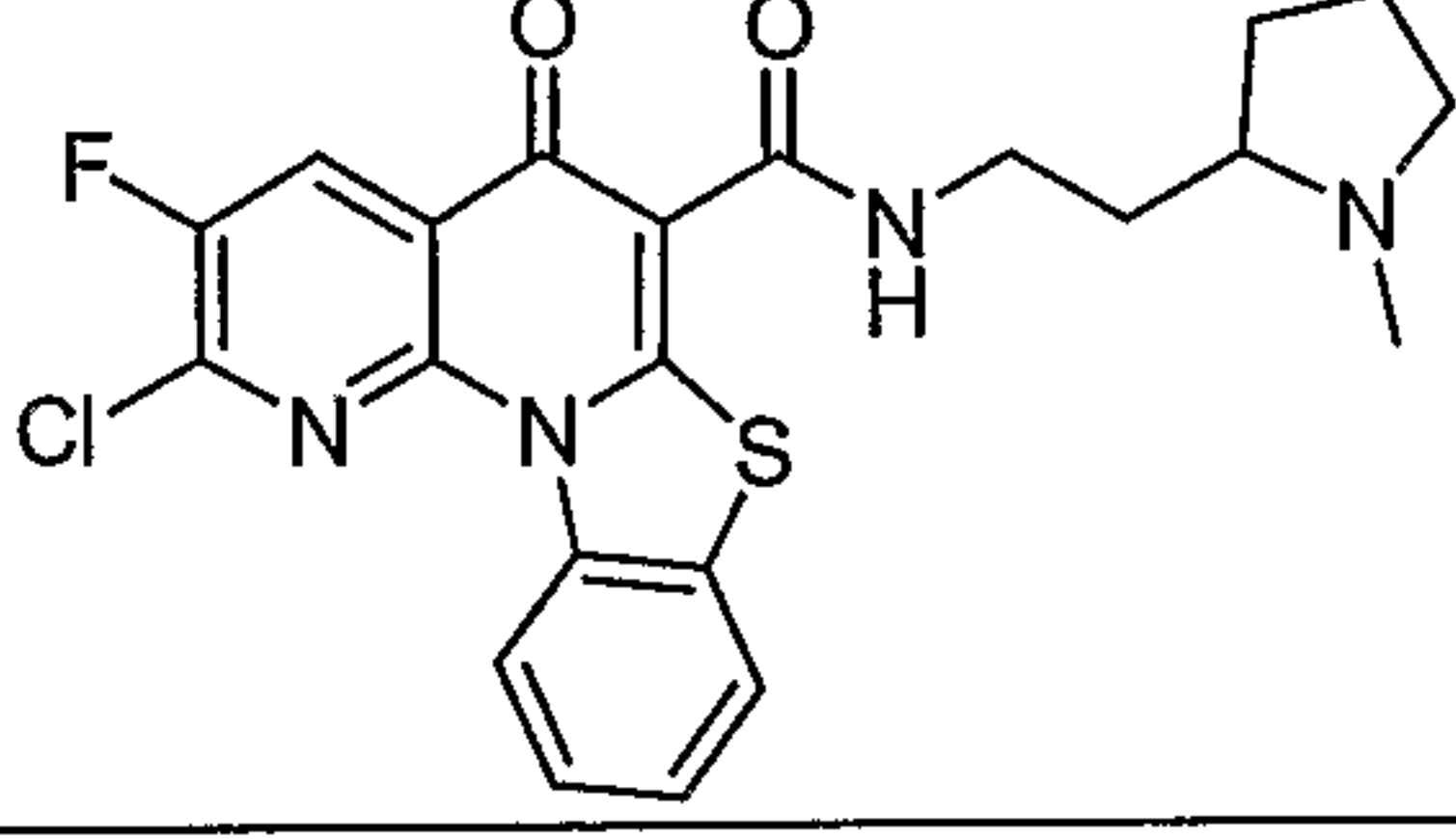
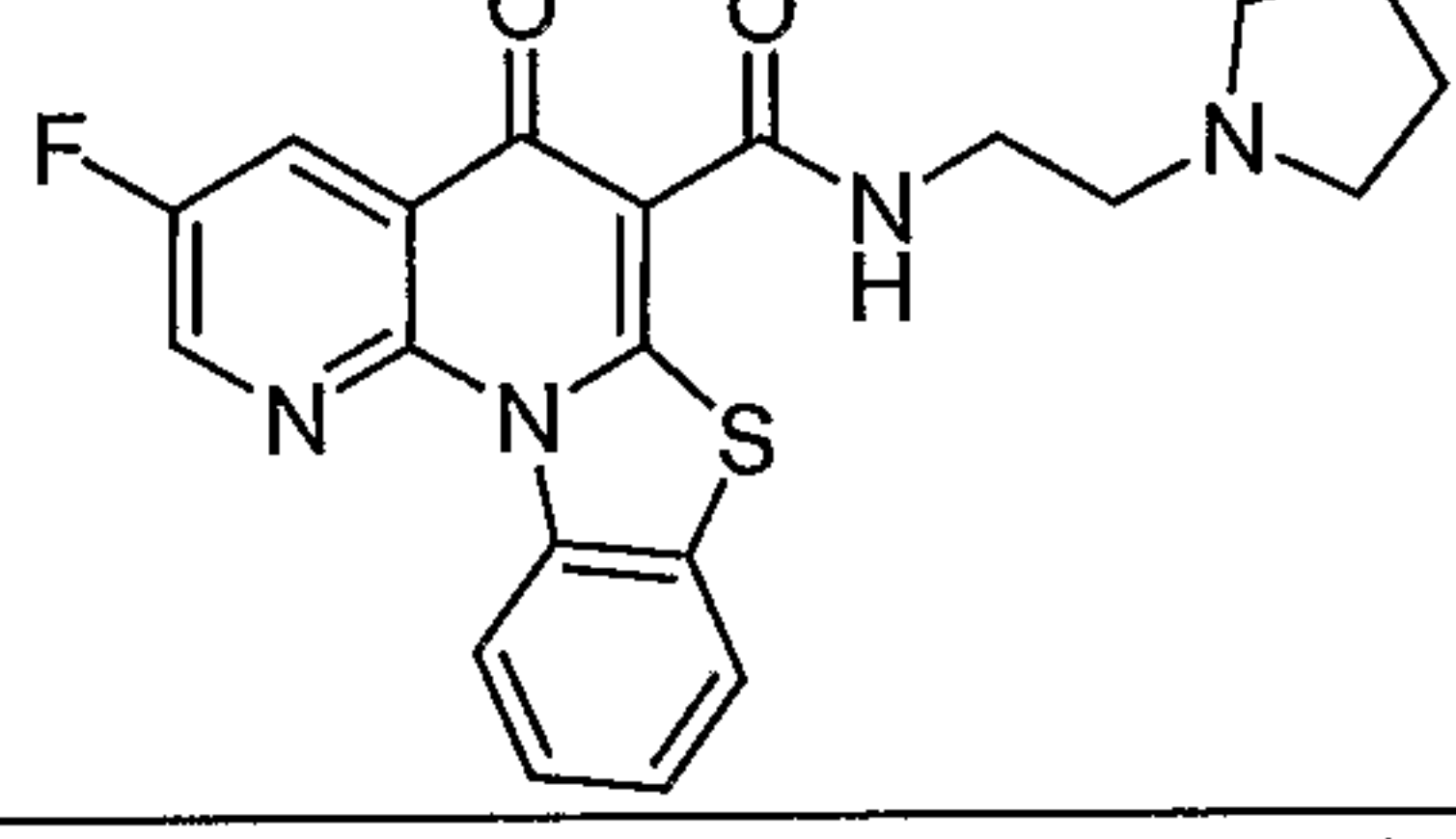
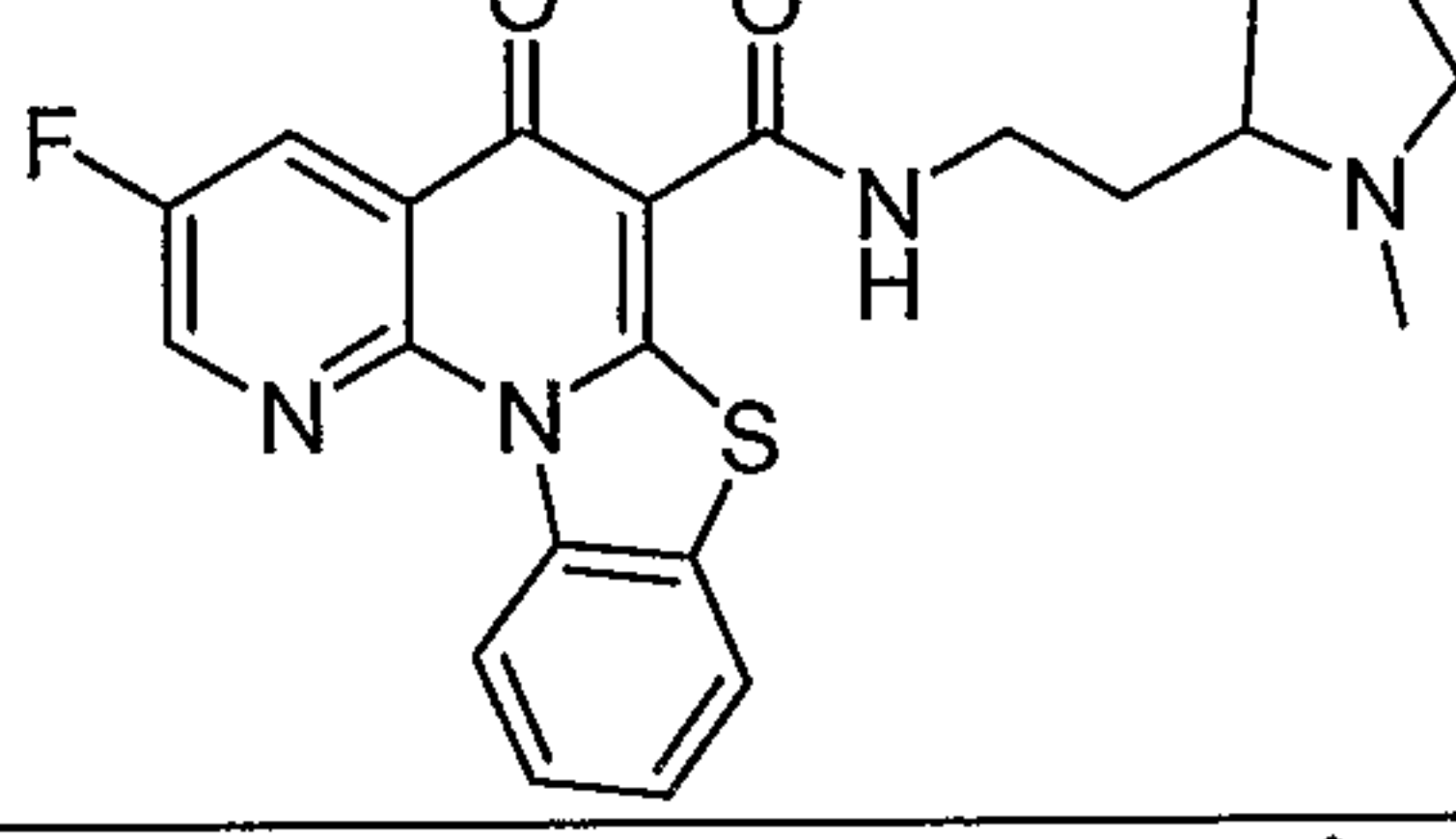
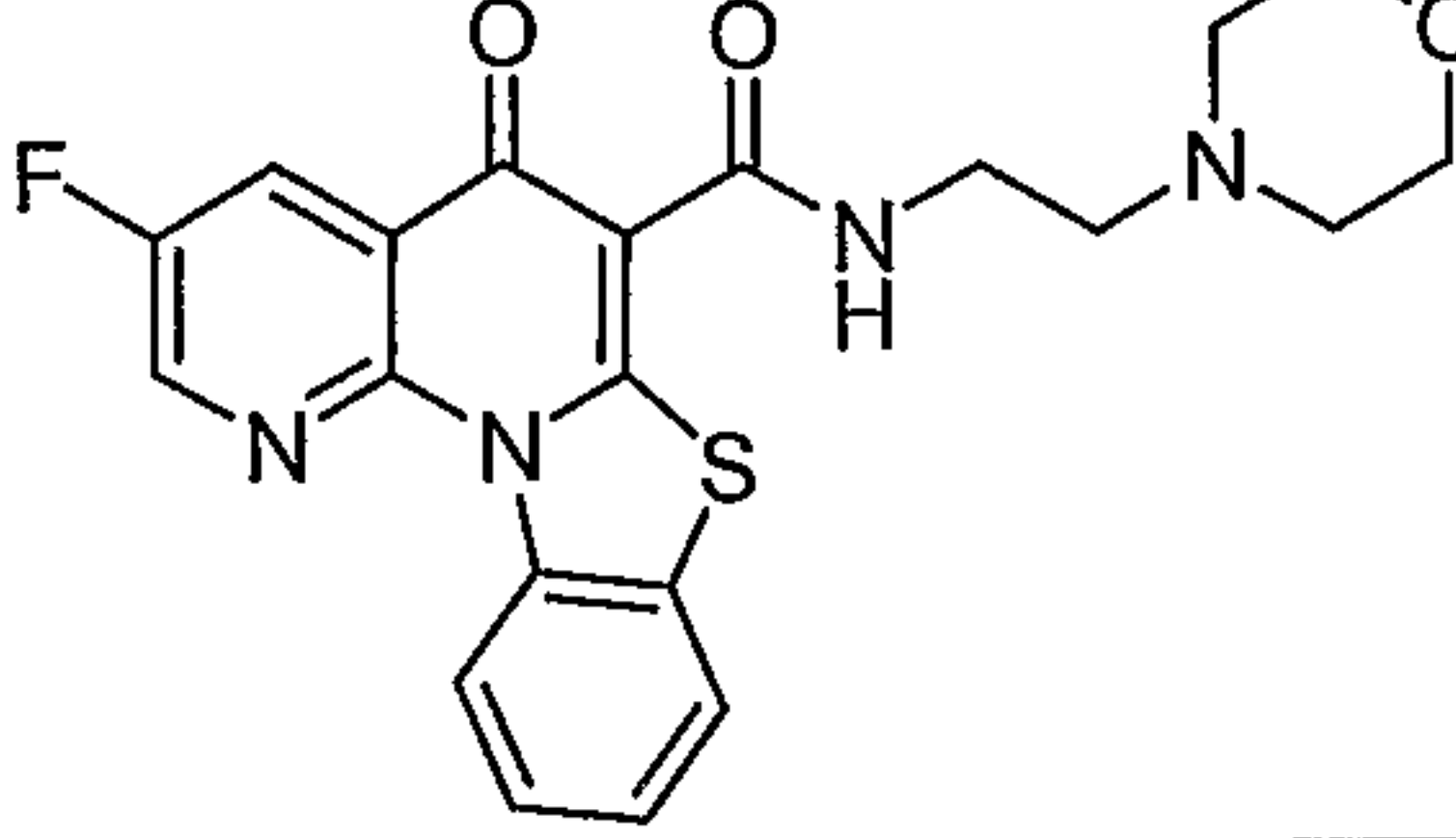
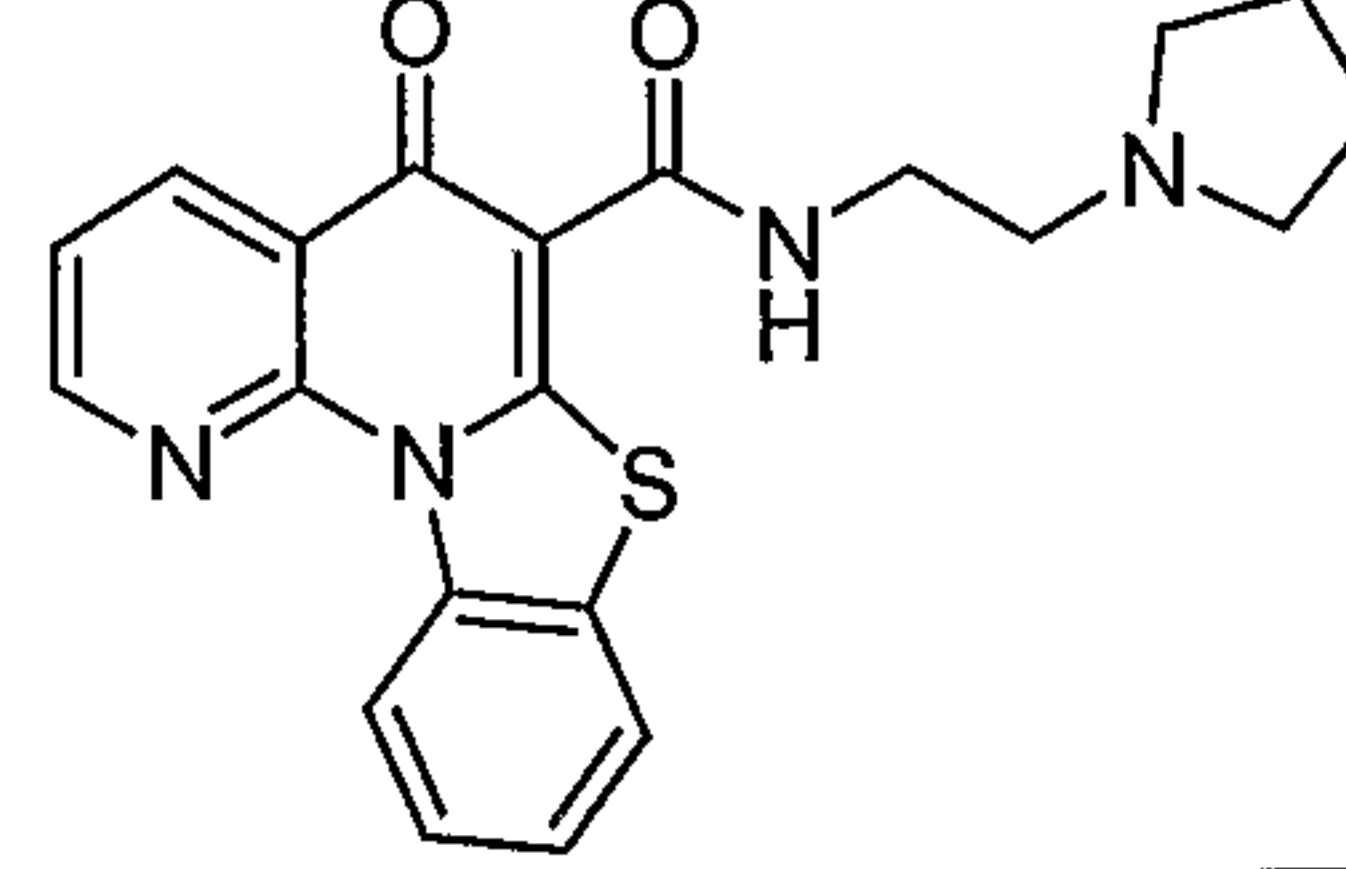
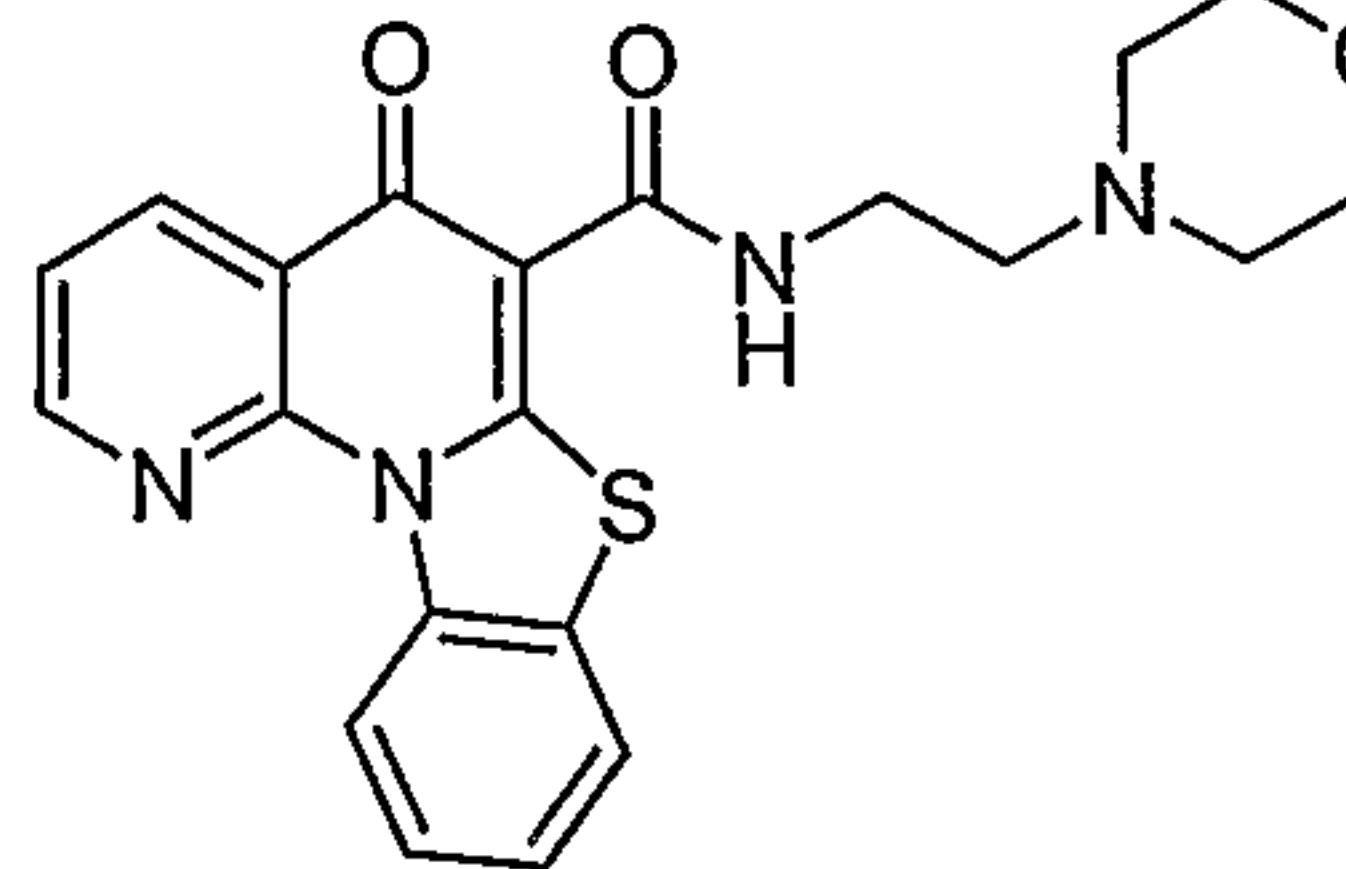
[0187] In a round bottom flask, compound **4** (1.0 eq, 1.28 g, 3.397 mmol) and 2-(pyrrolidin-1-yl)ethanamine (2.0 eq, 0.86 ml, 6.786 mmol) were mixed in CH<sub>2</sub>Cl<sub>2</sub> (50 ml). AlCl<sub>3</sub> (1.5 eq, 680 mg, 5.10 mmol) was added and the mixture was vigorously stirred at room temperature for 1 hour. After removal of CH<sub>2</sub>Cl<sub>2</sub> *in vacuo*, the resulting slurry was treated with a saturated aqueous tartaric acid solution (*ca.* 20 ml) and stirred until all solid disappeared (about 1 hr for completion of the hydrolysis). Water was added and the pH was adjusted to 14 by adding NaOH. The yellow precipitate was filtered and washed with water. The solid was dissolved in a large amount of a CH<sub>2</sub>Cl<sub>2</sub>/MeOH mixture and the cloudy solution filtered through a pad of celite. The volatiles were removed *in vacuo* and the solid material triturated in hot MeOH. Filtration and drying afforded compound **36** (1.09 g, 72% yield) as a yellow solid. LCMS (ES): 95% pure, *m/z* 445 [M+1]<sup>+</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.84 (m, 4H), 2.65 (m, 4H), 2.80 (t, *J* = 7.2, 2H), 3.68 (t, *J* = 7.2, 2H), 7.52 (td, *J* = 1.2, *J* = 7.2, 1H), 7.62 (td, *J* = 1.2, *J* = 8.4, 1H), 7.81 (dd, *J* = 0.8, *J* = 8.0, 1H), 8.62 (d, *J* = 7.2, 1H), 9.49 (d, *J* = 8.8, 1H) ppm.

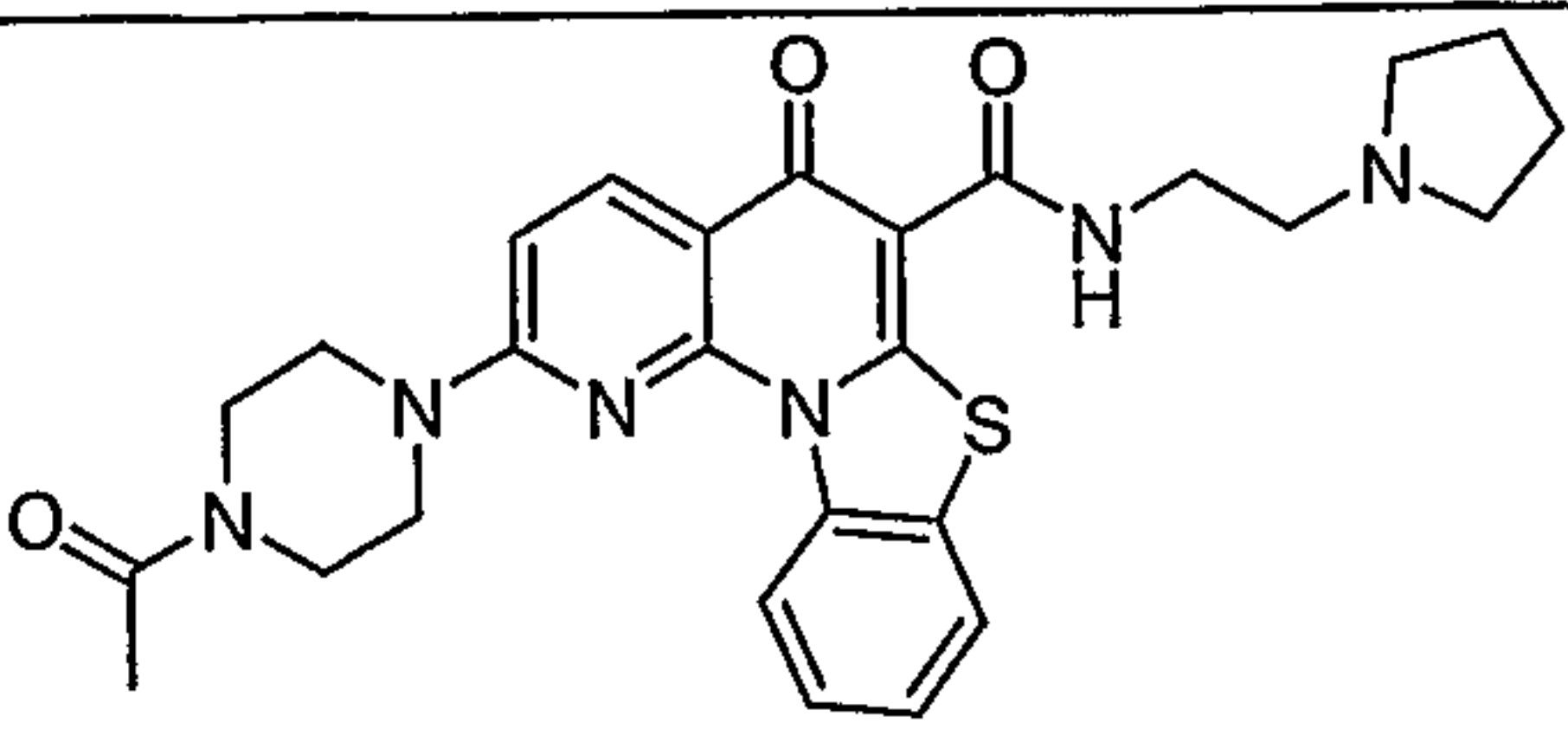
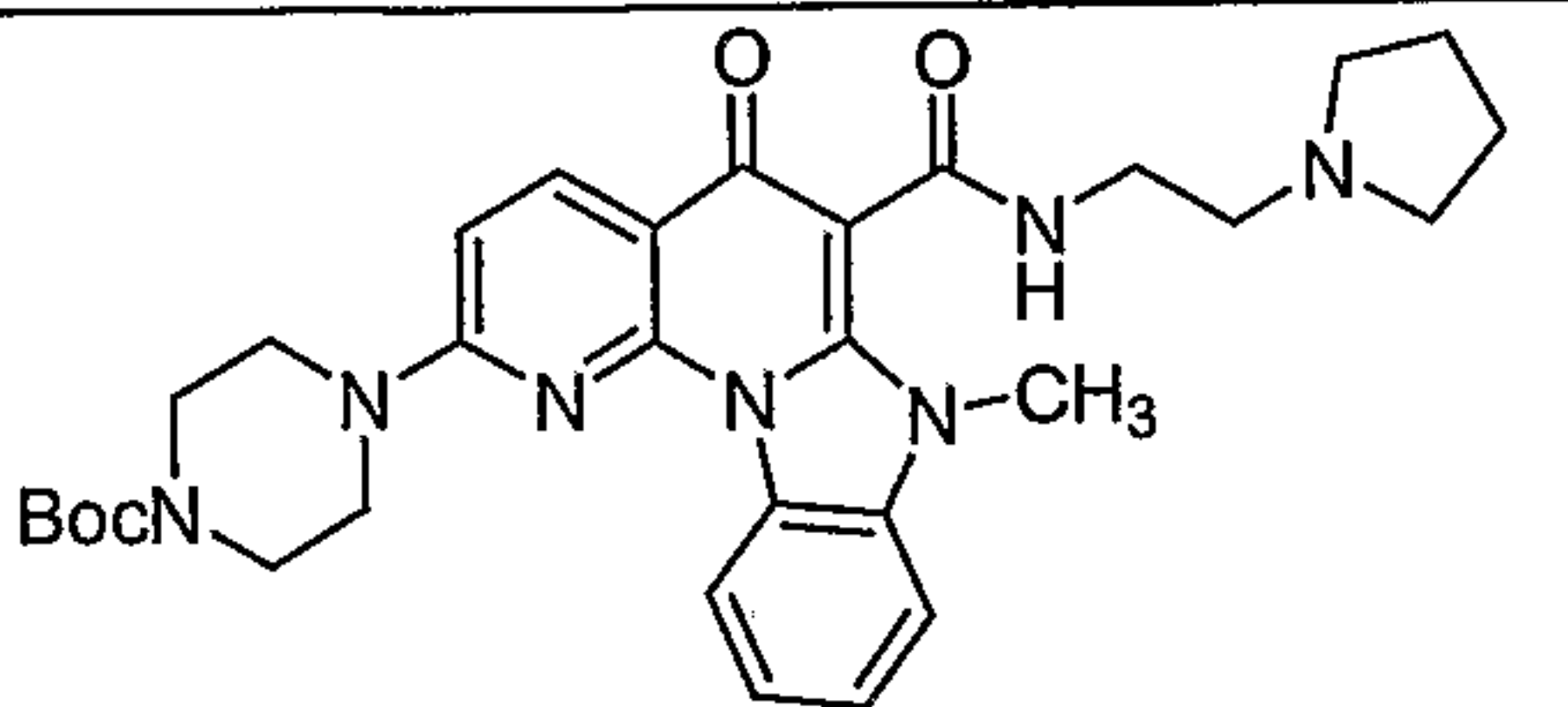
[0188] The following compounds were prepared by the same method, using the appropriate amines and azabenzofluorenone ethyl esters. Some compounds were alternatively isolated by extraction with CH<sub>2</sub>Cl<sub>2</sub> from the basic aqueous solution. Some compounds were purified by preparative TLC on alumina (eluted with 1 to 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>), preparative HPLC or trituration in EtOAc or CH<sub>2</sub>Cl<sub>2</sub>/hexanes mixtures.

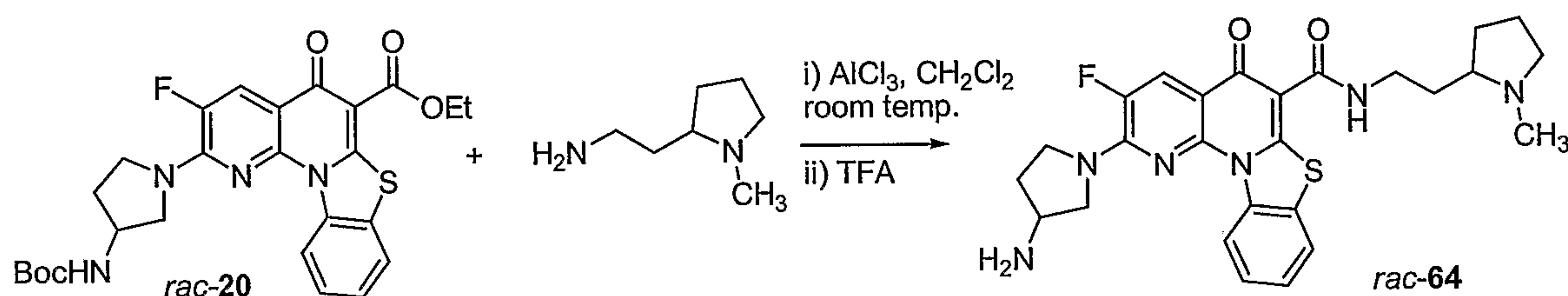
| Structure | M.W.   | LCMS (ES) <i>m/z</i>   |
|-----------|--------|------------------------|
|           | 550.69 | 551 [M+1] <sup>+</sup> |

| Structure                                                                            | M.W.   | LCMS (ES) m/z          |
|--------------------------------------------------------------------------------------|--------|------------------------|
|    | 571.67 | 572 [M+1] <sup>+</sup> |
|    | 550.65 | 551 [M+1] <sup>+</sup> |
|   | 586.68 | 587 [M+1] <sup>+</sup> |
|  | 562.68 | 563 [M+1] <sup>+</sup> |
|  | 547.67 | 548 [M+1] <sup>+</sup> |
|  | 572.68 | 573 [M+1] <sup>+</sup> |
|  | 568.64 | 569 [M+1] <sup>+</sup> |
|  | 559.66 | 560 [M+1] <sup>+</sup> |

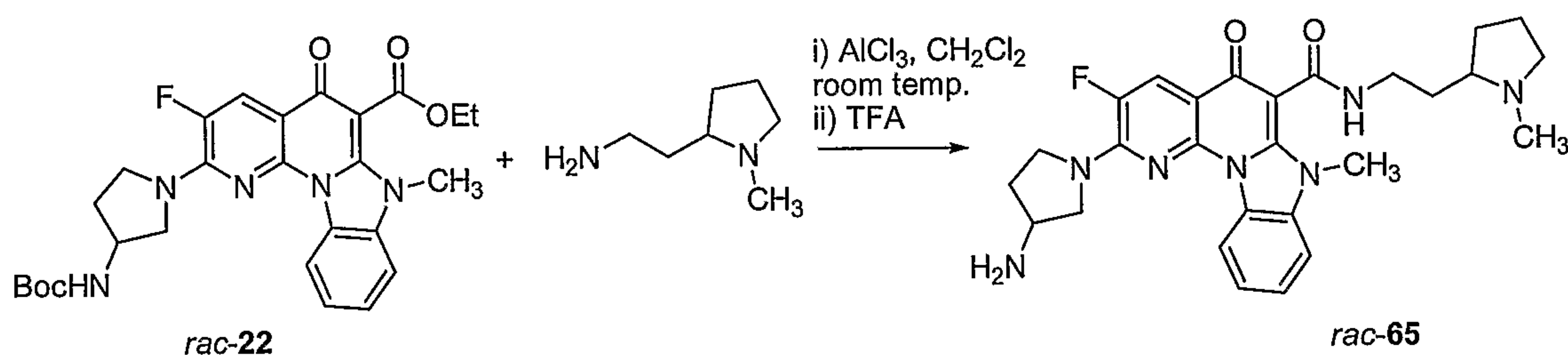
149

| Structure                                                                            | M.W.   | LCMS (ES) m/z          |
|--------------------------------------------------------------------------------------|--------|------------------------|
|    | 408.47 | 409 [M+1] <sup>+</sup> |
|    | 423.90 | 424 [M+1] <sup>+</sup> |
|   | 458.94 | 459 [M+1] <sup>+</sup> |
|  | 410.46 | 411 [M+1] <sup>+</sup> |
|  | 424.49 | 425 [M+1] <sup>+</sup> |
|  | 426.46 | 427 [M+1] <sup>+</sup> |
|  | 392.47 | 393 [M+1] <sup>+</sup> |
|  | 408.47 | 409 [M+1] <sup>+</sup> |

| Structure                                                                          | M.W.   | LCMS (ES) m/z          |
|------------------------------------------------------------------------------------|--------|------------------------|
|  | 518.63 | 519 [M+1] <sup>+</sup> |
|  | 573.70 | 574 [M+1] <sup>+</sup> |

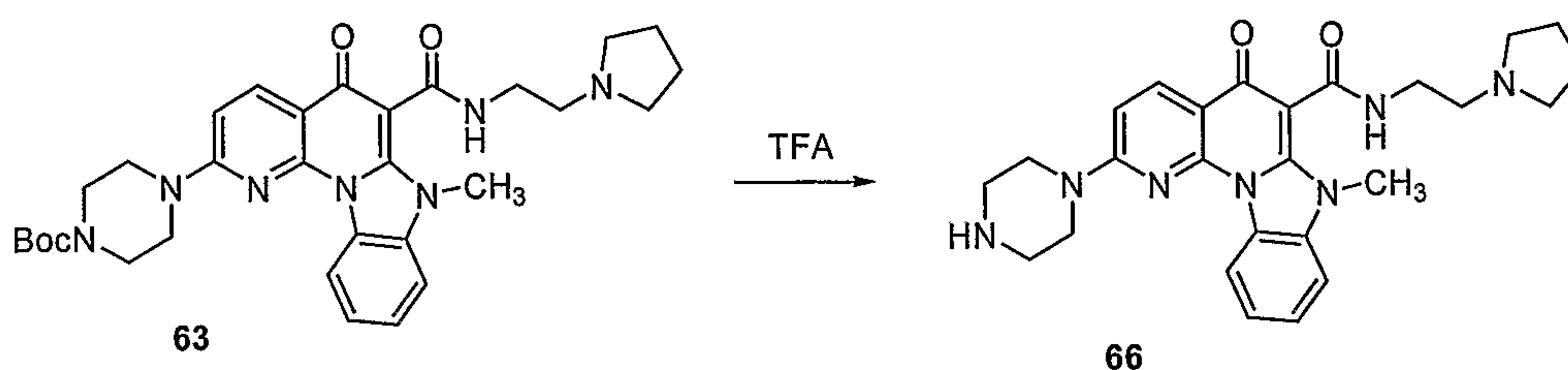
Example 54

[0189] Compound **64** protected by a Boc group was prepared according to the procedure used for product **36**. The crude boc-amine was treated with neat trifluoroacetic acid (0.5 ml) for 45 min. After evaporation of the acid, water was added and the pH was adjusted to 14 by adding 1N NaOH. After extraction with  $\text{CH}_2\text{Cl}_2$  (4x), the extracts were washed with water (1x) and dried over  $\text{Na}_2\text{SO}_4$ . Racemic compound **64** was purified by preparative TLC on alumina (1.5 mm plate, eluted 3 times with 4% MeOH in  $\text{CH}_2\text{Cl}_2$ ) to afford a yellow solid (6 mg, 37% yield). LCMS (ES): 95% pure, m/z 509 [M+1]<sup>+</sup>.

Example 55

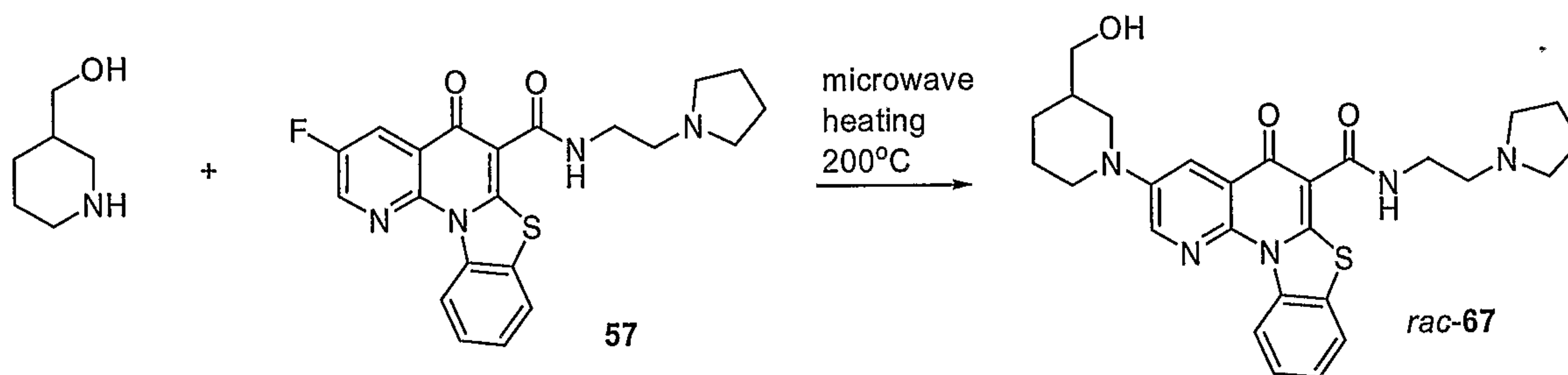
[0190] Compound **65** was prepared according to the procedure used for product **64**. Compound **65** was purified by preparative TLC on alumina (1.5 mm plate, eluted twice with 4% MeOH in CH<sub>2</sub>Cl<sub>2</sub> and four times with 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>) and precipitation with CH<sub>2</sub>Cl<sub>2</sub>/hexanes to afford a yellow solid (2.7 mg, 12 % yield). LCMS (ES): >85% pure, m/z 506 [M+1]<sup>+</sup>.

#### Example 56



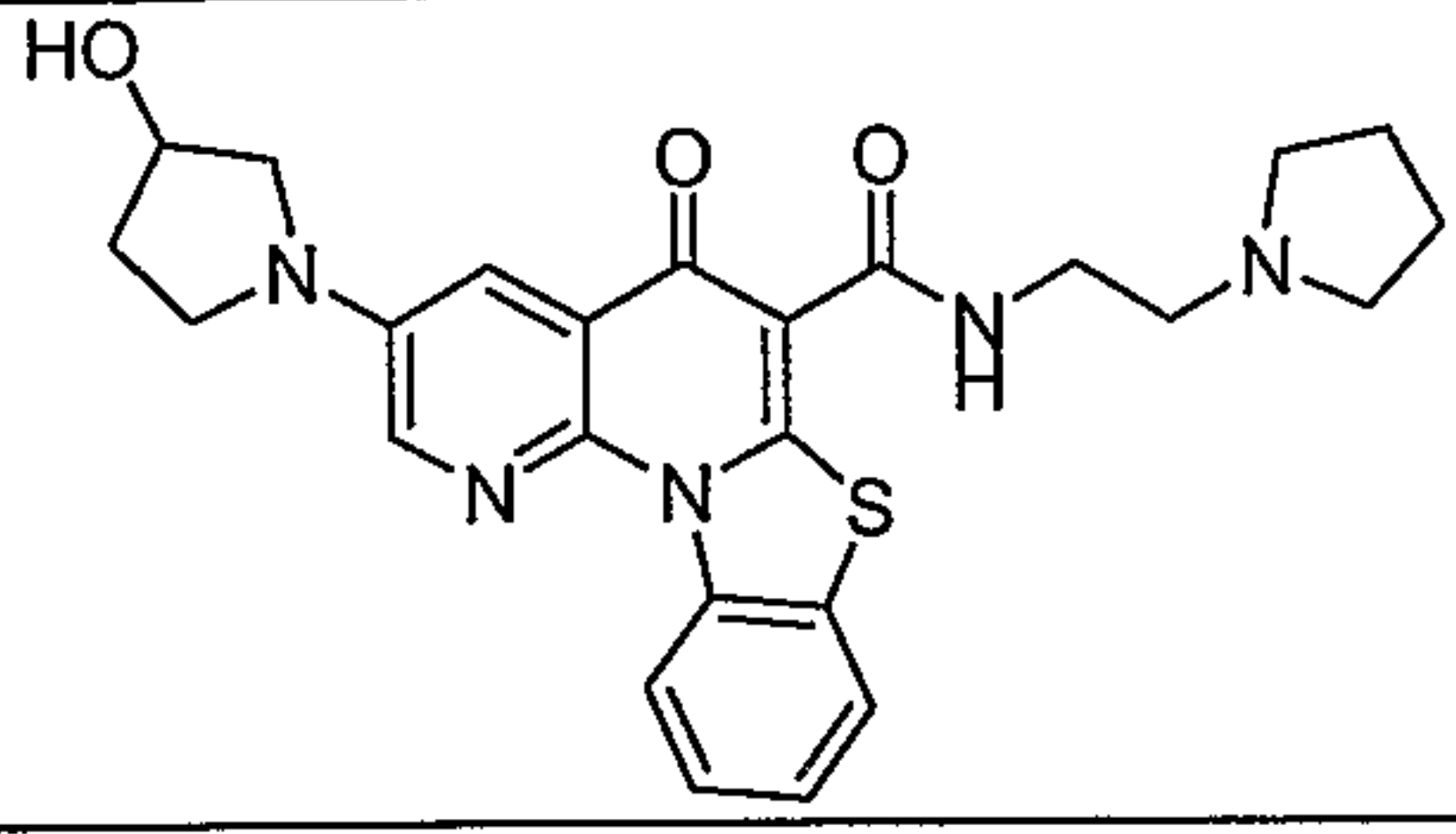
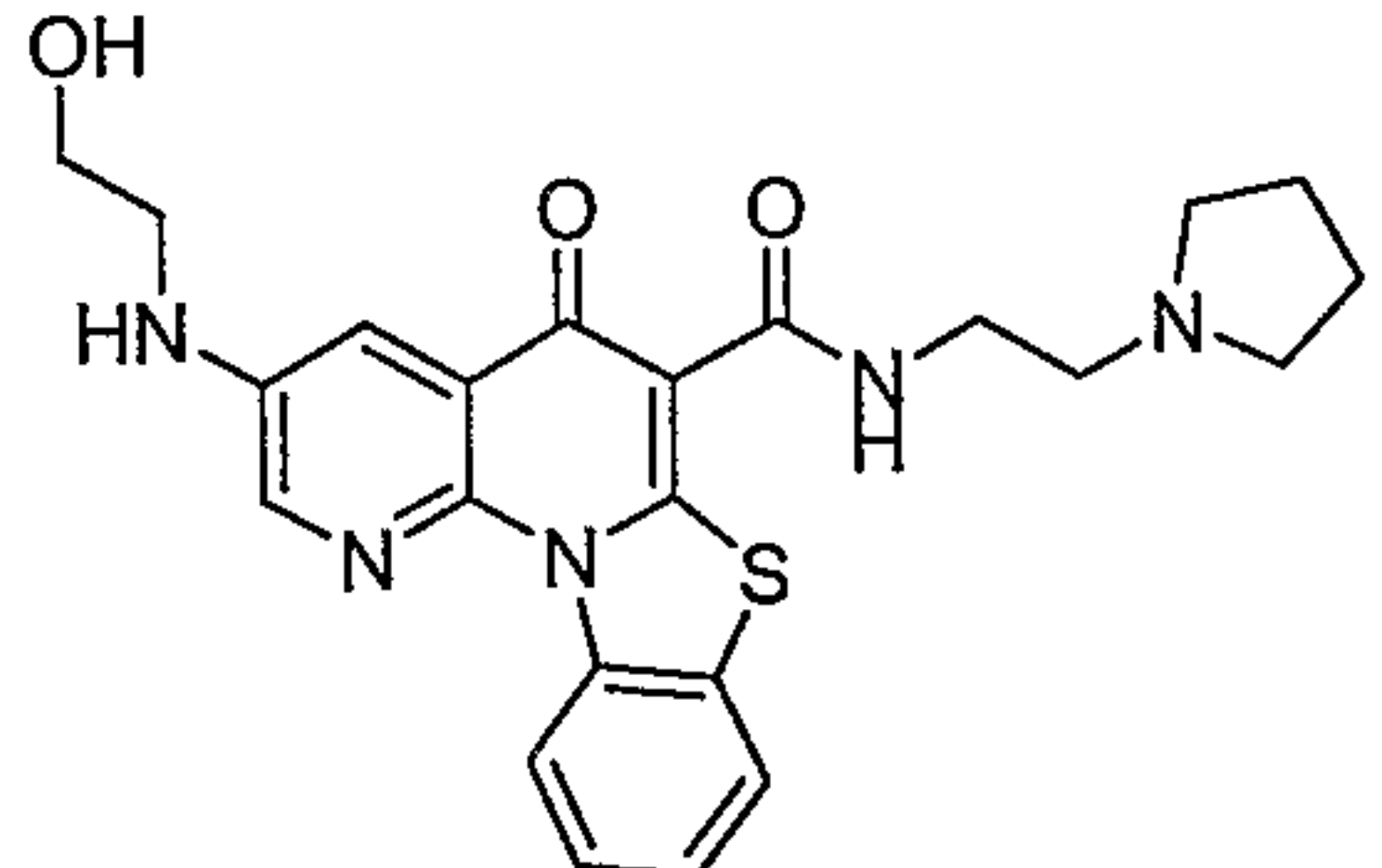
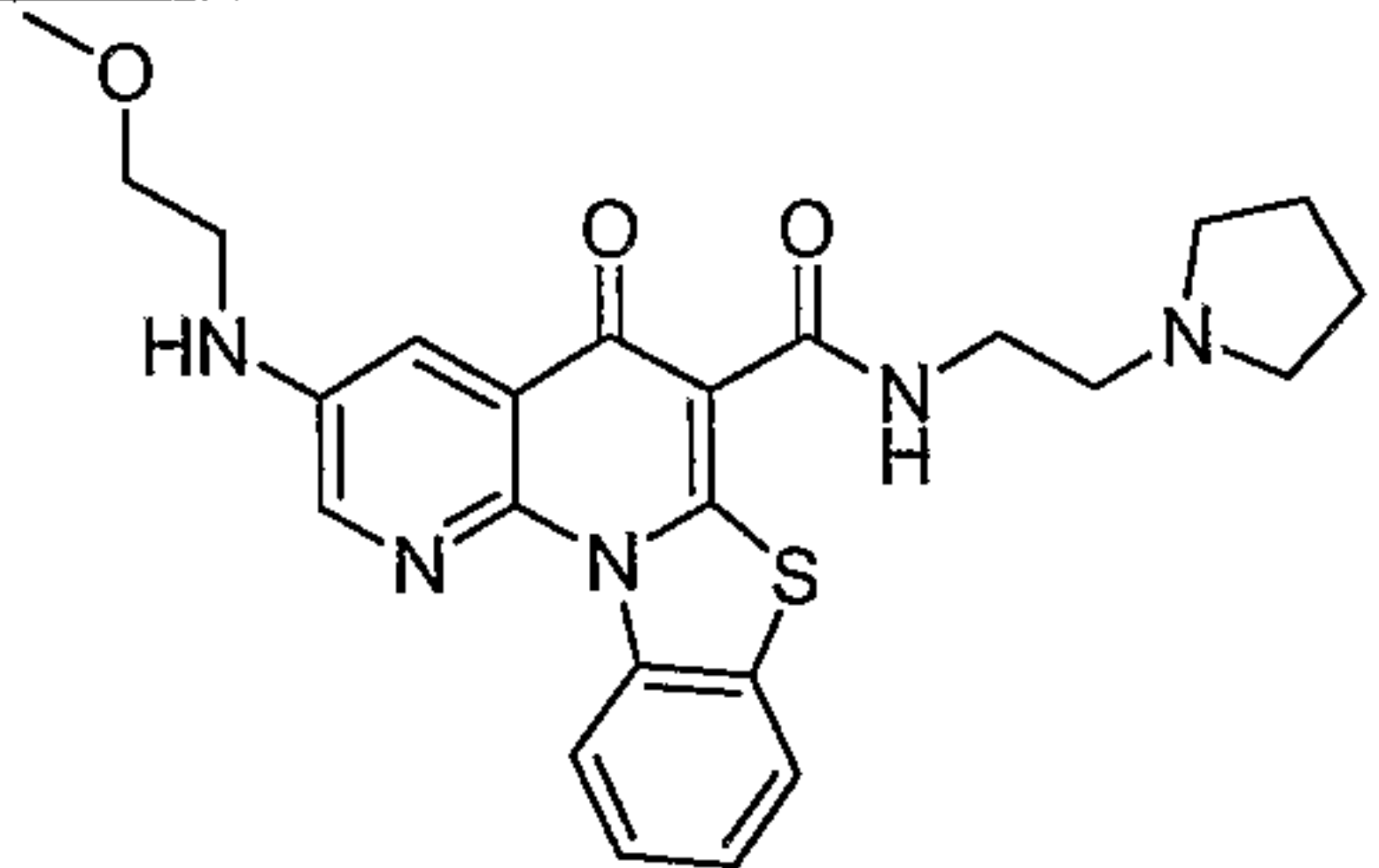
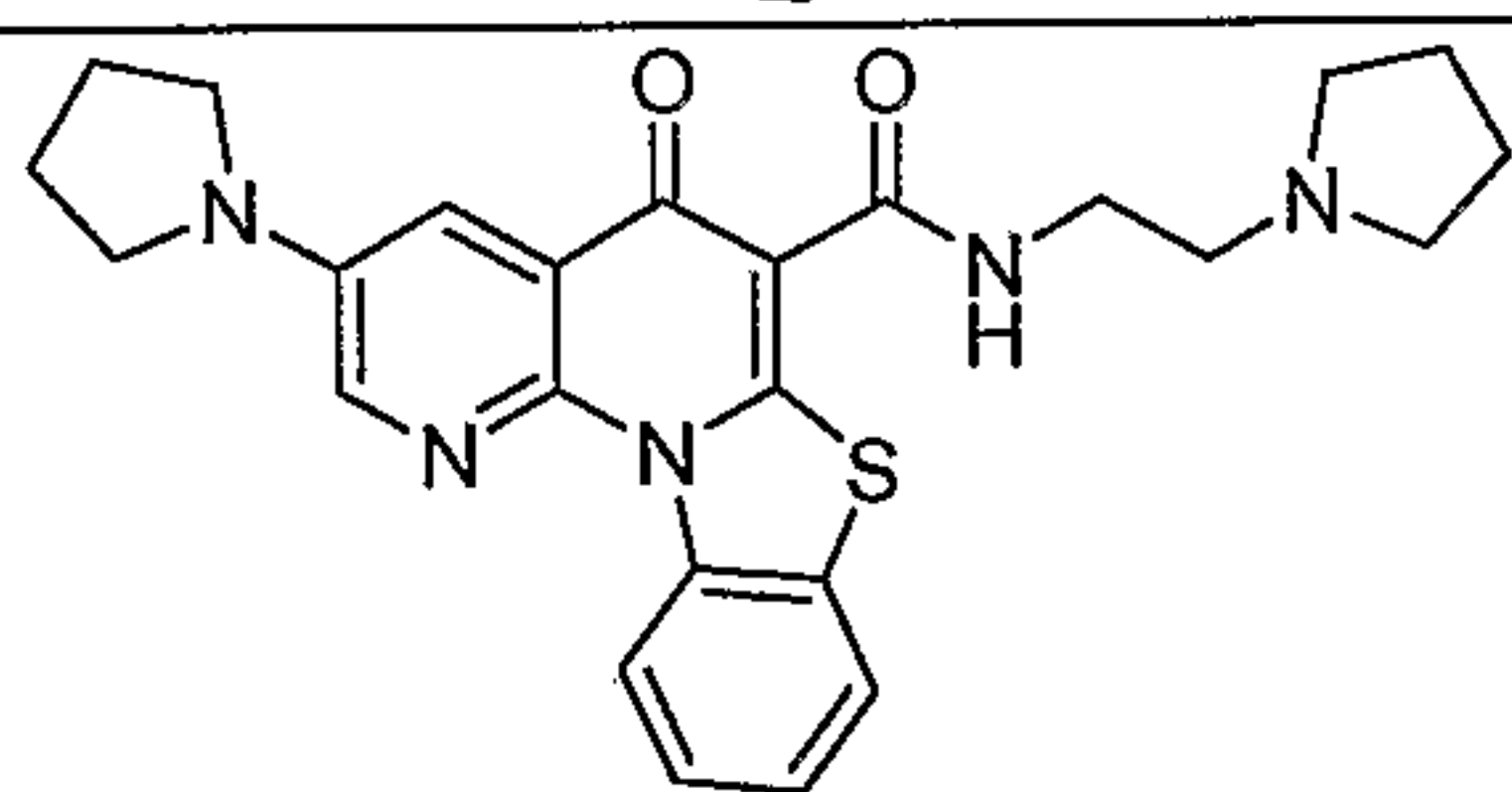
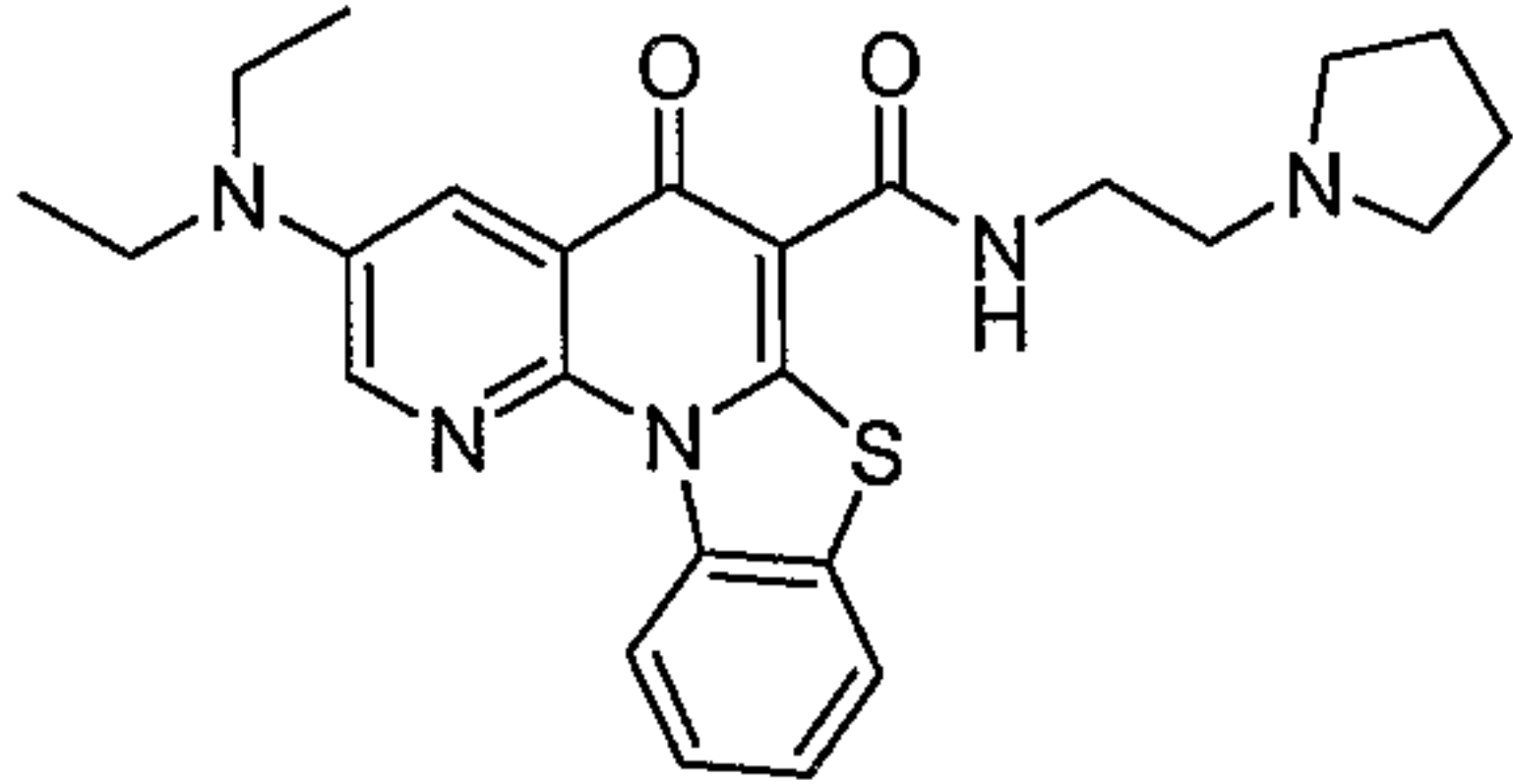
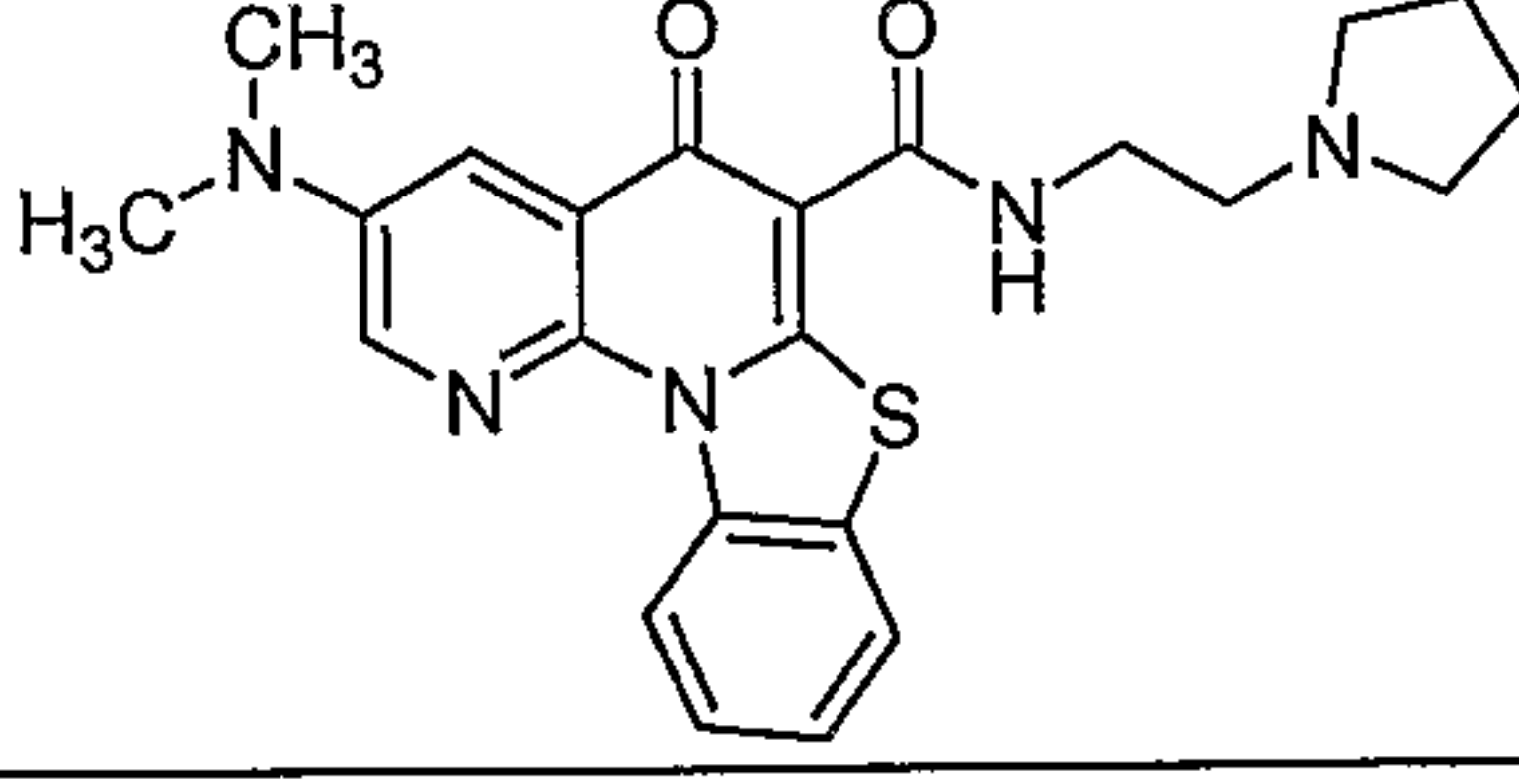
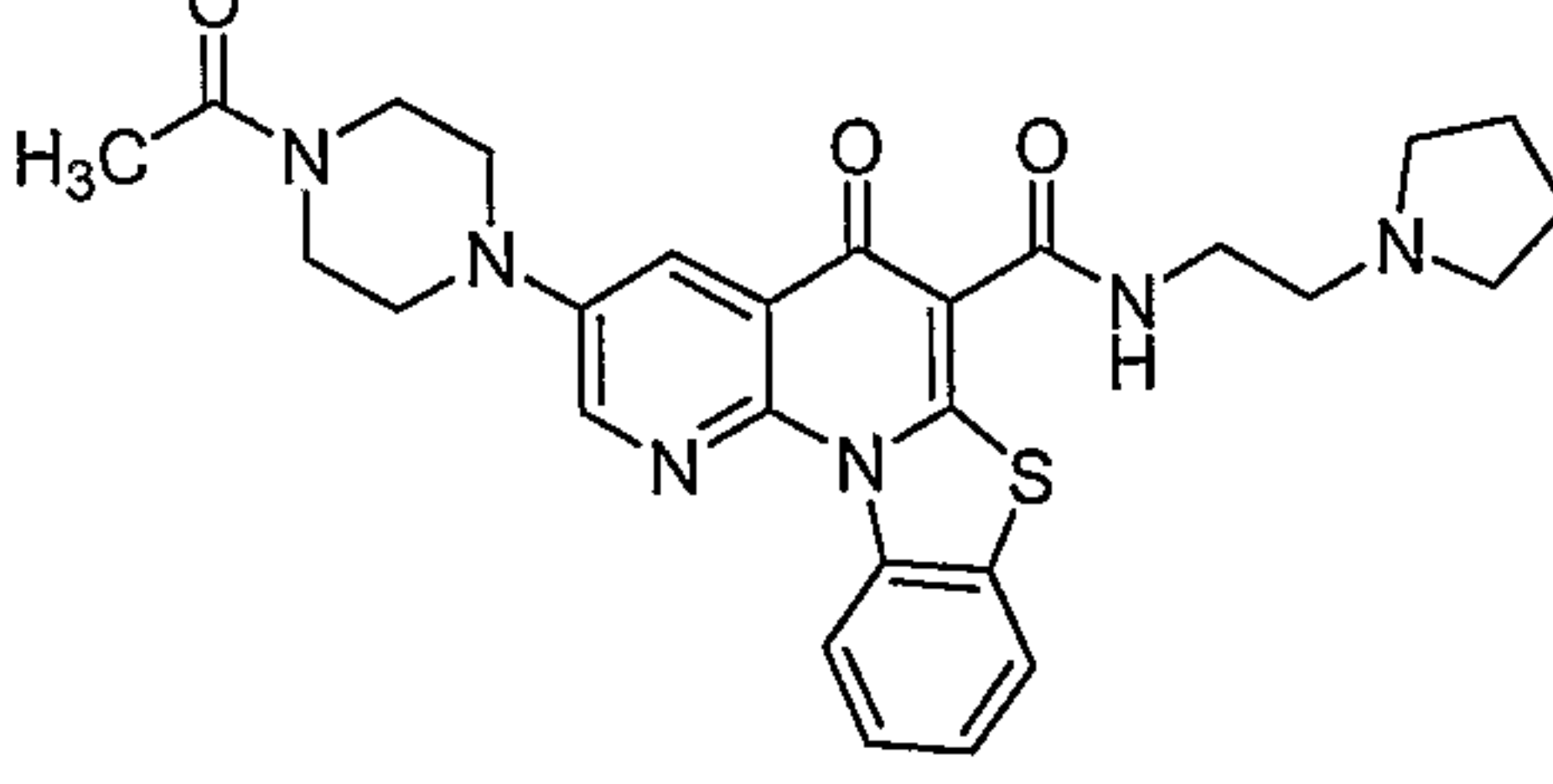
[0191] Compound **63** (300 mg, 0.523 mmol) was suspended in neat trifluoroacetic acid (2 ml). The mixture was stirred at *ca.* 50°C for a few minutes and the volatiles removed *in vacuo*. MeCN (5 ml) was added and the material crashed out with Et<sub>2</sub>O (200 ml). Compound **66** was purified by preparative HPLC and isolated after evaporation as a double TFA salt. (215 mg, 59 % yield). LCMS (ES): >95% pure, m/z 474 [M+1]<sup>+</sup>.

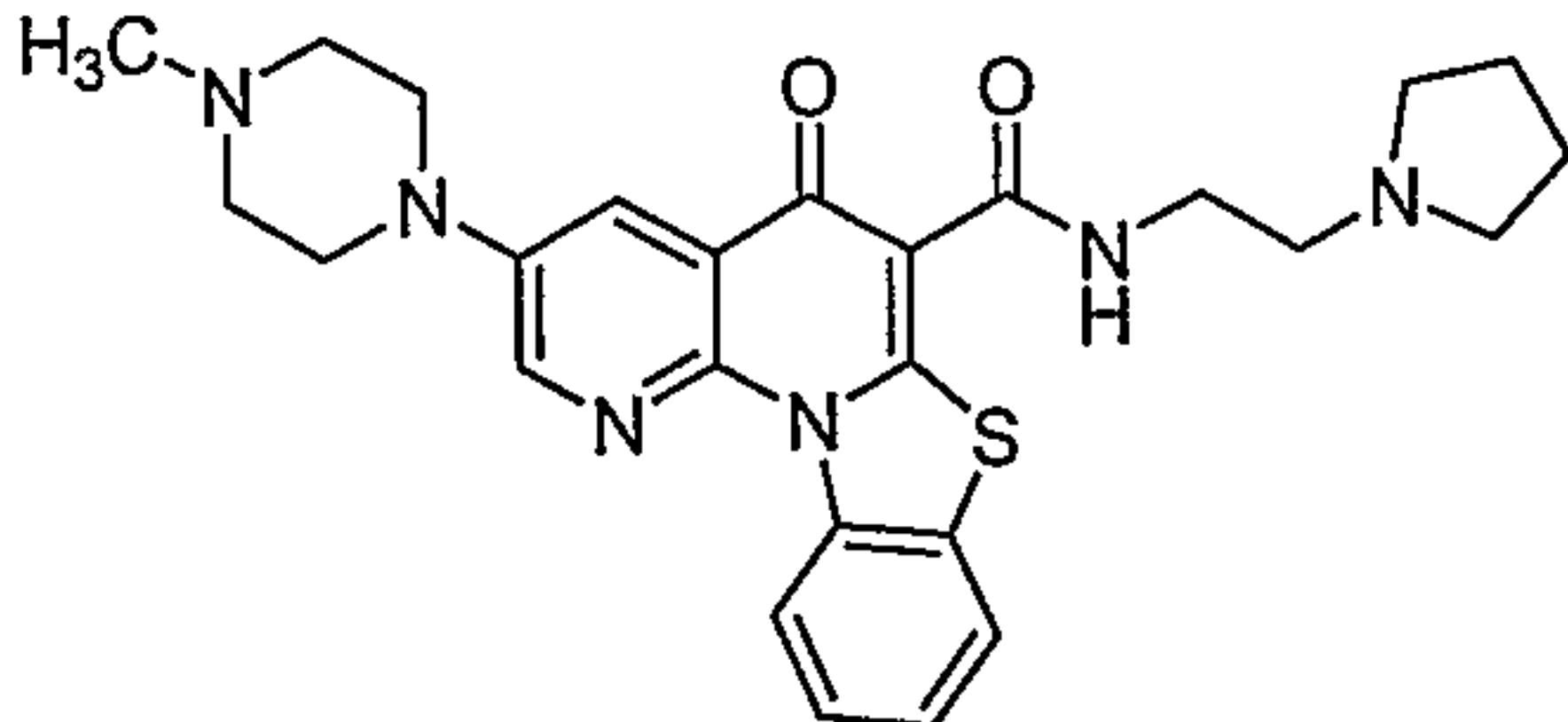
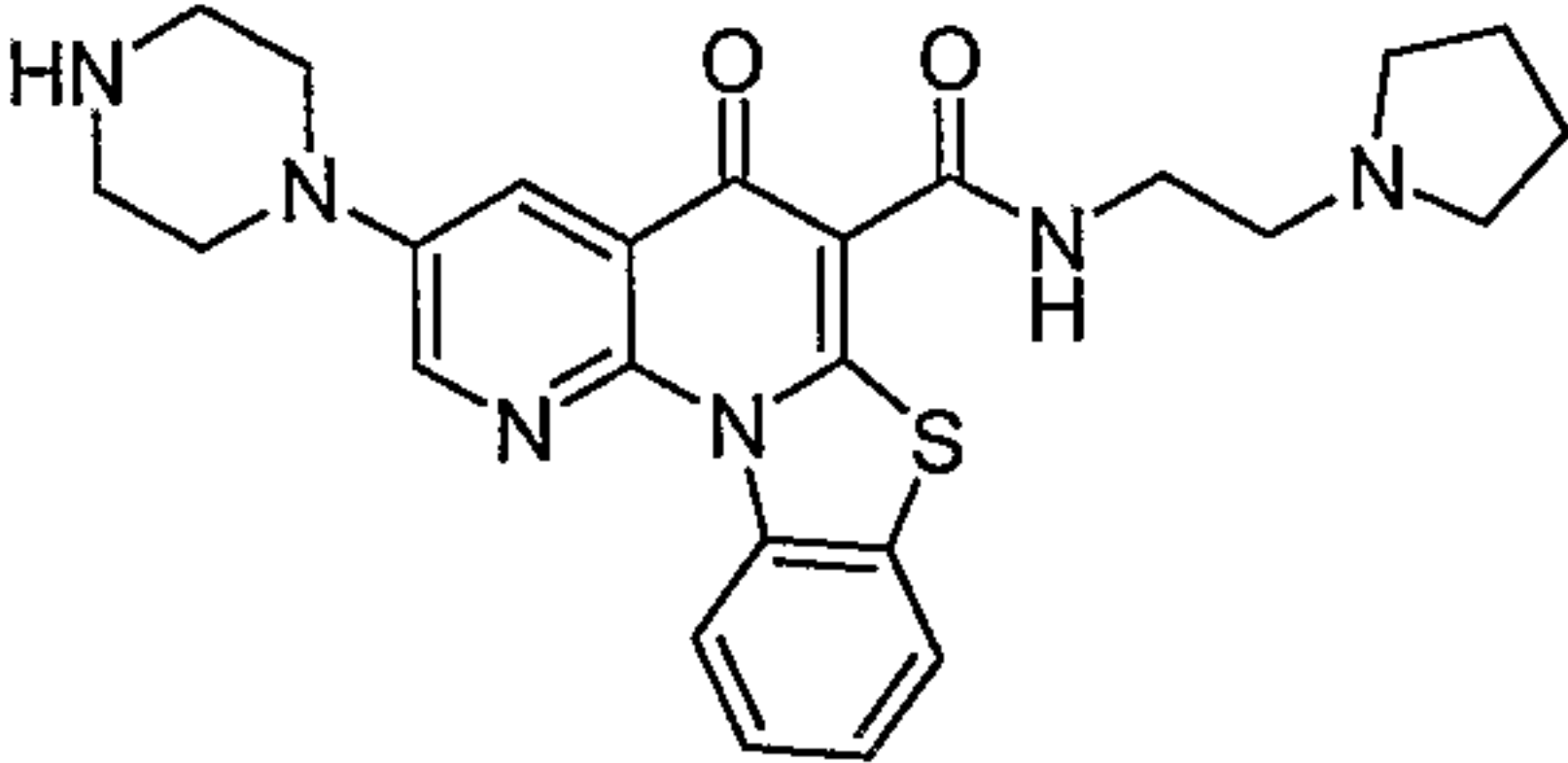
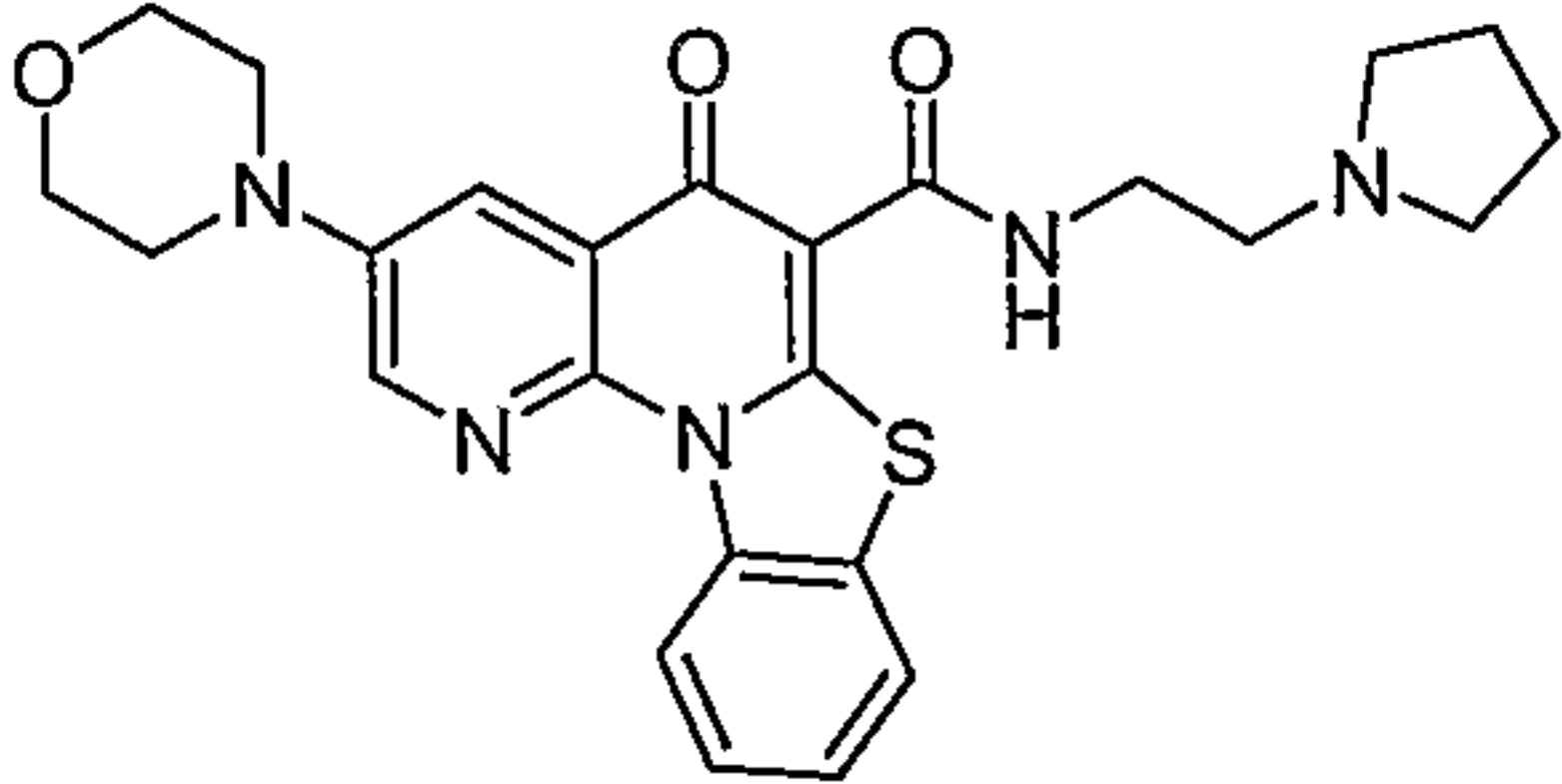
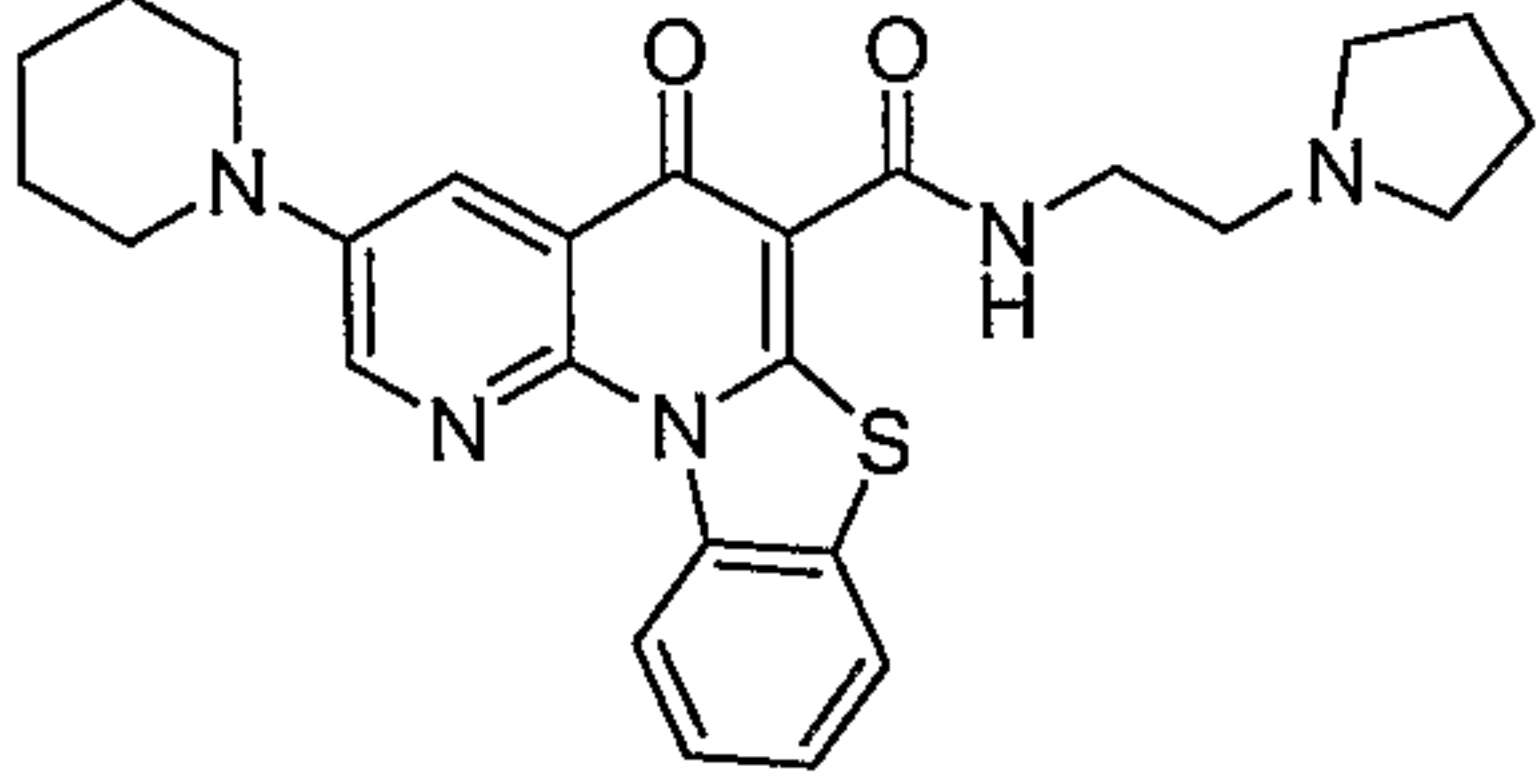
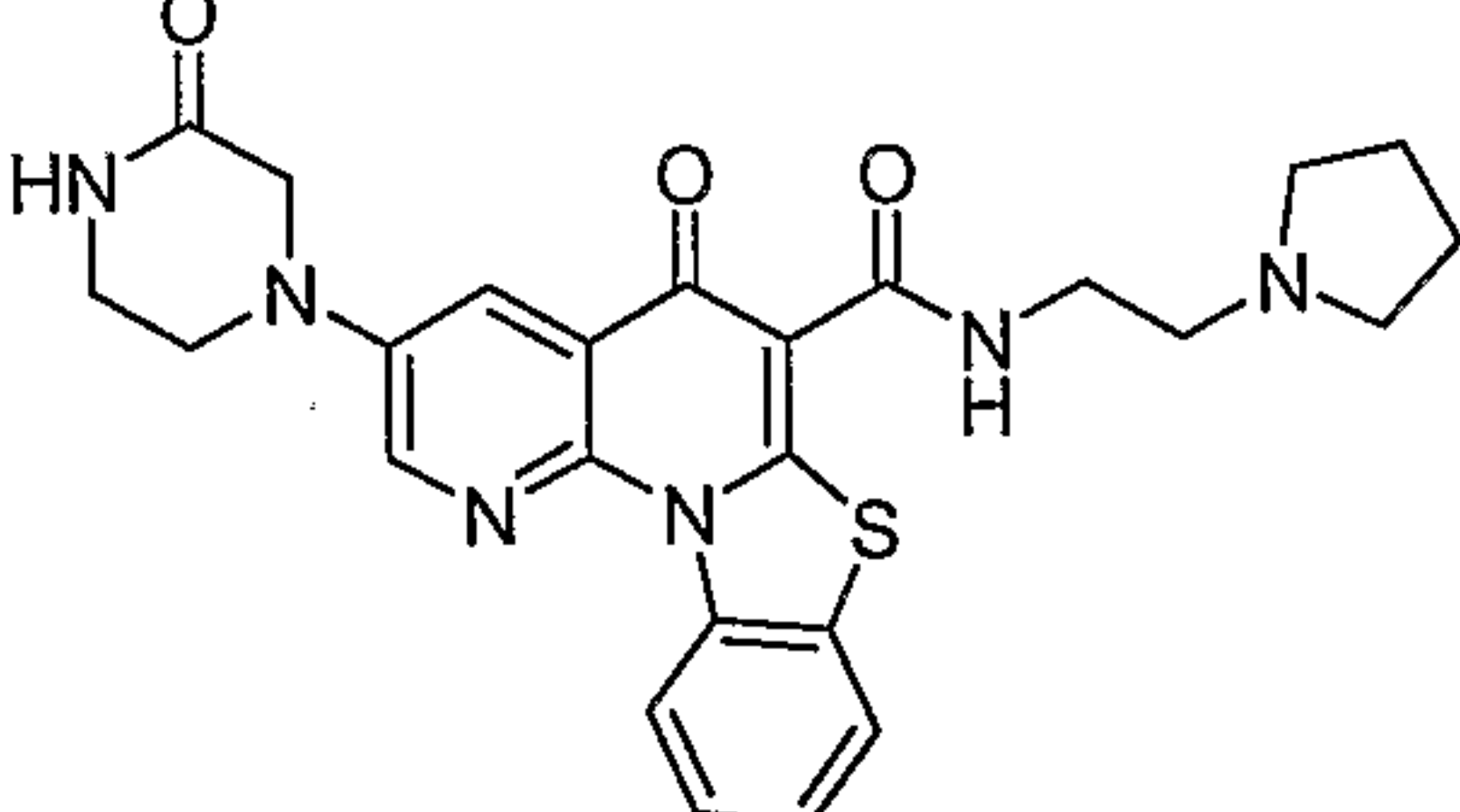
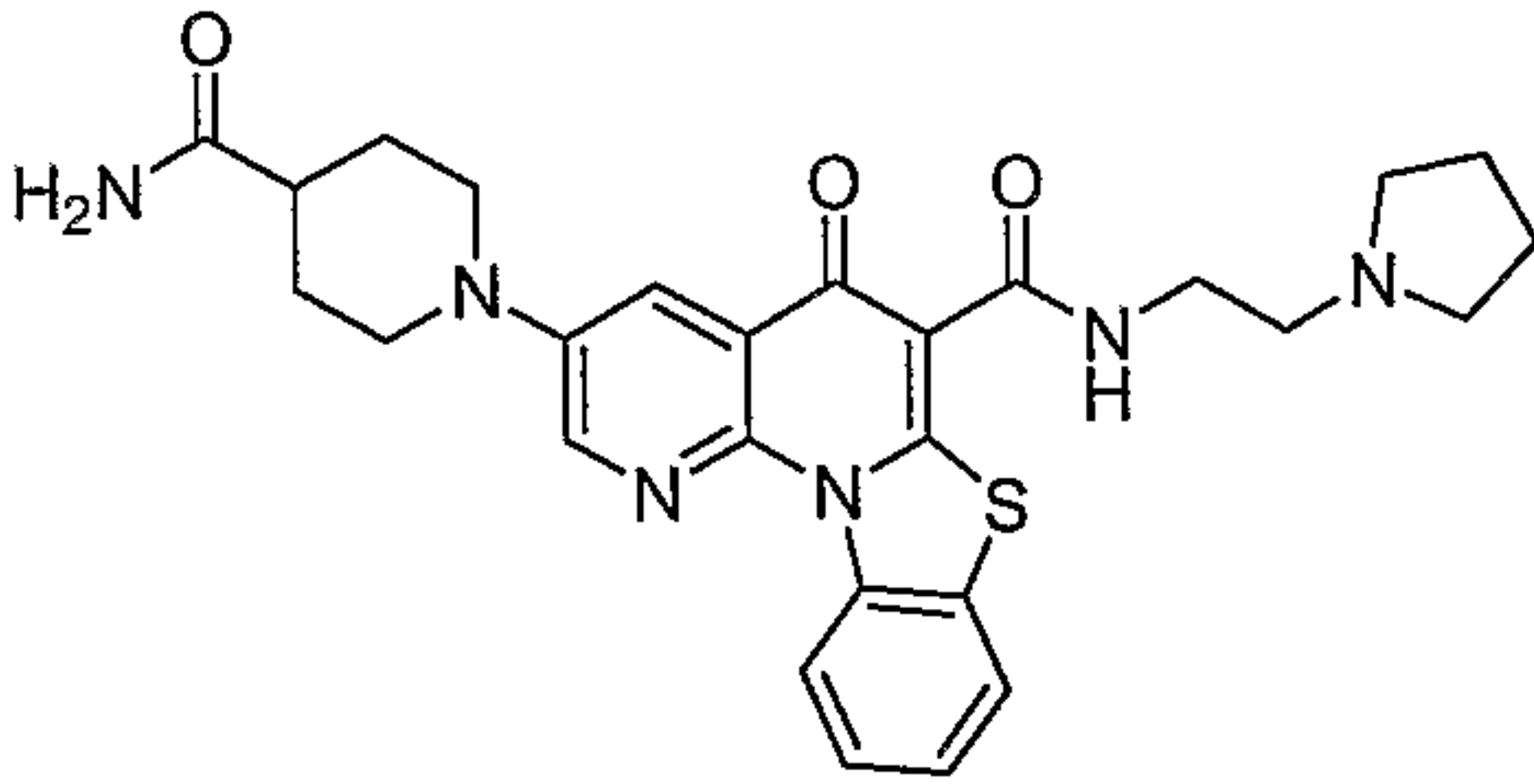
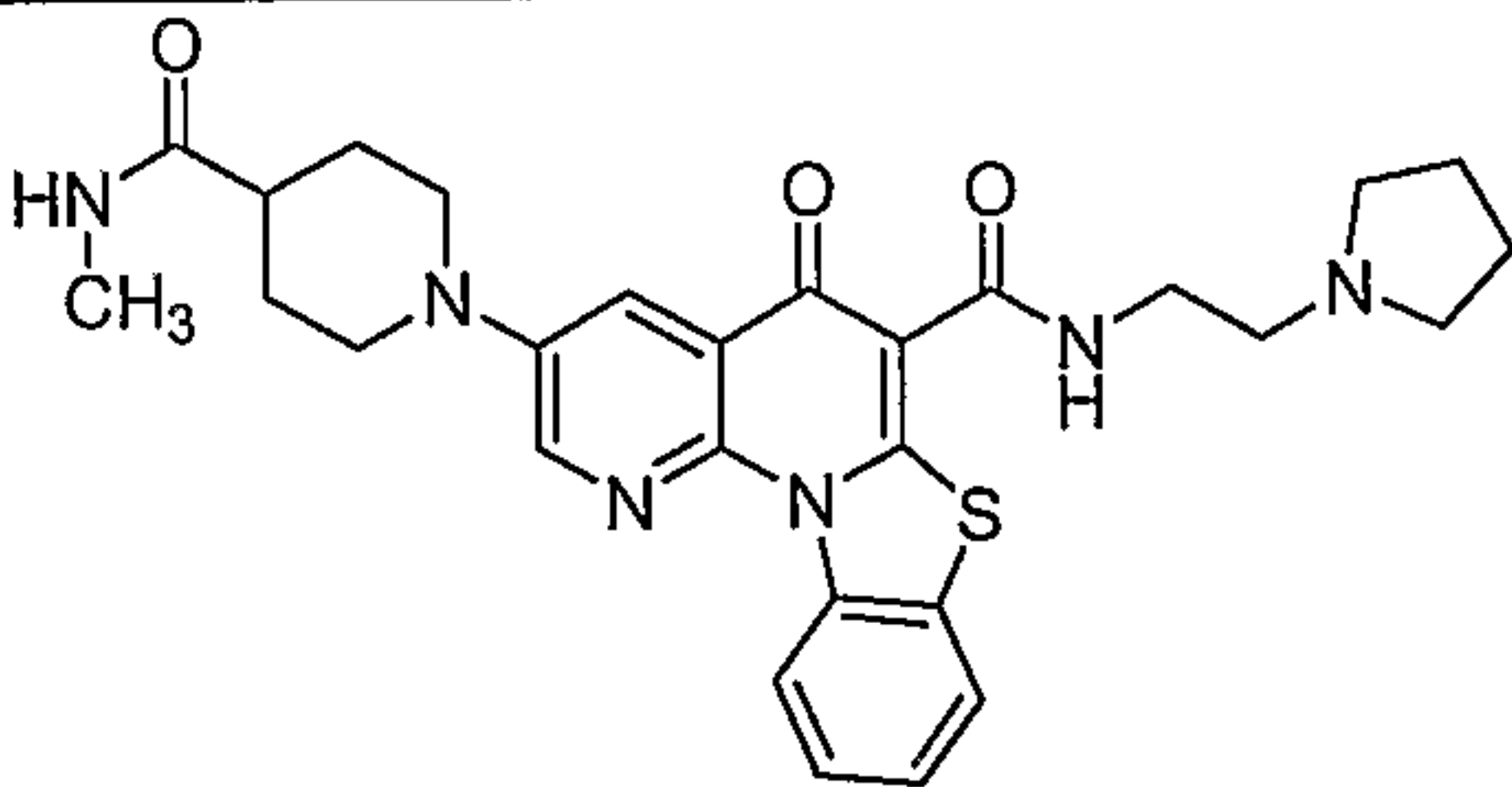
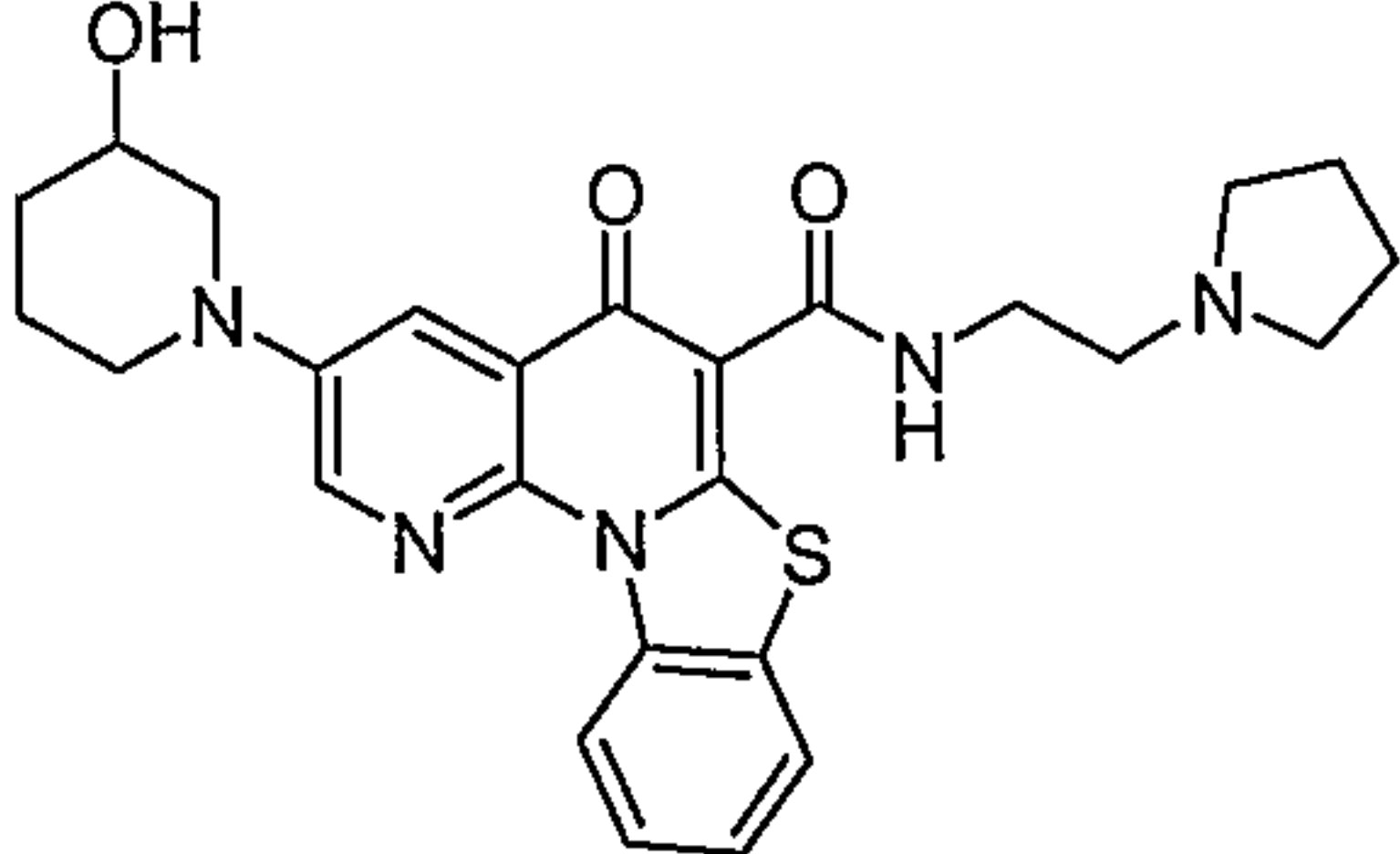
#### Example 57

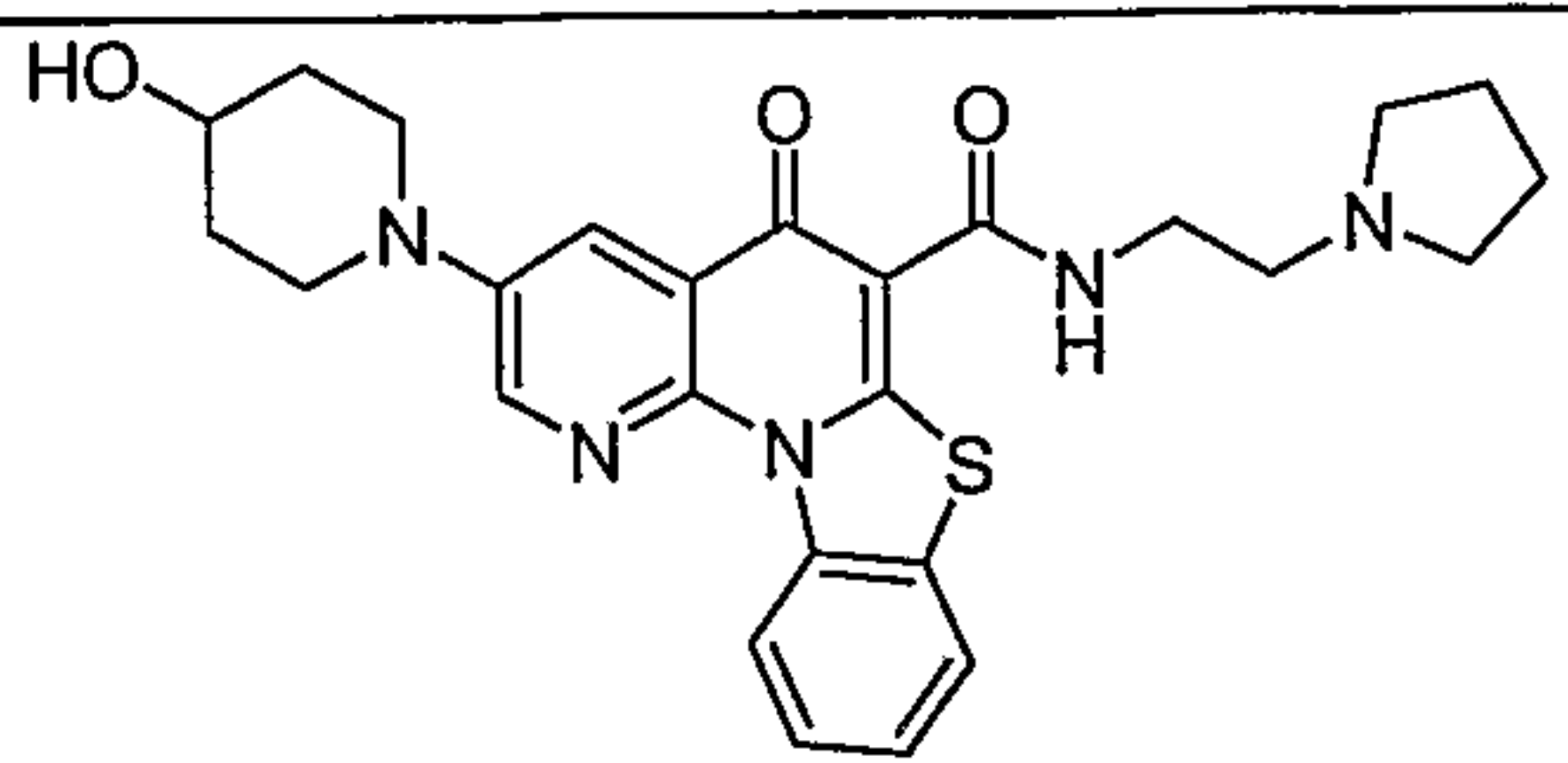
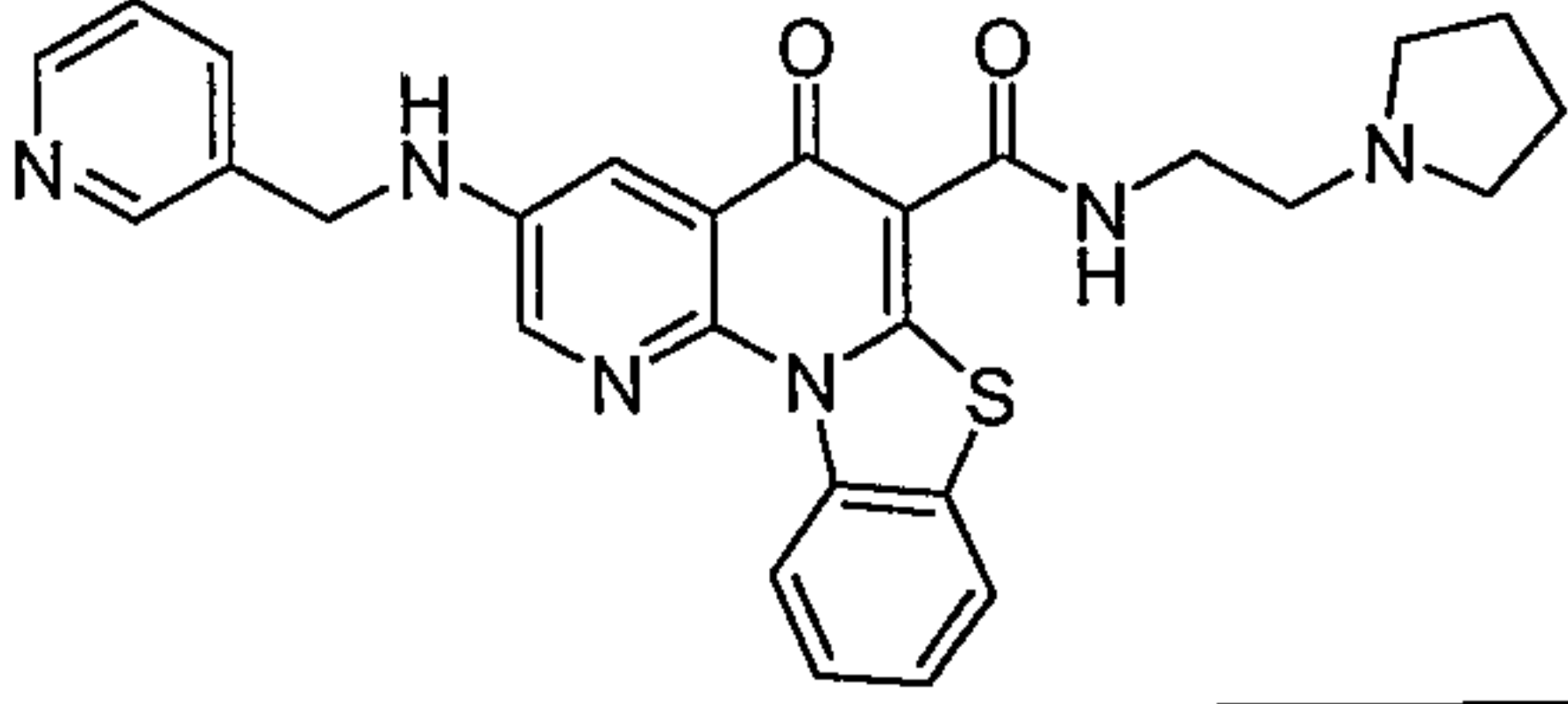
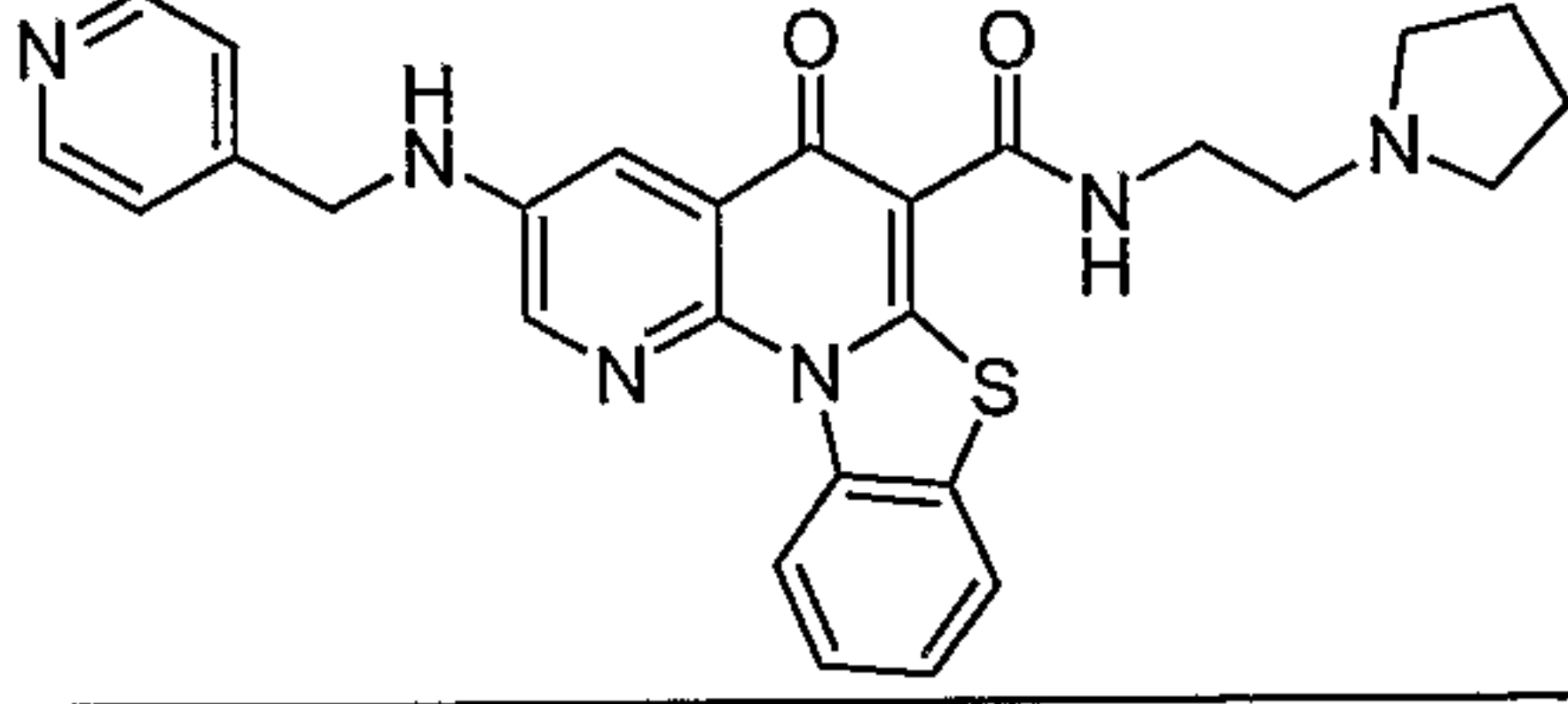
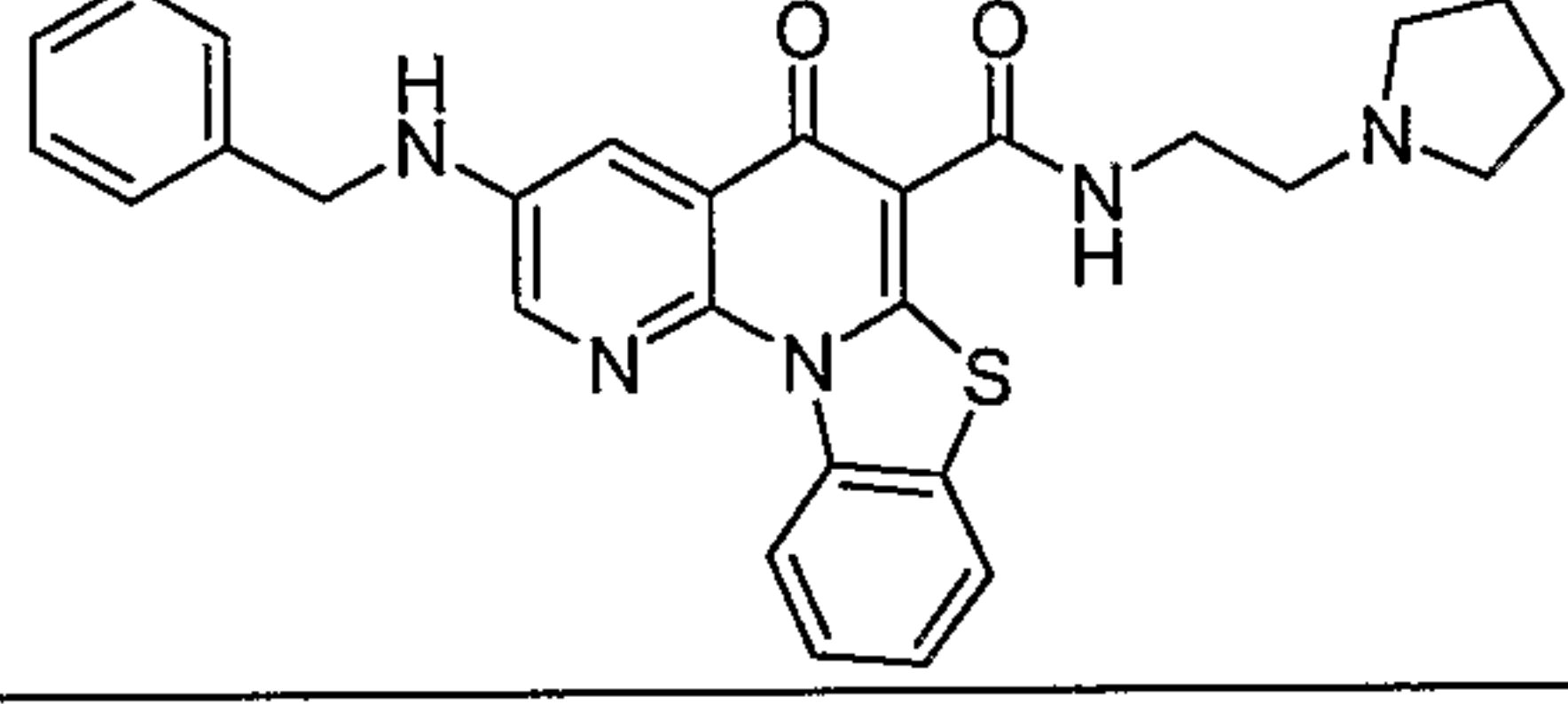
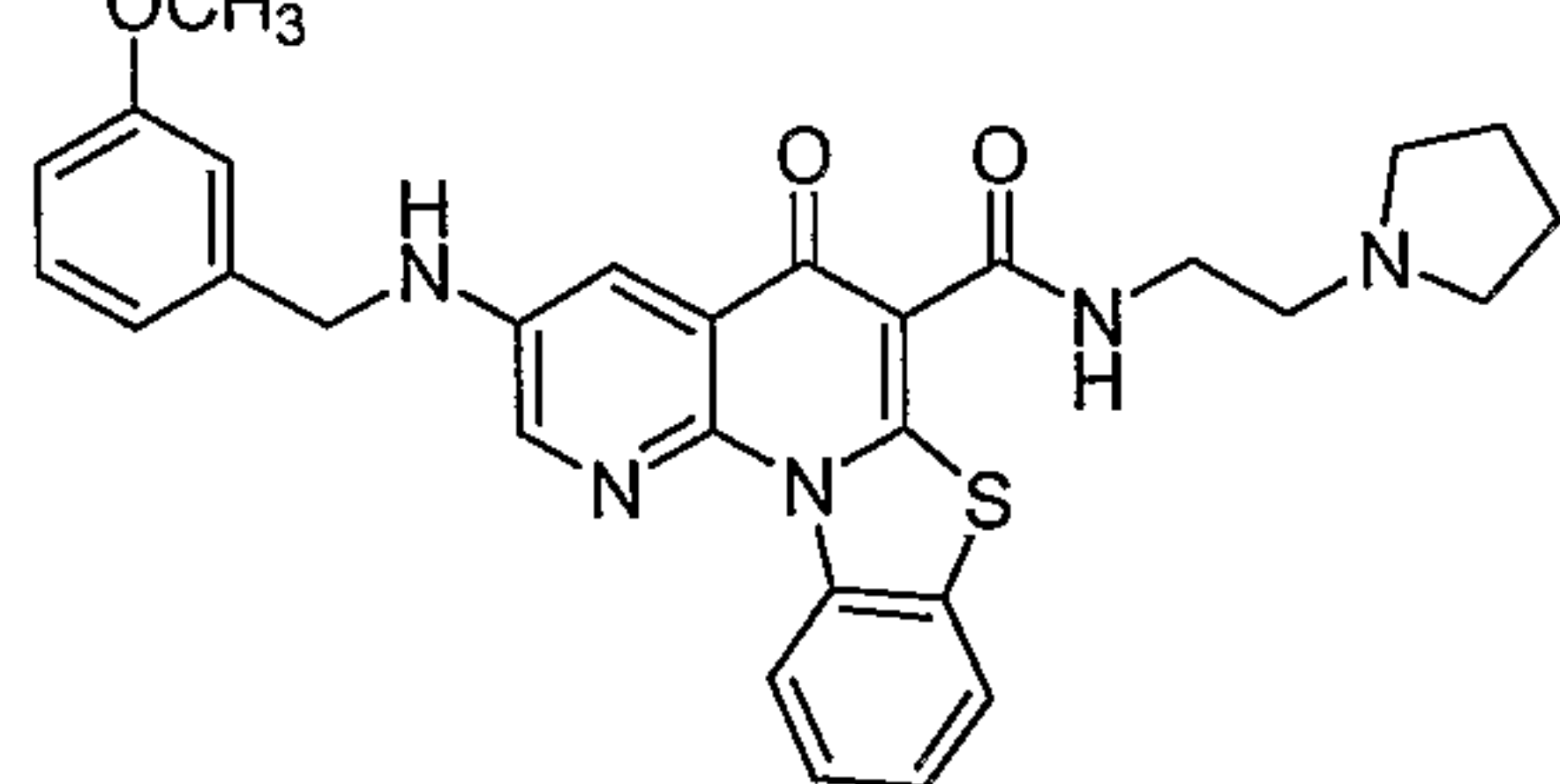
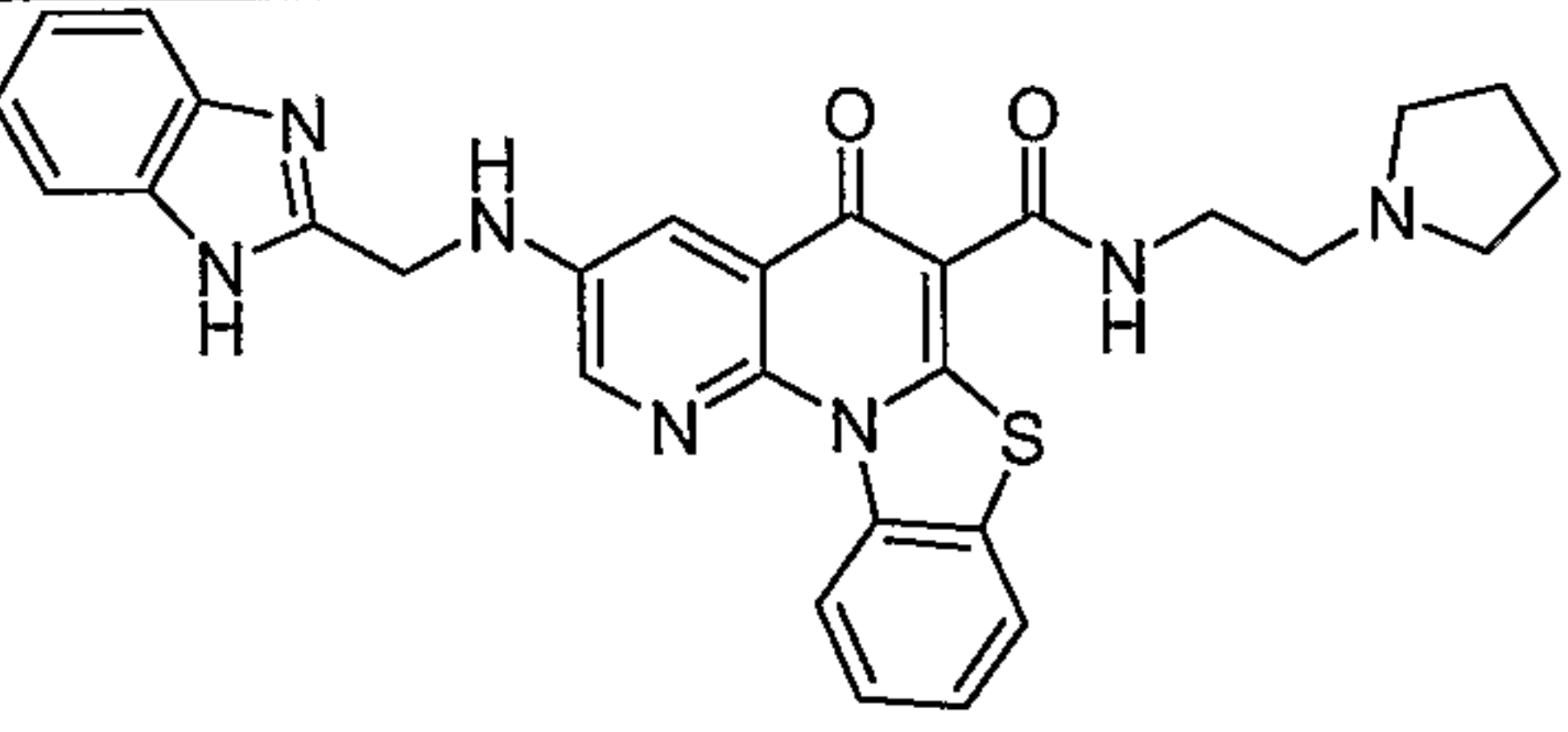
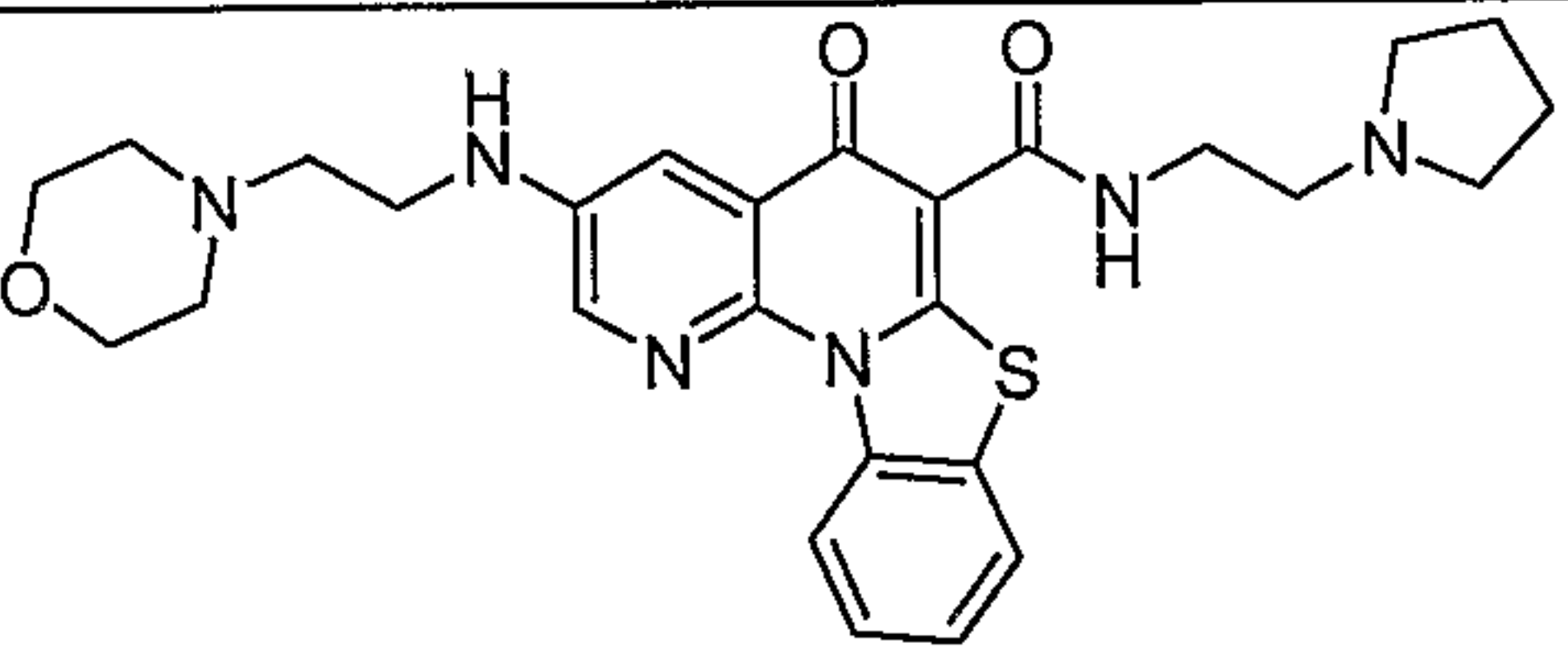
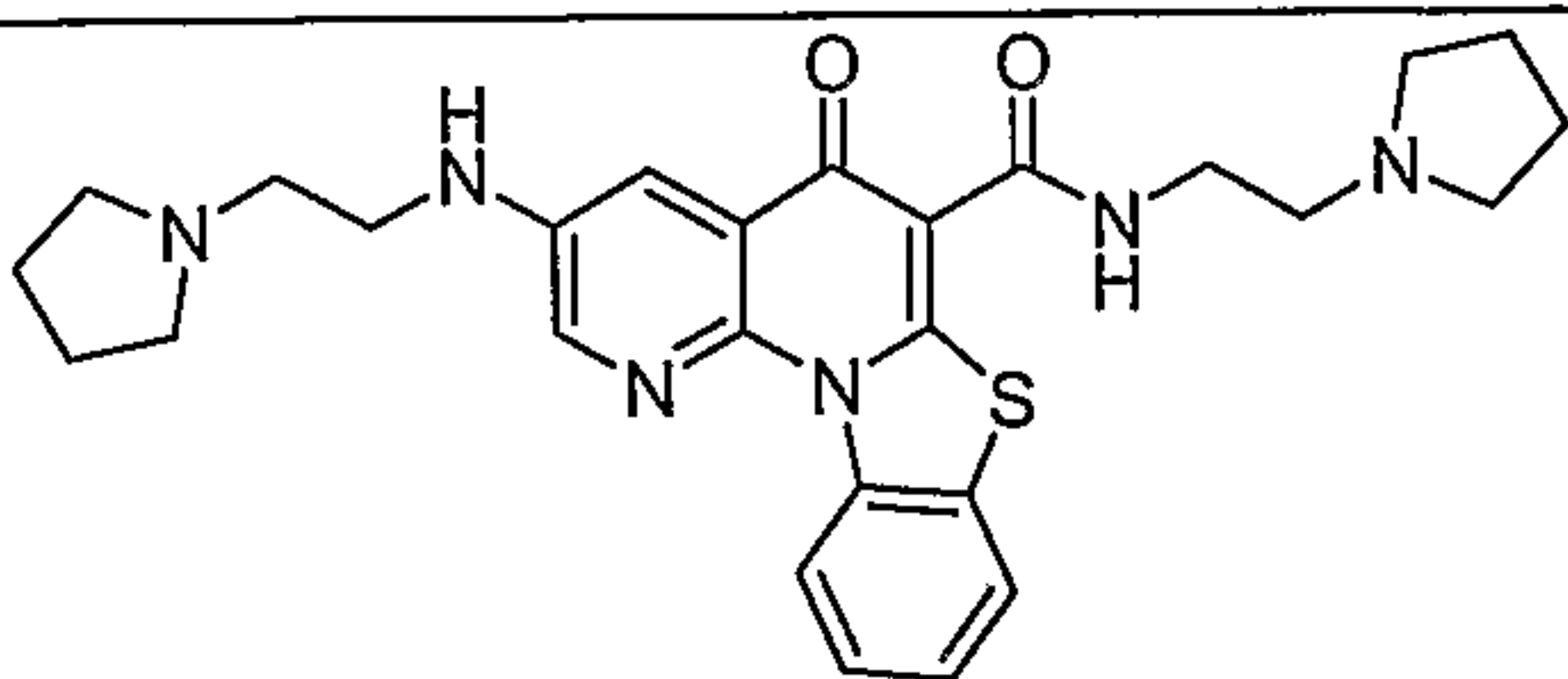


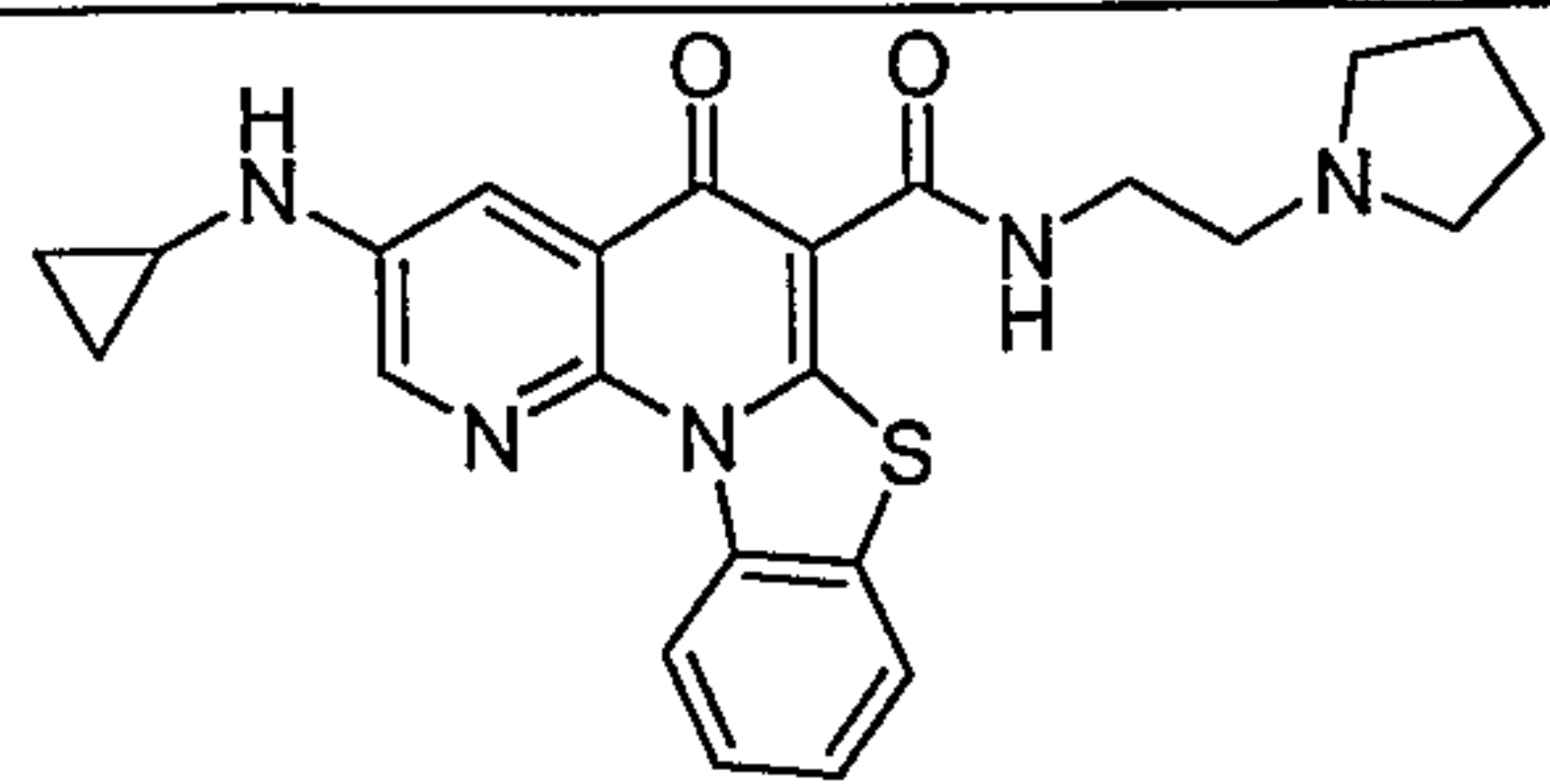
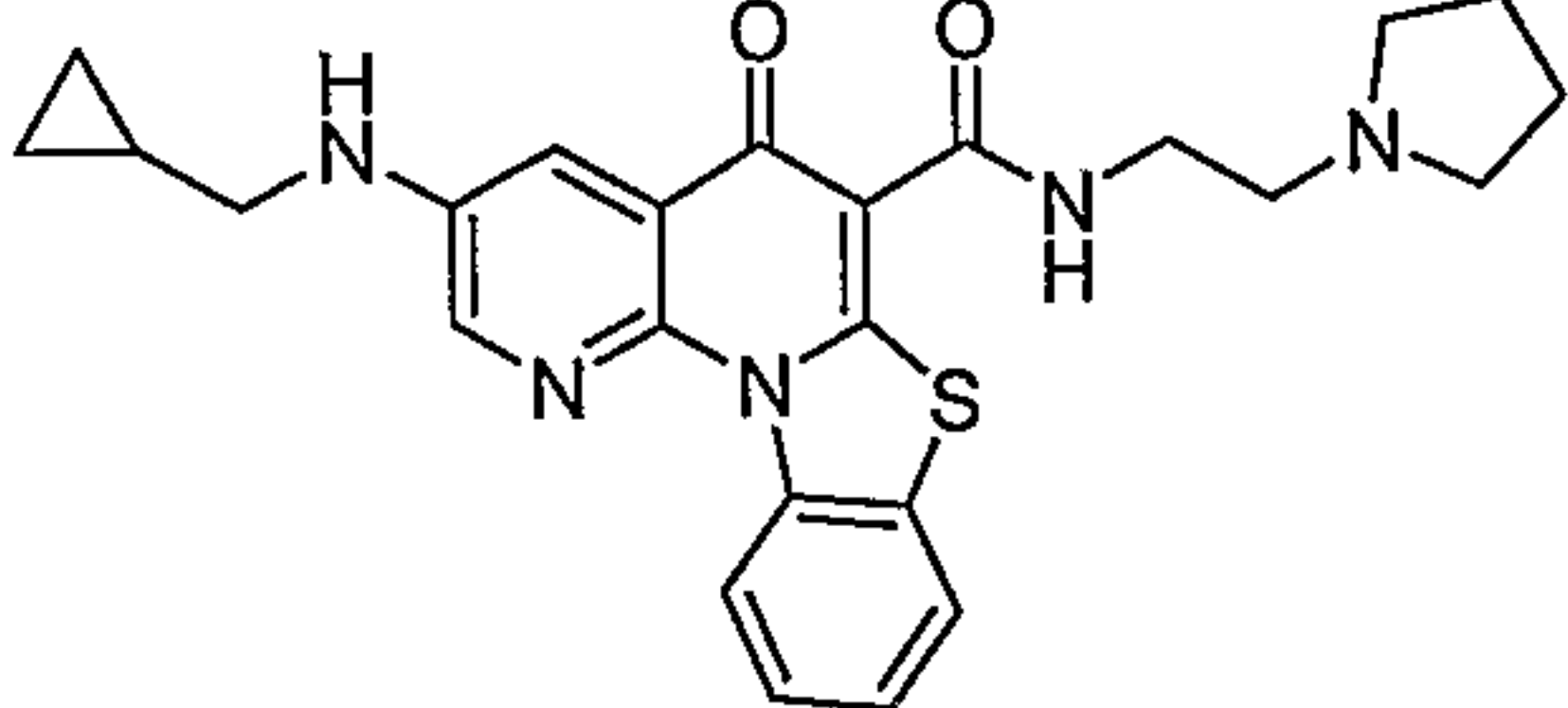
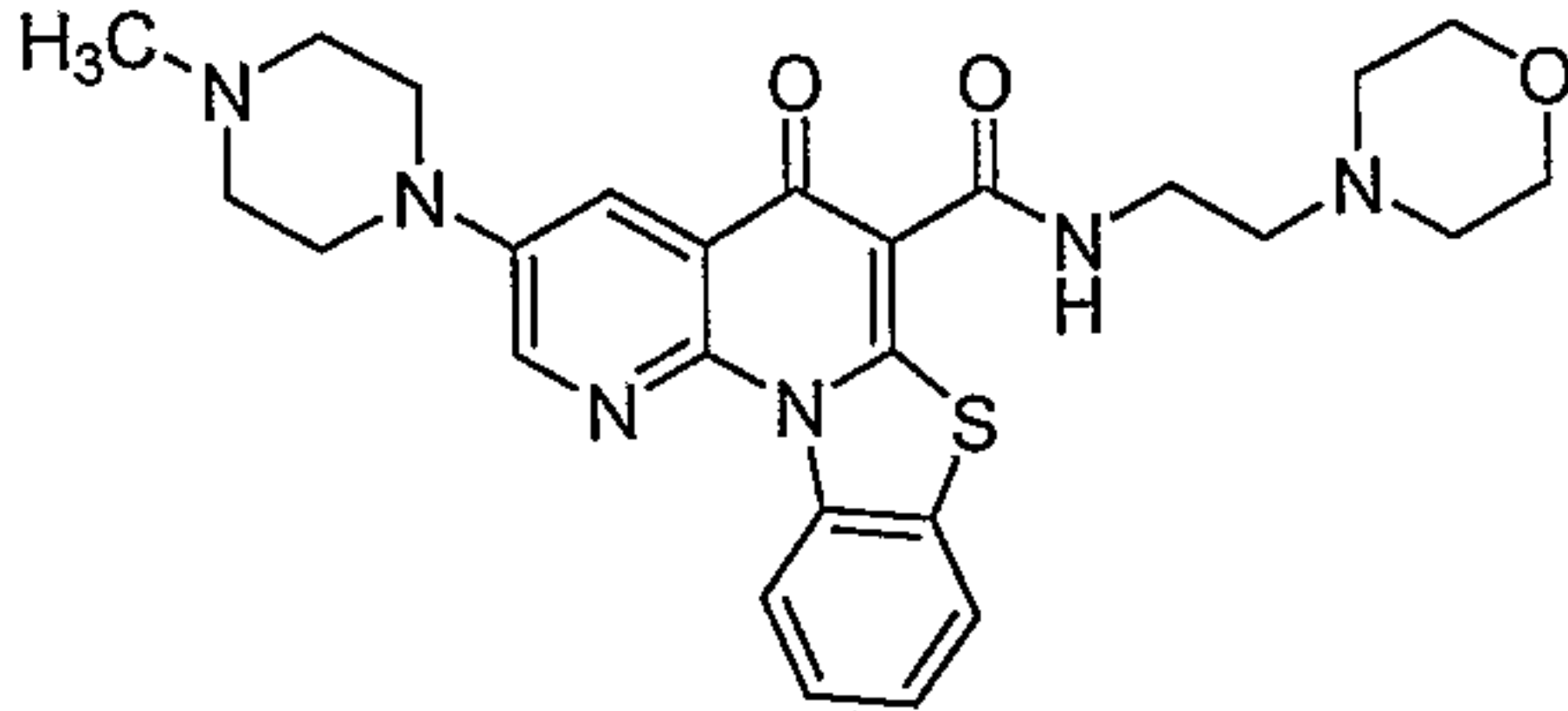
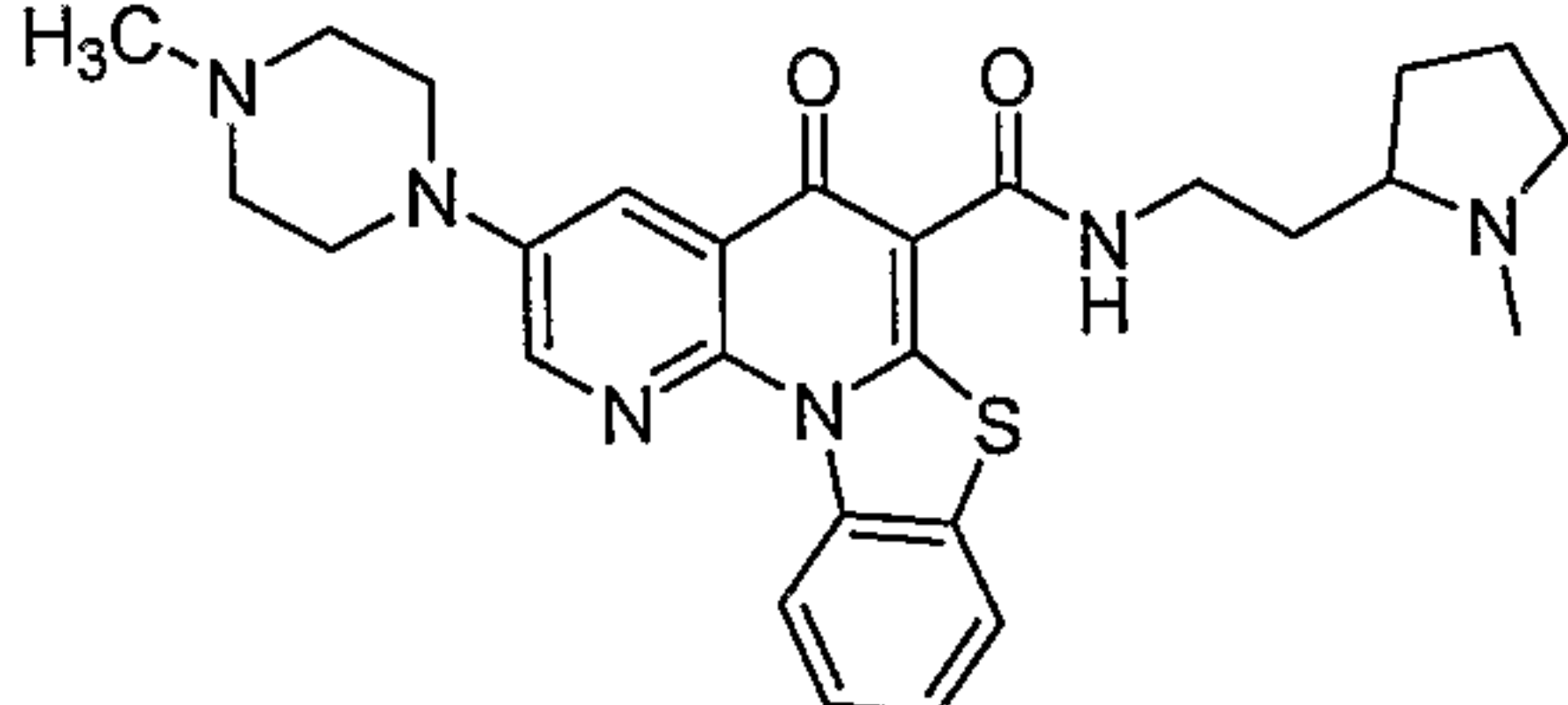
[0192] Compound **57** (20 mg) was suspended in NMP (0.2 ml) and racemic piperidin-3-ylmethanol (0.2 ml). The mixture was heated by microwaves at 200°C for 15 min. After purification by preparative HPLC, the H<sub>2</sub>O/MeCN solution of **67** was concentrated *in vacuo*, the pH adjusted to 9 by saturated NaHCO<sub>3</sub> and the compound extracted by CH<sub>2</sub>Cl<sub>2</sub> (4x). The combined extracts were dried over Na<sub>2</sub>SO<sub>4</sub> and the solvents removed *in vacuo*. The resulting solid was triturated in EtOAc/hexanes to afford racemic **67** as a tan solid (13 mg, 54% yield). LCMS (ES): >95% pure, m/z 506 [M+1]<sup>+</sup>.

[0193] The following compounds were prepared by the same method, using the appropriate amines and fluoro azabenzofluorenones. When using less reactive amines the reaction was carried out at 220°C for 20 min.

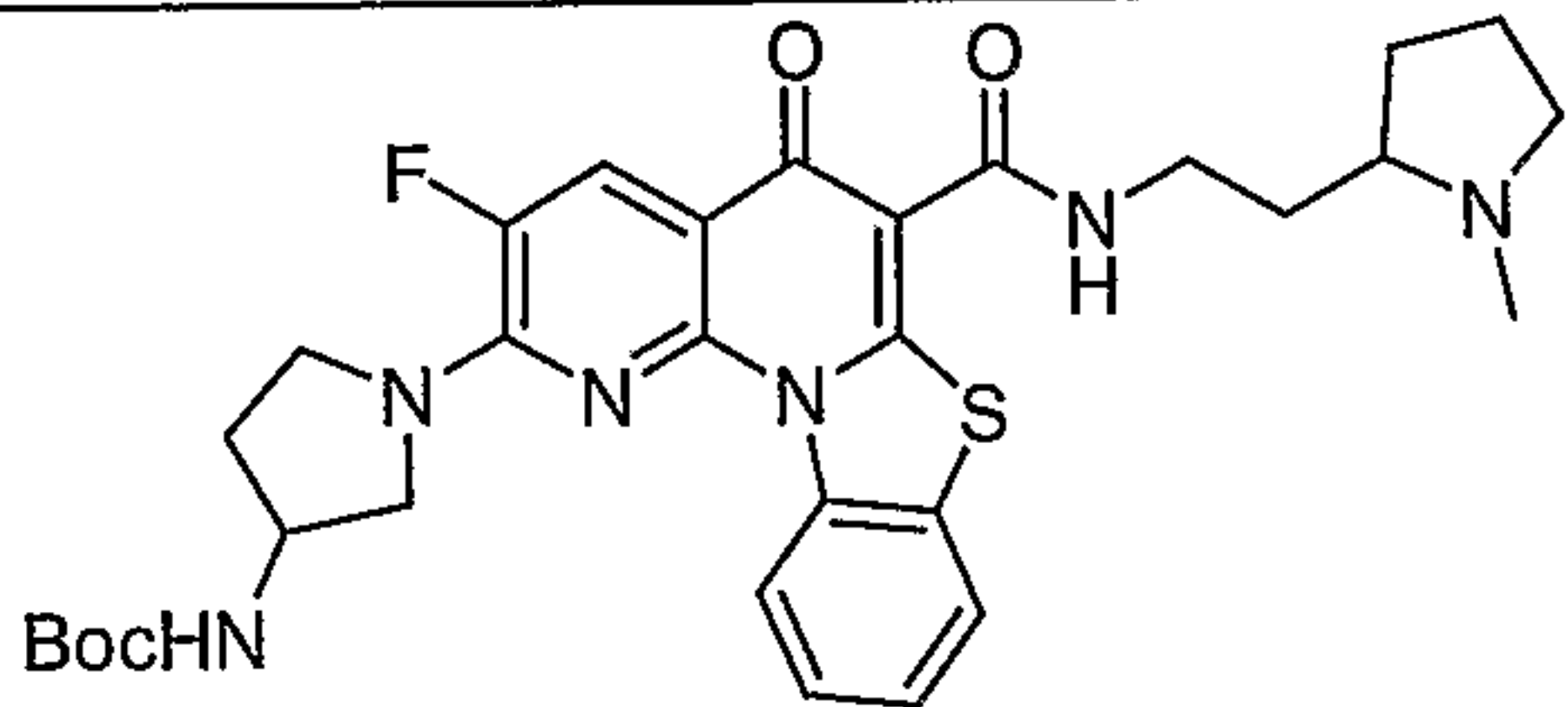
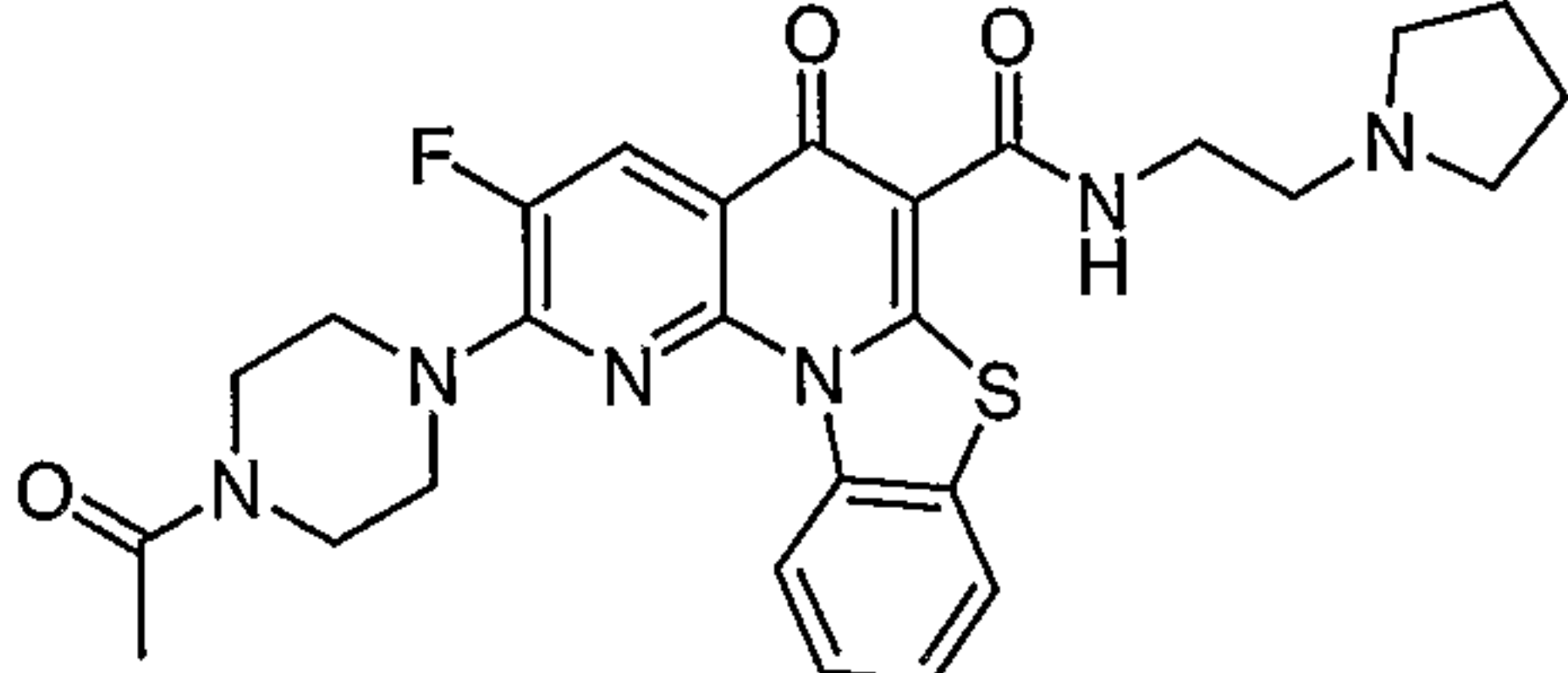
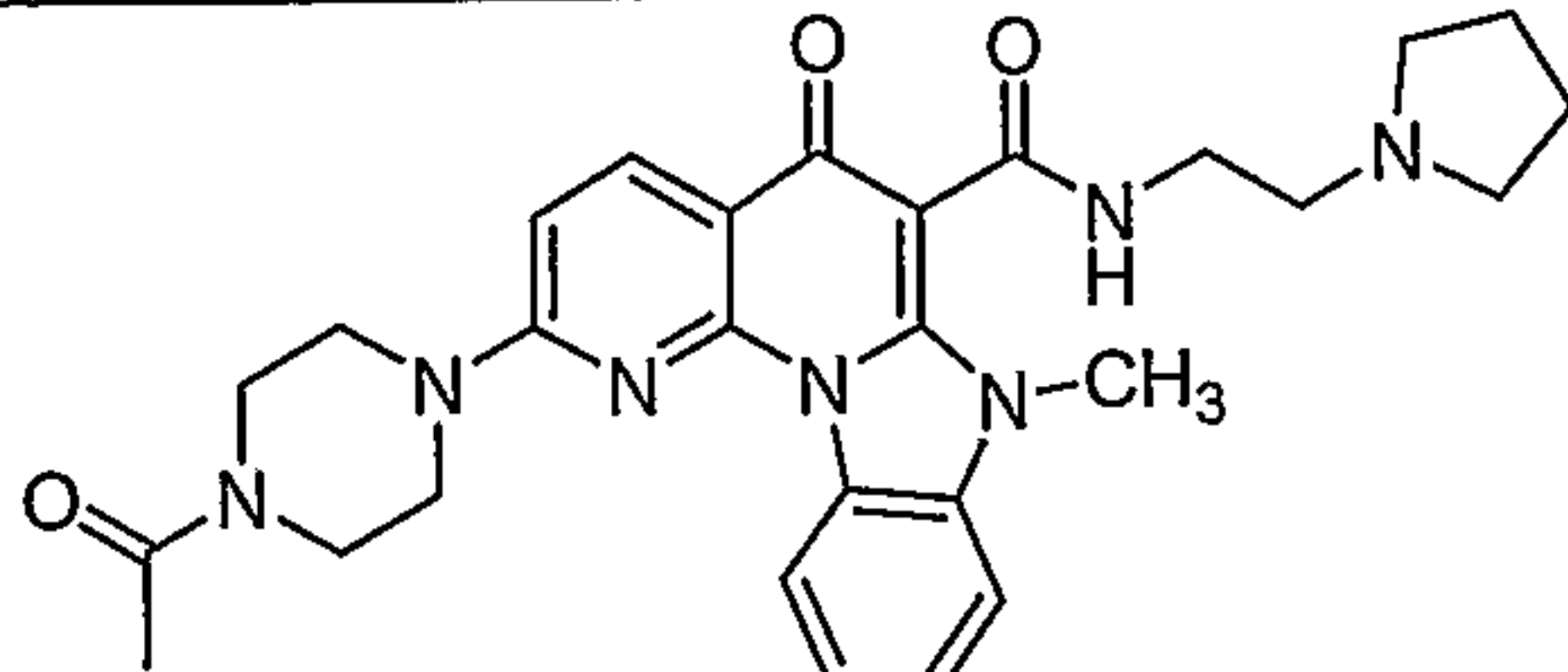
| Structure                                                                            | M.W.   | LCMS (ES) m/z          |
|--------------------------------------------------------------------------------------|--------|------------------------|
|    | 477.58 | 478 [M+1] <sup>+</sup> |
|   | 451.17 | 452 [M+1] <sup>+</sup> |
|  | 465.57 | 466 [M+1] <sup>+</sup> |
|  | 461.58 | 462 [M+1] <sup>+</sup> |
|  | 463.60 | 464 [M+1] <sup>+</sup> |
|  | 435.54 | 436 [M+1] <sup>+</sup> |
|  | 518.63 | 519 [M+1] <sup>+</sup> |

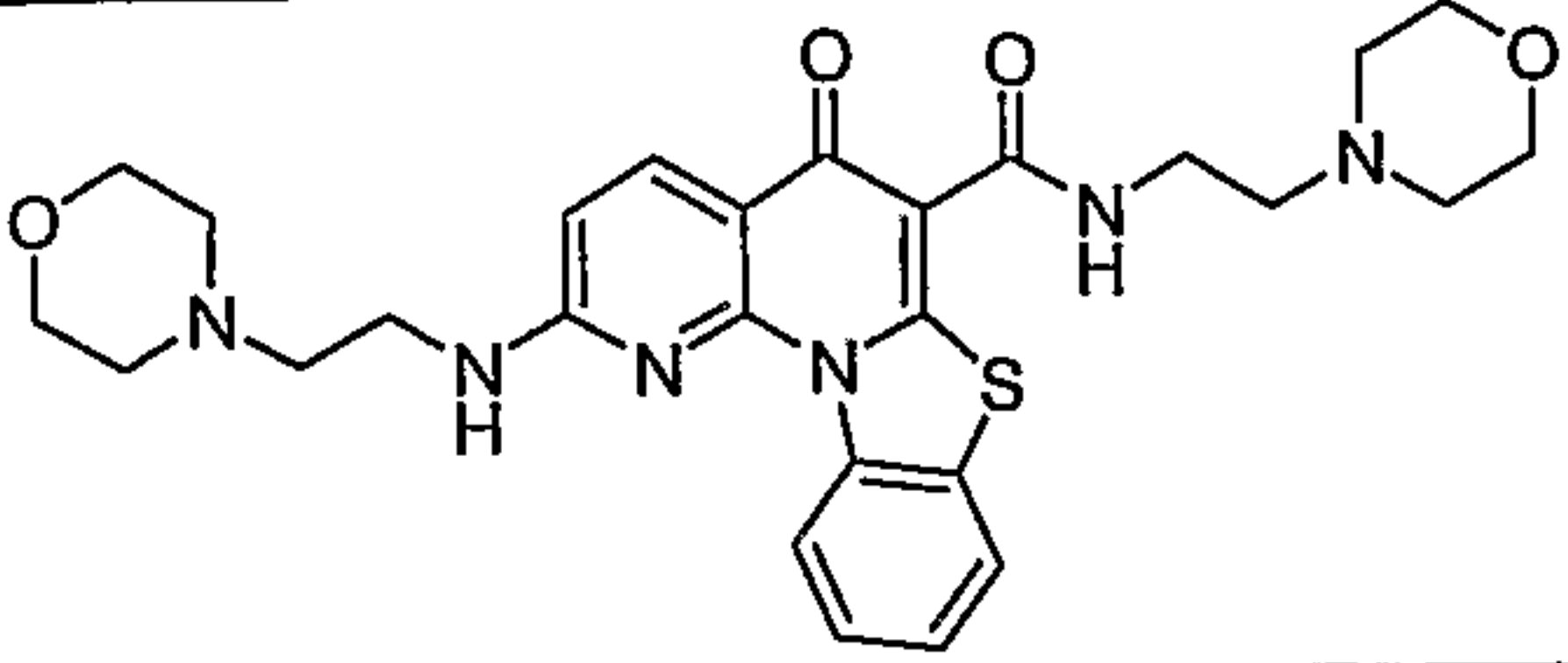
| Structure                                                                            | M.W.   | LCMS (ES) m/z          |
|--------------------------------------------------------------------------------------|--------|------------------------|
|    | 490.22 | 491 [M+1] <sup>+</sup> |
|    | 476.59 | 477 [M+1] <sup>+</sup> |
|   | 477.58 | 478 [M+1] <sup>+</sup> |
|  | 475.61 | 476 [M+1] <sup>+</sup> |
|  | 490.58 | 491 [M+1] <sup>+</sup> |
|  | 518.63 | 519 [M+1] <sup>+</sup> |
|  | 532.66 | 533 [M+1] <sup>+</sup> |
|  | 491.61 | 492 [M+1] <sup>+</sup> |

| Structure                                                                            | M.W.   | LCMS (ES) m/z          |
|--------------------------------------------------------------------------------------|--------|------------------------|
|    | 491.61 | 492 [M+1] <sup>+</sup> |
|    | 498.60 | 499 [M+1] <sup>+</sup> |
|   | 498.60 | 499 [M+1] <sup>+</sup> |
|  | 497.61 | 498[M+1] <sup>+</sup>  |
|  | 527.64 | 528 [M+1] <sup>+</sup> |
|  | 537.64 | 538 [M+1] <sup>+</sup> |
|  | 520.65 | 521 [M+1] <sup>+</sup> |
|  | 504.65 | 505 [M+1] <sup>+</sup> |

| Structure                                                                            | M.W.   | LCMS (ES) m/z          |
|--------------------------------------------------------------------------------------|--------|------------------------|
|    | 447.55 | 448 [M+1] <sup>+</sup> |
|    | 461.58 | 462 [M+1] <sup>+</sup> |
|   | 506.62 | 507 [M+1] <sup>+</sup> |
|  | 504.65 | 505 [M+1] <sup>+</sup> |

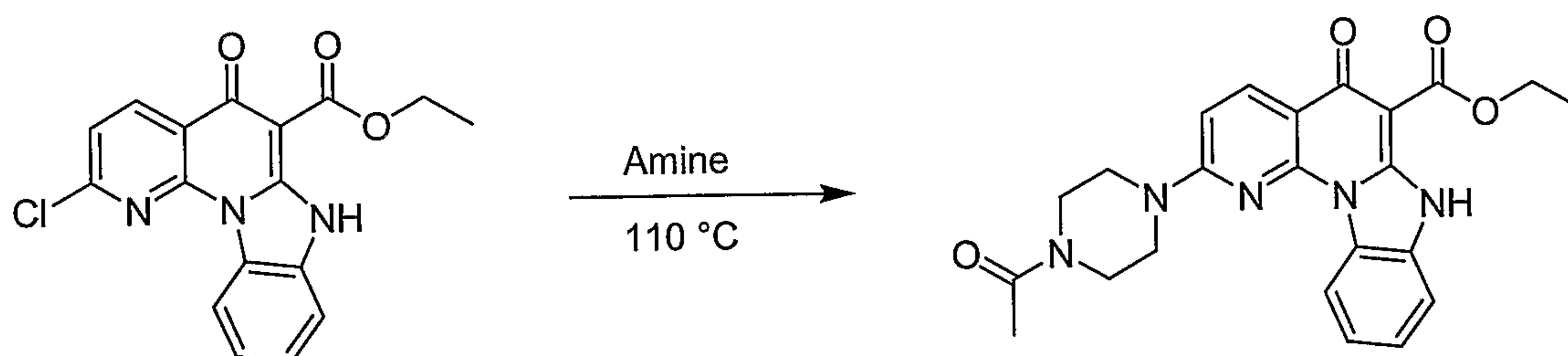
[0194] The following compounds were prepared by the same method, using the appropriate amines and chloro azabenzofluorenones. The reaction was carried out using microwave heating at 100°C for 5 min or 150°C for 3 min.

| Structure                                                                            | M.W.   | LCMS (ES) m/z          |
|--------------------------------------------------------------------------------------|--------|------------------------|
|  | 608.73 | 609 [M+1] <sup>+</sup> |
|  | 536.62 | 537 [M+1] <sup>+</sup> |
|  | 515.61 | 516 [M+1] <sup>+</sup> |

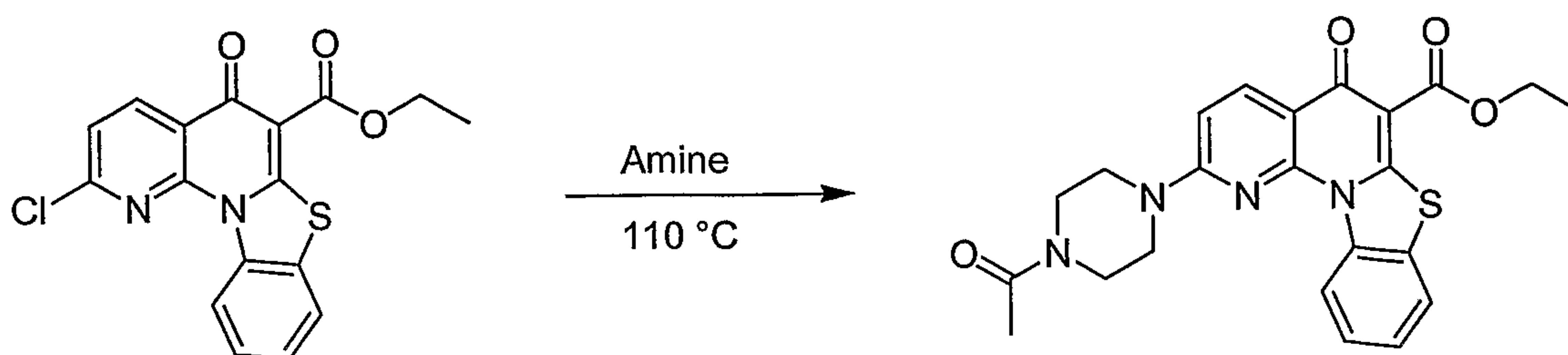
| Structure                                                                          | M.W.   | LCMS (ES) m/z          |
|------------------------------------------------------------------------------------|--------|------------------------|
|  | 536.65 | 537 [M+1] <sup>+</sup> |

Example 58

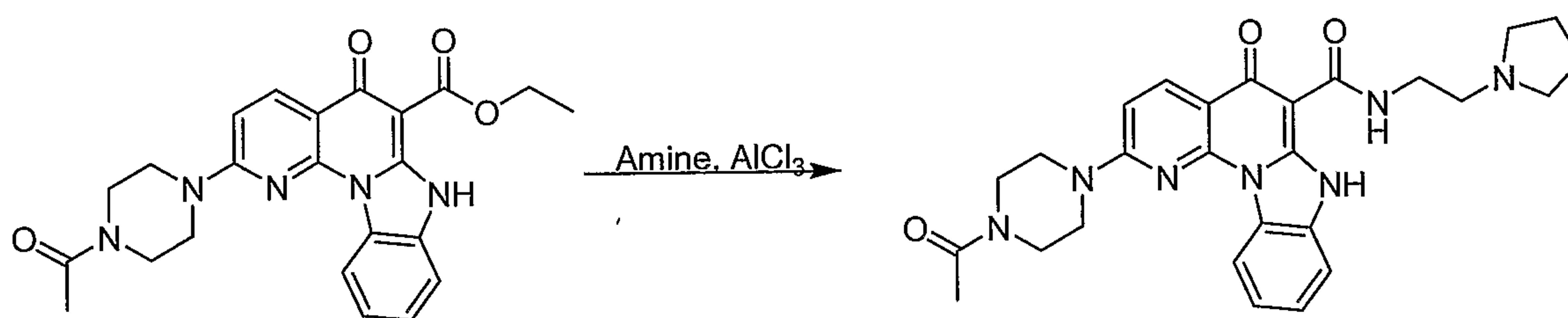
[0195] To a solution of the chloroester (1.0g, 2.81 mmol) and 1-(2-aminoethyl) pyrrolidine (0.50 g, 4.4 mmol) in methylene chloride (20 mL) was added aluminum chloride (585 mg, 4.4 mmol) and the reaction was allowed to stir for 4 hours at room temperature. The reaction was then quench with a saturated solution of Rochelle's salt and kept basic with 1N NaOH and stirred for an additional hour. The mixture was extracted with methylene chloride, dried over magnesium sulfate and the solvent was removed *in vacuo*. The resulting crude solid was triturated from ethyl acetate to afford the chloroamide as a tan solid (0.5 g, 1.1 mmol); LCMS (ES): 95% pure, m/z 437 [M+1]<sup>+</sup>.

Example 59

[0196] To a solution of the chloroester (1.0 g, 2.81 mmol) in N-methylpyrrolidinone (10 mL) was added N-acetylpiperazine (540 mg, 4.2 mmol) and the mixture was heated at 110 °C for 4 hours. Water was added and the crude product was collected by filtration. The resulting solid was dissolved in methylene chloride, dried over sodium sulfate and the solvent was removed *in vacuo*. The resulting crude solid was triturated from ethyl acetate to afford the aminoester as a tan solid (0.70 g, 1.62 mmol); LCMS (ES): 95% pure, m/z 434 [M+1]<sup>+</sup>.

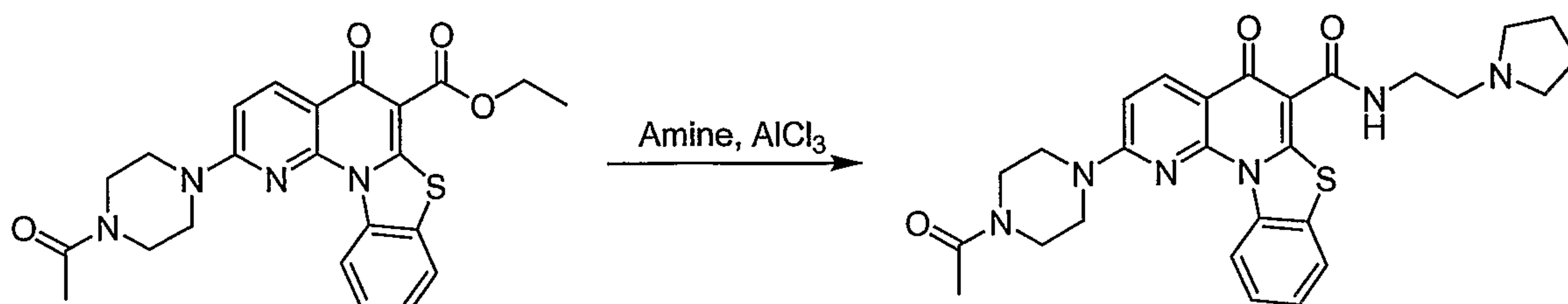
Example 60

[0197] To a solution of the chloroester (1.0 g, 2.81 mmol) in N-methylpyrrolidinone (10 mL) was added N-acetylpiperazine (540 mg, 4.2 mmol) and the mixture was heated at 110 °C for 4 hours. Water was added and the crude product was collected by filtration. The resulting solid was dissolved in methylene chloride, dried over sodium sulfate and the solvent was removed *in vacuo*. The resulting crude solid was triturated from ethyl acetate to afford the aminoester as a tan solid (0.75 g, 1.67 mmol); LCMS (ES): 95% pure,  $m/z$  451  $[M+1]^+$ .

Example 61

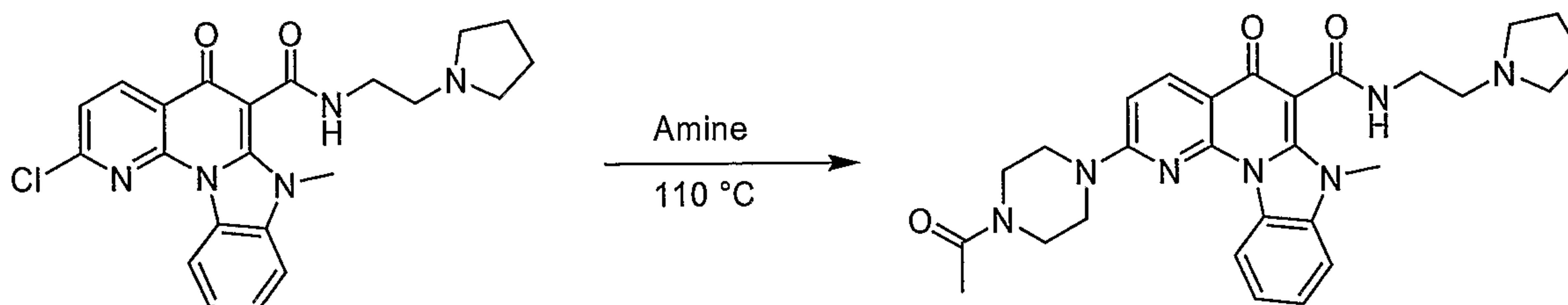
[0198] The following procedure was used to generate a library of analogs.

[0199] To a solution of the aminoester (200 mg, 0.46 mmol) and 1-(2-aminoethyl)pyrrolidine (80 mg, 1.5 eq.) in methylene chloride (3 mL) was added aluminum chloride (100 mg, 1.5 eq.) and the reaction was allowed to stir for 4 hours at room temperature. The reaction was then quenched with a saturated solution of Rochelle's salt and kept basic with 1N NaOH and stirred for an additional hour. The mixture was extracted with methylene chloride, dried over magnesium sulfate and the solvent was removed *in vacuo*. The resulting crude solid was purified by mass selective LCMS to afford the amide as a white solid (71 mg, 0.14 mmol); LCMS (ES): 95% pure,  $m/z$  502  $[M+1]^+$ .

Example 62

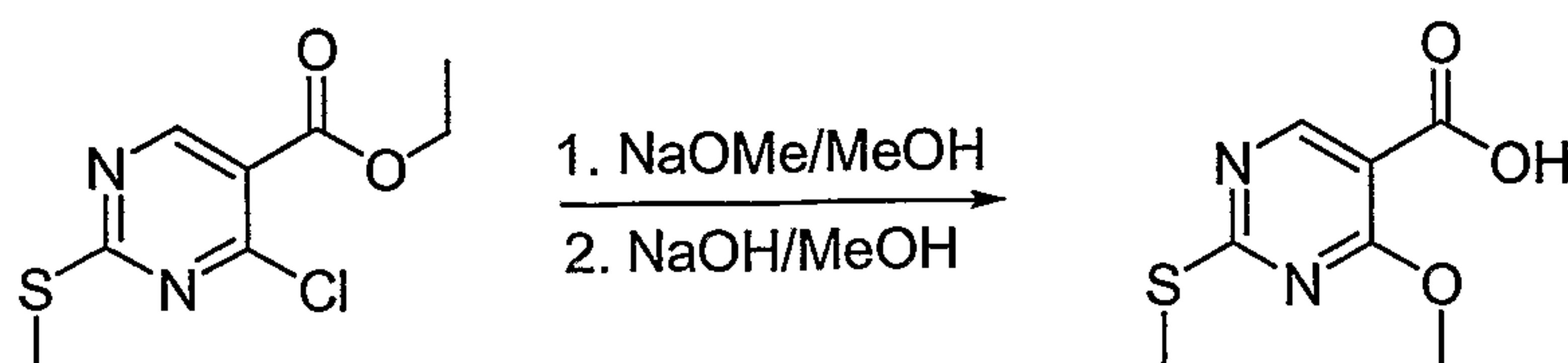
[0200] The following procedure was used to generate a library of analogs.

[0201] To a solution of the aminoester (200 mg, 0.46 mmol) and 1-(2-aminoethyl) pyrrolidine (80 mg, 1.5 eq.) in methylene chloride (3 mL) was added aluminum chloride (100 mg, 1.5 eq.) and the reaction was allowed to stir for 4 hours at room temperature. The reaction was then quenched with a saturated solution of Rochelle's salt and kept basic with 1N NaOH and stirred for an additional hour. The mixture was extracted with methylene chloride, dried over magnesium sulfate and the solvent was removed *in vacuo*. The resulting crude solid was purified by mass selective LCMS to afford the amide as a white solid (200 mg, 0.38 mmol); LCMS (ES): 95% pure,  $m/z$  519  $[\text{M}+1]^+$ .

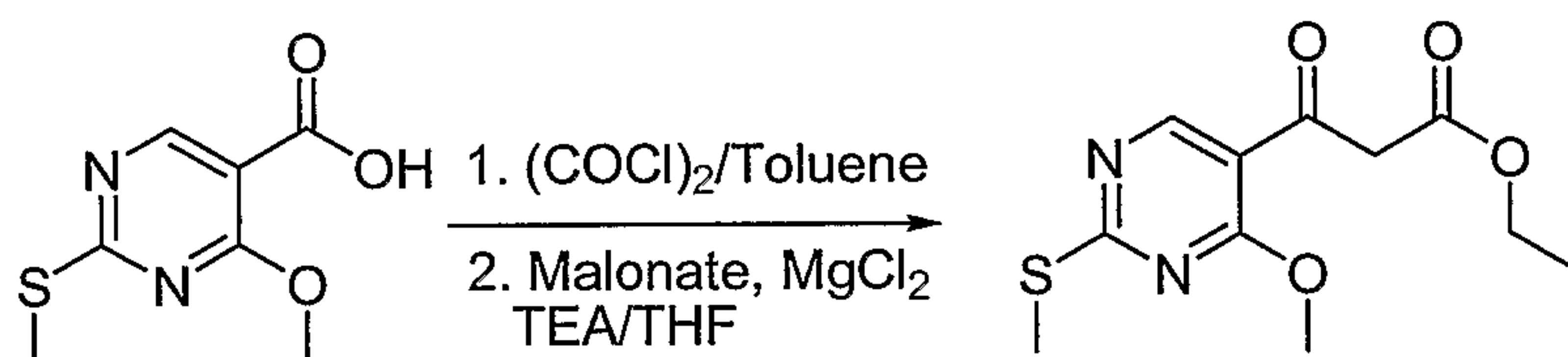
Example 63

[0202] The following procedure was used to generate a library of analogs.

[0203] To a solution of the chloroamide (350 mg, 0.83 mmol) in N-methylpyrrolidinone (2 mL) was added N-acetylpiperazine (160 mg, 1.24 mmol) and the mixture was heated at  $110\text{ }^\circ\text{C}$  for 4 hours. Water was added and the crude product was collected by filtration. The resulting solid was dissolved in methylene chloride, dried over sodium sulfate and the solvent was removed *in vacuo*. The resulting crude solid was triturated from ethyl acetate to afford the amide as a white solid (200 mg, 0.4 mmol); LCMS (ES): 98% pure,  $m/z$  516  $[\text{M}+1]^+$ .

Example 64

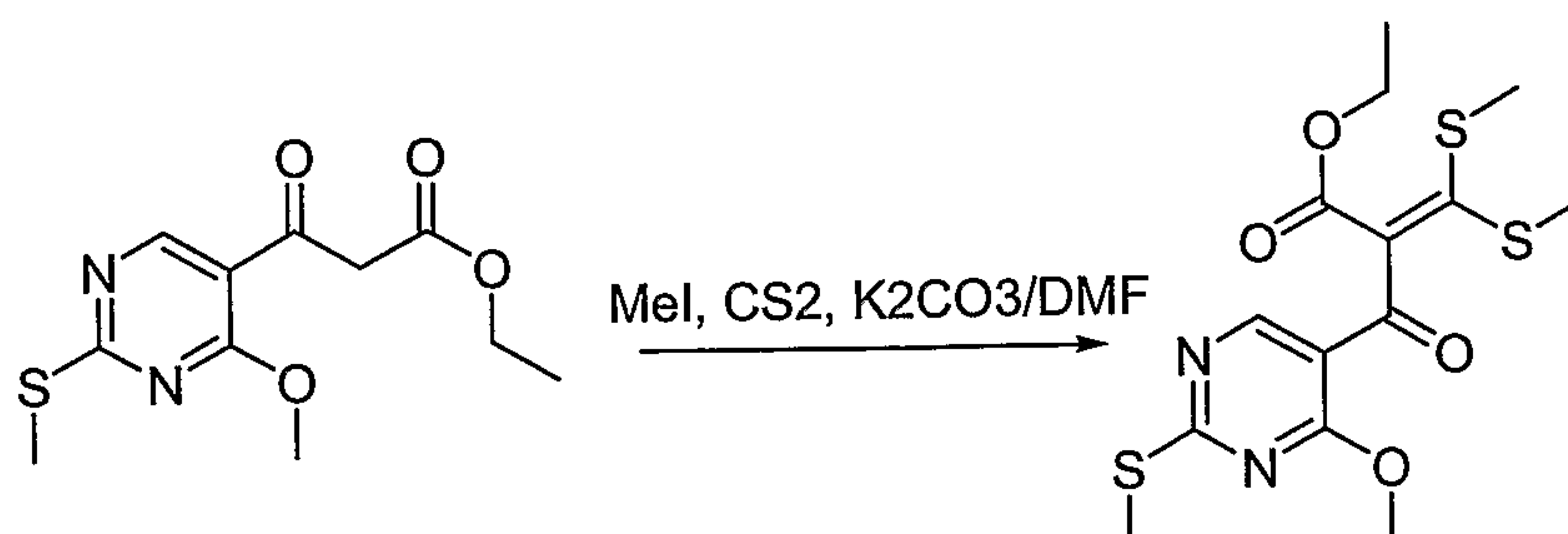
[0204] **4-Methoxy-2-(methylthio)pyrimidine-5-carboxylic acid.** To a solution of ethyl 4-chloro-2-(methylthio)pyrimidine-5-carboxylate (24.40 g, 104.9 mmol) in MeOH (125 mL) was added solid sodium methoxide (11.40 g, 211.0 mmol) at rt. The reaction mixture was stirred at rt for 5h and then treated with NaOH (17.70 g, 315.5 mmol) in H<sub>2</sub>O (100 mL). It was then stirred at the same temperature for an additional 1h. The reaction mixture was concentrated to half of the volume and then acidified to pH =4 with HCl (6N). The reaction was extracted with EtOAc (5 x 200 mL) and the organic layer was washed with brine (200 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated to yield the desired product as a white solid (19.28 g, 92 %). MS (*m/z*): 201 (MH<sup>+</sup>). <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) δ: 8.72 (s, 1H), 3.98 (s, 3H), 2.54 (s, 3H).

Example 65

[0205] **Ethyl 3-(4-methoxy-2-(methylthio)pyrimidin-5-yl)-3-oxopropanoate.** A solution of 4-methoxy-2-(methylthio)pyrimidine-5-carboxylic acid (10.92 g, 54.6 mmol) and oxalyl chloride (19.0 mL, 217.8 mmol) in toluene (100 mL) was heated at 100°C for 1h. The solvent was removed under reduced pressure and the crude acid chloride was used without purification in the next step. To a solution of MgCl<sub>2</sub> (7.80 g, 81.9 mmol) and ethyl magnesium malonate (14.10 g, 82.6mmol) in THF (100 mL) was added the above acid chloride in THF (100 mL) at 0 °C followed by TEA (15.0 mL, 107.6 mmol). The reaction mixture was stirred at 0 °C for 30 min and at rt for 2h. EtOAc (200 mL) and H<sub>2</sub>O (100 mL) were added and stirred for additional 30min. The layers were separated and the organic layer was washed with brine (100 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. The crude was purified by flash chromatography (15 % EtOAc/hexanes) to yield the desired compound as a white solid (7.73 g, 52 %). MS (*m/z*): 271 (MH<sup>+</sup>). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 12.65 (s, 1Hx2/3), 8.85 (s, 1Hx1/3), 8.84

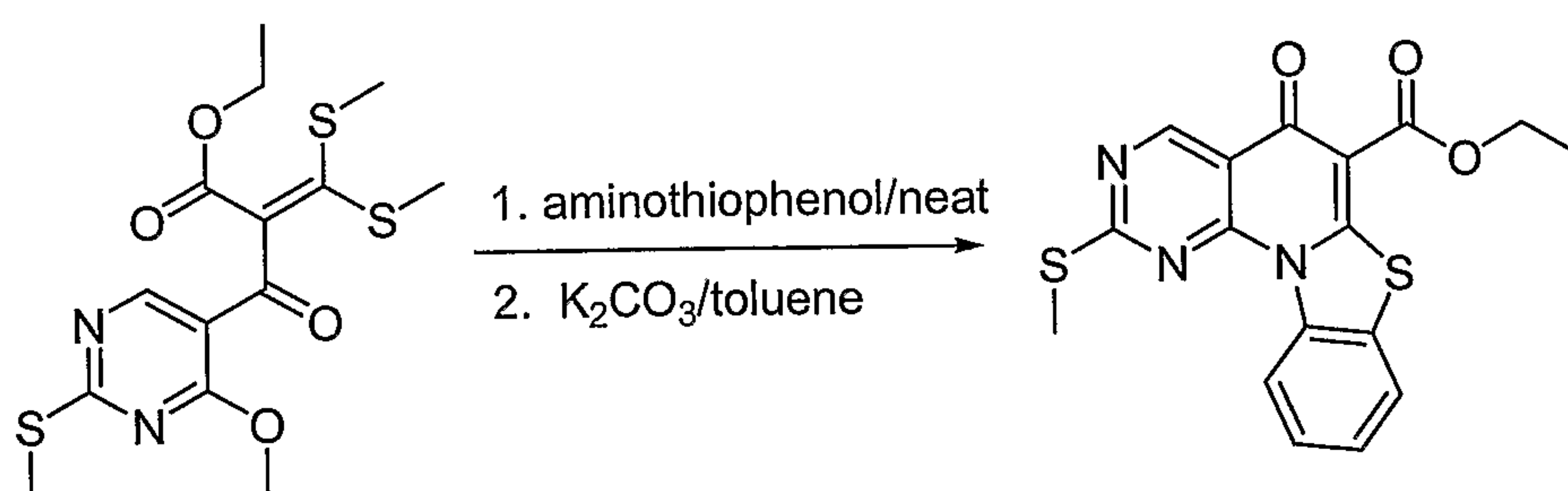
(s, 1Hx2/3), 6.03 (s, 1Hx2/3), 4.26 (q, 2Hx2/3), 4.19 (q, 2Hx1/3), 4.10 (s, 3Hx2/3), 4.09 (s, 3Hx1/3), 3.92 (s, 2Hx1/3), 2.60 (s, 3Hx1/3), 2.59 (s, 3Hx2/3), 1.34 (t, 3Hx2/3), 1.26 (3Hx1/3).

### Example 66



[0206] **Ethyl 2-(4-methoxy-2-(methylthio)pyrimidine-5-carbonyl)-3,3-bis(methylthio)acrylate.** To a solution of ethyl 3-(4-methoxy-2-(methylthio)pyrimidin-5-yl)-3-oxopropanoate in DMF (85 mL) was added methyl iodide (5.3 mL, 85.1 mmol), carbon disulfide (2.6 mL, 43.0 mmol) at -5 °C. Solid K<sub>2</sub>CO<sub>3</sub> was slowly added keeping the internal temperature below -3 °C. The reaction mixture was stirred at -5 °C for 1h and at rt for 1h. It was diluted with EtOAc (300 mL), washed with H<sub>2</sub>O (2x200 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed under reduced pressure and the crude was purified by flash chromatography (20 % EtOAc/hexanes) to yield the desired product as a yellow oil (7.60 g, 71 %). MS (*m/z*): 375 (MH<sup>+</sup>). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 8.82 (s, 1H), 4.16 (q, 2H), 4.03 (s, 3H), 2.61 (s, 3H), 2.51 (br s, 3H), 2.28 (br s, 3H), 1.15 (t, 3H).

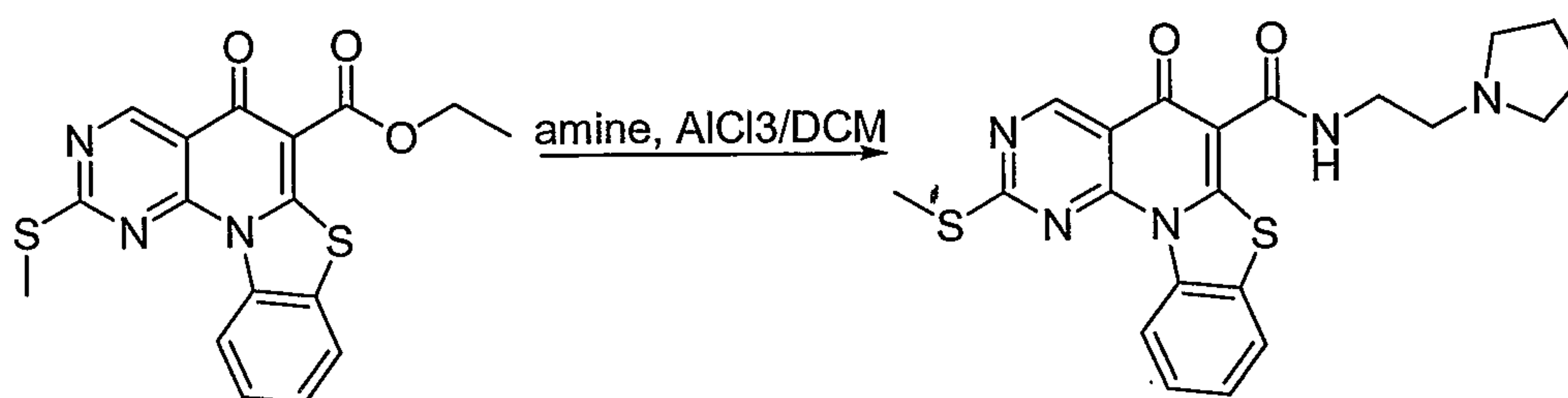
### Example 67



[0207] **2-Methylsulfanyl-5-oxo-5H-7-thia-1,3,11b-triaza-benzo[c]fluorene-6-carboxylic acid ethyl ester.** A solution of ethyl 2-(4-methoxy-2-(methylthio)pyrimidine-5-carbonyl)-3,3-bis(methylthio)acrylate (475 mg, 1.27 mmol) and 2-aminobenzenethiol (200 mg, 1.60 mmol) in toluene (5 mL) was heated to dryness and continued heating neat at 140 °C for 1h. Toluene (30 mL) and K<sub>2</sub>CO<sub>3</sub> (350 mg, 2.53 mmol) were added to the reaction and it was heated for an additional 1h. The reaction mixture was cooled to rt and EtOAc (100 mL) and H<sub>2</sub>O (50 mL)

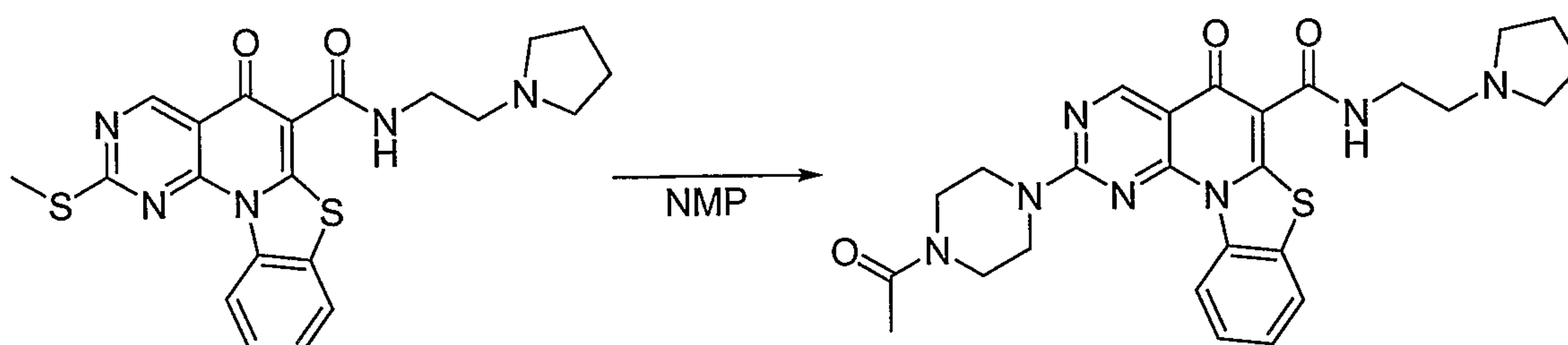
were added. The layers were separated and the organic layer was washed with brine (100 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. The crude solid was triturated in Et<sub>2</sub>O to afford the desired product as a yellow solid (260 mg, 55 %). MS (*m/z*): 372 (MH<sup>+</sup>). <sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ: 9.37 (d, 1H), 9.30 (s, 1H), 8.06 (m, 1H), 7.65 (m, 1H), 7.54 (m, 1H), 4.35 (q, 2H), 2.78 (s, 3H), 1.34 (t, 3H).

### Example 68



[0208] **2-Methylsulfanyl-5-oxo-5H-7-thia-1,3,11b-triaza-benzo[c]fluorene-6-carboxylic acid (2-cyclopentyl-ethyl)-amide.** To a solution of 2-methylsulfanyl-5-oxo-5H-7-thia-1,3,11b-triaza-benzo[c]fluorene-6-carboxylic acid ethyl ester (230 mg, 0.619 mmol) and 2-(pyrrolidin-1-yl)ethanamine (0.24 mL, 1.854 mmol) in DCM (15 mL) was added solid AlCl<sub>3</sub> (250 mg, 1.875 mmol) at rt. The reaction was stirred for 1h at rt and diluted with DCM (100 mL), conc. sodium potassium tartrate (30 mL), and NaOH (6N, 10 mL). The mixture was stirred for 15 min and the layers were separated. The aqueous layer was extracted with DCM (50 mL) and the combined organic layer was washed with brine (50 mL). The crude reaction mixture was dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated to yield the desired product as a yellow solid (250mg, 92%). MS (*m/z*): 440 (MH<sup>+</sup>). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 10.26 (br, 1H), 9.61 (s, 1H), 9.55 (m, 1H), 7.77 (m, 1H), 7.57 (m, 1H), 7.50 (m, 1H), 3.67 (q, 2H), 2.81 (s, 3H), 2.77 (t, 2H), 2.63 (m, 4H), 1.82 (m, 4H).

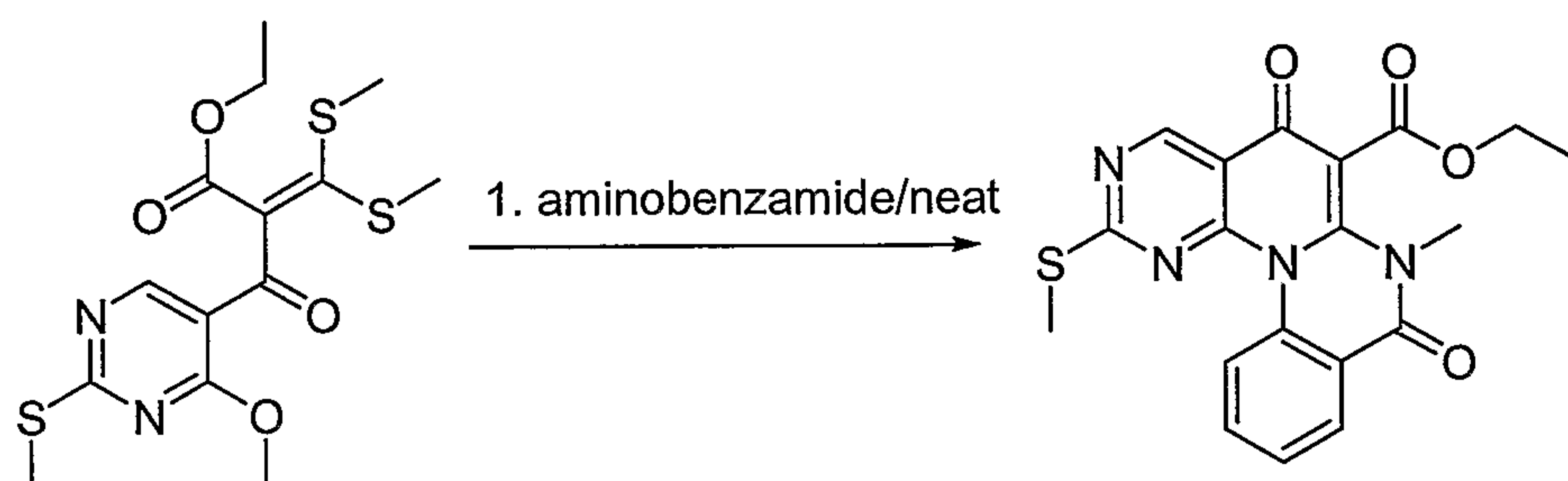
### Example 69



[0209] **2-(4-Acetyl-piperazin-1-yl)-5-oxo-5H-7-thia-1,3,11b-triaza-benzo[c]fluorene-6-carboxylic acid (2-pyrrolidin-1-yl-ethyl)-amide.** A solution of 2-methylsulfanyl-5-oxo-5H-

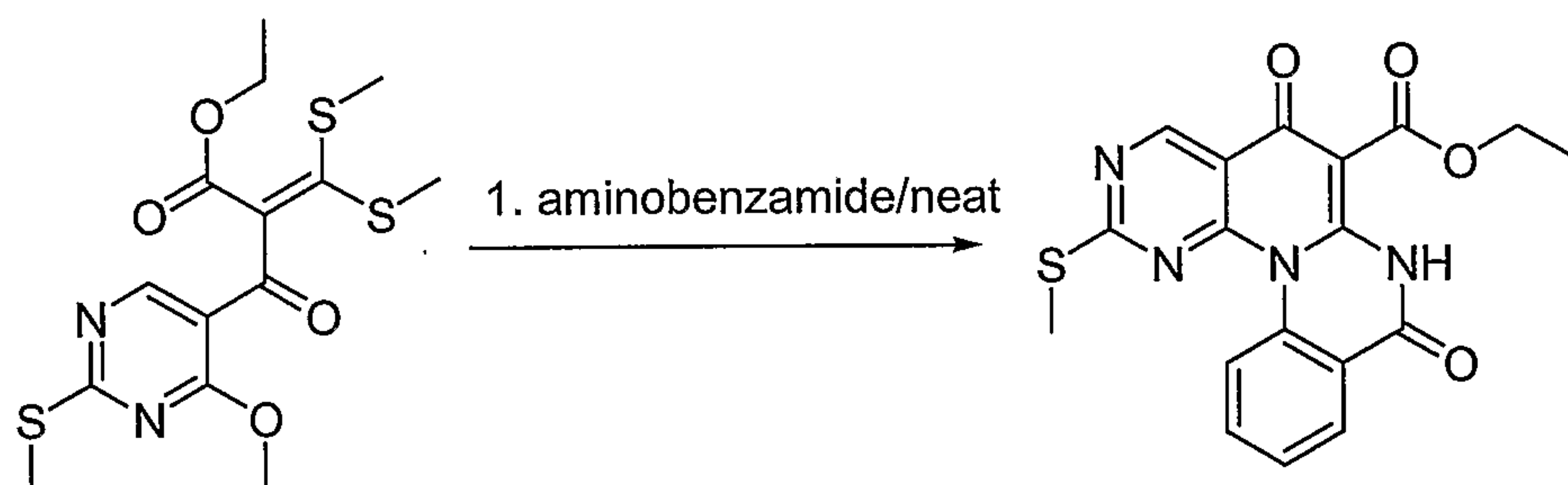
7-thia-1,3,11b-triaza-benzo[*c*]fluorene-6-carboxylic acid (2-cyclopentyl-ethyl)-amide (25 mg, 0.057 mmol) and 1-(piperazin-1-yl)ethanone (75 mg, 0.585 mmol) in NMP (1.0 mL) was heated for 20 min at 200 °C in a microwave. The crude reaction mixture was purified by reverse phase HPLC. MS (*m/z*): 520 (MH<sup>+</sup>).

### Example 70



[0210] **7-Methyl-2-methylsulfanyl-5,8-dioxo-7,8-dihydro-5H-1,3,7,12b-tetraaza-benzo[*c*]phenanthrene-6-carboxylic acid ethyl ester.** A solution of ethyl 2-(4-methoxy-2-(methylthio)pyrimidine-5-carbonyl)-3,3-bis(methylthio)acrylate (775 mg, 2.069 mmol) and 2-amino-N-methylbenzamide (475 mg, 3.163 mmol) in toluene (5 mL) was heated over stream of nitrogen allowing toluene to evaporate and continued heating as neat at 140 °C for over night. Cooled to rt and precipitate formed from DCM (10 mL) was filtered off. The solvent of mother liquor was removed under reduced pressure and repeated the precipitation process with Et<sub>2</sub>O (10mL). Trituration in methanol (10mL) afforded the desired product as a yellow solid (30 mg, 4 %). MS (*m/z*): 397 (MH<sup>+</sup>). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 9.37 (s, 1H), 8.37 (d, 1H), 8.26 (dd, 1H), 7.70 (m, 1H), 7.49 (m, 1H), 4.46 (q, 2H), 3.61 (s, 3H), 2.60 (s, 3H), 1.43 (t, 3H).

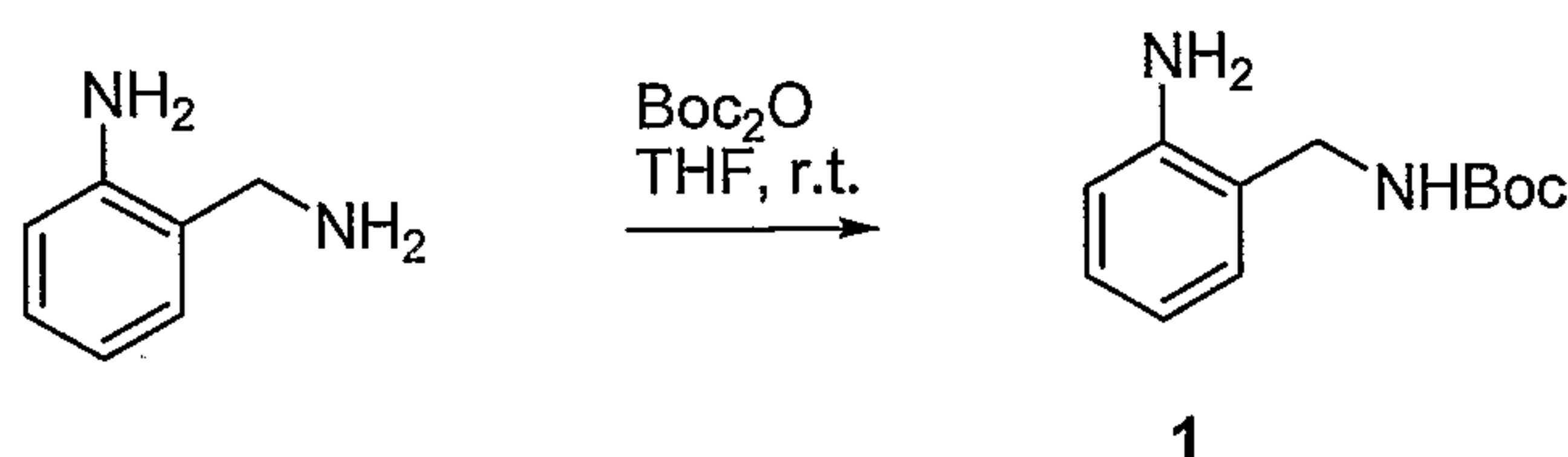
### Example 71



[0211] **2-Methylsulfanyl-5,8-dioxo-7,8-dihydro-5H-1,3,7,12b-tetraaza-benzo[*c*]phenanthrene-6-carboxylic acid ethyl ester.** A solution of ethyl 2-(4-methoxy-2-(methylthio)pyrimidine-5-carbonyl)-3,3-bis(methylthio)acrylate (250 mg, 0.668 mmol) and 2-aminobenzamide (118 mg, 0.867 mmol) in DCM (5 mL) was heated over stream of nitrogen

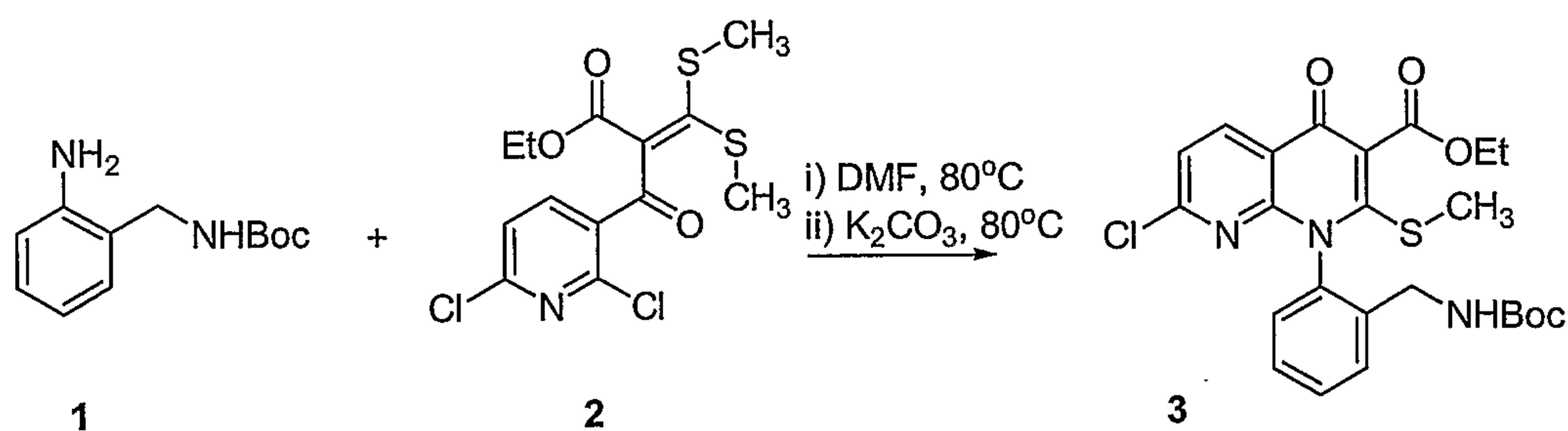
allowing DCM to evaporate and continued heating as neat at 140 °C for over night. The residue was dissolved in DMF (7 mL) after cooling to rt and treated with NaH (60 mg, 1.500 mmol). The reaction was stirred at rt for 1h and diluted with DCM (20 mL) and H<sub>2</sub>O (20mL). The layers were separated and DCM was removed under reduced pressure. Trituration of the crude in Et<sub>2</sub>O (10mL) afforded the desired product as a yellow solid (50 mg, 20 %). MS (*m/z*): 383 (MH<sup>+</sup>). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ: 13.58 (br s, 1H), 9.43 (s, 1H), 8.60 (d, 1H), 8.33 (dd, 1H), 7.79 (m, 1H), 7.59 (m, 1H), 4.48 (q, 2H), 2.62 (s, 3H), 1.46 (t, 3H).

### Example 72



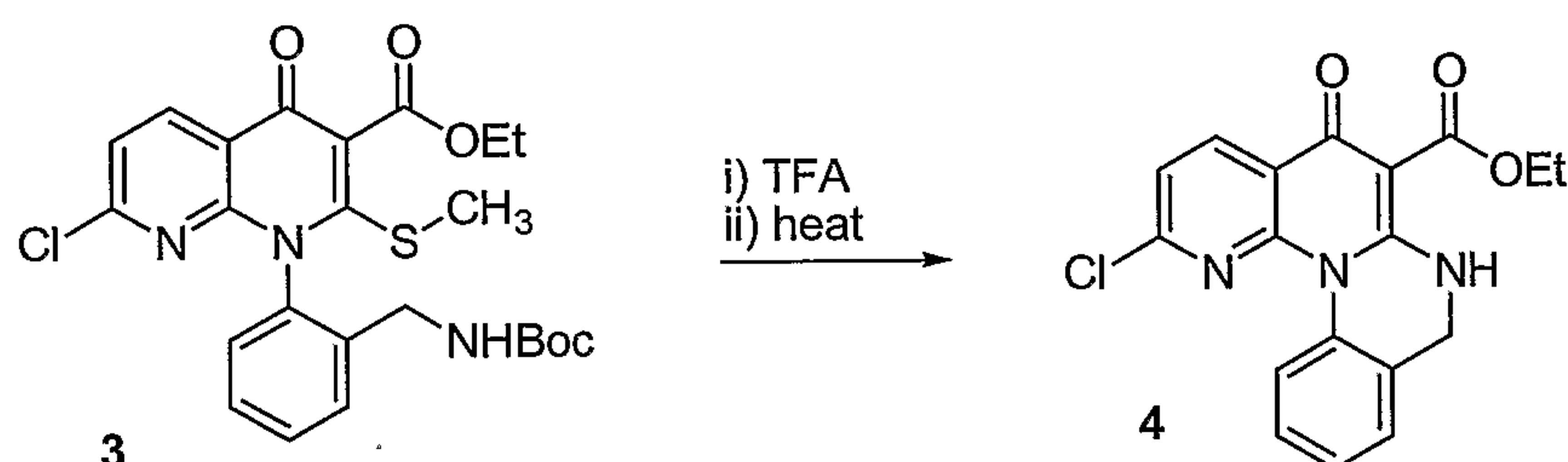
[0212] The material was prepared according to a published procedure, which was slightly modified (*J. Med. Chem.* 2003, 46, 1661-1669). Under nitrogen atmosphere, 2-aminobenzylamine (2.0 eq., 2.45 g, 20.05 mmol) was dissolved in THF (25 ml). A solution of di-*tert*-butyldicarbonate (1.0 eq, 2.19 g, 10.03 mmol) in THF (25 ml) was added slowly via a syringe pump over 30 min. After a few minutes, extra THF (25 ml) was added to enable efficient stirring of the thick suspension. After stirring at room temperature overnight, a white solid that formed was filtered off. The filtrate was concentrated *in vacuo* and poured on top of a silica gel column and purified by chromatography (10 to 20% gradient of EtOAc in hexanes). The resulting solid was recrystallized in EtOAc/hexanes to afford a white crystalline solid (1.95 g, 87% yield based on Boc<sub>2</sub>O). LCMS (ES): 95% pure, *m/z* 224 [M+H]<sup>+</sup>, 245 [M+Na]<sup>+</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.44 (s, 9H), 4.23 (d, *J* = 6.0, 2H), 4.2 (br s, 2H), 4.80 (br s, 1H), 6.68 (t, *J* = 7.6, 2H), 7.02 (d, *J* = 6.8, 1H), 7.10 (dt, *J* = 8.0, *J* = 1.6, 1H) ppm.

### Example 73

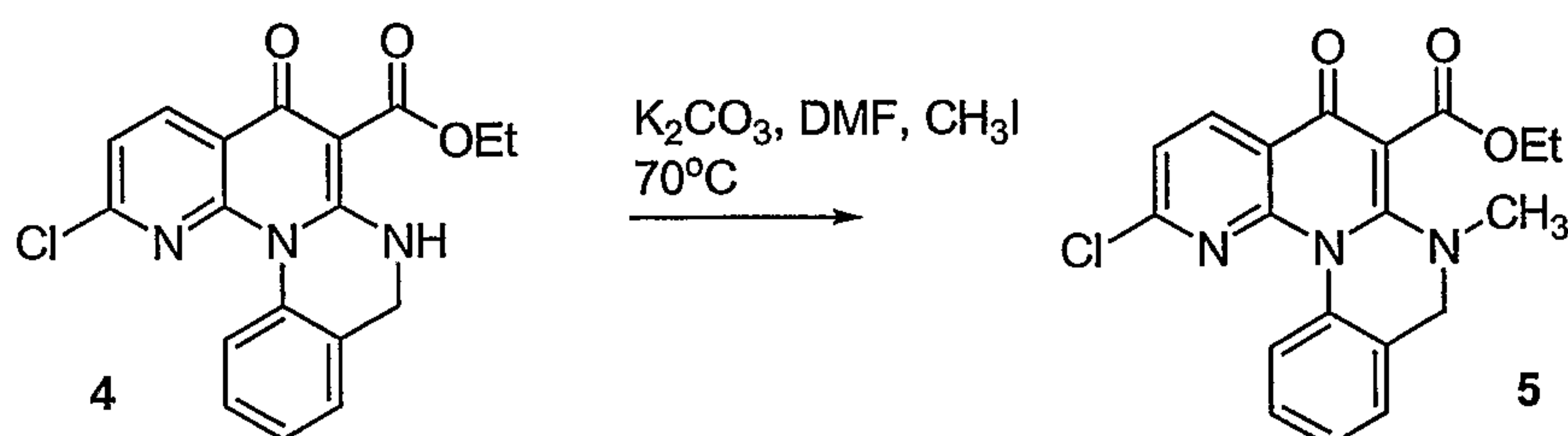


[0213] A mixture of compound **1** (1.22 g, 5.49 mmol) and **2** (2.01 g, 5.49 mmol) in DMF (10 ml) was stirred at 80°C for 5 hours, while maintaining bubbling of nitrogen into the solution. K<sub>2</sub>CO<sub>3</sub> (1.2 eq, 910 mg, 6.58 mmol) was added and the mixture stirred at 80°C for 40 minutes. Water was added and the resulting gummy material extracted with CH<sub>2</sub>Cl<sub>2</sub> (3x). The combined extracts were washed with water (1x), dried over Na<sub>2</sub>SO<sub>4</sub> and the volatiles removed *in vacuo* to afford a thick oil (3.2 g of solvent-containing material, >100% yield). The obtained material was over 85% pure and used for the following step without any further purification. A small part was purified by preparative HPLC for characterization. LCMS (ES): 95% pure, m/z 504 [M]<sup>+</sup>, 506 [M+2]<sup>+</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.38 (s, 9H), 1.40 (m, 3H), 2.32 (s, 3H), 3.93 (dd, *J* = 15.2, *J* = 5.5, 1H), 4.24 (dd, *J* = 14.8, *J* = 4.2, 1H), 4.43 (m, 2H), 4.84 (br t, 1H), 7.15 (m, 1H), 7.29 (m, 1H), 7.45 (m, 1H), 7.57 (m, 2H), 8.61 (d, *J* = 8.8, 1H) ppm.

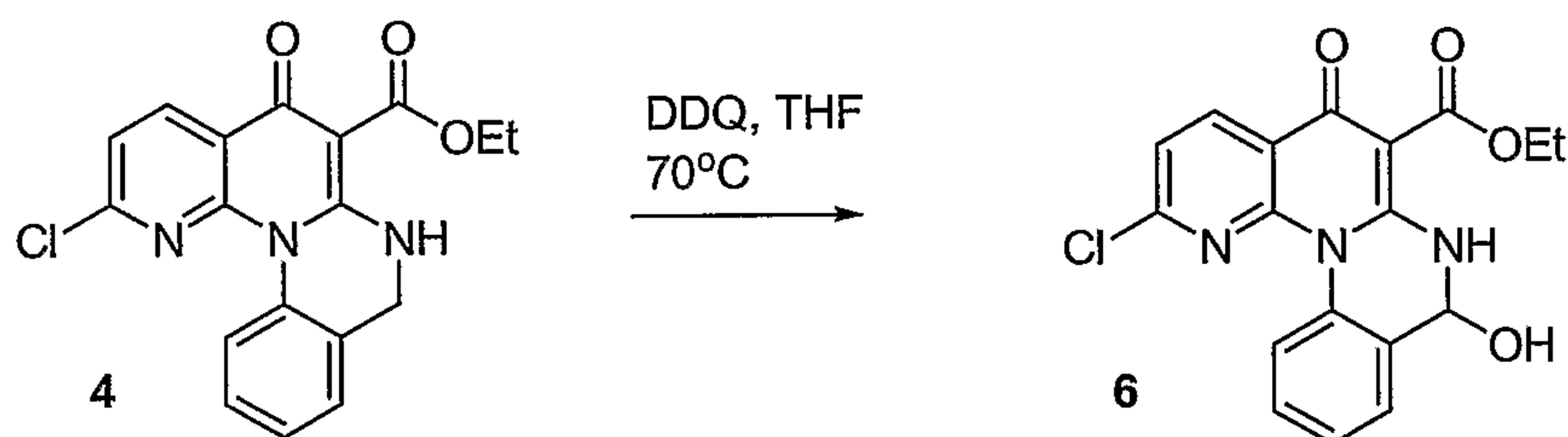
#### Example 74



[0214] Compound **3** (1.5 g, 2.98 mmol) was dissolved in 5 ml of trifluoroacetic acid. The mixture was stirred at room temperature for 2 hours. The volatiles were removed *in vacuo* and the resulting thick oil was heated with a heat gun while being kept *in vacuo*. This operation was pursued until the formation of **4** by cyclization was complete (LCMS monitoring). The compound was purified by flash chromatography on silica gel (30 to 80% gradient of EtOAc in hexanes). The solid that precipitated upon concentration of the solvents was filtered and dried to afford compound **4** as a white microcrystalline solid (434 mg, 41% yield). LCMS (ES): 95% pure, m/z 356 [M+H]<sup>+</sup>, 378 [M+Na]<sup>+</sup>, 310 [M+1-EtOH]<sup>+</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz) δ 1.43 (t, *J* = 6.8, 3H), 4.40 (q, *J* = 7.2, 2H), 4.46 (d, *J* = 2.4, 2H), 7.24-7.40 (m, 4H), 7.69 (d, *J* = 8.4, 1H), 8.62 (d, *J* = 8.0, 1H), 10.74 (br s, 1H) ppm.

Example 75

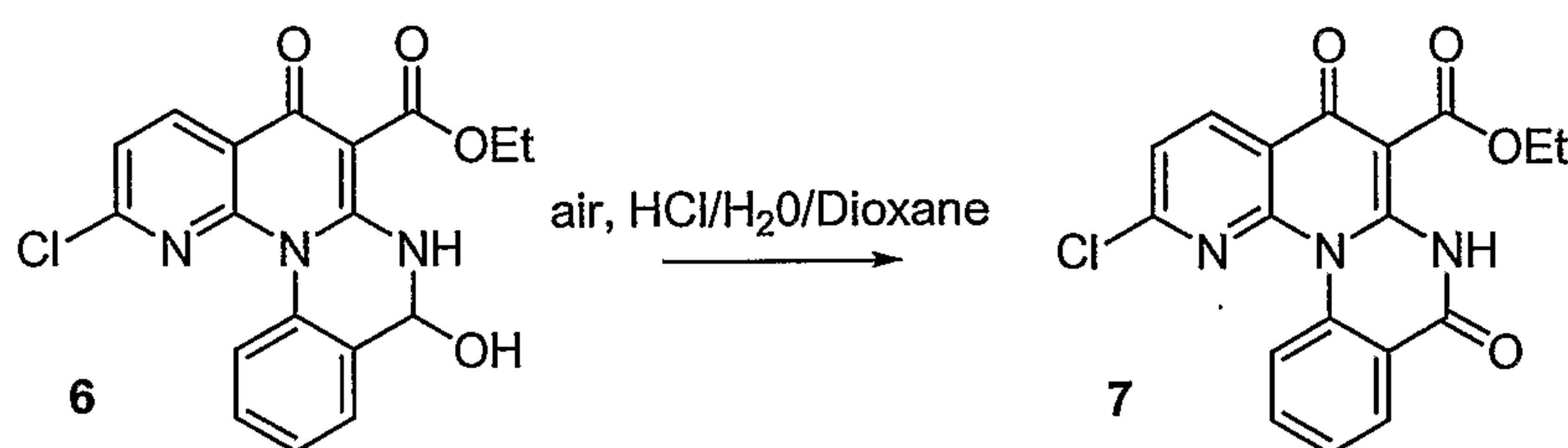
[0215] Compound 4 (1.0 eq, 198 mg, 0.556 mmol) and  $K_2CO_3$  (2.0 eq, 154 mg, 1.11 mmol) were suspended in DMF (1.5 ml). Iodomethane (1.4 eq, 0.05 ml, 0.80 mmol) was added and the mixture was stirred at  $70^\circ C$  for 45 min. Water was added and the material was extracted with  $CH_2Cl_2$  (3x). Combined extracts were washed with water, dried over  $Na_2SO_4$  and the volatiles removed *in vacuo*. After purification by flash chromatography on silica gel (40 to 80% gradient of EtOAc in hexanes), compound 5 was isolated as an off white solid (181 mg, 88% yield). LCMS (ES): 95% pure,  $m/z$  370  $[M+H]^+$ , 324  $[M+1-EtOH]^+$ ;  $^1H$  NMR ( $CDCl_3$ , 400 MHz)  $\delta$  1.41 (t,  $J = 7.2$ , 3H), 3.17 (s, 3H), 4.29 (s, 2H), 4.41 (q,  $J = 7.2$ , 2H), 7.27 (d,  $J = 7.2$ , 1H), 7.33 (t,  $J = 7.6$ , 1H), 7.36 (d,  $J = 8.4$ , 1H), 7.39 (td,  $J = 7.2$ ,  $J = 1.2$ , 1H), 7.57 (d,  $J = 8.4$ , 1H), 8.61 (d,  $J = 8.4$ , 1H) ppm.

Example 76

[0216] Compound 4 (1.0 eq, 104 mg, 0.29 mmol) was suspended in THF. A solution of DDQ (1.1 eq, 73 mg, 0.32 mmol) in THF (1ml) was added dropwise and the resulting mixture stirred at  $70^\circ C$  for 24 hours. An aqueous solution of 1N NaOH was added and the solution was extracted with  $CH_2Cl_2$  (4x). The combined extracts were washed with a saturated  $NaHCO_3$  aqueous solution, dried over  $Na_2SO_4$  and the solvents removed *in vacuo*. The compound was purified by preparative HPLC (MeCN/water). MeCN was evaporated,  $NaHCO_3$  was added and the substance extracted with  $CH_2Cl_2$ . Combined extracts were dried over  $Na_2SO_4$  and the solvent removed *in vacuo* to afford 6 as an off-white solid (18 mg, 17% yield). LCMS (ES): 95% pure,  $m/z$  372  $[M+H]^+$ , 326  $[M+1-EtOH]^+$ ;  $^1H$  NMR ( $CDCl_3$ , 400 MHz)  $\delta$  1.44 (t,  $J = 6.8$ ,

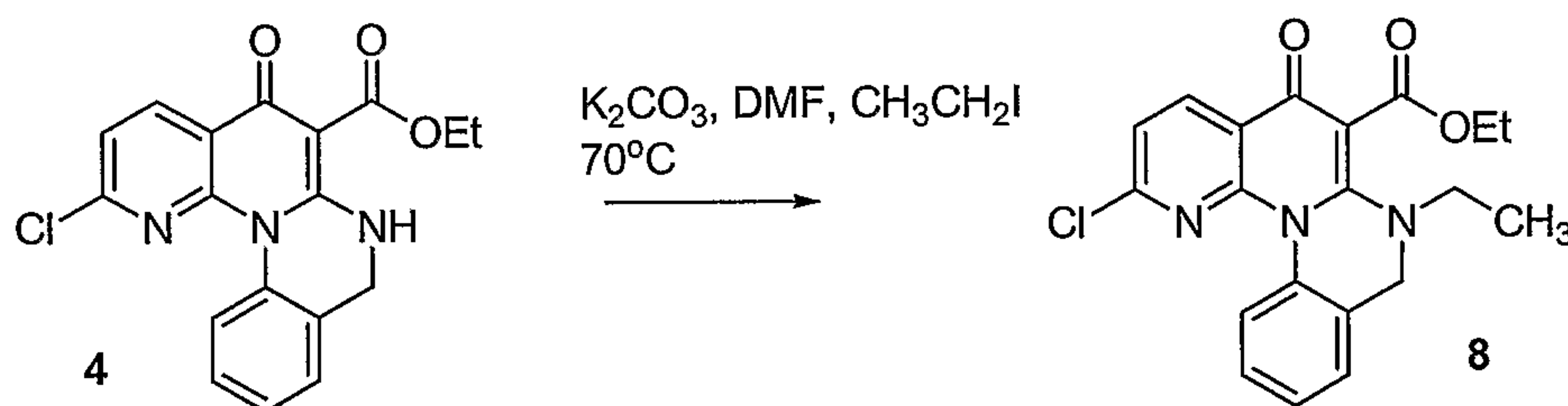
3H), 4.42 (q,  $J = 7.2$ , 2H), 6.01 (d,  $J = 4.4$ , 1H), 7.4-7.55 (m, 4H), 7.98 (d,  $J = 8.8$ , 1H), 8.64 (d,  $J = 8.4$ , 1H), 11.53 (br s, 1H) ppm.

### Example 77



[0217] Compound 6 (8 mg, 0.021 mmol) was suspended in dioxane (0.5 ml). One drop of concentrated aqueous HCl was added and the resulting yellow solution stirred at room temperature. After 2 hours, one extra drop of concentrated HCl was added and some air was bubbled into the solution. The solution was stirred at room temperature for 24 hours. The solvent were removed *in vacuo*, NMP was added and the compound was purified by preparative HPLC (MeCN/water). Acetonitrile was evaporated, the product was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3x) and the combined extracts dried over Na<sub>2</sub>SO<sub>4</sub>. Solvents were removed to afford 7 as a solid (5 mg, 62% yield). LCMS (ES): 95% pure,  $m/z$  370 [M+H]<sup>+</sup>, 392 [M+23], 324 [M+1-EtOH]<sup>+</sup>; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz)  $\delta$  1.46 (t,  $J = 7.2$ , 3H), 4.48 (q,  $J = 7.2$ , 2H), 7.52 (d,  $J = 8.0$ , 1H), 7.58 (t,  $J = 6.8$ , 1H), 7.81 (dt,  $J = 6.8$ ,  $J = 1.6$ , 1H), 8.33 (dd,  $J = 7.6$ ,  $J = 1.2$ , 1H), 8.56 (d,  $J = 8.8$ , 1H), 8.70 (d,  $J = 8.0$ , 1H), 13.58 (br s, 1H) ppm.

### Example 78

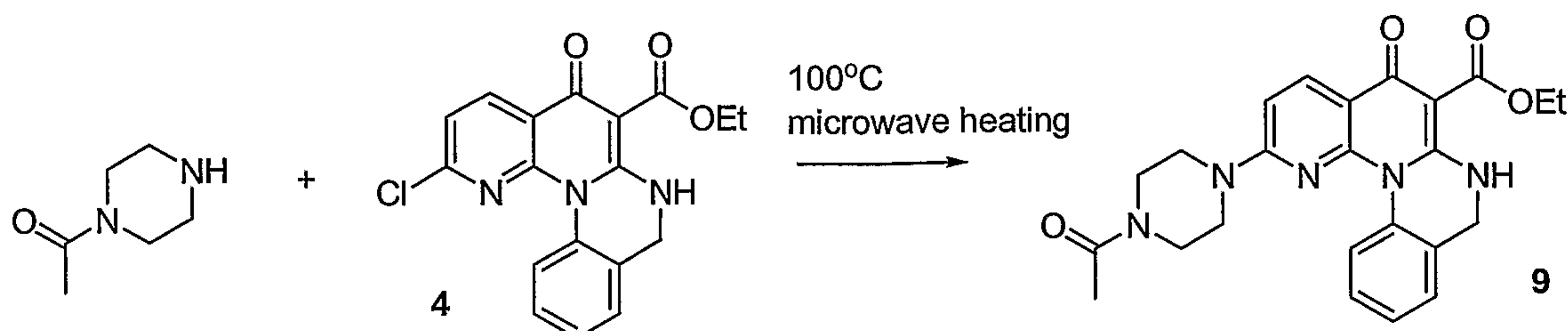


[0218] Compound 4 (1.0 eq, 218 mg, 0.613 mmol) and K<sub>2</sub>CO<sub>3</sub> (2.0 eq, 169 mg, 1.22 mmol) were suspended in DMF (1.5 ml). Iodomethane (1.2 eq, 0.059 ml, 0.74 mmol) was added and the mixture was stirred at 70°C for 35 min. Water was added and the material was extracted with CH<sub>2</sub>Cl<sub>2</sub> (4x). Combined extracts were dried over Na<sub>2</sub>SO<sub>4</sub> and the volatiles removed *in vacuo*. After purification by preparative HPLC, the pH of the resulting MeCN/water solution was adjusted by adding NaHCO<sub>3</sub> and MeCN was evaporated. The

material was extracted with  $\text{CH}_2\text{Cl}_2$  and the combined extracts were dried over  $\text{Na}_2\text{SO}_4$ .

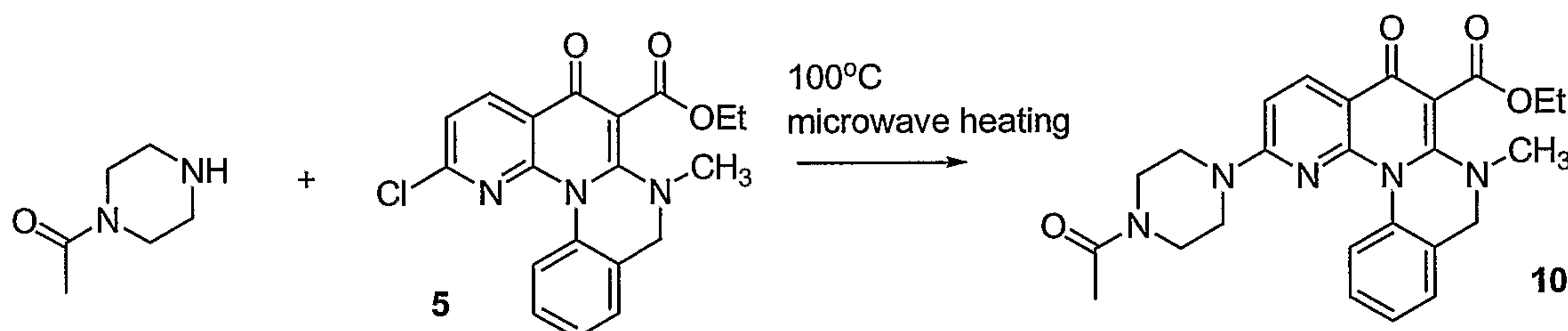
Precipitation using  $\text{CH}_2\text{Cl}_2$ /hexanes afforded **8** as an off-white solid (77mg, 33% yield). LCMS (ES): 95% pure,  $m/z$  384  $[\text{M}+\text{H}]^+$ , 406  $[\text{M}+\text{Na}]^+$ , 338  $[\text{M}+1-\text{EtOH}]^+$ .

### Example 79



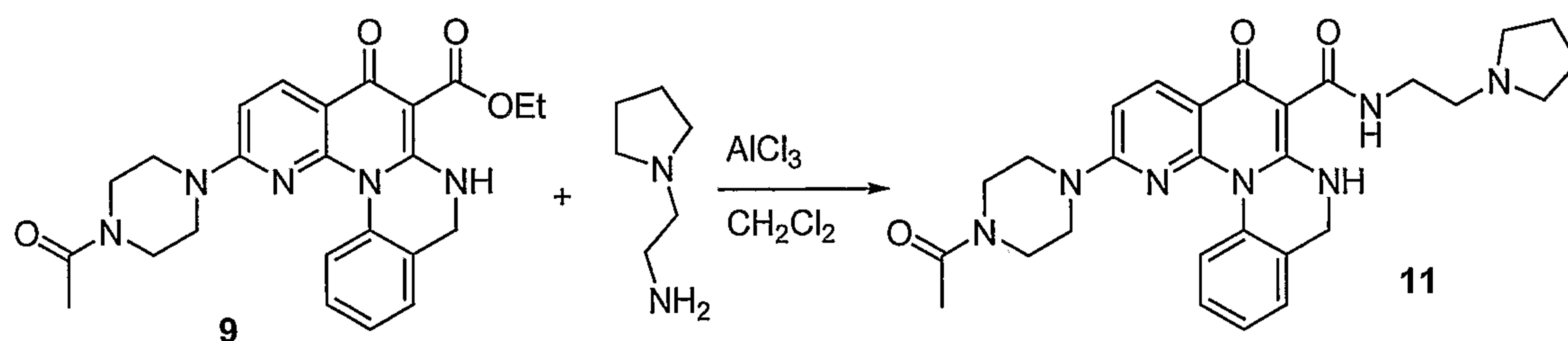
[0219] Compound **4** (1.0 eq, 110 mg, 0.309 mmol) and N-acetyl piperazine (4.0 eq, 158 mg, 1.233 mmol) were mixed in NMP (0.3 ml) and the resulting solution heated by microwave at  $100^\circ\text{C}$  for 5 min. A mixture of EtOAc and hexanes was added and the resulting solid filtered and dried. Compound **9** was isolated as an off-white solid (145 mg, 100% yield). LCMS (ES): 95% pure,  $m/z$  448  $[\text{M}+\text{H}]^+$ .

### Example 80



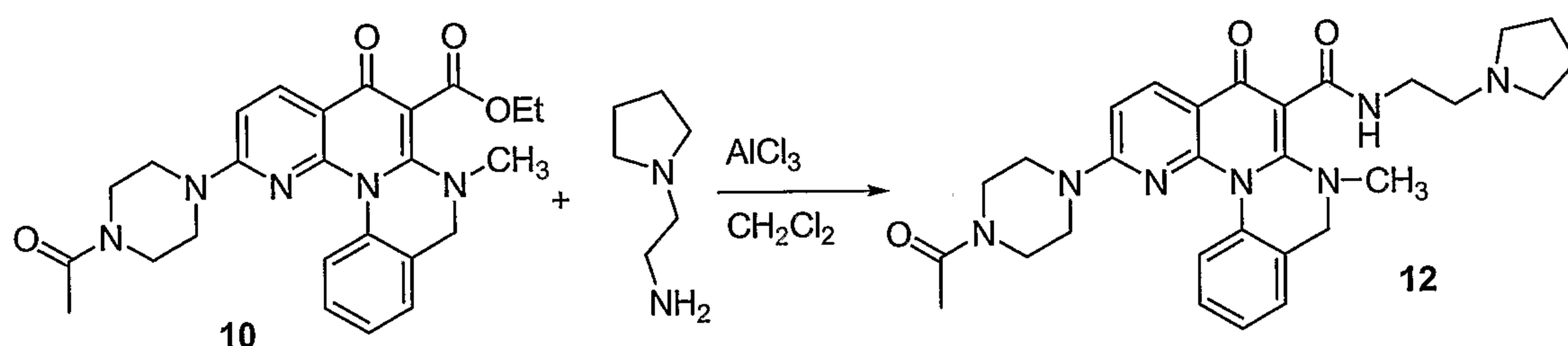
[0220] Compound **10** was prepared according to the procedure used for preparation of compound **9**. Compound **10** was isolated as an off-white solid (147 mg, 70% yield). LCMS (ES): 95% pure,  $m/z$  462  $[\text{M}+\text{H}]^+$ , 416  $[\text{M}+\text{H}-\text{EtOH}]^+$ .

### Example 81



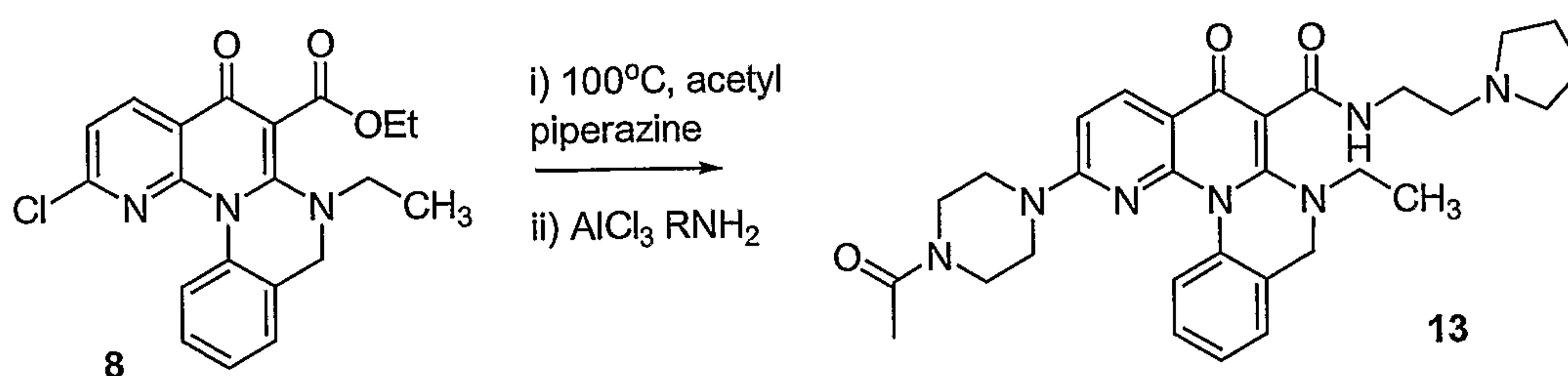
[0221] Compound **9** (1.0 eq, 131 mg, 0.293 mmol) and 1-(2-aminoethyl)pyrrolidine (1.5 eq, 0.05 ml, 0.394 mmol) were mixed in CH<sub>2</sub>Cl<sub>2</sub> (5 ml). AlCl<sub>3</sub> (2.0 eq, 80 mg, 0.60 mmol) was added and the resulting solution was vigorously stirred at room temperature. After 3 hours, another 0.1 ml of the 1-(2-aminoethyl)pyrrolidine and 2 ml of CH<sub>2</sub>Cl<sub>2</sub> were added and the reaction was stirred overnight. After removal of CH<sub>2</sub>Cl<sub>2</sub> *in vacuo*, the resulting slurry was treated with a saturated aqueous tartaric acid solution and stirred until all solid disappeared (about 1 hr for completion of the hydrolysis). Water was added and the pH was adjusted to 14 by adding NaOH. The product was extracted by CH<sub>2</sub>Cl<sub>2</sub> (4x). Combined extracts were washed with brine (1x) and dried over Na<sub>2</sub>SO<sub>4</sub>. After removal of the solvent, the crude material was dissolved in a mixture of NMP and trifluoroacetic acid and purified by preparative HPLC (MeCN, water). After adjusting pH with NaOH and evaporation of MeCN, the material was extracted with CH<sub>2</sub>Cl<sub>2</sub>. After drying over Na<sub>2</sub>SO<sub>4</sub> and evaporation of the volatiles, compound **11** was precipitated by EtOAc/hexanes, filtered and dried to provide a white solid (60 mg, 40% yield). LCMS (ES): 95% pure, m/z 516 [M+H]<sup>+</sup>.

#### Example 82



[0222] Compound **12** was prepared according to the procedure used for preparation of compound **11**. Compound **12** was isolated as an off-white solid (28 mg, 17% yield). LCMS (ES): 95% pure, m/z 530 [M+H]<sup>+</sup>.

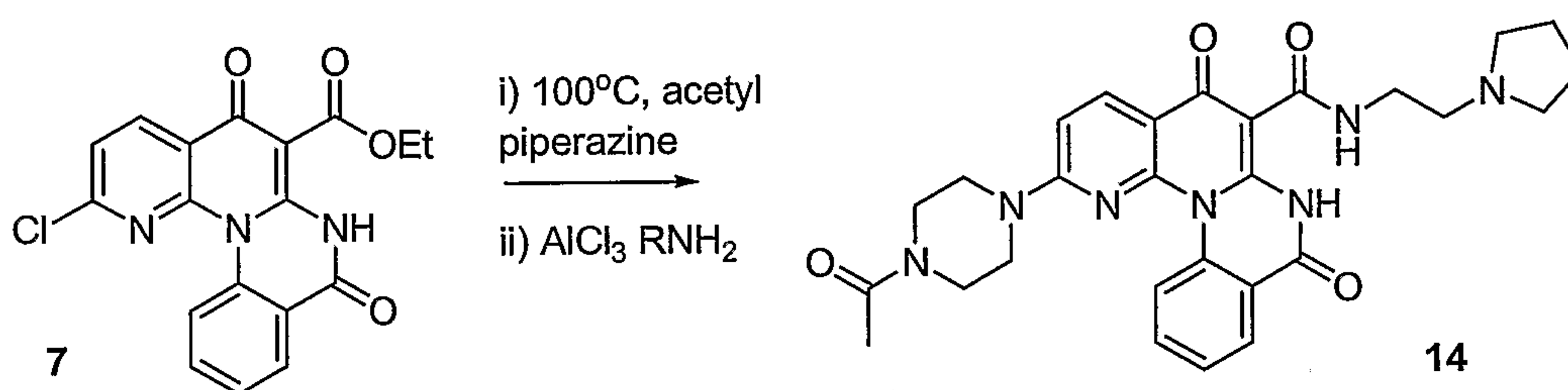
#### Example 83



[0223] Compound **8** (1.0 eq, 77 mg, 0.201 mmol) and N-acetyl piperazine (4.0 eq., 103 mg, 0.803 mmol) were mixed in NMP (0.5 ml) and heated by microwave at 100°C for 10 min.

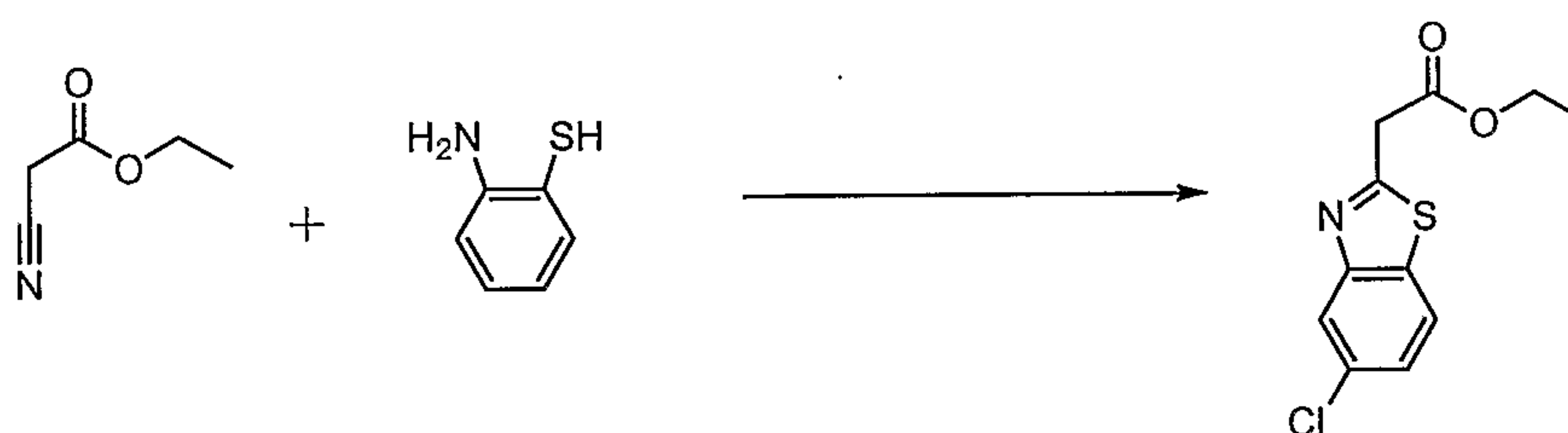
Water and brine were added and a gummy solid was isolated by filtration. This crude material was dissolved in  $\text{CH}_2\text{Cl}_2$ , the solution dried over  $\text{Na}_2\text{SO}_4$  and the volatiles removed *in vacuo*. The final reaction step was then carried out on the crude ester according to the procedure used for the preparation of compound **11**. Compound **13** was isolated as an off-white solid (7 mg, 6% yield). LCMS (ES): 95% pure,  $m/z$  544  $[\text{M}+\text{H}]^+$ .

#### Example 84

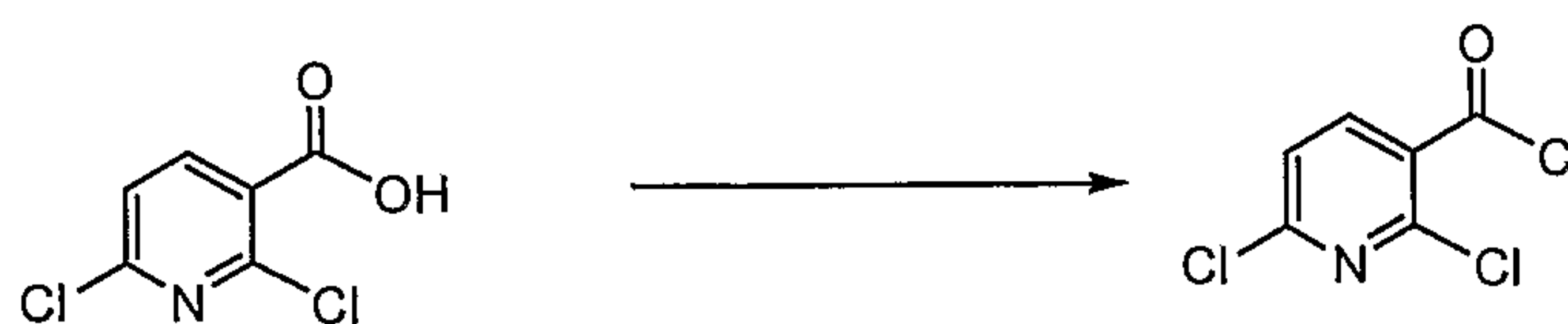


[0224] Compound **14** was prepared according to the procedure used for preparation of compound **13** as described in Example 83. Compound **14** was isolated as an off white solid. LCMS (ES): 91% pure,  $m/z$  530  $[\text{M}+\text{H}]^+$ .

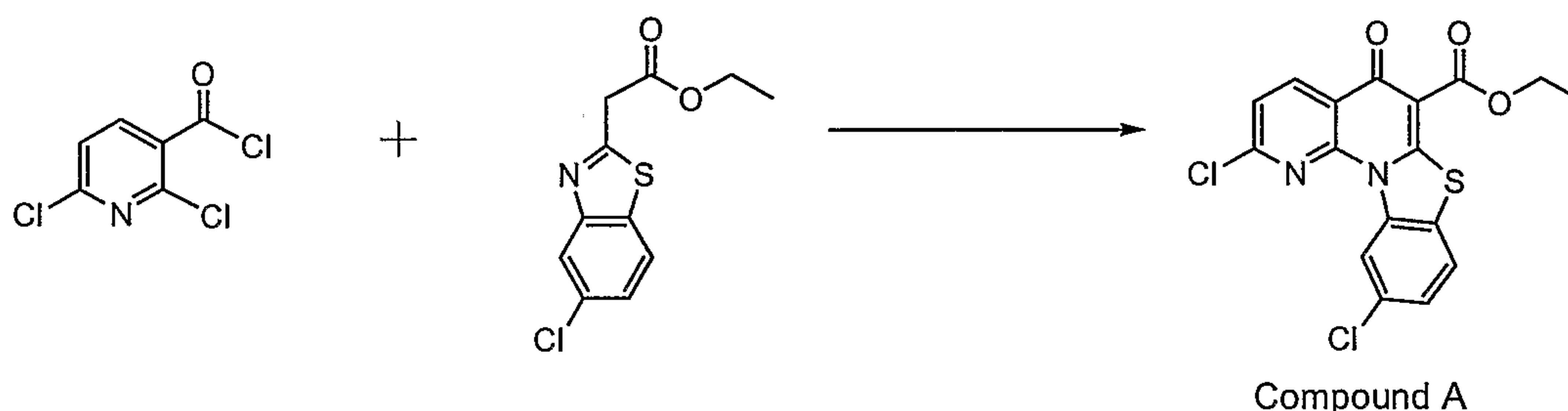
#### Example 85



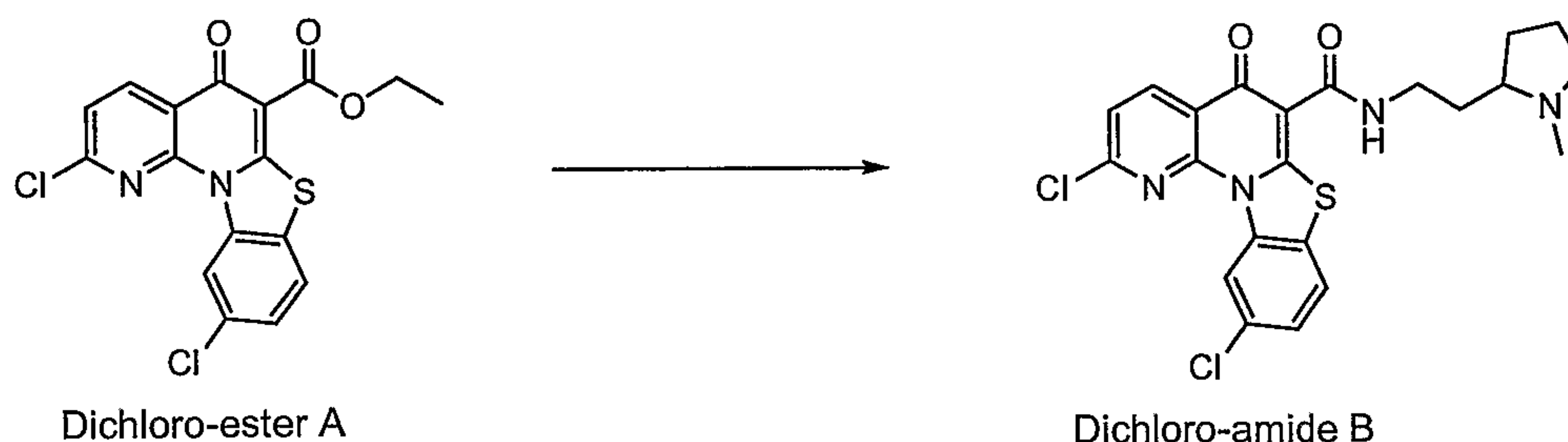
[0225] Ethyl 2-(4-chloro-benzothiazol-2-yl)acetate was prepared by the method of Abbotto, Bradamante *et. al.* (*J. Org. Chem.* **2002**, *16*, 5753. A neat mixture of 4-chloro-2-aminothiophenol (6.36g, 40 mmol) and ethyl cyanoacetate (6.8g, 60 mmol) was heated at  $125^\circ\text{C}$  for 2 hours at which time TLC analysis indicated that the reaction was complete as judged by the disappearance of starting material. The mixture was triturated with diethyl ether and hexanes to give 7.91g of yellow crystals in the first batch and 0.75g of crystals in a second batch for a total of 8.66g (85%). LCMS: 256.2  $(\text{M}+\text{H})^+$ .



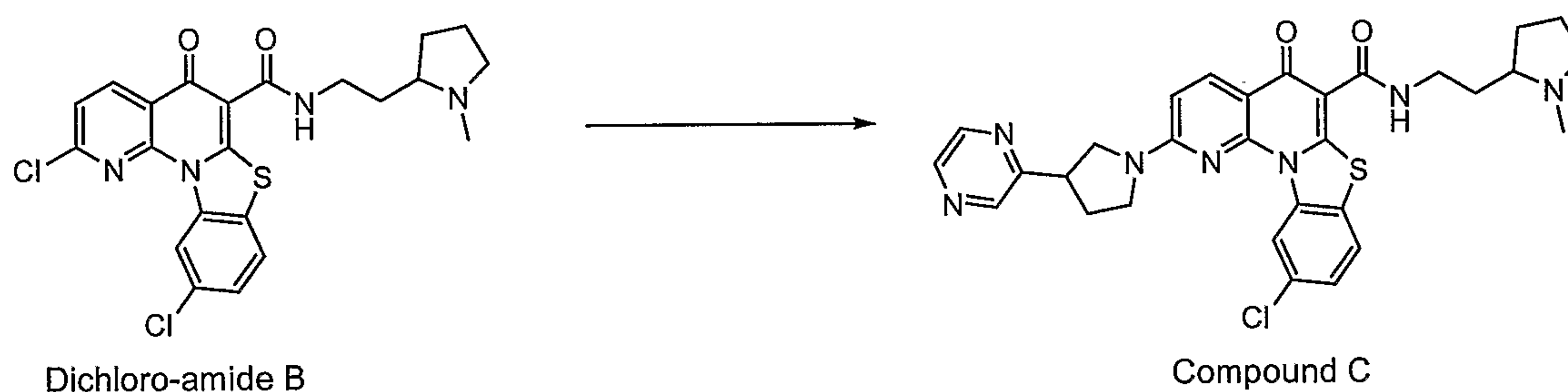
[0226] 2,6-dichloropicolinic acid (1.80g, 9.5 mmol) was suspended in dichloromethane (10 ml) and treated with oxalyl chloride (1.53g, 12.0 mmol). The mixture was cooled in an ice bath and 2 drops of dimethylformamide was added. After an initial vigorous out gassing, the ice bath was removed and the solution was stirred for 18 hours at room temperature. An aliquot was quenched with methanol and analyzed by LCMS indicating that all the acid had been converted to the acid chloride. The solution was concentrated on a rotary evaporator to give the acid chloride as a light brown crystalline solid which was used in the subsequent step without further purification. LCMS: 206.2 (methyl ester M+H)<sup>+</sup>.



[0227] Tetrahydrofuran (25 ml) was added to a mixture of ethyl 2-(4-chloro-benzothiazol-2-yl)acetate (2.2g, 8.6 mmol), magnesium chloride (1.19g, 12.9 mmol) and 2,6-dichloropicolinyl chloride from the previous step. The resulting suspension was cooled in an ice bath and triethylamine (2.4g, 17.2 mmol) was added drop wise at such a rate that the internal temperature did not go over 10°C as measured with an internal thermocouple probe. Once the addition was complete, the ice bath was removed and the mixture was allowed to stir while warming to room temperature over 2 hours. The reaction was diluted with dimethylformamide and potassium carbonate (1.19g, 8.6 mmol) and warmed to 80°C for 1 hour at which time LCMS indicated that the reaction was done. The reaction was diluted with water and filtered. The solid was dissolved in a mixture of dichloromethane and chloroform washed with water and the organic phase was dried over sodium sulfate. After concentration on a rotary evaporator, the product was purified by trituration with diethyl ether to give 2.5g (74%) of the dichloro-ester as a beige solid. LCMS: 393.2 (M+H)<sup>+</sup>.

Example 86

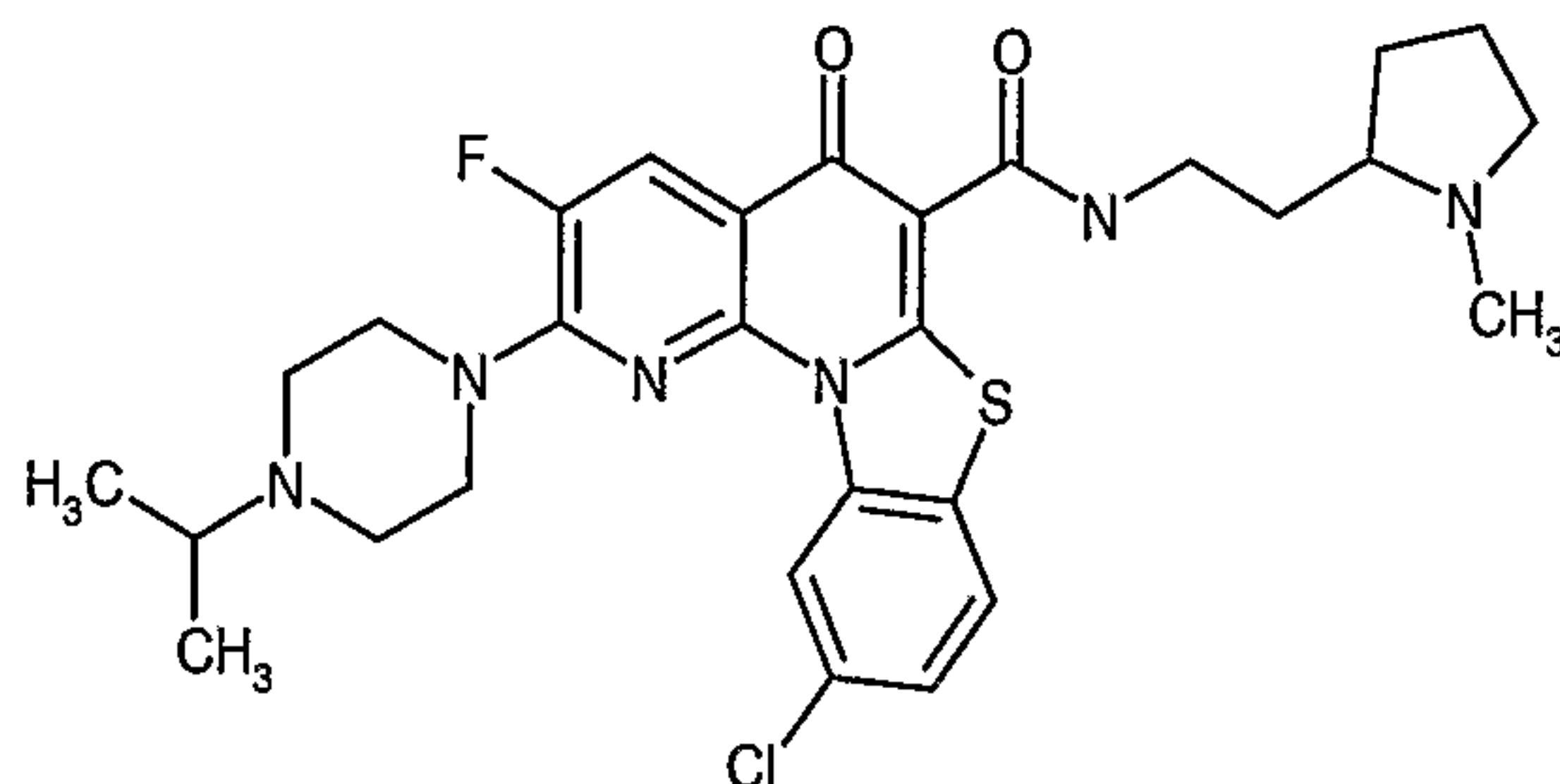
**[0228]** A solution of 2-(1-methylpyrrolidin-2-yl)ethylamine (295mg, 2.3 mmol) in dichloromethane (5ml) was treated with trimethylaluminum (0.99 ml, 25% in hexanes, 2.37 mmol). This solution was added to a solution containing dichloro-ester A (600mg, 1.53 mmol) and dichloromethane (10ml) at such a rate that the internal temperature did not exceed 10°C. The solution was allowed to stir for 18 hours at which time the reaction was judged to be complete by LCMS. The reaction was treated with Rochelle's salt and extracted three times with dichloromethane. The organic extracts were dried over sodium sulfate to give a residue which was purified by trituration with dichloromethane and diethyl ether to give 450mg (62%) of dichloro-amide B as a beige solid. LCMS: 475.5 (M+H)<sup>+</sup>.

Example 87

**[0229]** A mixture of boc-amine (250mg, 1.0mmol) was deprotected by treatment with trifluoroacetic acid (1 ml) and dichloromethane (1ml) at 50°C for 2 hours. The resulting residue was concentrated on a rotary evaporator followed by high vacuum. To this residue was added dichloro-amide B (200mg, 0.42mmol) and N-methyl pyrrolidinone (0.8ml) and diisopropylethylamine (0.5ml). The mixture was allowed to stir at 80°C for 72 hours at which time LCMS indicated that the reaction was done. The mixture was diluted with water (containing 10% trifluoroacetic acid) and purified by preparative HPLC to give 286mg (97%) of compound C as the corresponding trifluoroacetate salt. LCMS: 588.3 (M+H)<sup>+</sup>.

### Example 88

[0230] This example evaluates the activity of the test compound below administered intravenously,



### Methodology

[0231] 6 wk old female *nu/nu* mice were purchased from Taconic Farms, Germantown NY. They were injected with  $5 \times 10^6$  HCT116 cells SQ in right flank. When tumors reached sufficient size for study, they were randomized into groups.

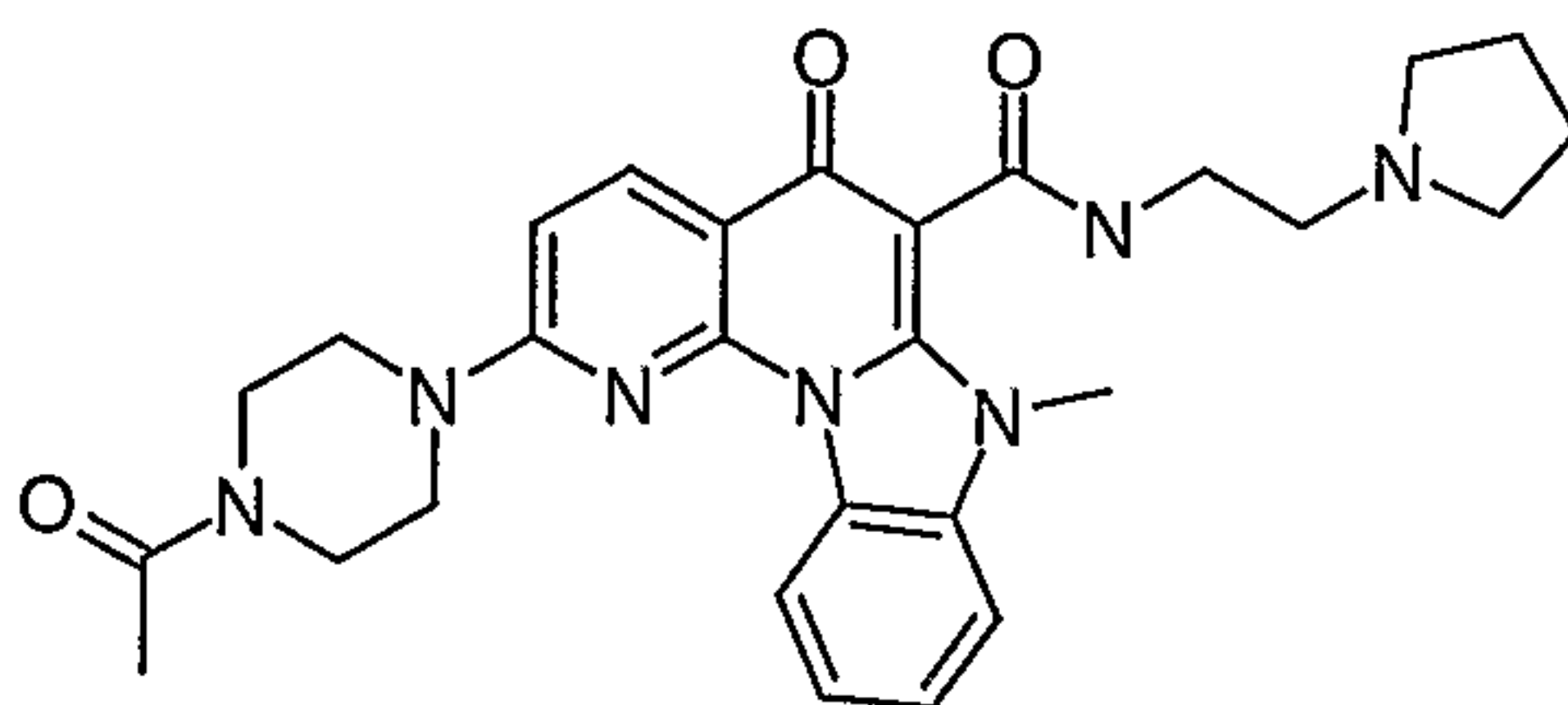
Vehicle: 242.9 mm<sup>3</sup>

Test compound: 251.0 mm<sup>3</sup>

[0232] Animals dosed by bolus injection - IV through lateral tail vein for five consecutive days. Caliper measurements were taken on Day 1, 4, 6. Figure 3 shows the activity of the test compound at a 25 mg/kg dose with no adverse effects observed.

### Example 89

[0233] This example evaluates the activity of the test compound below administered intravenously.



Methodology

[0234] 6 wk old female *nu/nu* mice were purchased from Simonsen Labs, Gilroy, CA. They were injected with  $5 \times 10^6$  HCT116 cells SQ in right flank. When tumors reached sufficient size for study, they were randomized into groups.

Vehicle: 101.9 mm<sup>3</sup>

Test compound: 152.8 mm<sup>3</sup>

[0235] Animals dosed by bolus injection - IV through lateral tail vein for fourteen consecutive days. Caliper measurements were taken on Day 1, 3, 5, 7, 10, 12, 14, and 18. Figure 4 shows the activity at a 12.5 mg/kg dose with no adverse effects observed.

Example 90

[0236] This example evaluates the activity of the test compound in Example 89, administered intravenously at different dose levels.

Methodology

[0237] 6 wk old female *nu/nu* mice were purchased from Simonsen Labs, Gilroy, CA. They were injected with  $5 \times 10^6$  HCT116 cells SQ in right flank. When tumors reached sufficient size for study, they were randomized into groups.

Vehicle: 114.6 mm<sup>3</sup>

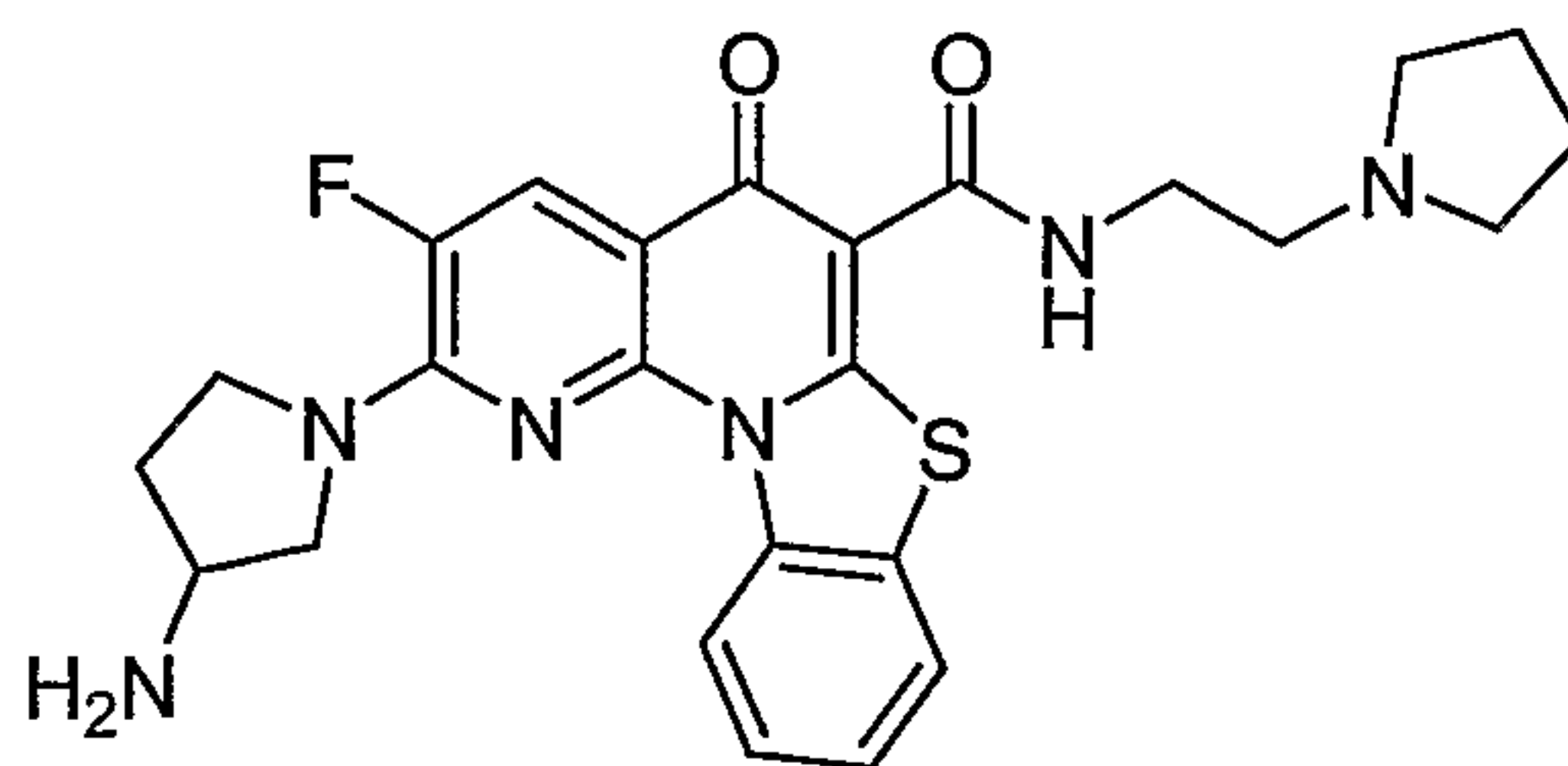
Test compound: 106.1 mm<sup>3</sup>

Test compound: 84.1 mm<sup>3</sup>

[0238] Animals dosed by bolus injection - IV through lateral tail vein for fourteen consecutive days. Caliper measurements were taken on Day 1, 4, 6, 8, 12, and 15. Figure 5 shows dose dependent activity with no adverse effects observed.

Example 91

[0239] This example evaluates the activity of the test compound below administered intravenously.



### Methodology

[0240] 6 wk old female *nu/nu* mice were purchased from Taconic Farms, Germantown NY. They were injected with  $5 \times 10^6$  HCT116 cells SQ in right flank. When tumors reached sufficient size for study, they were randomized into groups.

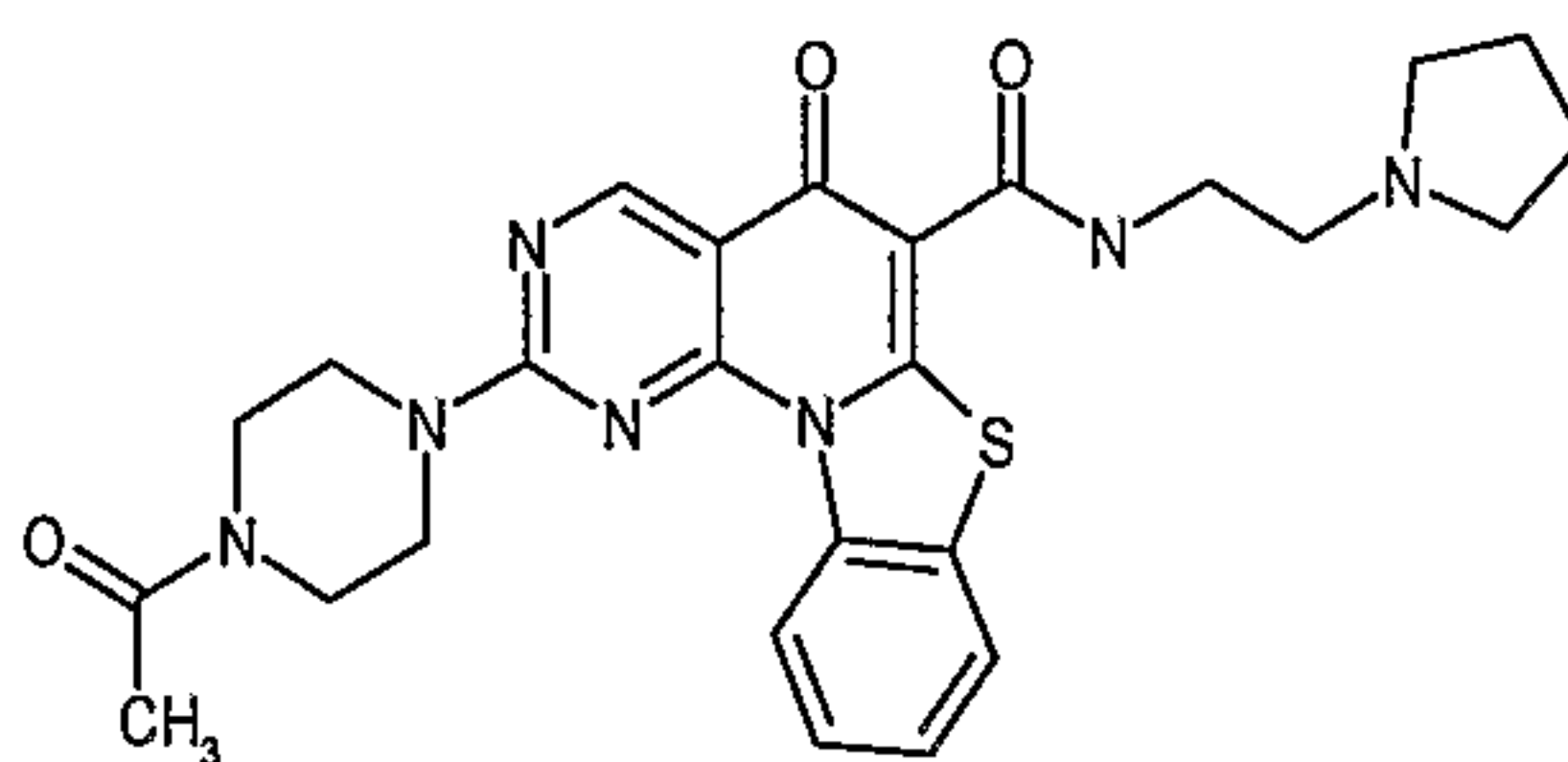
Vehicle: 242.9 mm<sup>3</sup>

Test compound: 252.1 mm<sup>3</sup>

[0241] Animals dosed by bolus injection - IV through lateral tail vein for five consecutive days. Caliper measurements were taken on Day 1, 4, and 6. Figure 6 shows the activity of the test compound at a 25 mg/kg dose with no adverse effects observed.

### Example 92

[0242] This example evaluates the activity of the test compound below administered intravenously.



### Methodology

[0243] 6 wk old female *nu/nu* mice were purchased from Taconic Farms, Germantown NY. They were injected with  $5 \times 10^6$  HCT116 cells SQ in right flank. When tumors reached sufficient size for study, they were randomized into groups.

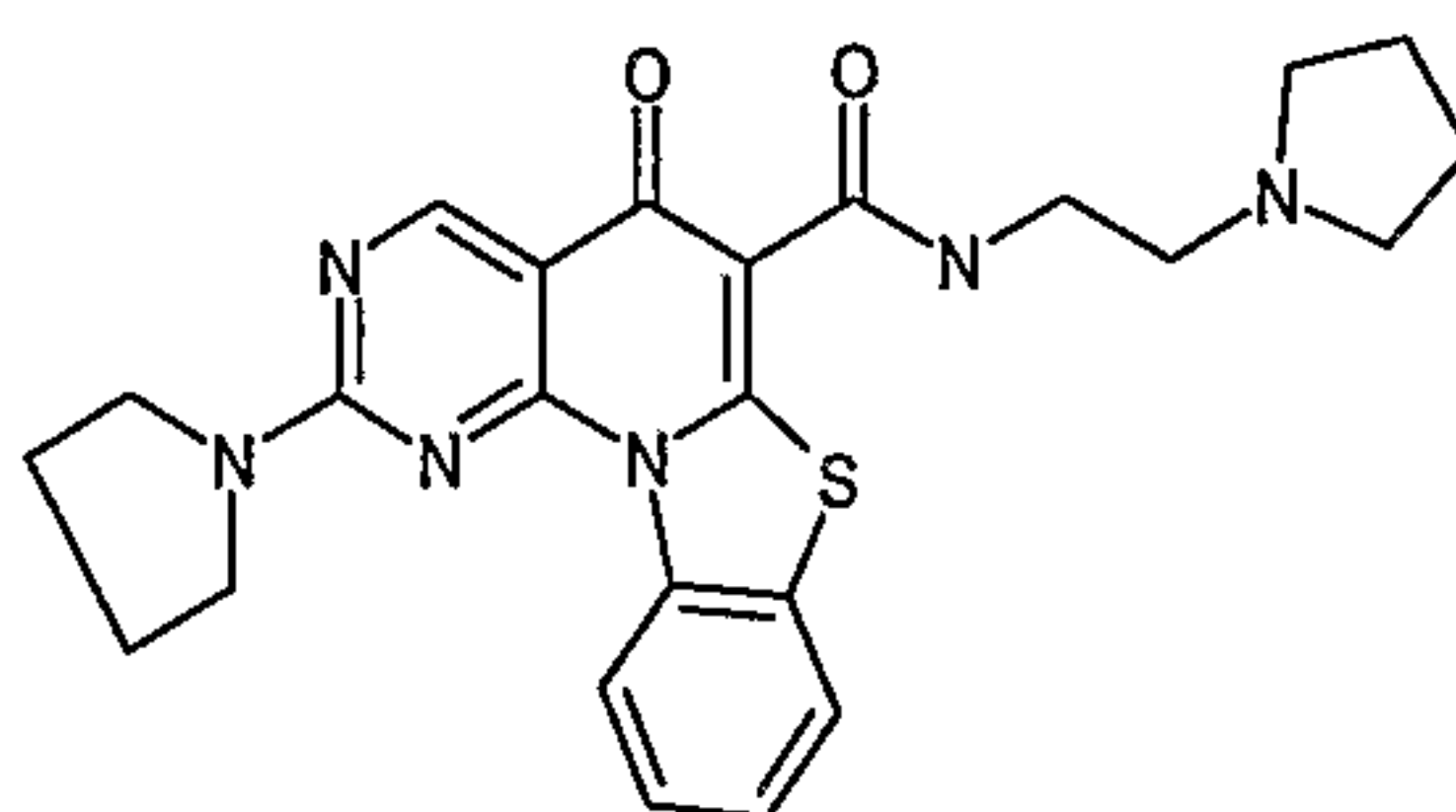
Vehicle: 242.9 mm<sup>3</sup>

Test compound: 236.1 mm<sup>3</sup>

[0244] Animals dosed by bolus injection - IV through lateral tail vein for five consecutive days. Caliper measurements were taken on Day 1, 4, and 6. Figure 7 shows the activity of the test compound at a 25 mg/kg dose with no adverse effects observed.

#### Example 93

[0245] This example evaluates the activity of the test compound below administered intravenously.



#### Methodology

[0246] 6 wk old female *nu/nu* mice were purchased from Taconic Farms, Germantown NY. They were injected with  $5 \times 10^6$  HCT116 cells SQ in right flank. When tumors reached sufficient size for study, they were randomized into groups.

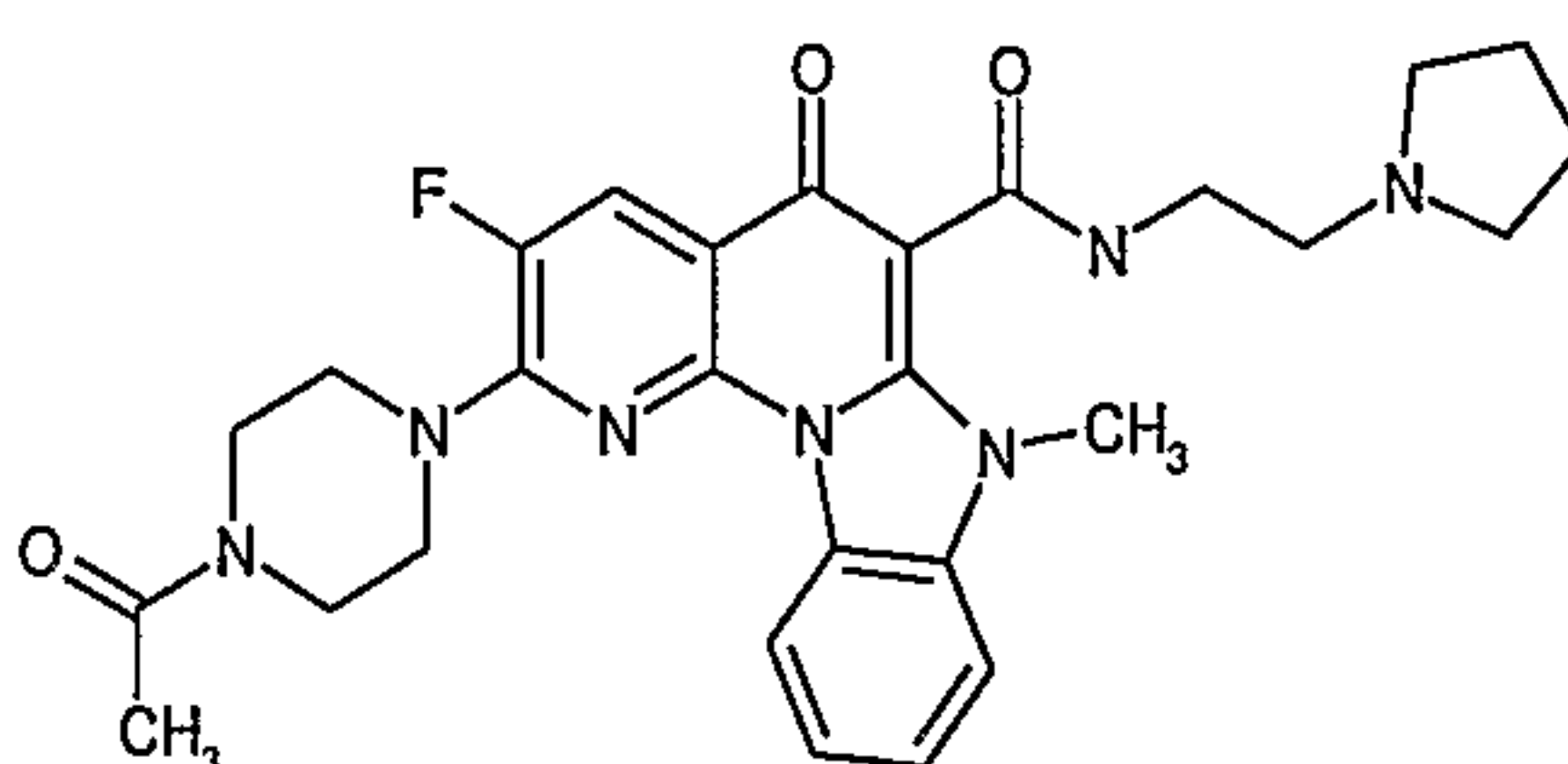
Vehicle: 242.9 mm<sup>3</sup>

Test compound: 254.7 mm<sup>3</sup>

[0247] Animals dosed by bolus injection - IV through lateral tail vein for five consecutive days. Caliper measurements were taken on Day 1, 4, and 6. Figure 8 shows the activity of the test compound at a 25 mg/kg dose with no adverse effects observed.

#### Example 94

[0248] This example evaluates the activity of the test compound below administered intravenously.



Methodology

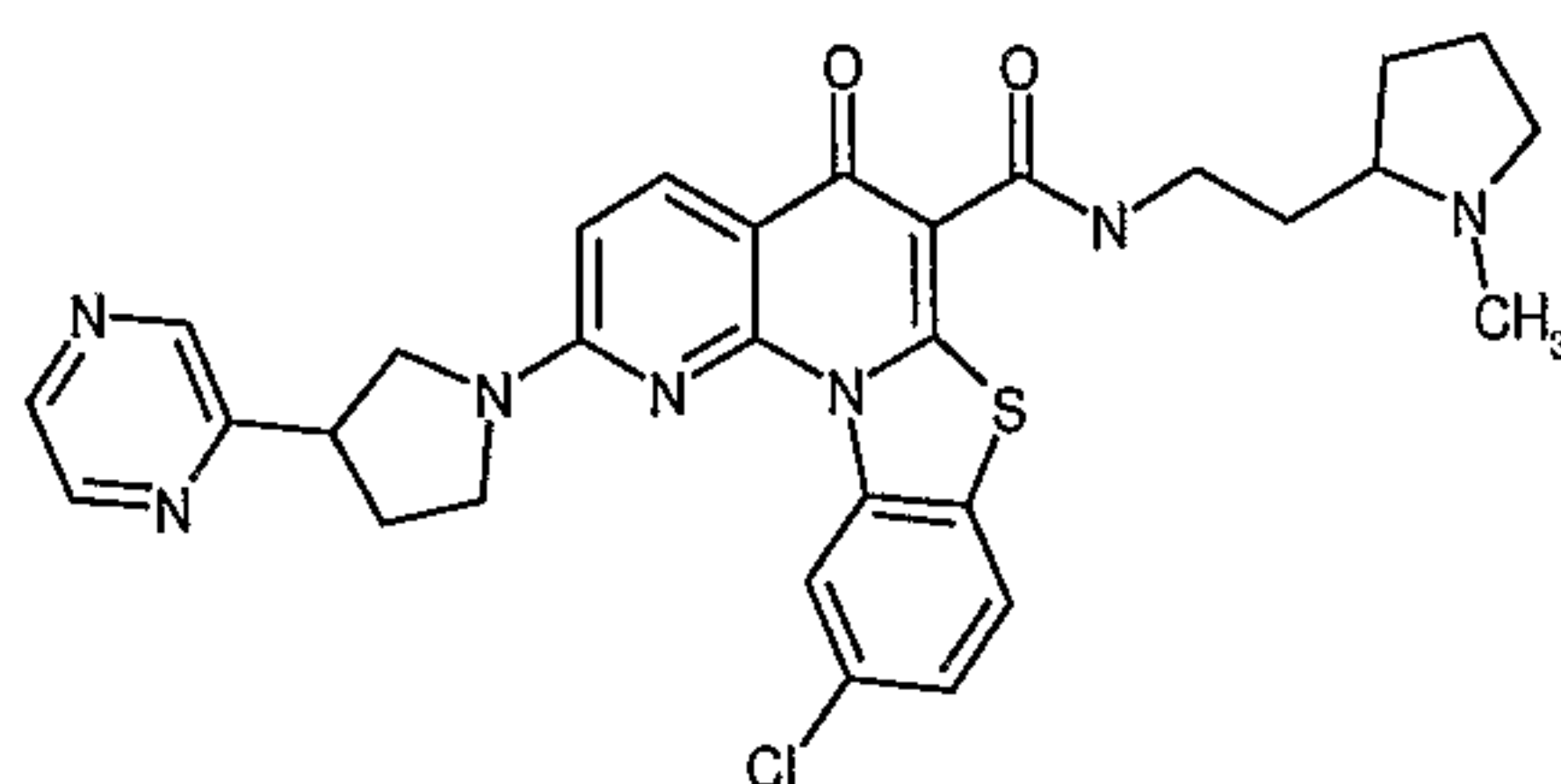
[0249] 6 wk old female *nu/nu* mice were purchased from Taconic Farms, Germantown NY. They were injected with  $5 \times 10^6$  HCT116 cells SQ in right flank. When tumors reached sufficient size for study, they were randomized into groups.

|                |                       |            |
|----------------|-----------------------|------------|
| Vehicle:       | 112.7 mm <sup>3</sup> |            |
| Test compound: | 113.1 mm <sup>3</sup> | 25 mg/kg   |
| Test compound: | 110.1 mm <sup>3</sup> | 12.5 mg/kg |
| CPT11:         | 109.4 mm <sup>3</sup> |            |

[0250] Animals dosed by bolus injection - IV through lateral tail vein for nine consecutive days. Caliper measurements were taken on Day 1, 3, 5, 7, and 9. Figure 9 shows limited activity at the high dose and no activity at the mid dose with no adverse effects observed.

Example 95

[0251] This example evaluates the activity of the test compound below administered intravenously.

Methodology

[0252] 6 wk old female *nu/nu* mice were purchased from Taconic Farms, Germantown NY. They were injected with  $5 \times 10^6$  HCT116 cells SQ in right flank. When tumors reached sufficient size for study, they were randomized into groups.

|                |                       |            |
|----------------|-----------------------|------------|
| Vehicle:       | 112.7 mm <sup>3</sup> |            |
| Test compound: | 107.1 mm <sup>3</sup> | 25 mg/kg   |
| Test compound: | 108.9 mm <sup>3</sup> | 12.5 mg/kg |
| CPT11:         | 109.4 mm <sup>3</sup> |            |

[0253] Animals dosed by bolus injection - IV through lateral tail vein for seven consecutive days. Caliper measurements were taken on Day 1, 3, 5, and 7. Figure 10 shows excellent activity with minimal dose dependency. No adverse effects were observed.

### Example 96

#### Cell Proliferation and/or Cytotoxicity Assay

[0254] The antiproliferative effects of the present compounds may be tested using a cell proliferation and/or cytotoxicity assay, following protocols described below.

[0255] Cell culture. Human cervical epithelial cells (HeLa cells) are obtained from American Type Culture Collection (Manassas, VA). Cells are grown in Eagle's minimum essential medium (MEM, Hyclone, Utah) supplemented with 2 mM Glutamine, 0.1 mM nonessential amino acid, 1 mM Na Pyruvate, 1.5 g/L NaHCO<sub>3</sub>, 50 mg/L gentamicin, and 10% fetal bovine serum (Hyclone, USA) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

[0256] MTS assays. Antiproliferative effects of anticancer drugs are tested by the CellTiter 96 AQueous assay (Promega, WI), which is a colorimetric assay for determining the number of viable cells. (See, e.g., Wang, L., *et al.*, *Methods Cell Sci* (1996) 18:249-255). Generally, cells (2,000 to 5,000 cells/well) are seeded on 96 well flat bottom plates (Corning, NY) in 100 µl of culture medium without any anticancer drug on day 0, and the culture medium is exchanged for that contained anticancer drugs at various concentrations on day 1. After incubation for 3 days under normal growth conditions (on day 4), the monolayers are washed once in PBS, and the medium is switched to 100 µl of PBS in each of the 96 well plate. After mixing MTS and PMS at the ratio of 20:1, 20 µl of MTS/PMS solution is added to each of the 96 well plate and incubated for 4 hours in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. The absorbance is read at 490 nm using FLUOstar Galaxy 96 well plate reader (BMG Labtechnologies, Germany).

### Example 97

#### Measurement of mRNA values in Cell Assays

[0257] Real-time quantitative PCR (QPCR) method may be used to detect the changes of the target c-myc and the endogenous reference GAPDH gene copies in the same tube. Generally, cells (15,000 cells/well) are seeded on 96 well flat bottom plates (Corning, NY) and incubated under normal growth conditions for overnight. The next day, the culture medium is exchanged for that containing anticancer drugs at various concentrations and incubated for 4 hrs in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Total RNA (tRNA) is extracted using the RNeasy 96 Kit (QIAGEN, CA). The concentration of the tRNA is determined by the RiboGreen RNA Quantitation Reagent (Molecular Probes, OR).

[0258] A reverse-transcription (RT) reaction may be conducted using 50 ng of tRNA from each well in a 25  $\mu$ l reaction containing 1x TaqMan RT buffer, 2.5  $\mu$ M random hexamers, 5.5 mM MgCl<sub>2</sub>, 0.5 mM each deoxynucleoside triphosphate (dNTP), 30 U MultiScribe Reverse Transcriptase, and 10 U RNase inhibitor. RT reactions are incubated for 10 min at 25°C, reverse-transcribed for 30 min at 48°C, inactivated for 5 min at 95°C, and placed at 4°C. All RT reagents may be purchased from Applied Biosystems, CA.

[0259] Real-Time QPCR reaction may be performed in a 50  $\mu$ l reaction containing the 5  $\mu$ l of cDNA, 1x Universal PCR Master Mix, 1x c-myc Pre-Developed Primers and Probe set, and 0.8 x GAPDH Pre-Developed Primers and Probe set. Because of the relative abundance of GAPDH gene in Hela, GAPDH primers and probe concentration may be adjusted to get accurate threshold cycles ( $C_T$ ) for both genes in the same tube. The threshold cycle ( $C_T$ ) indicates the fractional cycle number at which the amount of amplified target reaches a fixed threshold. By doing so, the GAPDH amplification is stopped before it can limit the common reactants available for amplification of the c-myc. The  $\Delta R_n$  value represents the normalized reporter signal minus the baseline signal.  $\Delta R_n$  increases during PCR as amplicon copy number increases until the reaction approaches a plateau.

[0260] The c-myc probe is labeled with 6FAM™ dye-MGB and the GAPDH probe is labeled with VIC™ dye-MGB. Preincubation is performed for 2 min at 50°C to activate AmpErase UNG enzyme and then for 10 min at 95°C to activate AmpliTaq DNA Polymerase. DNA is amplified for 40 cycles of 15 sec at 95°C and 1 min at 60°C. Human c-myc and GAPDH cDNA are amplified, detected, and quantitated in real time using the ABI Prism 7000 Sequence Detection system (Applied Biosystems, CA), which is set to detect both 6-FAM and VIC reporter dyes simultaneously.

[0261] The data may be analyzed using the ABI PRISM Sequence Detection System and Microsoft Excel. Relative quantitation is done using the standard curve and comparative  $C_T$  method at the same time, and both methods gave equivalent results. The cycle at which the amplification plot crosses the  $C_T$  is known to accurately reflect relative mRNA values. (See, Heid, *et al.*, *Genome Res.* (1996) 6:986-994; Gibson, *et al.*, *Genome Res.* (1996) 6:995-1001). QPCR reactions are set up in triplicate at each cDNA sample and the triplicate  $C_T$  values are averaged. All reagents including Pre-Developed Primers and probe set may be purchased from Applied Biosystems, CA.

### Example 98

#### In vitro Characterization

[0262] Various methods may be used for *in vitro* characterization of the compounds of the present invention, including but not limited to i) stop assays; ii) quadruplex/duplex competition assay; iii) quadrome footprints; and iv) direct assay in the absence of a competitor molecule.

[0263] Stop Assays. Stop assays are high throughput, first-pass screens for detecting drugs that bind to and stabilize the target G-quadruplex. Generally, DNA template oligonucleotide is created, which contains the nucleotide sequence of the “target” quadruplex against which drug screening is desired. A fluorescently labeled primer DNA is then annealed to the 3' end of the template DNA. A DNA polymerase such as Taq polymerase is then introduced to synthesize a complementary strand of DNA by extending from the fluorescently labeled primer. When the progress of the Taq polymerase is unhindered, it synthesizes a full-length copy of the template. Addition of a test drug that merely binds to duplex DNA but does not bind selectively the quadruplex region results in a decrease in synthesis of full length product and a concomitant increase in variable-length DNA copies. If, however, the test drug selectively binds to and stabilizes the quadruplex, the progress of polymerase arrests only at the quadruplex, and a characteristic “Stop Product” is synthesized.

[0264] Compounds are initially screened at a single concentration, and “hits” are re-assayed over a range of doses to determine an IC<sub>50</sub> value (*i.e.*, the concentration of drug required to produce an arrest product/full-length product ratio of 1:1). These products are visualized by capillary electrophoresis.

[0265] Quadruplex/Duplex Competitor Assay. The selectivity of compounds for the target quadruplex sequence relative to duplex DNA may be measured using a competition assay (*i.e.*, “selectivity screen”). This selectivity screen uses the stop assay as a reporter system to measure the relative ability of an externally added DNA sequence to compete with the target quadruplex structure formed in the DNA template for binding of the drug. For example, the competitors are the c-myc quadruplex sequence, which is identical to the quadruplex sequence present in the template DNA; or a plasmid DNA which mimics complex genomic duplex DNA. The degree to which each competitor successfully “soaks up” drug in solution is reflected by the quantitative decrease in synthesis of the stop product. In this manner, the relative binding affinities of drug to both the target quadruplex and duplex DNA are determined.

[0266] Quadrome Footprints. Compounds may also be evaluated for their ability to bind to other native quadruplex structures of biological relevance, including quadruplex control elements that regulate a range of different oncogenes. The resulting data are used to create a Quadrome footprint.

[0267] Direct Interaction Assay. Compounds may be evaluated for their ability to interact directly with nucleic acids capable of forming a quadruplex structure, wherein the nucleic acid is not a telomeric nucleic acid. The assay may be performed in the same or different vessels. For example, a compound may be contacted with each nucleic acid in the same vessel. Alternatively, a compound may be separately contacted with each of the nucleic acids tested in a different vessel. A telomeric nucleic acid as used herein represents a region of highly repetitive nucleic acid at the end of a chromosome. As used herein, a direct interaction is measured without the presence of a competitor nucleic acid.

[0268] An interaction between the compound and the nucleic acid may be determined for example, by measuring  $IC_{50}$  values, which are indicative of the binding and/or quadruplex stabilization. The selectivity of interactions may be determined, for example, by comparing measured  $IC_{50}$  values. For example, the lowest  $IC_{50}$  values may be used to indicate a strong interaction between the compound and the nucleic acid, while highest  $IC_{50}$  values show a poor interaction; thus, showing selectivity of interaction. The reaction products may be characterized by capillary electrophoresis.

#### Example 99

##### Direct Interaction Assay

[0269] Generally, a 5'-fluorescent-labeled (FAM) primer (P45, 15 nM) is mixed with template DNA (15 nM) in a Tris-HCL buffer (15 mM Tris, pH 7.5) containing 10 mM  $MgCl_2$ , 0.1 mM EDTA and 0.1 mM mixed deoxynucleotide triphosphates (dNTP's). The mixture is denatured at 95°C for 5 minutes and, after cooling down to room temperature, is incubated at 37°C for 15 minutes. After cooling down to room temperature, 1 mM  $KCl_2$  and the test compound (various concentrations) are added and the mixture incubated for 15 minutes at room temperature.

[0270] The primer extension is performed by adding 13 mM KCl and Taq DNA Polymerase (2.5 U/reaction, Promega) and incubating at 70°C for 20 minutes. The reaction is stopped by adding 1  $\mu$ l of the reaction mixture to 10  $\mu$ l Hi-Di Formamide mixed and 0.25  $\mu$ l LIZ120 size standard. The method is repeated with the addition of various concentrations of

competitor nucleic acids at the first step, along with the primer and template sequences. The G-quadruplex binding ligand is added at the concentration previously established to produce a 1:1 ratio of stop-product to full-length product. A CC50 for each nucleic acid competitor is defined as the concentration of competitor required to change the ratio of arrest product to full-length product from 1:1 to 1:2. The nucleic acid sequences of quadruplexes that may be used for this assay are set forth in Table 4.

**Table 4 (STOP TEMPLATES)**

**TGFB3-81**

TATACGGGGTGGGGGAGGGAGGGATTAGCGACACGCAATTGCTATAGTGAGTCGT  
ATTAGCTACGTACAGTCAGTCAGACT

**HRAS-85**

TATACCGGGGCGGGGCGGGGGCGGGGGCTTAGCGACACGCAATTGCTATAGTGAG  
TCGTATTAGCTACGTACAGTCAGTCAGACT

**BCL2-97(full)**

TAGGGGCGGGGCGCGGGAGGAAGGGGGCGGGAGCGGGGCTGTAGCGACACGCAA  
TTGCTATAGTGAGTCGTATTAGCTACGTACAGTCAGTCAGACT

**HMGA-97**

TTAGAGAAGAGGGGAGGAGGAGGAGGAGGAGGAGGAGGCGCTTAGCGACACGCAA  
TTGCTATAGTGAGTCGTATTAGCTACGTACAGTCAGTCAGACT

**MYC99**

TCCAACCTATGTATACTGGGGAGGGTGGGGAGGGTGGGGAAGGTTAGCGACACGCA  
ATTGCTATAGTGAGTCGTATTAGCTACGTACAGTCAGTCAGACT

**IMOTIF99**

TCCAACCTATGTATACCCTTCCCCACCCTCCCCACCCTCCCCATTAGCGACACGCAA  
TTGCTATAGTGAGTCGTATTAGCTACGTACAGTCAGTCAGACT

**Humtel-95**

TCATATATGACTACTTAGGGTTAGGGTTAGGGTTAGGGTTACTGCCACGCAATTGC  
TATAGTGAGTCGTATTAGCTACGTACAGTCAGTCAGACT

**SRC89**

ATGATCACCGGGAGGAGGAGGAAGGAGGAAGCGCGCTGCCACGCAATTGCTATA  
GTGAGTCGTATTAGCTACGTACAGTCAGTCAGACT

**Primer:**

(45 MER)

AGTCTGACTGACTGTACGTAGCTAATACGACTCACTATAGCAATT

### Example 100

#### Cytochrome P450 (CYP450) Inhibition Assay

[0271] The compounds of the present invention may be evaluated for potential inhibitory activity against cytochrome P450 isoenzymes. Generally, six reaction tubes with 100  $\mu$ L of a solution containing 50 mM potassium phosphate, pH 7.4, 2.6 mM NADP<sup>+</sup>, 6.6 mM glucose 6-phosphate, 0.8 U of glucose 6-phosphate dehydrogenase/mL and 1:6 serial dilutions of the test compound will be prepared along with six tubes of 1:6 serial dilutions of a suitable positive control inhibitor. The reactions will be initiated by adding 100  $\mu$ L of a pre-warmed enzyme/substrate solution to the reaction tubes. A zero time-point control reaction will be prepared by adding 50  $\mu$ L of acetonitrile to 100  $\mu$ L of cofactor solution to inactivate the enzymes, then adding 100  $\mu$ L of enzyme/substrate solution. A control reaction with no inhibitor may also be prepared. After a suitable incubation at 37 C, the reactions will be terminated by the addition of 50  $\mu$ L of acetonitrile. The reactions will be analyzed for the metabolite forms of the probe substrate using LC/MS/MS.

### Example 101

#### Evaluation of Compound Efficacy in Tumor Suppression

[0272] A representative experiment for evaluating the efficacy of compounds of the present invention in athymic nude mouse models of human carcinoma may be designed as follows. Male or female animals (mouse, Sim) (NCR, nu/nu) aged five to six weeks and weighing more than 20 grams will be used. The animals will be purposely bred and will be experimentally naïve at the outset of the study. Tumors will be propagated either from injected cells or from the passage of tumor fragments. Cell lines to be used include, but are not limited to, alia Paca-2, HPAC, Hs700T, Panc10.05, Panc 02.13, PL45, SW 190, Hs 766T, CFPAC-1 and PANC-1.

[0273] Cell implantation. One to ten million cells suspended in 0.1 ml culture media with or without Matrigel (Collaborative Biomedical Products, Inc, Bedford, MA) will be inoculated subcutaneously in the right flank of sixty animals. There will only be one injection per animal. Within 7-14 days of injection tumors will develop to a study use size of approximately 1.0 cm<sup>3</sup>. A small subset (<10/60) animals will be considered. Donors and tumors will be grown 10-28 days and to a size of 1.5 cm<sup>3</sup> in order to be used for serial transplantation.

[0274] Fragment transplantation. Donor animals will be euthanized and tumors surgically excised and cut into 2 mm<sup>3</sup> size fragments using aseptic technique. Animals to be implanted will be lightly anesthetized with isoflurane. The area to implanted will be cleansed with 70% alcohol and betadine. A single fragment will then be implanted subcutaneously using a trocar.

[0275] Efficacy studies. Groups of 50-60 tumor bearing animals will be randomly divided. For example, in a representative study, animals may be randomly divided into three to eight groups containing 7 animals each, as described in Table 5.

**Table 5**

| Group No.    | Number of Males/<br>Females | Dose Level                                                | Dose Vol.<br>( $\mu$ L)                    | Dose Solution<br>Conc.<br>(mg/mL)   | Number Euthanized<br>on:<br>Day 28-42 |
|--------------|-----------------------------|-----------------------------------------------------------|--------------------------------------------|-------------------------------------|---------------------------------------|
| 1            | N = 7                       | Negative Control<br>*                                     | 250                                        |                                     | all                                   |
| 2            | N = 7                       | Positive Control<br>**                                    | 10 – 400 IP<br>10 – 250 IV<br>125 – 500 PO | 2 to 5 IP<br>2.5 to 5 IV<br>10 PO   | all                                   |
| Groups 3 - 8 | N = 7/grp<br><56 total      | Test Compound<br>1 to 25 IP<br>1 to 50 IV<br>50 to 200 PO | 10 – 400 IP<br>10 – 250 IV<br>125 – 500 PO | 2.5 to 5 IP<br>2.5 to 5 IV<br>10 PO | all                                   |

\*Vehicle/Diluent

\*\*Commercially available anticancer compounds including, but not limited to, Taxol, CPT11 and Gemcitabine will be used as positive controls.

[0276] Dosing Procedure. Compounds will be administered QD, QOD, Q3D or once weekly via IP, IV (lateral tail vein) or PO. Animals will be dosed in a systematic order that distributes the time of dosing similarly across all groups. For bolus IP and PO dosing, animals will be manually restrained. For IV bolus dosing or short term IV infusion (one minute), animals will be mechanically restrained but not sedated. Disposable sterile syringes will be used for each animal/dose. A test compound in combination with about 10-100 mg/kg (e.g., about 40 mg/kg) chemotherapeutic agent such as gemcitabine also will be tested, normally by IP administration once per week.

Example 102Evaluation of Maximum Tolerated Doses

**[0277]** A representative experiment for evaluating the maximum tolerate dose (MTD) of compounds of the present invention may be designed as follows. Selection for animal models is as described in Example 101.

**[0278]** Acute Toxicity Studies. In a representative study to determine the MTD after a single dose, sixty naive animals, for example, will be randomly divided into groups containing 10 animals (5 male and 5 female) and will receive either one compound via two routes of administration or two compounds via a single route of administration. A single 50 mg/kg IV dose has been shown to be tolerated, and is used as the preliminary low dose levels. The low dose for oral studies is based on projected tolerability and will be adjusted downward if necessary. A representative design of dose levels, dose volumes and dose solution concentration are described in Table 6.

**Table 6**

| Group No. | Number of Males and Females | Dose Level (mg/kg)                   | Dose Vol. (μL)   | Dose Solution Conc. (mg/mL) | Number Euthanized on: Day 7 |
|-----------|-----------------------------|--------------------------------------|------------------|-----------------------------|-----------------------------|
| 1         | N = 5 M<br>N = 5 F          | Test compound #1<br>50IV<br>100 PO   | 250 IV<br>500 PO | 5 IV<br>5 PO                | all                         |
| 2         | N = 5 M<br>N = 5 F          | Test compound #1<br>75IV<br>200 PO   | 250 IV<br>500 PO | 8.25 IV<br>10 PO            | all                         |
| 3         | N = 5 M<br>N = 5 F          | Test compound #1<br>100 IV<br>300 PO | 250 IV<br>500 PO | 10 IV<br>15 PO              | all                         |
| 4         | N = 5 M<br>N = 5 F          | Test compound #2<br>50IV<br>100 PO   | 250 IV<br>500 PO | 5 IV<br>5 PO                | all                         |
| 5         | N = 5 M<br>N = 5 F          | Test compound #2<br>75IV<br>200 PO   | 250 IV<br>500 PO | 8.25 IV<br>10 PO            | all                         |
| 6         | N = 5 M<br>N = 5 F          | Test compound #2<br>100 IV<br>300 PO | 250 IV<br>500 PO | 10 IV<br>15 PO              | all                         |

**[0279]** SubChronic Studies. In a representative study to characterize dose-response relationships following repeated dosing, twenty-five naive animals, for example, will be

randomly divided into groups containing 5 animals each as described in Table 7. Each two week study will test only one compound via a single route of administration at an optimal dose derived from data collected in prior acute toxicity studies.

**Table 7**

| Group No. | Number of Males or Females | Dose Level (mg/kg)                         | Dose Vol. (μL)   | Dose Solution Conc. (mg/mL) | Number Euthanized on: Day 14 |
|-----------|----------------------------|--------------------------------------------|------------------|-----------------------------|------------------------------|
| 1         | N = 5                      | Negative Control                           | 250 IV<br>500 PO | Depends on Dose Level       | all                          |
| 2<br>QD   | N = 5                      | Test Compound As Determined in MTD Studies | 250 IV<br>500 PO | Depends on Dose Level       | all                          |
| 3<br>QOD  | N = 5                      | Test Compound As Determined in MTD Studies | 250 IV<br>500 PO | Depends on Dose Level       | all                          |
| 4<br>Q3D  | N = 5                      | Test Compound As Determined in MTD Studies | 250 IV<br>500 PO | Depends on Dose Level       | all                          |
| 5<br>Q7D  | N = 5                      | Test Compound As Determined in MTD Studies | 250 IV<br>500 PO | Depends on Dose Level       | all                          |

[0280] Dosing Procedure. Compounds will be administered QD, QOD, Q3D or Q7D via IV (lateral tail vein) or PO. Animals will be dosed in a systematic order that distributes the time of dosing similarly across all groups. For PO dosing, animals will be manually restrained. For IV bolus dosing or short term IV infusion (one minute), animals will be mechanically restrained but not sedated. Disposable sterile syringes will be used for each animal/dose.

### Example 103

#### Evaluation of Pharmacokinetic Properties

[0281] A representative pharmacokinetic study for evaluating pharmacokinetic properties of the compounds herein may be designed as follows. Male animals (mouse, Balb/c or rat, SD) aged five to six weeks. For rat models, rats weighing more than 200 grams will be used. In a representative study, twenty animals, for example, will randomly divided into 4 groups, as shown in Table 8. One group will be untreated and samples taken to be used as a base line. The other three groups will be and administered a single dose of compounds by intravenous injection.

**Table 8**

| Group No. | No. of Animals | Time followed by injection (h) |
|-----------|----------------|--------------------------------|
| 1         | 2              | Naïve                          |
| 2         | 6              | .25, 2, 8                      |
| 3         | 6              | .5, 4, 12                      |
| 4         | 6              | 1, 6, 24                       |

[0282] Dosing Procedure. Compounds will be administered via IV (lateral tail vein), IP or PO. Animals will be dosed in a systematic order that distributes the time of dosing similarly across all groups. For IP and PO dosing, animals will be manually restrained. For IV bolus dosing or short term IV infusion (one minute), animals will be mechanically restrained but not sedated. Disposable sterile syringes will be used for each animal/dose.

[0283] Approximately 0.5 ml of blood will be collected from the naive animals via cardiac puncture prior to the first dose. Terminal blood samples (0.5 ml) will be collected via cardiac puncture from two animals per group per time point according to the above chart. All samples will be placed in tubes containing lithium heparin as anticoagulant and mixed immediately by inverting. They will be centrifuged and the plasma flash frozen in liquid nitrogen, stored at –70 °C or greater and analyzed for drug levels.

#### Example 104

##### Determination of *in vitro* Metabolic Stability in Hepatocytes

[0284] A representative protocol to determine the stability of a new chemical entity in the presence of hepatocytes (human, rat, dog, monkey) in *in vitro* incubations may be designed as follows. The test article will be incubated with hepatocytes and suitable media for various times at 37°C. The reaction mixtures will be extracted and analyzed by LC/MS/MS for the parent compound and anticipated metabolites. If applicable, a half-life will be calculated for the consumption of the test article. Metabolism controls will be run for comparison of the half-life values with that obtained for the test article. The metabolism controls may be tolbutamide, desipramine and naloxone, which have defined pharmacokinetics corresponding to low, moderate and high *in vivo* clearance values, respectively.

[0285] Metabolic Stability Study. Generally, solutions of the test compounds will be prepared along with a cocktail solution of metabolism controls that are intended to provide a reference for enzyme activity. The reactions will be initiated by combining these pre-warmed

solutions with hepatocyte suspensions and with a media control solution. Control zero samples will be taken from these reactions immediately after initiation. Additional samples may be taken at appropriate time points. Each sample will be immediately placed in a terminating solution (acidified MeCN containing IS) to stop the reaction. Hepatocyte blank suspensions and test compound standard solutions will be prepared.

[0286] Samples and standards for the test compound as well as appropriate blanks may be subjected to a custom sample preparation procedure and analyzed for the parent and/or metabolite form of the test compound using HPLC coupled with tandem mass spectrometry. Samples and standards for the metabolism controls may be subjected to the analytical method described herein. Where Krebs Henseleit buffer will be added, the buffer is bubbled with 5% CO<sub>2</sub> in air at room temperature for 5-10 minutes before adding BSA to a final concentration of 0.2% w/v. The volume of terminating solution and the method of sample preparation will be determined for the test article during method development.

[0287] Test Article/Media Solution. A solution of the test article will be prepared by adding an appropriate volume of the stock solution to 0.2% BSA in Krebs Henseleit buffer equilibrated with 5% CO<sub>2</sub> in air. The final concentration will be between 5 μM and 20 μM, and the final assay concentration at initiation of the reactions will be between 1 μM and 10 μM.

[0288] Metabolism Controls/Media Solution. A solution of tolbutamide, desipramine and naloxone will be prepared by adding an appropriate volume of each 10 mM stock solution to 0.2% BSA in Krebs Henseleit buffer equilibrated with 5% CO<sub>2</sub> in air. The final concentration will be 20 μM for each metabolism control and the final assay concentration will be 10 μM at initiation of the reactions.

[0289] Hepatocyte Suspension Solution. The hepatocytes will be thawed and isolated according to the vendor (Invitrotech, Inc.) instructions. During the final step of the procedure, the viability of the cells will be determined using the method of trypan blue exclusion. Then, the hepatocytes will be resuspended with 0.2% BSA in Krebs Henseleit buffer equilibrated with 5% CO<sub>2</sub> in air so the final concentration is 0.5 million viable cells/mL. The concentration at the initiation of the reactions will be 0.25 million viable cells/mL.

[0290] Initiating Test Article Incubation. Equal volumes of the test article solution prepared in step 2.1.3 will be dispensed into four polypropylene scintillation vials. The vials are pre-warmed for 5-10 minutes at 37°C with 95% humidity and 5% CO<sub>2</sub>. Equal volumes of 0.2% BSA in Krebs Henseleit buffer equilibrated with 5% CO<sub>2</sub> in air will be added to two of the vials and mixed thoroughly. Immediately after initiating the reaction, a timer is started and

a 100  $\mu$ L sample is removed from each vial and placed into a 1.7-mL centrifuge tube containing a suitable volume of terminating solution. These samples will serve as media controls to check for non-enzymatic degradation and non-specific binding to the vessel.

**[0291]** Equal volumes of the hepatocyte suspension prepared above will be added to two of the vials and mixed thoroughly. Immediately after initiating the reaction, a timer is started and a 100  $\mu$ L sample is removed from each vial and placed into a 1.7-mL centrifuge tube containing a suitable volume of terminating solution. All vials are placed in an incubator maintained at 37°C, 95% humidity and 5% CO<sub>2</sub>.

**[0292]** Initiating Metabolism Control Incubation. Equal volumes of the metabolism control solution prepared above will be dispensed into two polypropylene scintillation vials. The vials are pre-warmed for 5-10 minutes at 37°C with 95% humidity and 5% CO<sub>2</sub>. Equal volumes of the hepatocyte suspension prepared above will be added to each of the two vials and mixed thoroughly. Immediately after initiating the reaction, a timer is started and a 100  $\mu$ L sample is removed from each vial and placed into a 1.7-mL centrifuge tube containing an equal volume of terminating solution. All vials are placed in an incubator maintained at 37°C, 95% humidity and 5% CO<sub>2</sub>.

**[0293]** Sample Collection. The vials will be gently shaken and samples (100  $\mu$ L) will be removed and placed into a 1.7-mL centrifuge tube containing an appropriate volume of terminating solution according to the following schedule: Test article samples are taken after 5, 10, 15, 30, 60, 90 and 120 minutes; metabolism control samples are taken after 30, 60, 90 and 120 minutes. Immediately after removal of the samples, the vials are placed back in the incubator until the last sample is collected.

**[0294]** Blank Preparation. A sample (100  $\mu$ L) of the hepatocyte suspension will be added to an equal volume of 0.2% BSA in Krebs Henseleit buffer and mixed thoroughly. A 100  $\mu$ L sample of this solution will be removed and placed into a 1.7-mL centrifuge tube containing the same volume of terminating solution used for the test article reaction. A sample of the incubation medium (0.2% BSA in Krebs Henseleit buffer) will be placed into a 1.7-mL centrifuge tube containing the same volume of terminating solution used for the test article reaction.

**[0295]** Sample Preparation and Analysis. All vials will be centrifuged at 16,000 g for 3 minutes. The supernatants will be placed into polypropylene autosampler vials and stored at 4°C (<1 day) or -70°C (>1 day) until analysis. The test article solutions will be analyzed using HPLC/MS/MS conditions according to standard procedures. In one example, the following

HPLC conditions may be used: column (Phenomenex Synergi Hydro-RP, 100.0 × 2.0 mm, 5 μm); guard column (Phenomenex C18, 4.0 × 2.0 mm, 5 μm); flow rate (0.3 mL/min); column temperature at 45°C; injection volume at 10 μL; and ambient autosampler temperature.

#### Example 105

##### Determination of *in vitro* Metabolic Stability in Microsomes

[0296] A representative protocol to determine the stability of a new chemical entity in the presence of liver microsomes (human, rat, dog, monkey) in *in vitro* incubations may be designed as follows. The test article will be incubated with microsomes and suitable media for various times at 37°C. The reaction mixtures will be extracted and analyzed by LC/MS/MS for the parent compound and anticipated metabolites. If applicable, a half-life will be calculated for the consumption of the test article. Metabolism controls will be run for comparison of the half-life values with that obtained for the test article. The metabolism controls are tolbutamide, desipramine and testosterone, and these compounds have defined pharmacokinetics corresponding to low, moderate and high *in vivo* clearance values, respectively.

[0297] Metabolic Stability Study. Generally, six pre-warmed reaction vials with 100 μL of a solution containing 50 mM potassium phosphate, pH 7.4, 2.6 mM NADP<sup>+</sup>, 6.6 mM glucose 6-phosphate, 0.8 U/mL of glucose 6-phosphate dehydrogenase and 1, 10 or 50 μM of the test compound are prepared. Similar reactions with metabolic controls representing low (tolbutamide), moderate (desipramine), and high (testosterone) clearance compounds are run simultaneously with the same enzyme solution. The reactions are initiated by adding 100 μL of a pre-warmed enzyme solution and incubated at 37°C. The zero time-point reaction is prepared by adding 50 μL of acetonitrile (containing internal standard) to the test compound/cofactor solution prior to adding the enzyme solution. After 15, 30, 60, 90 and 120 minutes, a reaction tube is removed from the water bath and the reaction is terminated with 50 μL of acetonitrile containing internal standard. The reactions are extracted and the samples are analyzed for the parent form of the test compound and one metabolite using a C18 column with MS/MS detection. Each assay is performed in duplicate.

[0298] Cofactor/Test compound Solution Concentrations. A stock solution of 10 mM NCE will be prepared in 10% DMSO (v/v). For all assays, a 2, 20 or 100 μM solution of the test

article will be prepared in 50 mM potassium phosphate, pH 7.4, 2.6 mM NADP<sup>+</sup>, 6.6 mM glucose 6-phosphate and 0.8 U/mL of glucose 6-phosphate dehydrogenase (cofactor solution).

[0299] Cofactor/Metabolism Control Solution Concentrations. Stock solutions of the metabolism controls (tolbutamide, desipramine, and testosterone) will be used to prepare a 6  $\mu$ M solution of the metabolism control in cofactor solution described in step

[0300] Enzyme Solution Concentrations. The enzyme solutions will be prepared by adding liver microsomes to 50 mM potassium phosphate, pH 7.4, to a final concentration of 1 mg/mL. All microsomes were purchased from XenoTech or InvitroTech, Inc.

[0301] Initiating the Reactions. All the reaction tubes will be pre-warmed at 37°C in a water bath for about 3-5 minutes. The zero time-point control reaction will be prepared for each replicate by adding 50  $\mu$ L of acetonitrile containing 15.9  $\mu$ M nebularine (internal standard) to 100  $\mu$ L of cofactor solution to inactivate the enzymes, and then vortex mixing. The reactions will be initiated by adding 100  $\mu$ L of the enzyme solution to each of the tubes and vortex mixing. All the tubes, including the zero time-point control, will be incubated in a 37°C water bath. The final concentrations of all components in the tubes after initiating the reactions are 50 mM potassium phosphate, pH 7.4, 1.3 mM NADP<sup>+</sup>, 3.3 mM glucose 6-phosphate, 0.4 U/mL of glucose 6-phosphate dehydrogenase, 0.5 mg/mL liver microsomes and 1, 10 or 50  $\mu$ M test article.

[0302] Terminating and Extracting the Reactions. After 15, 30, 60, 90 and 120 minutes at 37°C, the reactions will be terminated by the addition of 150  $\mu$ L of acetonitrile containing 15.9  $\mu$ M nebularine (internal standard). The zero time-point control is removed from the water bath after 120 minutes. All vials will be centrifuged at 16,000 g for 3 minutes. The supernatants will be placed into polypropylene autosampler vials and stored at 4°C (<1 day) or -70°C (>1 day) until analysis.

[0303] Analysis of Test Article Solutions. The test article solutions will be analyzed using HPLC/MS/MS conditions according to standard procedures, such as those described in Example 39.

#### Example 106

##### Bacterial Mutagenicity Test

[0304] This Mutagenicity Assessment assay (Ames Assay) will evaluate the potential of the test article extracts to induce histidine (his) reversion in *S. typhimurium* (his- to his+) or

tryptophan (trp) reversion in *E. coli* (trp<sup>-</sup> to trp<sup>+</sup>) caused by base changes or frameshift mutations in the genome of tester organisms. Generally, a plate incorporation assay will be conducted with five strains of *Salmonella typhimurium* (TA97a, TA98, TA100, TA102, and TA1535) and one strain of *Escherichia coli* (WP2-uvrA<sup>-</sup>) in the presence and absence of an exogenous mammalian activation system (S9). The test article will be dissolved in 5% dextrose. A series of dilutions will then be prepared in saline just prior to testing. A Range Finding Study will also be conducted for this assay to determine the appropriate doses for definitive mutagenicity assessment.

#### Test Material Preparation

[0305] A stock solution of test article will be prepared at 20.0 mg/mL as follows: 1.0 g test article will be added to 15.0 mL of 0.1 HCl for 1 minute. The test article will be stirred for 15 minutes at room temperature. Next 33.0 mL of deionized water will be added and allowed to stir for 30 minutes. The pH will then be adjusted to 3.53. Lower doses will be prepared by dilution in 5% dextrose from this stock immediately prior to use. To minimize any change of degradation, the test article solutions will be kept on ice after preparation and until just prior to dosing procedures. The test article will be administered *in vitro*, through a solvent compatible with the test system.

#### Genotypic Characterization of the Test Strains

[0306] Working stocks of test strains will be confirmed for genotypic markers and acceptable spontaneous reversion rates. All working stocks should demonstrate a requirement for histidine or tryptophan (*E. coli* only). Additionally, the following conformations will be made with each assay, as appropriate: sensitivity to crystal violet due to the *rfa* wall mutation; sensitivity to ultraviolet light due to the deletion of the *uvrB* gene (*uvrA* in *E. coli*), resistance to ampicillin due to the presence of the pKM101 plasmid; and resistance to tetracycline due to the presence of the pAQ1 plasmid. Spontaneous reversion rates for the strains will be determined using the negative controls.

[0307] Test articles that are water-soluble will be dissolved in isotonic saline or other suitable solvent. Test articles that are not water-soluble will be dissolved in dimethylsulfoxide (DMSO) or other suitable solvent. If DMSO is anticipated to cause adverse reactions with the test article, the test article will be suspended in carboxymethylcellulose. In order to aid in dissolution, heating, vigorous vortexing or alternative solvents may be employed.

### Test System

[0308] This assay will be conducted in accordance with the plate incorporation methodology originally described by Ames (Ames *et al.*, *Mutation Research* (1975) 31:347-364) and updated by Maron and Ames (Maron *et al.*, *Mutation Research* (1983) 113:173-215). This assay has historically been used to detect mutation in a gene of a histidine requiring strain to produce a histidine independent strain or concordantly, to detect mutation in a gene of a tryptophan requiring strain to produce a tryptophan independent strain. In addition, it has been shown to detect diverse classes of chemical mutagens which produce heritable DNA mutations of a type which are associated with adverse effects.

[0309] The *Salmonella typhimurium* strains that may be used in this assay, TA97a, TA98, TA100, and TA102 are described by Maron and Ames, *supra*; Green *et al.*, *Mutation Research* (1976) 38:33-42); and Brusick *et al.*, *Mutation Research* (1980) 76:169-190)). *S. typhimurium* strain TA1535 and *E. coli* strain Wp2-uvrA<sup>-</sup> may be obtained from American Type Culture Collection, Manassas, VA (ATCC numbers: 29629 and 49979, respectively). All working stocks of test strains will be confirmed for genotypic markers and acceptable reversion rates. Working stocks should demonstrate a requirement for histidine or tryptophan (*E. coli* only).

### Experimental Methods

[0310] Master plates of the tester strains will be prepared from frozen working stocks. To create working cultures for each bacterial strain used in the assay, a single colony will be transferred from the master plate into Oxoid nutrient broth and incubated, with shaking, at  $37 \pm 2^{\circ}\text{C}$  until an optical density (at 650 nm) of 0.6-1.6 is reached. This overnight culture will be used for the mutagenicity test and for genotypic confirmation. Genotype tests will be performed as described in the protocol.

[0311] For both the dose range and mutagenicity test, a top agar consisting of 0.6% Difco agar in 0.5% NaCl will be melted and a solution of 0.5 mM L-histidine/0.5 mM biotin or 0.5 mM L-tryptophan will be added to the melted top agar at a ratio of 10 mL per 100 mL agar. The supplemented agar will be aliquotted, 2 mL per tube and held at 45-47°C. To prepare the top agar for treatment, 0.1 mL of the test article or control, 0.1 mL of the bacterial culture and 0.5 mL of phosphate buffered saline will be added to the molten agar. The mixture will be briefly vortexed and poured onto a room temperature minimal glucose agar plate (1.5% Difco agar, 2% glucose, in Vogel-Bonner medium E). Metabolic activation will be provided by adding 0.5 mL of the S9 mix in place of the PBS. The plates will be allowed to harden and

then incubated 48-72 hours at  $37 \pm 2^{\circ}\text{C}$ . All plates will be counted using an automatic image analysis system. Negative control and test article treated plates will also be examined for the presence of a bacterial lawn.

#### Exogenous Metabolic Activation

[0312] The *in vitro* metabolic activation system used in this assay is comprised of Sprague Dawley rat liver enzymes and a cofactor pool. The enzymes will be contained in a preparation of liver microsomes (S9 fraction) from rates treated with Arochlor to induce the production of enzymes capable of transforming chemicals to more active forms. Immediately prior to use, the S9 will be thawed and mixed with a cofactor pool to contain 5% S9, 5 mM glucose 6-phosphate, 4 mM  $\beta$ -nicotine-adenine dinucleotide phosphate, 8 mM  $\text{MgCl}_2$  and 33 mM KCl in a 200 mM phosphate buffer at pH 7.4.

#### Dose Levels and Replicates

[0313] The test article will be tested in triplicate at five dose levels (20.0, 10.0, 5.0, 2.5, and 1.25 mg/mL) along with appropriate vehicle (5% dextrose) and positive controls in the dose range assay. This is equivalent to 2.0, 1.0, 0.5, 0.25, and 0.125 mg/plate.

[0314] For the definitive assay, three dose levels will be chosen (10.0, 10.0, and 5.0 mg/mL), which is equivalent to 2.0, 1.0, and 0.5 mg/plate. All treatments, including negative and positive control, will be plated in triplicate against test strains TA97a, TA98, TA100, TA102, TA1535, and WP2-uvrA<sup>-</sup> in the presence and absence of metabolic activation. These doses will be chosen based on inducing a range of test article toxicity and maximizing the applied dose.

#### Control Substances

[0315] Control substances may be prepared and used in the mutagenicity assay as described in Table 9.

**Table 9**

| <b>Control</b>                      | <b>Strain</b>               | <b>Concentration</b> |
|-------------------------------------|-----------------------------|----------------------|
| ICR-191 Acridine                    | TA97a                       | 1.0 µg/plate         |
| 2-nitrofluorene                     | A98                         | 10.0 µg/plate        |
| Sodium azide                        | TA100 and TA1535            | 1.5 µg/plate         |
| 1-methyl-3-nitro-1-nitrosognanidine | WP2-uvrA <sup>-</sup>       | 4.0 µg/plate         |
| 2-aminoanthracene                   | all strains (except TA1535) | 10.0 µg/plate        |
| 2-aminoanthracene                   | TA1535                      | 1.6 µg/plate         |

Negative (Vehicle) Control

[0316] Tester strains will be plated with untreated dextrose solution at the corresponding maximum concentration (0.1 mL), with and without S9. These plates serve as the negative controls and provide information regarding background lawn and revertant colony formation.

Dose Range Assay

[0317] The initial dose range assay starts at the maximum concentration of 2.0 mg/plate. The four lower doses to be tested will be diluted in a 1:2 dilution series.

Reverse Mutation Assay

[0318] Each separate bacterial strain, with and without S9, is considered a separate experiment with its own concurrent positive and vehicle controls. All plates will be scored with an automated colony counter and a printout of the data was made. The positive controls will consist of direct-acting mutagens and mutagens requiring metabolic transformation. A two-fold or greater increase in reversion rates may be observed for all strains with the appropriate positive control. The negative control article reversion rates for each strain should be within or slightly below the expected ranges from laboratory historical data. An induced positive result for any strain would be demonstrated by at least a two-fold increase in the number of revertant colonies per plate over the negative control values.

Example 107In Vitro Chromosome Aberration Assay in CHO Cells

[0319] The Chromosomal Aberration Assay may be one of several *in vitro* tests that can be used to screen materials for their potential genetic toxicity. Chromosome aberrations are mutations which have been associated with carcinogenesis. Therefore, the chromosome aberration assay is relevant for testing potential mutagens and carcinogens (Galloway *et al.*, *Environ. Mut.* (1985) 7:1-51; Galloway *et al.*, *Environ. Mut.* (1987) 10:1-175). This Chromosome Aberration Assay evaluates the potential of the test article extracts to induce damage in Chinese Hamster Ovary Cells (CHO). This test will be conducted in the presence and absence of an exogenous mammalian activation system (S9) over three treatment periods. All negative control treated preparations should demonstrate normal levels of spontaneously occurring aberrations while positive control treated cultures should demonstrate dramatic, dose dependent increases in aberrant chromosomes.

[0320] A representative assay to determine whether a test material is clastogenic, *i.e.*, whether it has the capacity to break chromosomes may be designed as follows. Clastogenicity is an important endpoint because it is through chromosomal breakage and inappropriate rejoining that certain oncogenes (*e.g.*, *myc*) can be activated and certain tumor suppressor genes (*e.g.*, those suppressing retinoblastoma) can be inactivated). In this test, mammalian Chinese Hamster Ovary (CHO) cells will be exposed to the test material and blocked in metaphase using a spindle poison. Visualization of chromosomes will be performed microscopically after hypotonic swelling, fixing and staining the treated CHO cells. Agents found to be capable of inducing chromosome breakage have a high probability of being carcinogens and also have the potential for inducing heritable chromosomal defects.

[0321] The CHO-K<sub>1</sub> cell line (ATCC number: CCL-61) is a proline auxotroph with a modal chromosome number of 20 and a population doubling time of 10-14 hours. This system has been shown to be sensitive to the clastogenic activity of a variety of chemicals (Preston *et al.*, *Mutation Res.* (1981) 87:143-188). CHO cells will be grown and maintained in McCoy's 5A medium supplemented with 10% fetal calf serum, 1% L-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 µg/mL). Cultures will be incubated in 5-7% CO<sub>2</sub> with loose caps in a humidified incubator at 37 ± 2°C.

### Test Procedures

[0322] A stock solution will be prepared at 5 mg/mL. Lower doses will be prepared by dilution in 5% dextrose from this stock immediately prior to use. To minimize any chance of degradation, the test article solutions will be kept on ice after preparation and until just prior to dosing procedures. Cells will be seeded at approximately  $1-1.5 \times 10^6$  cells per 75 cm<sup>2</sup> tissue culture flask in 10 mL fresh medium one day prior to treatment. For treatment, spent medium will be replaced with fresh growth medium and the test article extract, negative or positive control will be added to each flask. Positive controls will be dosed in 0.1 mL volumes to minimize vehicle toxicity. The test article dilutions and negative control will be dosed in 1 mL volumes. Fresh medium will be added to bring the total treatment volume to 10 mL. For the portion of the test with metabolic activation, the S9 activation mix will be added to serum free medium at 1.5%, (v/v) final concentration. All treatments will be carried out in duplicate. The cells will be incubated at  $37 \pm 2^\circ\text{C}$  in the presence of the test article extract, the S9 reaction mixture (metabolic activation portion of the study only) and growth medium. The assay will be divided into three treatment periods: 3 hours, 3 hours with S9 activation, and 20 hours.

[0323] After the treatment period, all flasks will be evaluated microscopically for gross manifestations of toxicity. *i.e.*, morphological changes in cells or significant cell detachment. All flasks will be washed twice with phosphate buffered saline (PBS). Normal growth medium containing 10% fetal bovine serum (FBS) will be added to the freshly washed cells and the flasks will be returned to the incubator for an additional 14.5-15.5 hours. Microscopic evaluation will be performed immediately prior to harvest. Two hours prior to harvest, 1 µg of colcemid will be added (0.1 µg/mL final concentration) to all flasks to accumulate dividing cells.

[0324] The test article extracts will be tested in duplicate at six dose levels (0.5, 0.16, 0.05, 0.016, 0.005, and 0.0016 mL/mL final concentration in culture) along with appropriate vehicle and positive controls.

### Metabolic Activation System

[0325] The use of a metabolic activation system is an important aspect for evaluation of a test article, as some compounds exist only in a promutagenic state. That is, they become mutagenic only after being acted upon by an outside metabolic source. *In vitro* test systems lack this ability to metabolize compounds unless an outside system such as S9 is added.

[0326] The *in vitro* metabolic activation system to be used in this assay may comprise Sprague Dawley rat liver enzymes and an energy producing system necessary for their function (NADP and isocitric acid; core reaction mixture). The enzymes will be contained in a preparation of liver microsomes (S9 fraction) from rats treated with Arochlor 1254 to induce enzymes capable of transforming chemicals to more active forms. The S9 may be purchased from Moltox (Boone, NC) and retained frozen at less than -70°C until use. This S9 fraction will be thawed immediately before use and added to the core reaction mixture.

#### Cell Fixation, Staining and Scoring

[0327] Metaphase cells will be collected by mitotic shake off, swollen with 75 mM KCl, fixed in methanol : glacial acetic acid (3:1 v/v). Cells will be pipetted onto glass slides after resuspension in fresh fixative and air dried. The slides will be labeled with a blind code. Three slides will be prepared from each treatment flask. Slides will be stained with Giemsa and permanently mounted. All slides will be read under blind code with the exception of the high dose positive controls, which are evaluated first to ensure the aberration frequency was adequate. Two hundred cells per dose (100 from each of the duplicate flasks) will be read from each of the doses. One hundred cells will be read from each of the high dose positive controls in accordance with the following definitions and were scored as such.

#### Chromatid Type

TG (Chromatid Gap): "Tid Gap". An achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations, as they may not all be true breaks.

IG (Isochromatid Gap): "Chromosome Gap". The gaps are at the same locus in both sister chromatids. These are noted but are not usually included in final totals of aberrations, as they may not all be true breaks.

TB (Chromatid Break): An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced, or missing.

ID (Chromatid Deletion): Length of chromatid "cut" from midregion of a chromatid resulting in a small fragment or ring lying beside a shortened chromatid or a gap in the chromatid.

TR (Triradial): An exchange between two chromosomes, which results in a three-armed configuration. May have an associated acentric fragment.

QR (Quadriradial): The same as the triradial, but resulting in a four-armed configuration.

CR (Complex Rearrangement): An exchange among more than two chromosomes which is the result of several breaks and exchanges.

TI (Chromatid Interchange): Exchange within a chromosome involving one or both arms.

### Chromosome Type

SB (Chromosome Break): Terminal deletion. Chromosome has a clear break forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated and may remain associated or may appear anywhere in the cell.

DM (Double Minute Fragment): Chromosome interstitial deletion. These appear as small double “dots” or may be paired rings. In some cases, they cannot be distinguished from acentric fragments that result from exchanges or terminal deletions.

D (Dicentric): An exchange between two chromosomes that results in a chromosome with two centromeres. This is often associated with an acentric fragment in which it is classified as Dicentric with Fragment (DF).

MC (Multi-centric Chromosome): An exchange among chromosomes that results in a chromosome with more than two centromeres.

R (Ring): A chromosome that forms a circle containing a centromere. This is often associated with an acentric fragment, in which case it is classified as Ring with Fragment (RF). Acentric rings are also included in this category.

Ab (Abnormal Monocentric Chromosome): This is a chromosome whose morphology is abnormal for the karyotype, and often the result of such things as a translocation or pericentric inversion. Classification used if abnormally cannot be ascribed to, e.g., a reciprocal translocation.

T (Translocation): Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes. When identifiable, scored at “T”, not as “2 Ab”.

### Other

SD (Severely Damaged Cell): A cell with 10 or more aberrations of any type. A heavily damaged cell should be analyzed to identify the type of aberrations and may not have 10 or more, e.g., because of multiple fragments such as those found associated with a tricentric.

PU (Pulverized Chromosome): Despiralized or fragmented chromosome. This may simply be at a different stage of chromosome condensation.

P (+ Pulverized Cell): More than one chromosome, up to the whole nucleus, is “pulverized”.

PP (Polyploid Cell): A cell containing multiple copies of the haploid number of chromosomes. Polyploid cells are occasionally observed in normal bone marrow or cell culture. These are recorded but are not included in final totals of structural aberrations.

#### Control Substances

[0328] Control substances are prepared and used in this assay as described in published reports. Positive controls which may be used are: cyclophosphamide – High dose 15 µg/mL; cyclophosphamide – Low dose 5 µg/mL; mitomycin C – High dose 1.0 µg/mL; and citomycin C – Low dose 0.25 µg/mL. For negative (vehicle) control, the CHO cells are treated with the 5% dextrose negative controls with and without S9 activation. These treatments provide information regarding background numbers of aberrant cells.

#### Assay Validity Evaluation and Statistical Analysis

[0329] The total number of aberrations (%CA) of the solvent control culture(s) should fall within 1-14%. High dose positive controls should produce a statistically significant increase in the number of aberrations at the 95% confidence level ( $p < 0.05$ ) as determined by statistical analysis. Analysis of Variance (ANOVA) may be used to identify significant differences between positive and negative control groups or test article and negative control groups. A difference is considered significant when the p value obtained is less than 0.05.

#### Example 108

##### Safety and Tolerance Determination in Dogs

[0330] A representative study for determining the safety and tolerance of compounds at dose levels administered intravenously once daily to beagle dogs for five consecutive days, for example, may be designed as follows. Safety parameters will be monitored through observation, clinical pathology, and microscopic histopathology assessments.

### Experimental Design

[0331] Table 10 summarizes a representative study. For example, the study will be conducted using three (3) test article and one (1) control article group. The control article will be the solution (5% dextrose in water) used to dilute the test article prior to administration and will be administered at the same volume as the high dose. The test article dosage levels for this study will be approximately 12, 3.8, and 1.2 mg/kg. Test and control articles will be administered once by intravenous (IV) infusion over approximately a one hour period on five consecutive days.

[0332] Blood samples for test article blood level analysis will be taken as follows (*i.e.*, pk/tk sampling). Approximately 1.0 mL of blood will be taken from three male and three female dogs in the low dose group at approximately 20 minutes and 40 minutes from the start of the infusion, and then at the end of infusion (Time 0) and at 5, 10, 15, and 30 minutes, and 1, 2, 4, 8, 12, and 24 hours from the end of the infusion after the first and fifth doses. Also, prior to and immediately after Dose 1 and after Dose 5 for all animals, and for recovery animals prior to necropsy, approximately 5-10 second ECG tracings in a lead II configuration will be obtained. Animals will be terminated one (1) or 15 days after the last dose. Blood for hematology and clinical chemistry analysis will be drawn pre-dose and prior to euthanasia at termination. Following euthanasia, a necropsy will be performed to include collection of major organs for microscopic evaluation.

**Table 10**

| GROUP No. | ARTICLE <sup>a</sup> | DOSAGE (MG/KG) | PRIMARY NO. ANIMALS (MALE/FEMALE) | RECOVERY (15 DAY) NO. ANIMALS (MALE/FEMALE) |
|-----------|----------------------|----------------|-----------------------------------|---------------------------------------------|
| 1         | Control              | 0.0            | 3/3                               | 1/1                                         |
| 2         | Test Article         | 12.0           | 3/3                               | 1/1                                         |
| 3         | Test Article         | 3.8            | 3/3                               | 1/1                                         |
| 4         | Test Article         | 1.2            | 3/3                               | 1/1                                         |

<sup>a</sup> Delivered as an approximate 1 hour infusion

### Test Methods

[0333] In a representative study, animals will be assigned to groups as follows: The heaviest dog for a sex will be assigned to Group 1, the next heaviest for that sex will be assigned to Group 2, the next heaviest to Group 3, the next heaviest to Group 4, then continue with Groups 2, 3, 4, and 1, then Groups 3, 4, 1, and 2, continuing with this pattern until each group had a full complement of animals. The test and control article will be administered at

each dosing as an intravenous infusion into a cephalic or saphenous vein over approximately one hour.

[0334] Animals will be weighed daily prior to dosing and prior to necropsy. All animals will be observed for signs of pharmacological activity, behavioral changes, and toxicity immediately and one hour after dosing. Recovery animals will be also observed once daily during the recovery period. Prior to and immediately after Doses 1 and 5 for all animals, and for recovery animals prior to necropsy, approximately five second ECG tracings in a lead II configuration will be obtained. These tracings will be used to provide data for interpretation of the rhythm and amplitude changes of the QRS-complex and T-wave and to measure QT intervals on a number of segments per tracing (approximately 5-10).

#### Blood Collection

[0335] PK/TK. Blood samples for test article blood level analysis will be taken. Approximately 1 mL of blood will be taken from three males and three females in the low dose group at approximately 20 minutes and 40 minutes from the start of the infusion, and then at the end of infusion (Time 0) and at 5, 10, 15, and 30 minutes, and 1, 2, 4, 8, 12, and 24 hours from the end of the infusion after the first and fifth dose. Plasma (lithium heparin anticoagulant) samples will be prepared for analysis.

[0336] Clinical Pathology. After overnight fasting and prior to the first dose (baseline; all animals) and then prior to each necropsy, blood samples will be taken for hematology and clinical chemistry. For hematology assays, blood collected at baseline and prior to necropsy (fasted) are analyzed for erythrocyte count, hematocrit, MCH, leukocyte count, differential WC, MCHC, hemoglobin, MCV, platelet count, PT, and APTT. For clinical chemistry assays, blood collected at baseline and prior to necropsy (fasted) will be tested for: aspartate aminotransferase (ASP), globulin & A/G ratio, Alanine aminotransferase (ALT), sodium, alkaline phosphatase, potassium, gamma glutamyltransferase (GGT), chloride, glucose, calcium, blood urea nitrogen (BUN), total bilirubin, creatinine, inorganic phosphorus, total protein, cholesterol, albumin, and triglycerides.

#### Necropsy

[0337] Following blood sample collection, primary treatment and recovery group animals will be sacrificed at their respective termination times and are necropsied. Major organs will be collected, weighed, and preserved for microscopic evaluation. Necropsy will include

examination of the cranial, thoracic, abdominal and pelvic cavities, their viscera, the tissues, organs, and the carcass.

#### Statistical Methods

[0338] Statistical analysis of the clinical chemistry and hematology values and organ and body weight data will be performed to compare the test article groups to the control group. The statistical methods used for the data will be selected as appropriate: parametric data will be analyzed using a one way Analysis of Variance, non-parametric data will be analyzed using the Kurskai-Wallis test. A paired t-test will also be used to compare baseline and post treatment clinical chemistry and hematology values for each animal. Probability (p) values of 0.05 or less will be considered significant for all statistical tests.

#### Example 109

##### Safety and Tolerance Study in Rats

[0339] A representative study to determine the safety and tolerance of a test compound, for example, at three dose levels administered intravenously once daily to rats for five consecutive days may be designed as follows. Safety parameters will be monitored through observation, clinical pathology, and microscopic histopathology assessments. Selected animals will also undergo blood sample collection for pharmacokinetic/toxicokinetic evaluation.

#### Experimental Methods

[0340] Table 11 summarizes a representative study. The study will be conducted using three (3) test and one (1) control article groups. The high and low test article groups and the control group will consist of 28 animals each and will be used to assess tolerance. The medium test article group will consist of 64 animals, of which 28 animals will be used to assess tolerance and 36 animals will be used to determine the level of test article in the blood at various time points after the first and fifth doses in the PK/TK portion of the study. The control article will be the solution (5% dextrose in water; D5W) used to dilute the test article prior to administration and is administered at the same volume as the high dose test article group. The test article dosage levels for this study will be 24, 7.6, and 2.4 mg/kg. Test and control articles will be administered by intravenous (IV) injection into a tail vein over one minute on five consecutive days.

[0341] Blood samples for test article blood level analysis will be taken as follows. Approximately 0.3 - 0.5 mL of blood will be taken from three male and three female rats under anesthesia at each sample time point of pre-dose and at the end of injection (Time 0) and at approximately 0.08, 0.25, 0.5, 1, 2, 4, 8, 12, and 24 hours from the end of the injection after the first and fifth doses. Animals used to assess tolerance will be terminated one day (for the primary group) or 15 days (for the recovery group) after the last dose. At termination of the tolerance test animals, blood for hematology and clinical chemistry analysis will be drawn prior to euthanasia and following euthanasia. A necropsy will be performed to include collection of major organs for microscopic evaluation. The animals used for the pk/tk blood sampling only to determine the level of test article will be euthanized after the final blood sample is collected without any further sampling or observations.

**Table 11**

| GROUP No. | ARTICLE <sup>a</sup> | DOSAGE (MG/KG) | PRIMARY NO. ANIMALS (MALE/FEMALE) | RECOVERY (15 DAY) NO. ANIMALS (MALE/FEMALE) |
|-----------|----------------------|----------------|-----------------------------------|---------------------------------------------|
| 1         | Control              | 0.0            | 3/3                               | 1/1                                         |
| 2         | Test Article         | 12.0           | 3/3                               | 1/1                                         |
| 3         | Test Article         | 3.8            | 3/3                               | 1/1                                         |
| 4         | Test Article         | 1.2            | 3/3                               | 1/1                                         |

<sup>a</sup> Delivered as an approximate 1 hour infusion

### Test Methods

[0342] The test and control article will be administered at each dosing as an intravenous infusion into a tail vein over approximately one minute. Animals will be weighed daily prior to dosing and prior to necropsy. All animals will be observed for signs of pharmacological activity, behavioral changes, and toxicity immediately and one hour after dosing. Recovery animals will also be observed once daily during the recovery period. The control animals will be dosed with approximately 6 mL/kg of D5W. The high, mid, and low dose test article animals will be administered dosages of approximately 24 mg/kg, 7.6 mg/kg, and 2.4 mg/kg, respectively.

### Blood Collection

[0343] PK/TK. Blood samples for test article blood level analysis will be taken. Utilizing 18 male and 18 female medium dose animals, approximately 0.3-0.5 mL of blood will be taken from three male and three female rats under anesthesia at each sampling time point of pre-dose and at the end of injection (Time 0), and at approximately 0.08, 0.25, 0.5, 1, 2, 4, 8, 12, and 24

hours from the end of the injection after the first and fifth dose. Blood sampling will be via retro-orbital bleeding or cardiac puncture bleeding for an animal's terminal sample. Plasma (lithium heparin anticoagulant) samples will be prepared for analysis. General procedures for chemical pathology, necropsy, and histopathology, as well as statistical methods, such as those previously described, will be followed.

#### Example 110

##### Phosphorylated and Total p53 Assay Protocol

[0344] A phosphorylated and total p53 assay protocol may be designed as follows. On Day 1, cells are seeded at  $2 \times 10^6$  cells/10 cm dish/10 mL medium. On day two, cells will be treated as follows: control = 0.05% DMSO (5 $\mu$ l DMSO stock / 10ml medium); 1 $\mu$ M test compound (1 $\mu$ l Stock (10mM) / 10ml medium); 2 $\mu$ M test compound (2 $\mu$ l Stock (10mM) / 10ml medium); 3 $\mu$ M test compound (3 $\mu$ l Stock (10mM) / 10ml medium); 4 $\mu$ M test compound (4 $\mu$ l Stock (10mM) / 10ml medium) and 5 $\mu$ M test compound (5 $\mu$ l Stock (10mM) / 10ml medium).

[0345] On Day 3, cells will be harvested and attached and floating cells will be collected. Cells will be washed twice with PBS, counted and collected at  $4 \times 10^6$  cells/sample. The cell pellet will be frozen at -80°C until further use. On the same day or on Day 4, cells will be extracted using a cell extraction buffer (3 mL cell extraction buffer, 300  $\mu$ l protease inhibitor and 10  $\mu$ l 0.3M PMSF). To each sample will be added 200 $\mu$ l Buffer, and the solution will be vortexed and set on ice for 30 minutes, and subsequently vortexed after every 10 mins. The solution will be then centrifuged at 13,000rpm for 10min, and 100 $\mu$ l supernatant per tube will be aliquoted and stored at - 80°C.

[0346] Assay preparation (Day 5). An anti-rabbit IgG HRP solution will be prepared by diluting 10 $\mu$ l of 100x concentrate solution with 1ml HRP diluent for each 8-well strip. A wash buffer solution will be prepared by diluting the original vial (x25) using distilled water to make a x1 solution. Dilutions of p53 standard solution or p53 total solution can be prepared as described according to representative parameters of Table 12. To ensure complete reconstitution, standard 1 will be mixed gently and allowed to sit for 10 minutes at room temperature.

**Table 12**

|            | <b>Conc.</b>  | <b>Standard Soln.</b>                                   | <b>Dilution Buffer</b> |
|------------|---------------|---------------------------------------------------------|------------------------|
| Standard 1 | 100 Units/ml  | Reconstitute 1 Vial worth 0.7ml of standard Dil. Buffer |                        |
| Standard 2 | 50 Units/ml   | 250µl of Standard 1                                     | 250µl                  |
| Standard 3 | 25 Units/ml   | 250µl of Standard 2                                     | 250µl                  |
| Standard 4 | 12.5 Units/ml | 250µl of Standard 3                                     | 250µl                  |
| Standard 5 | 6.25 Units/ml | 250µl of Standard 4                                     | 250µl                  |
| Standard 6 | 3.12 Units/ml | 250µl of Standard 5                                     | 250µl                  |
| Standard 7 | 1.6 Units/ml  | 250µl of Standard 6                                     | 250µl                  |
| Standard 8 | 0             |                                                         | 250µl                  |

**[0347]** Test Procedure. Allow all solution to reach RT and mix gently before use. Take out and insert 8-well strips. Add 100µl of standard dilution buffer to standard 8 well (0 ng/ml/well or 0 Units/well). Add nothing to the chromogen blank well. Add 100µl of standard or diluted sample to the appropriate microtiter wells. Generally, the sample should be diluted with standard dilution buffer at least 1: 10 or greater. Each sample will be run in duplicates. Gently tap the side of the plate to thoroughly mix. Cover plate with plate cover and incubate for 2 hours at RT or o/n at 4C. Wash wells with 400µl working wash buffer 4 times. Let soak for 15-30sec., and then aspirate the liquid. After washing, the plate will be inverted and tapped dry on absorbance tissue. Add 100µl of anti-p53 [pS15] or anti-p53 (total) (detection antibody) to each well except chromogen blank. Tap gently to mix; cover plate and incubate 1 hour at RT. Aspirate solution from wells thoroughly.

**[0348]** Wash wells with 400µl working wash buffer four times. Let soak for 15-30sec., and then aspirate the liquid. After washing, the plate will be inverted and tapped dry on absorbance tissue. Add 100µl of anti-rabbit IgG HRP working solution. to each well except chromogen blank. Cover plate and incubate 30 min at RT. Wash wells with 400 µl working wash buffer four times. Let soak for 15-30 sec., and then aspirate the liquid. After washing, the plate will be inverted and tapped dry on absorbance tissue. Add 100µl of TMB (stabilized chromogen substrate) to each well and incubate for 30 min. at RT in the dark. The color will change to blue. Add 100µl Stop soln. Tap plate gently to mix. The color should change to yellow. Read the plate at A450nm by setting chromogen blank (= 100µl TMB + 100µl Stop soln) as blank. Read absorbance within 2 hours of assay completion.

### Example 111

#### Caspase-3/7 Assay Protocol

[0349] A representative Caspase-3/7 assay protocol may be designed as follows. On Day 1, seed  $0.015 \times 10^6$  HCT-116 cells / 50ul / well. Incubate o/n in 37°C CO<sub>2</sub> incubator. On Day 2, remove 25ul of medium from wells. Treat HCT-116 cells with 1, 3, and 5 uM test compound. Treat positive control group with Staurosporin 0.01, 0.1, 1 uM. Keep six negative control wells treated with medium only (add 25ul of diluted sample to appropriate wells). Incubate for 24h at 37°C in a CO<sub>2</sub> incubator. On Day 3, prepare Apo-ONE Homogeneous Caspase-3/7 assay reagent (Promega) at 10ul reagent / 1ml buffer. Add 50ul of diluted reagent. Incubate one hour at room temp. Measure fluorescence at 485/520.

### Example 112

#### Annexin V-Alexa 488 Staining Protocol

[0350] A representative Annexin V-Alexa 488 staining protocol may be designed as follows. Seed  $1.5 - 2.0 \times 10^6$  HCT-116 cells / 10cm dish/ 10ml medium. Incubate o/n or up to 24hrs at 37°C in CO<sub>2</sub> incubator. The following day, treat cells with 1, 2, 3, 4 and 5µM test compound. Keep one or two untreated plates (medium only) as control plates. The following controls are used: untreated samples (no Alexa or propidium iodide), controls treated with propidium iodide or Alexa 488 only, and controls treated with both Alexa 488 and propidium iodide. Harvest cells (collect attached as well as floating cells). Wash cells twice with cold PBS. Re-suspend cells in 1x Annexin binding buffer.

[0351] Count cells and dilute in 1x Annexin binding buffer to  $\sim 1 \times 10^6$  cells/0.1ml, preparing a sufficient volume to have 100µl per assay. Add 5µl of the Annexin V conjugate to each 100µl of cell suspension. Add 4µl of propidium iodide solution (stock = 1mg/ml) to each 100µl of cell suspension. Incubate sample at RT for 15 minutes. Add 400µl Annexin binding buffer, mix gently and keep samples on ice. Analyze stained cells immediately by flow cytometry.

### Example 113

#### DNA Cell Cycle Analysis Protocol

[0352] A representative DNA cell cycle analysis protocol will be designed as follows. Seed  $1.5 - 2.0 \times 10^6$  cells/ 10cm dish (seed one extra dish for unstained cells). Incubate cells in

37°C humidified 5% CO<sub>2</sub> incubator for 24 hours. For synchronizing cells in a low growth state to make cells quiescent, remove media and rinse once with serum-free media, add 10ml of serum-free media to each dish. Incubate the cells for 24 hr in a 37°C humidified 5% CO<sub>2</sub> incubator. Remove media and add treatment (diluted in serum contained media, 10ml): 1-5μM test compound plus control. Incubate the cells for 24 hr in a 37°C humidified 5% CO<sub>2</sub> incubator.

[0353] To trypsinize/isolate cells, remove treatment. Add 3 ml trypsin/EDTA solution. Keep floating cells and combine with attached cells. Incubate for 5 min in a 37°C humidified 5% CO<sub>2</sub> incubator. Add 3ml media (containing FBS) to wells and pipette into centrifuge tube. Centrifuge at 1000rpm for 5 minutes. Decant supernatant and re-suspend pellet in 2-3ml PBS. Count cells and wash cells once by putting  $2 \times 10^6$  cells/ tube, adding 2ml PBS and centrifuging at 1000rpm for 5 minutes. Re-suspend pelleted cells in 0.3ml cold PBS.

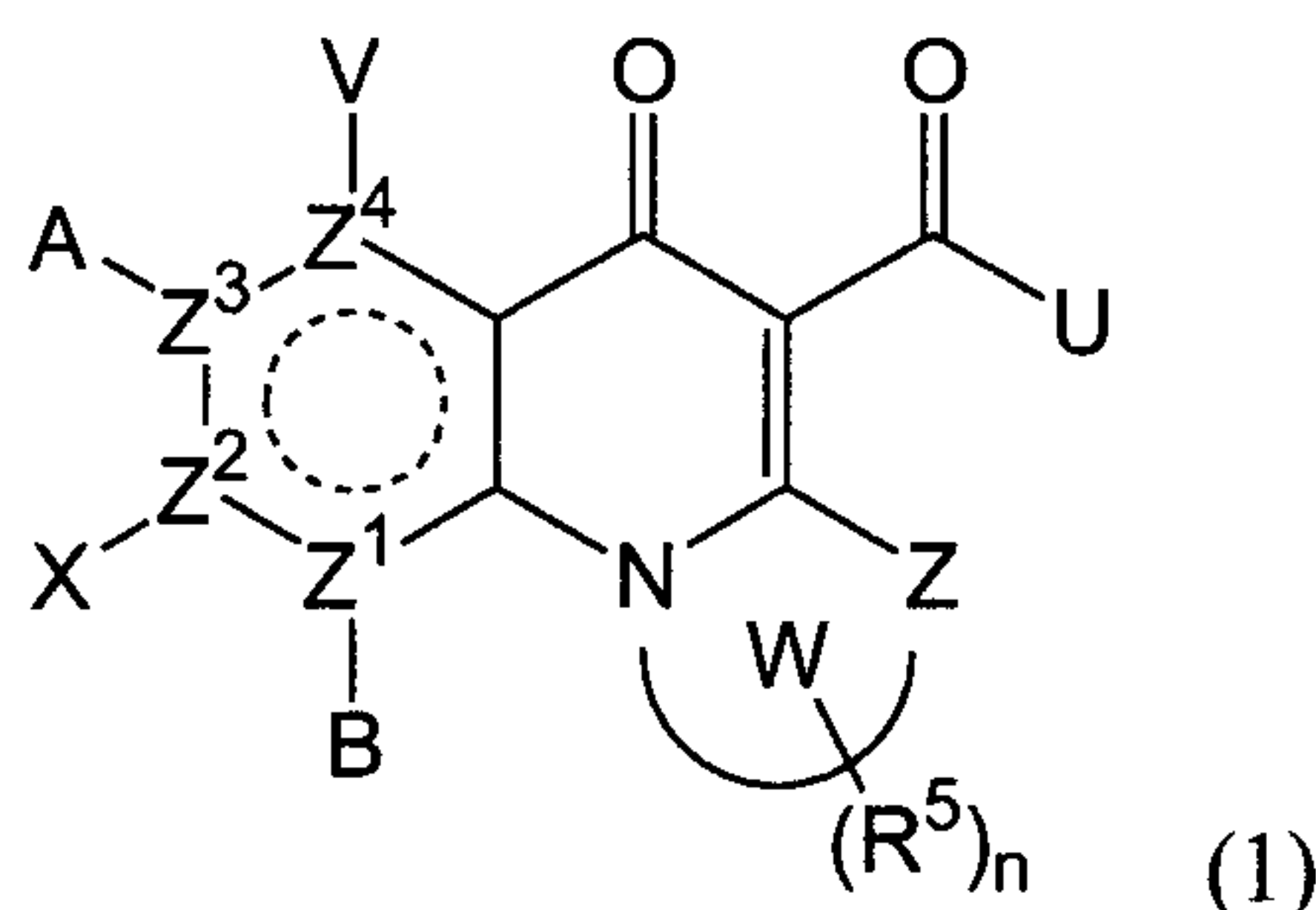
[0354] To fix cells, gently add 0.7ml ice cold 70% ethanol drop wise to tube containing 0.3ml of cell suspension in PBS while vortexing. Leave on Ice for one hour (or up to a few days at 4°C). Centrifuge at 1000rpm for 5 minutes. Wash one time with cold PBS (1-2 ml). Centrifuge at 1000rpm for 5 minutes. Re-suspend cell pellet in 0.25ml cold PBS, add 5μl of 10mg/ml RNase A (the final concentration being 0.2-0.5mg/ml). Incubate at 37°C for 1 hour. Add 10μl of 1mg/ml of propidium iodide solution in deionized water (the final concentration being 10μl/ml), and keep in the dark and at 4°C until analysis. Analyze on FACS by reading on cytometer at 488nm. Cells may be stained with propidium iodide on the same day of analysis.

[0355] It is understood that the foregoing detailed description and accompanying examples are merely illustrative, and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art. Such changes and modifications, including without limitation those relating to the chemical structures, substituents, derivatives, intermediates, syntheses, formulations and/or methods of use of the invention, may be made without departing from the spirit and scope thereof.

## Claims

### What is claimed is:

1. A compound having formula (1):



and pharmaceutically acceptable salts and esters thereof;

wherein B, X, A, or V is absent if  $Z^1$ ,  $Z^2$ ,  $Z^3$ , and  $Z^4$  are N, respectively, and independently H, halo, azido,  $R^2$ ,  $CH_2R^2$ ,  $SR^2$ ,  $OR^2$  or  $NR^1R^2$  when  $Z^1$ ,  $Z^2$ ,  $Z^3$ , and  $Z^4$  are C; or

A and V, A and X, or X and B may form a carbocyclic ring, heterocyclic ring, aryl or heteroaryl, each of which may be optionally substituted and/or fused with a cyclic ring;

Z is O, S,  $NR^1$ ,  $CH_2$ , or  $C=O$ ;

$Z^1$ ,  $Z^2$ ,  $Z^3$  and  $Z^4$  are C or N, provided any two N are non-adjacent;

W together with N and Z forms an optionally substituted 5- or 6-membered ring that is fused to an optionally substituted saturated or unsaturated ring that optionally contains a heteroatom and is monocyclic or fused with a single or multiple carbocyclic or heterocyclic rings;

U is  $NR^1R^2$ ,  $NR^1 - (CR^1_2)_n - NR^3R^4$ , or  $N=CR^1R^2$ , wherein in  $N=CR^1R^2$ ,  $R^1$  and  $R^2$  together with C may form a ring,

provided when U is  $NH_2$ , then at least one of  $Z^1$ - $Z^4$  is N;

in each  $NR^1R^2$ ,  $R^1$  and  $R^2$  together with N may form an optionally substituted ring;

in  $NR^3R^4$ ,  $R^3$  and  $R^4$  together with N may form an optionally substituted ring;

$R^1$  and  $R^3$  are independently H or  $C_{1-6}$  alkyl;

each  $R^2$  is H, or a  $C_{1-10}$  alkyl or  $C_{2-10}$  alkenyl each optionally substituted with a halogen, one or more non-adjacent heteroatoms, a carbocyclic ring, a heterocyclic ring, an

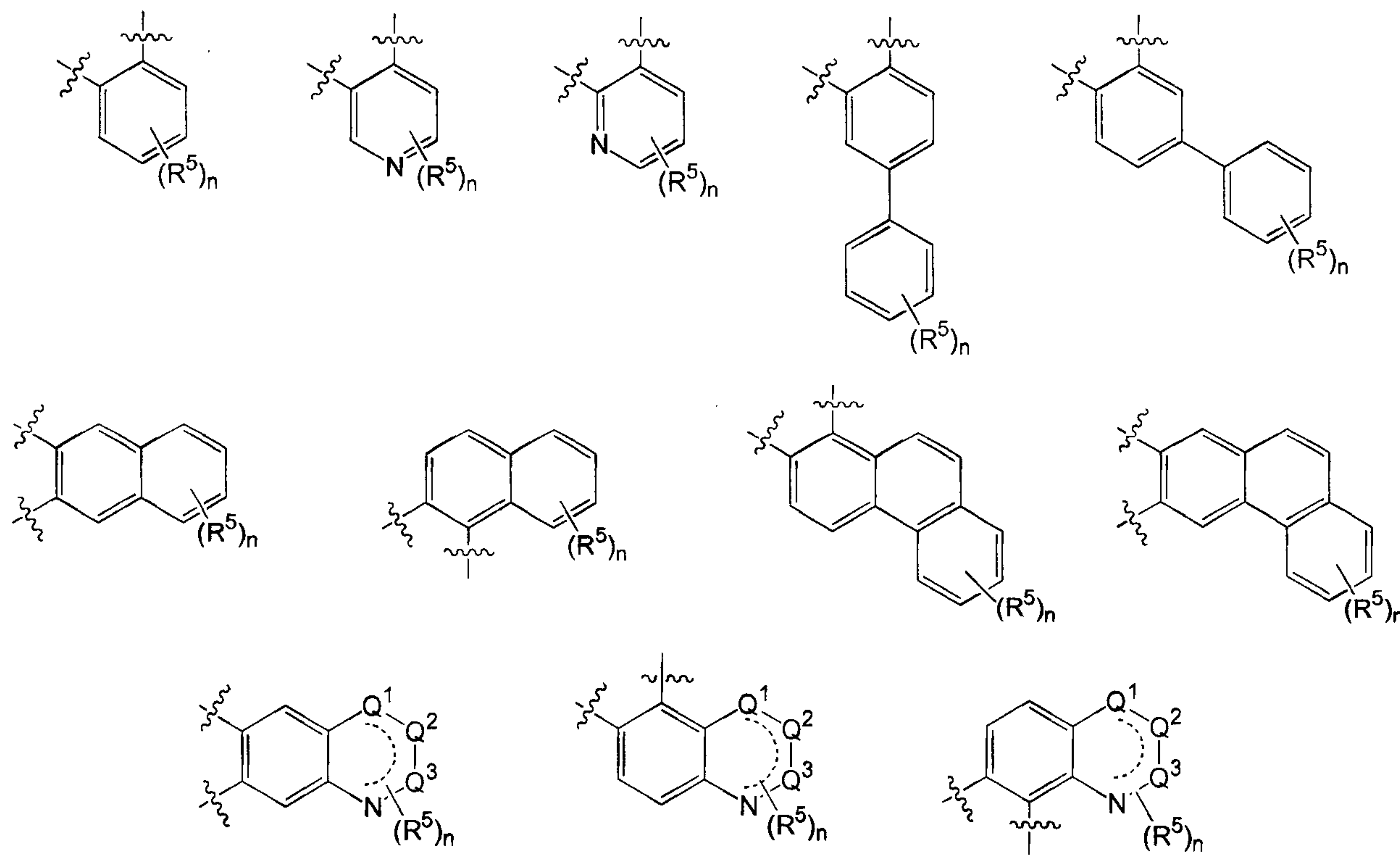
aryl or heteroaryl, wherein each ring is optionally substituted; or  $R^2$  is an optionally substituted carbocyclic ring, heterocyclic ring, aryl or heteroaryl;

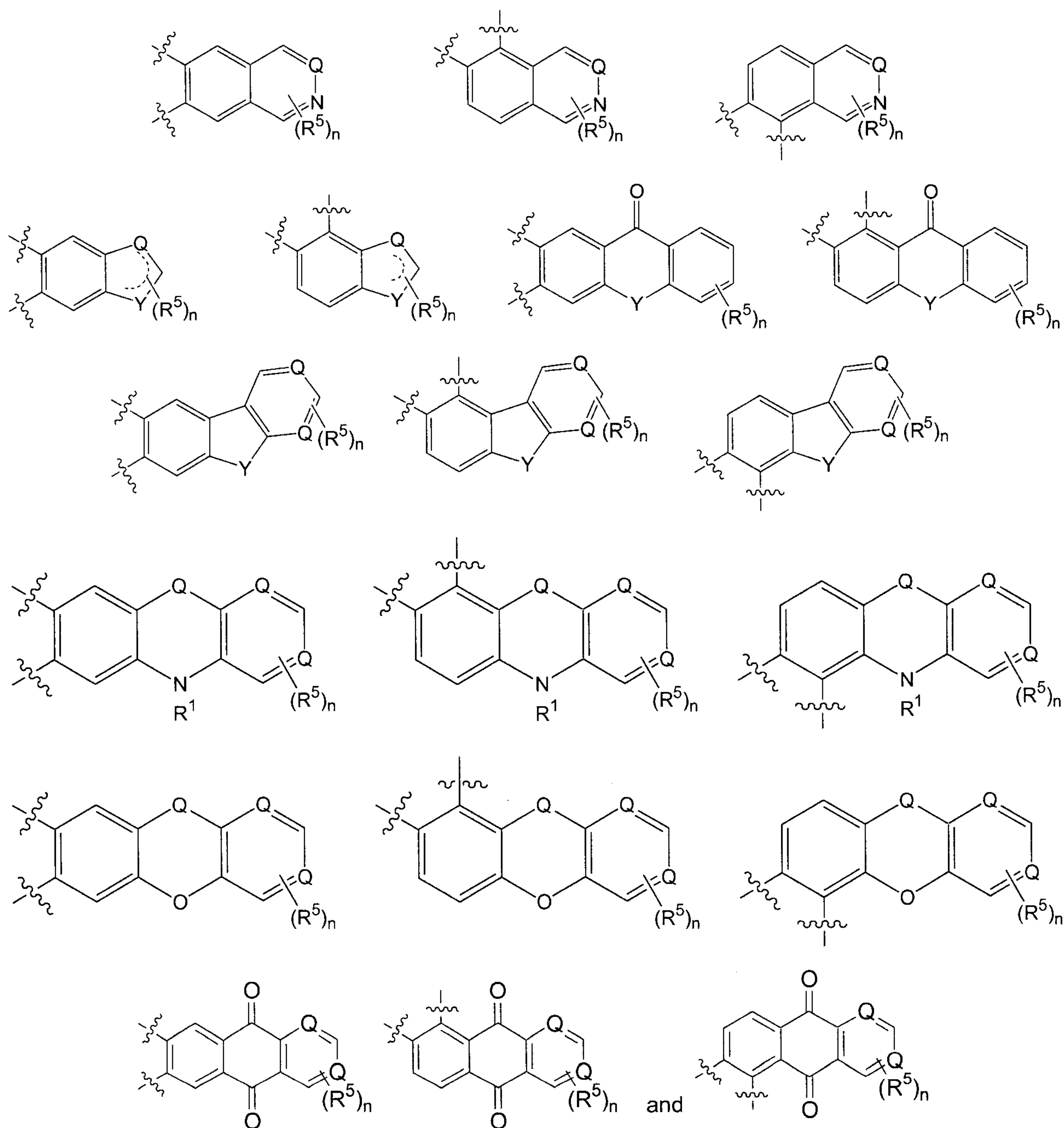
$R^4$  is H, a  $C_{1-10}$  alkyl or  $C_{2-10}$  alkenyl optionally containing one or more non-adjacent heteroatoms selected from N, O and S, and optionally substituted with a carbocyclic or heterocyclic ring; or  $R^3$  and  $R^4$  together with N may form an optionally substituted ring;

each  $R^5$  is a substituent at any position on ring W; and is H,  $OR^2$ , amino, alkoxy, amido, halogen, or cyano; or  $R^5$  is  $C_{1-6}$  alkyl,  $C_{2-6}$  alkenyl,  $C_{2-6}$  alkynyl,  $-CONHR^1$ , each optionally substituted by halo, carbonyl or one or more non-adjacent heteroatoms; or two adjacent  $R^5$  are linked to obtain a 5-6 membered optionally substituted carbocyclic or heterocyclic ring that may be fused to an additional optionally substituted carbocyclic or heterocyclic ring; and

n is 1-6.

2. The compound of claim 1, wherein W together with N and Z form an optionally substituted 5- or 6-membered ring that is fused to an optionally substituted aryl or heteroaryl selected from the group consisting of

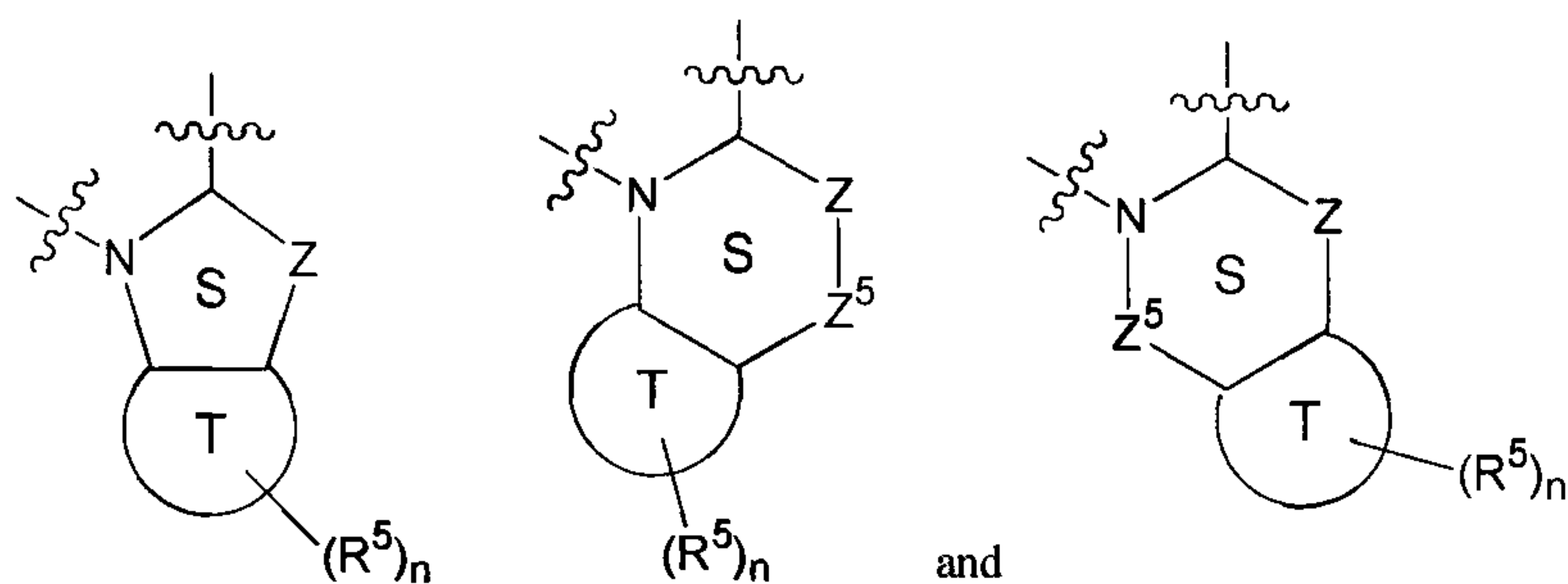




wherein each  $Q$ ,  $Q^1$ ,  $Q^2$ , and  $Q^3$  is independently CH or N; and

$Y$  is independently O, CH, C=O or  $NR^1$ ; or

$W$  together with N and Z form a ring selected from the group consisting of



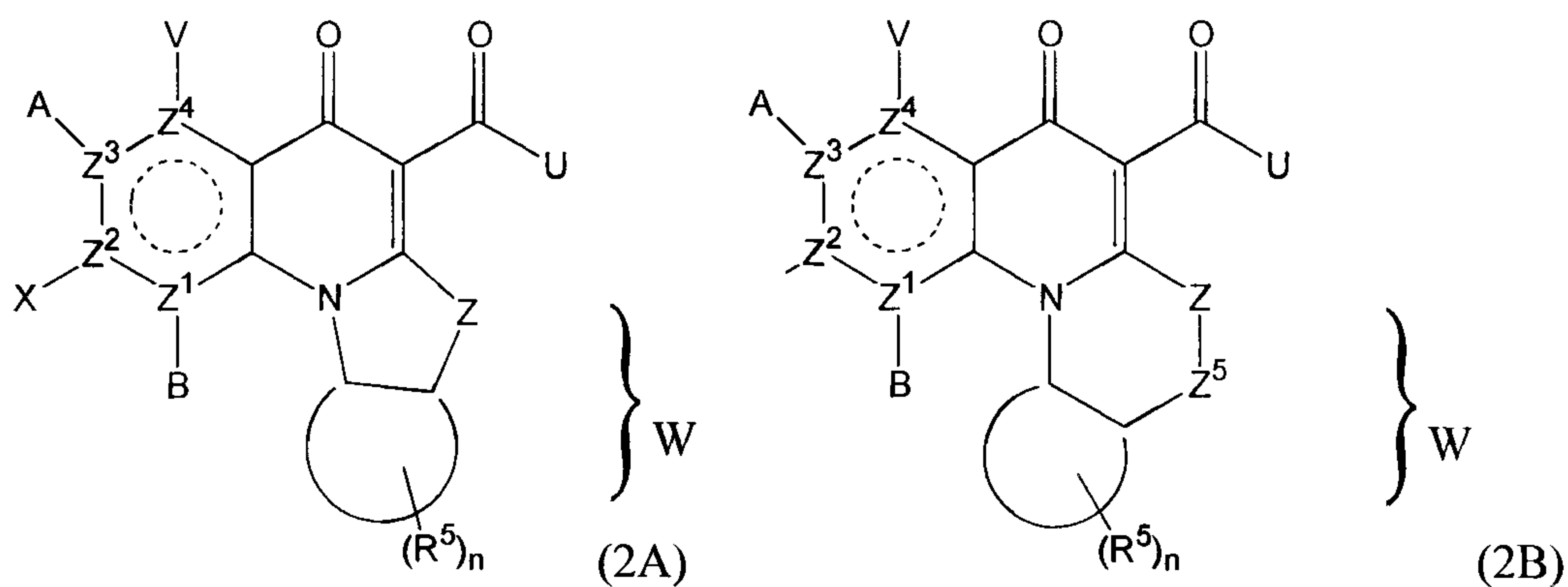
wherein each  $Z^5$  is  $CR^6$ ,  $NH$ , or  $C=O$ ;

$R^6$  is H, hydroxyl, alkyl, alkoxy, halo, amino, or amido; and

ring S and ring T may be saturated or unsaturated;

and wherein Z,  $R^1$  and  $R^5$  are as defined in claim 1 and  $n = 1-5$ .

3. The compound of claim 1, wherein said compound has formula (2A) or (2B):



wherein  $Z^5$  is O,  $NR^1$ ,  $CR^6$ , or  $C=O$ ;

$R^6$  is H,  $C_{1-6}$  alkyl, hydroxyl, alkoxy, halo, amino or amido; and

Z and  $Z^5$  optionally form a double bond;

and wherein A, B, V, X, U, Z,  $Z^1$ ,  $Z^2$ ,  $Z^3$ ,  $Z^4$ , W,  $R^1$ , and  $R^5$  are as defined in claim 1 and  $n = 1-5$ .

4. The compound of claim 1, wherein W together with N and Z forms a 5- or 6-membered ring that is fused to a phenyl.

5. The compound of any one of claims 1 to 4, wherein each of  $Z^1$ ,  $Z^2$ ,  $Z^3$  and  $Z^4$  are C.
6. The compound of any one of claims 1 to 4, wherein three of  $Z^1$ ,  $Z^2$ ,  $Z^3$  and  $Z^4$  is C, and the other is N.
7. The compound of any one of claims 1 to 4, wherein two of  $Z^1$ ,  $Z^2$ ,  $Z^3$  and  $Z^4$  are C, and the other two are non-adjacent nitrogens.
8. The compound of any one of claims 1 to 4, wherein  $Z^1$  and  $Z^3$  are C, and  $Z^2$  and  $Z^4$  are N; wherein  $Z^1$  and  $Z^3$  are N, and  $Z^2$  and  $Z^4$  are C; or wherein  $Z^1$  and  $Z^4$  are N, and  $Z^2$  and  $Z^3$  are C.
9. The compound of any one of claims 1 to 8, wherein at least one of B, A, X or V is halo, and the corresponding adjacent  $Z^1$ - $Z^4$  is C.
10. The compound of any one of claims 1 to 4, 6 and 7, wherein  $Z^1$  is N.
11. The compound of any one of claims 1 to 10, wherein V is H.
12. The compound of any one of claims 1 to 11, wherein Z is  $NR^1$  and  $R^1$  is  $C_{1-6}$  alkyl.
13. The compound of any one of claims 1 to 12, wherein  $R^1$  is methyl.
14. The compound of any one of claims 1 to 13, wherein A and X are independently halo or  $SR^2$ , wherein  $R^2$  is a  $C_{1-10}$  alkyl or  $C_{2-10}$  alkenyl optionally substituted with a heteroatom, a carbocyclic ring, a heterocyclic ring, an aryl or a heteroaryl.
15. The compound of any one of claims 1 to 14, wherein U is  $NR^1R^2$ , wherein  $R^1$  is H, and  $R^2$  is a  $C_{1-10}$  alkyl optionally substituted with a heteroatom, a  $C_{3-6}$  cycloalkyl, aryl or a 5-14 membered heterocyclic ring containing one or more N, O or S; or  $R^1$  and  $R^2$  together with

N form an optionally substituted piperidine, pyrrolidine, piperazine, morpholine, thiomorpholine, imidazole, or aminodithiazole.

16. The compound of any one of claims 1 to 15, wherein  $R^2$  is a  $C_{1-10}$  alkyl substituted with morpholine, thiomorpholine, imidazole, aminodithiadazole, pyrrolidine, piperazine, pyridine or piperidine.

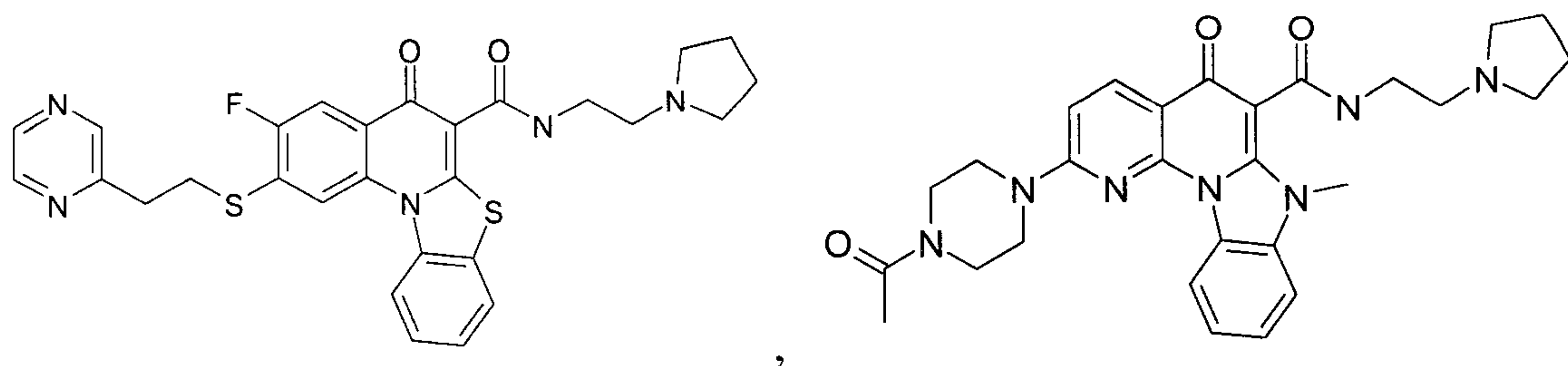
17. The compound of any one of claims 1 to 15, wherein  $R^2$  is a  $C_{1-10}$  alkyl substituted with phenyl or pyrazine.

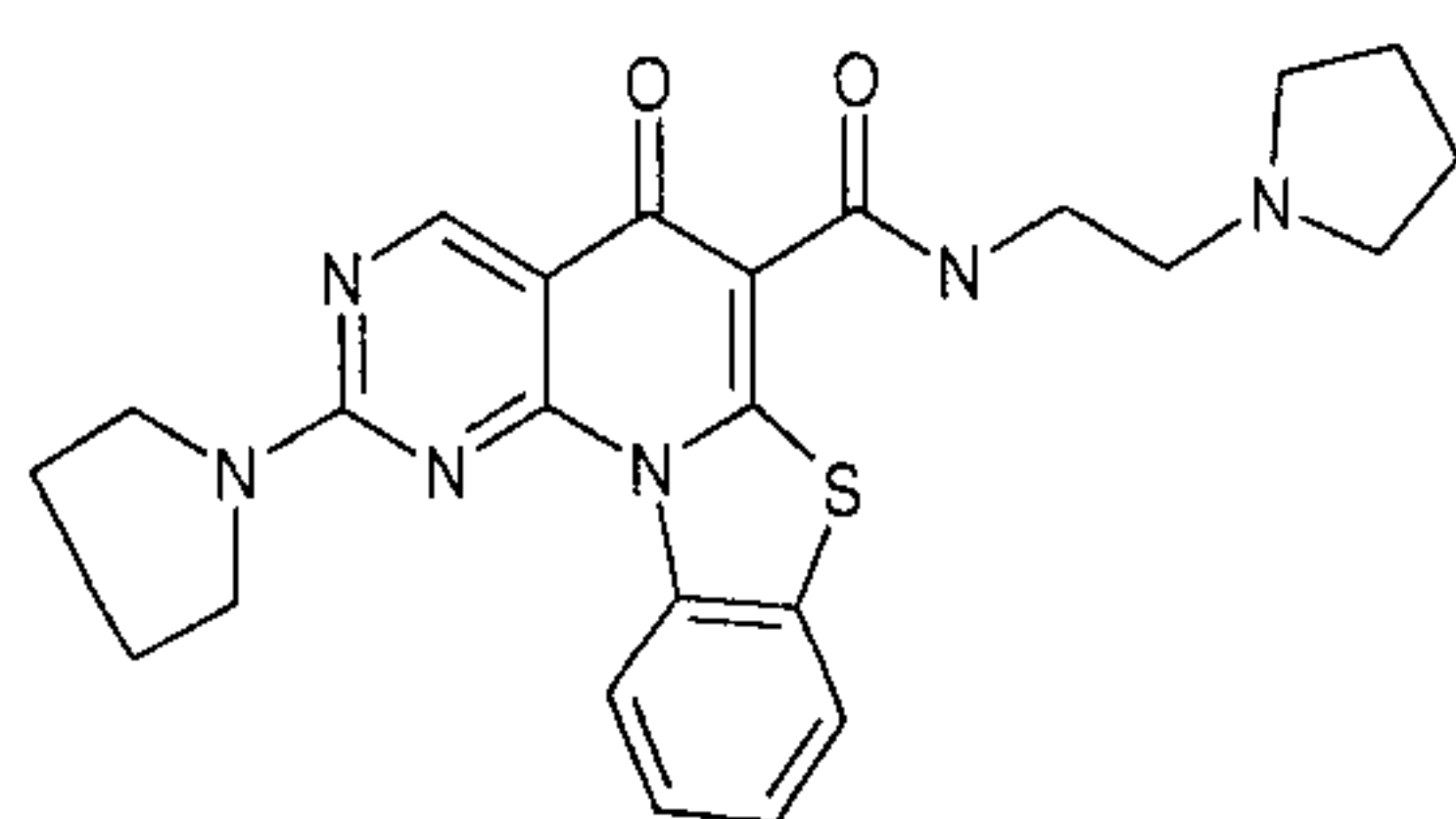
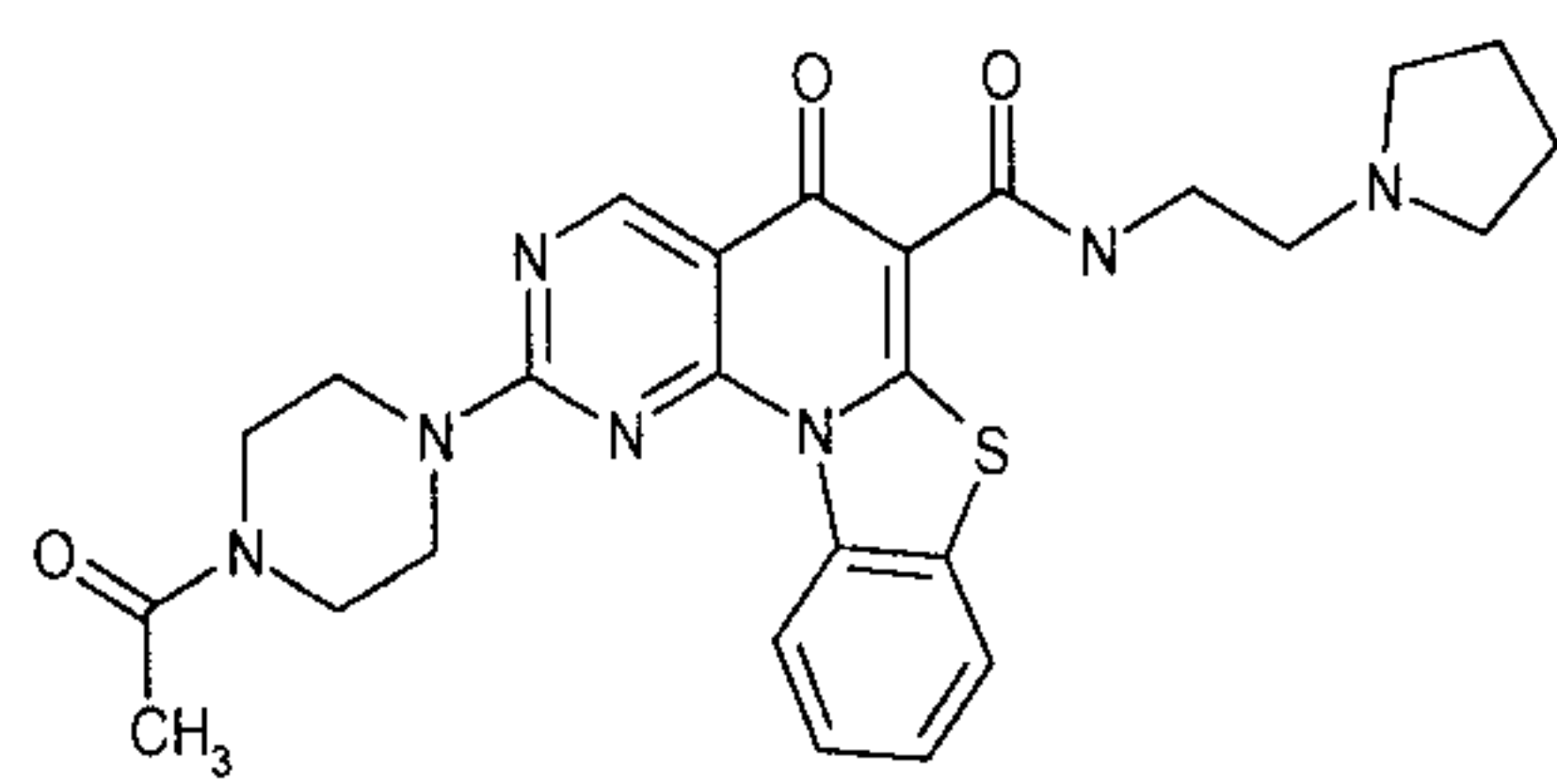
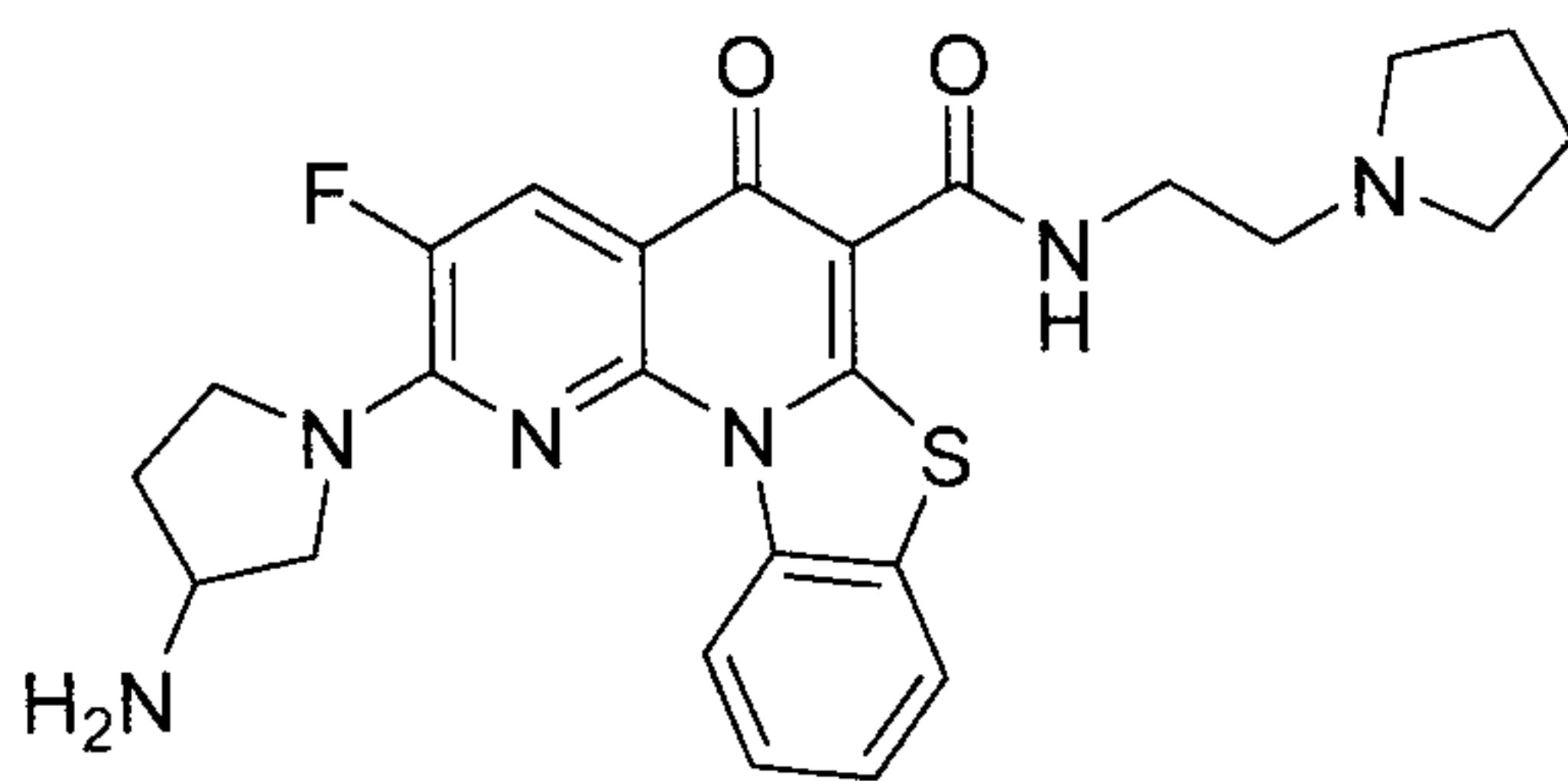
18. The compound of any one of claims 1 to 14, wherein U is  $NR^1 - (CR^2)_n - NR^3R^4$ ; n is 1-4; and  $R^3$  and  $R^4$  in  $NR^3R^4$  together form an optionally substituted piperidine, pyrrolidine, piperazine, morpholine, thiomorpholine, imidazole, or aminodithiazole.

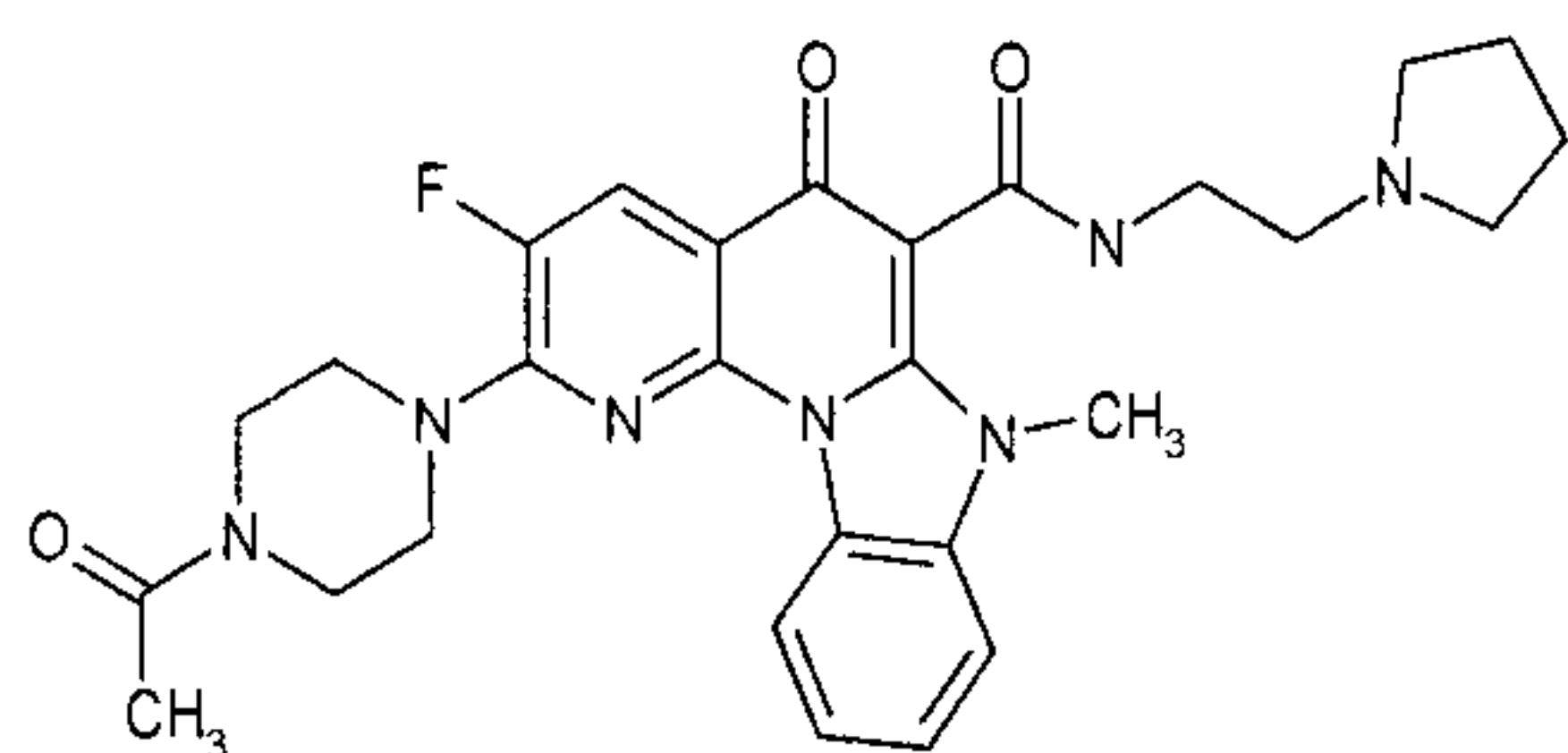
19. The compound of any one of claims 1 to 14, wherein U is  $NH-(CH_2)_n-NR^3R^4$  wherein  $R^3$  and  $R^4$  together with N form an optionally substituted pyrrolidine.

20. The compound of any one of claims 1 to 14, wherein U is 2-(N-methylpyrrolidin-2-yl)ethylamino or (2-pyrrolidin-1-yl)ethanamino.

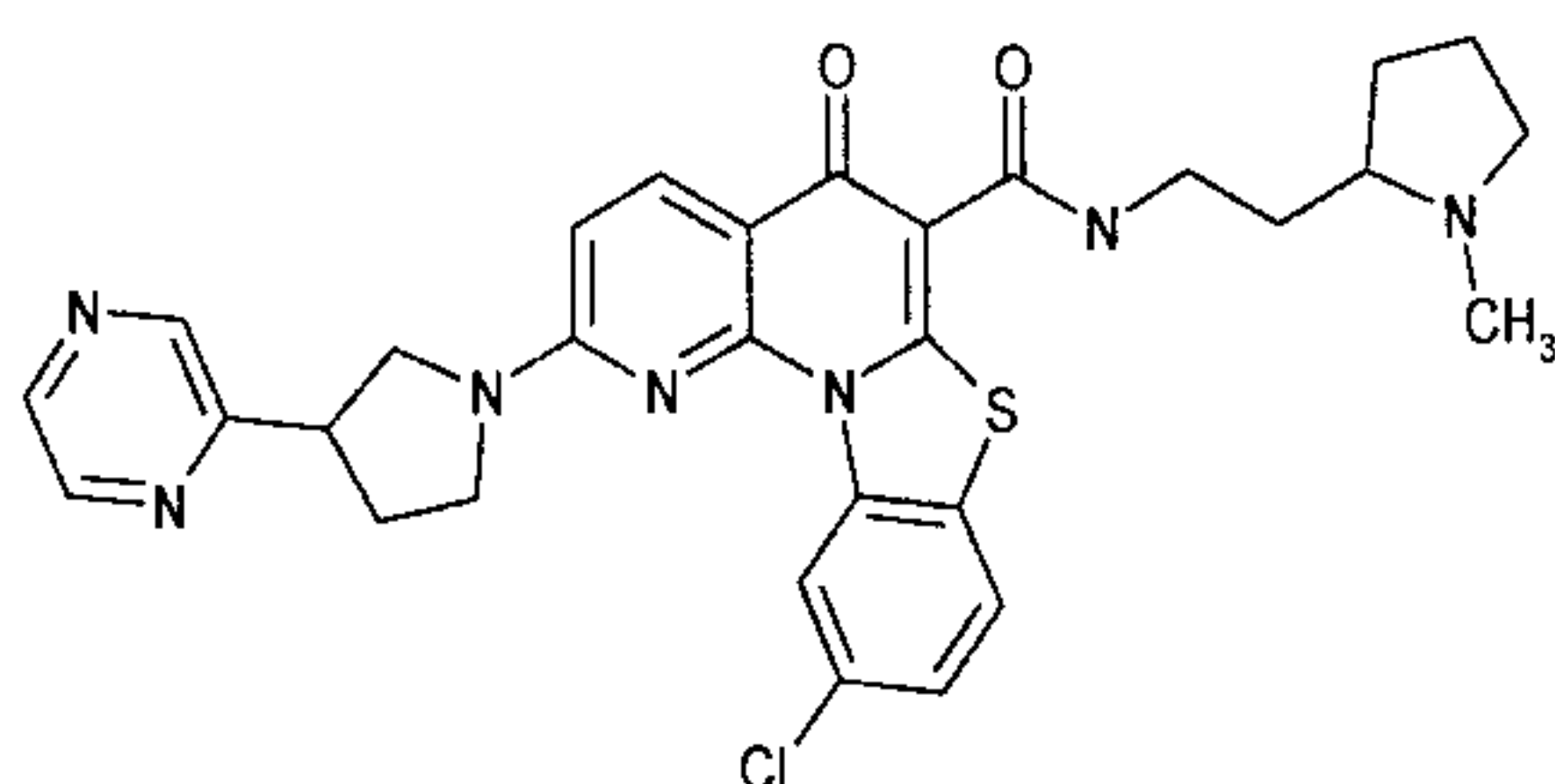
21. A compound having the formula







, or



22. A pharmaceutical composition comprising the compound of any one of claims 1 to 21, and a pharmaceutically acceptable carrier.

23. Use of the compound of any one of claims 1 to 21 or the pharmaceutical composition of claim 22 optionally with a procedure and/or a chemotherapeutic agent, to reduce cell proliferation and/or ameliorate a cell-proliferative disorder.

24. Use of the compound of any one of claims 1 to 21 or the pharmaceutical composition of claim 22 optionally with a chemotherapeutic agent, for preparation of a medicament to reduce cell proliferation and/or ameliorate a cell-proliferative disorder.

25. The use of claim 23 or 24, wherein said cell proliferative disorder is a tumor or cancer.

26. Use of the compound of any one of claims 1 to 21 or the pharmaceutical composition of claim 22 optionally with an antimicrobial agent, to reduce microbial titers and/or ameliorate a microbial infection.

27. Use of the compound of any one of claims 1 to 21 or the pharmaceutical composition of claim 22 optionally with an antimicrobial agent, for preparation of a medicament to reduce microbial titers and/or ameliorate a microbial infection.

28. The use of claim 26 or 27, wherein the microbial titers and/or microbial infection are viral, bacterial or fungal titers.

29. The use of any one of claims 23 to 28, wherein said compound or composition is for administration to a cell or tissue.

30. The use of any one of claims 23 to 29, wherein said reduction or amelioration is in a human or animal.

31. Use of the compound of any one of claims 1 to 21 or the pharmaceutical composition of claim 22 optionally with a procedure and/or a chemotherapeutic agent, to induce cell death and/or induce apoptosis.

32. Use of the compound of any one of claims 1 to 21 or the pharmaceutical composition of claim 22 optionally with a chemotherapeutic agent, for preparation of a medicament to induce cell death and/or induce apoptosis.

33. The use of claim 31 or 32, wherein said compound or composition is for administration to a cell or tissue.

34. The use of claim 31, 32 or 33, wherein said induction is in a human or animal.

35. The use of any one of claims 23 to 25 and 31 to 34, wherein said chemotherapeutic agent is gemcitabine.

36. The use of claim 23 or 31, wherein said procedure is radiotherapy or a surgical procedure.

37. An *in vitro* method for reducing cell proliferation comprising administering to a system an effective amount of the compound of any one of claims 1 to 21, or the pharmaceutical composition of claim 22 optionally with a procedure and/or a chemotherapeutic agent, thereby reducing cell proliferation in the system.

38. The method of claim 37, wherein said system comprises tumor or cancer cells.

39. An *in vitro* method for reducing microbial titers and/or ameliorating a microbial infection, comprising contacting a system with an effective amount of the compound of any one of claims 1 to 21, or the pharmaceutical composition of claim 22 optionally with an antimicrobial agent, thereby reducing microbial titers and/or ameliorating said microbial infection.

40. The method of claim 39, wherein the microbial titers and/or microbial infection are viral, bacterial or fungal titers.

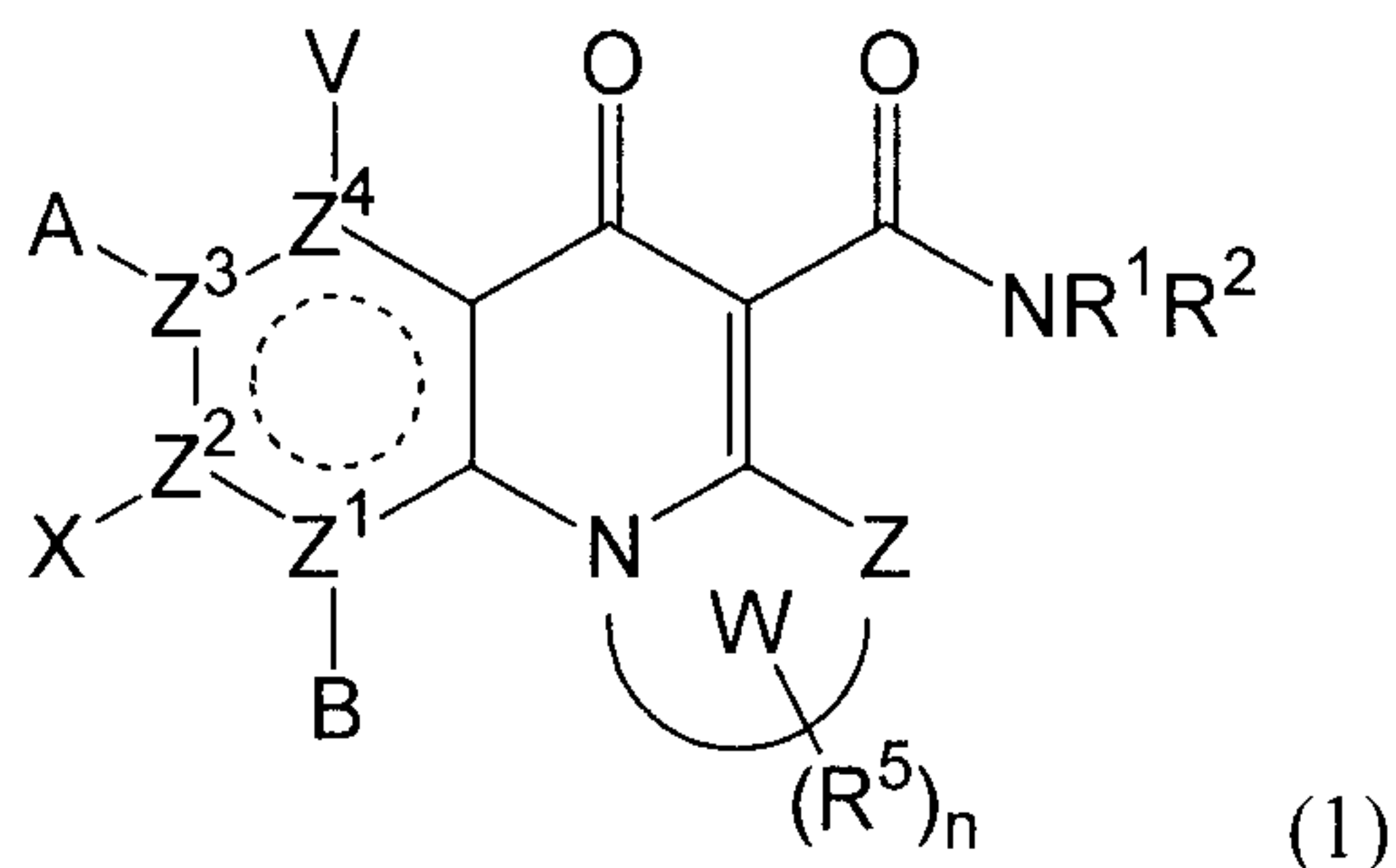
41. An *in vitro* method for inducing cell death and/or inducing apoptosis, comprising administering to a system an effective amount of the compound according to any one of claims 1 to 21, or the pharmaceutical composition of claim 22 optionally with a procedure and/or a chemotherapeutic agent, thereby inducing cell death and/or inducing apoptosis.

42. The method of claim 37, 38 or 41, wherein said chemotherapeutic agent is gemcitabine.

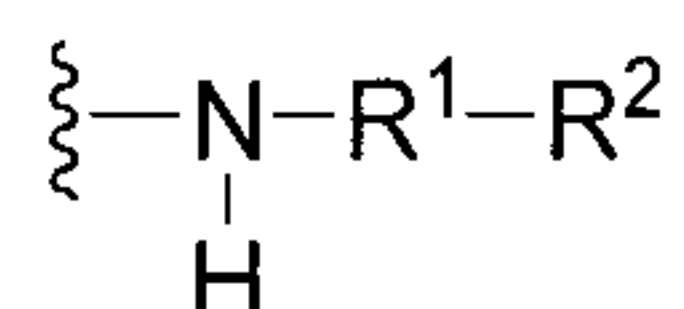
43. The method of any one of claims 37, 38, 41, and 42, wherein said procedure is radiation.

44. The method of any one of claims 37 to 43, wherein said system comprises cells or tissue or both.

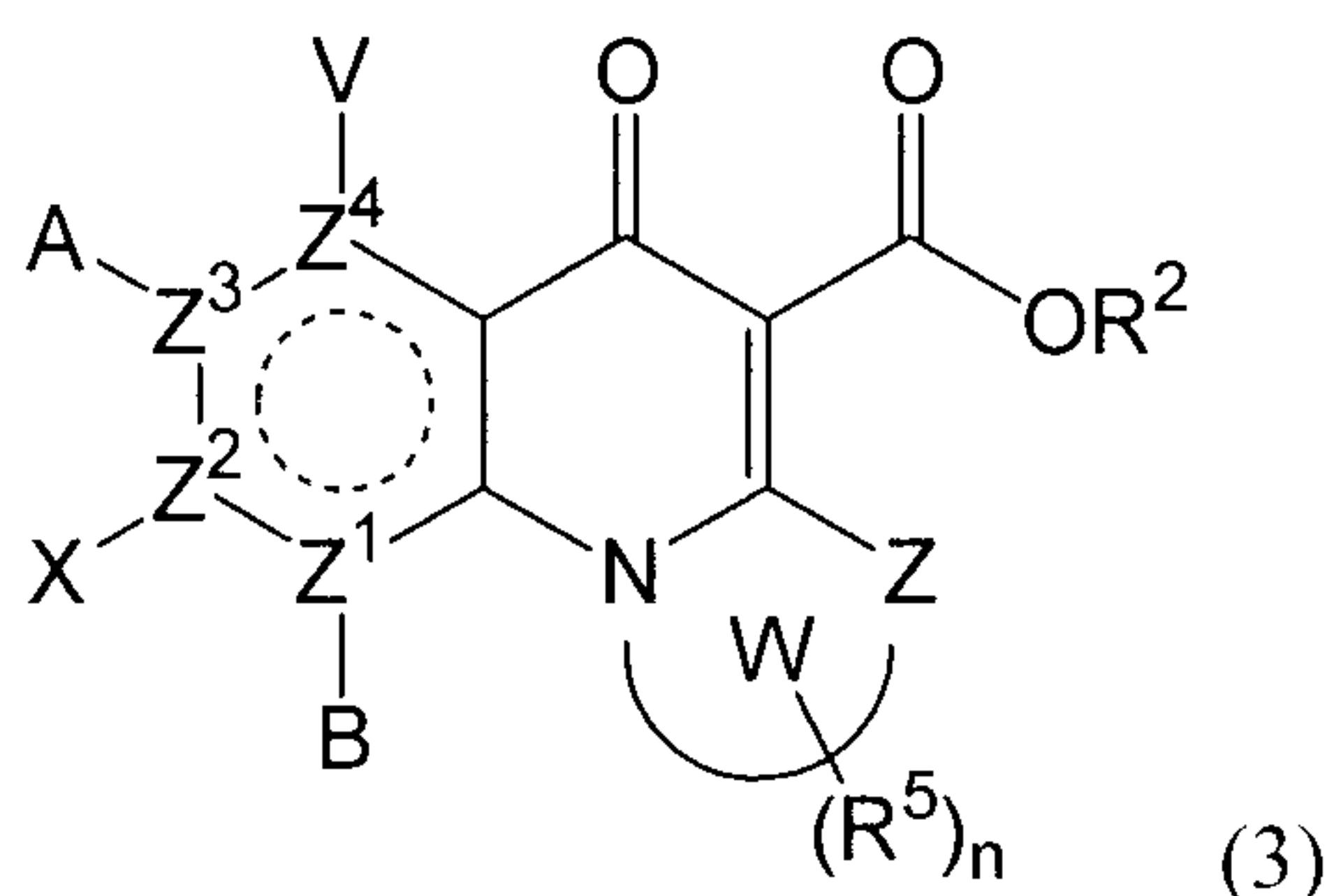
45. The method of claim 44, wherein the cells or tissue are human or animal.
46. A method for preparing compounds having formula (1),



comprising contacting an ester having formula 3, an amine having formula



and a Lewis acid having formula  $\text{ML}_{n'}$ , wherein L is a halogen atom or an organic radical,  $n'$  is 3-5, and M is a group III elemental atom, a group IV elemental atom, As, Sb, V or Fe,



wherein A, V, and X are independently H, halo, azido,  $\text{R}^2$ ,  $\text{CH}_2\text{R}^2$ ,  $\text{SR}^2$ ,  $\text{OR}^2$  or  $\text{NR}^1\text{R}^2$ ;  
or

wherein A and X, or A and V may form a carbocyclic ring, heterocyclic ring, aryl or heteroaryl, each of which may be optionally substituted and/or fused with a cyclic ring;

wherein in  $\text{NR}^1\text{R}^2$ ,  $\text{R}^1$  and  $\text{R}^2$  may form an optionally substituted ring;

each Z is  $\text{CH}_2$ , O, S, or  $\text{NR}^1$ ;

$\text{Z}^1$ ,  $\text{Z}^2$ ,  $\text{Z}^3$ , and  $\text{Z}^4$  are independently C or N, provided any two N are non-adjacent;

each  $\text{R}^1$  is H or a  $\text{C}_{1-6}$  alkyl;

each  $R^2$  is H or a  $C_{1-10}$  alkyl or  $C_{2-10}$  alkenyl optionally containing one or more non-adjacent heteroatoms selected from N, O, and S, and optionally substituted with a carbocyclic or heterocyclic ring; or  $R^2$  is an optionally substituted carbocyclic ring, heterocyclic ring, aryl or heteroaryl;

each B is H or halo;

W together with N and Z forms an optionally substituted 5- or 6-membered ring that is fused to an optionally substituted aryl or heteroaryl, wherein said aryl or heteroaryl is monocyclic or fused with a single or multiple ring which optionally contains a heteroatom;

and each  $R^5$  is a substituent at any position on W and is H,  $OR^2$ ,  $NR^1R^2$ ,  $C_{1-6}$  alkyl,  $C_{2-6}$  alkenyl, each optionally substituted by halo, C=O or one or more heteroatoms; or two adjacent  $R^5$  is linked to obtain a 5-6 membered substituted or unsubstituted carbocyclic or heterocyclic ring, optionally fused to an additional substituted or unsubstituted carbocyclic or heterocyclic ring; and

n is 1-6.

47. The method of claim 46, wherein said ester, amine, and Lewis acid are contacted at room temperature.

48. The method of claim 46 or 47, comprising contacting said ester and amine in an organic solvent to form a solution, and contacting said solution with the Lewis acid.

49. The method of claim 48, wherein said organic solvent is methylene chloride.

50. The method of any one of claims 46 to 49, wherein an excess of amine in relation to the ester is used.

51. The method of claim 50, wherein the ratio of the ester to the amine is 1:2; 1:1.5; or 1:1.25.

52. The method of any one of claims 46 to 51, wherein an equimolar amount of the Lewis acid to the amine is used.

53. The method of any one of claims 46 to 52, further comprising isolating the compound having formula 1 and optionally purifying an isolated compound having formula 1.

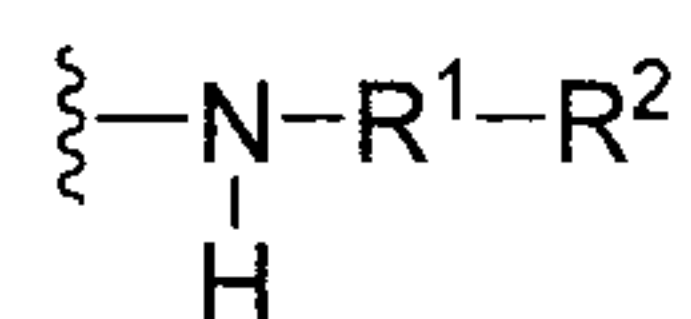
54. The method of claim 53, wherein said isolated compound is purified through column chromatography, recrystallization, or both.

55. The method of claim 54, wherein the purity of the isolated compound is between 90 and 99 %.

56. The method of any one of claims 46 to 55, wherein said Lewis acid is  $BL_3$ ,  $AlL_3$ ,  $FeL_3$ ,  $GaL_3$ ,  $SbL_5$ ,  $InL_3$ ,  $ZrL_4$ ,  $SnL_4$ ,  $TiL_4$ ,  $TiL_3$ ,  $AsL_3$ , or  $SbL_3$ , wherein L is halo or alkyl.

57. The method of any one of claims 46 to 55, wherein said Lewis acid is aluminum chloride.

58. The method of any one of claims 46 to 57, wherein said ester is contacted with said amine having the formula



wherein  $R^1$  is a  $(CR^3)_n$  group;

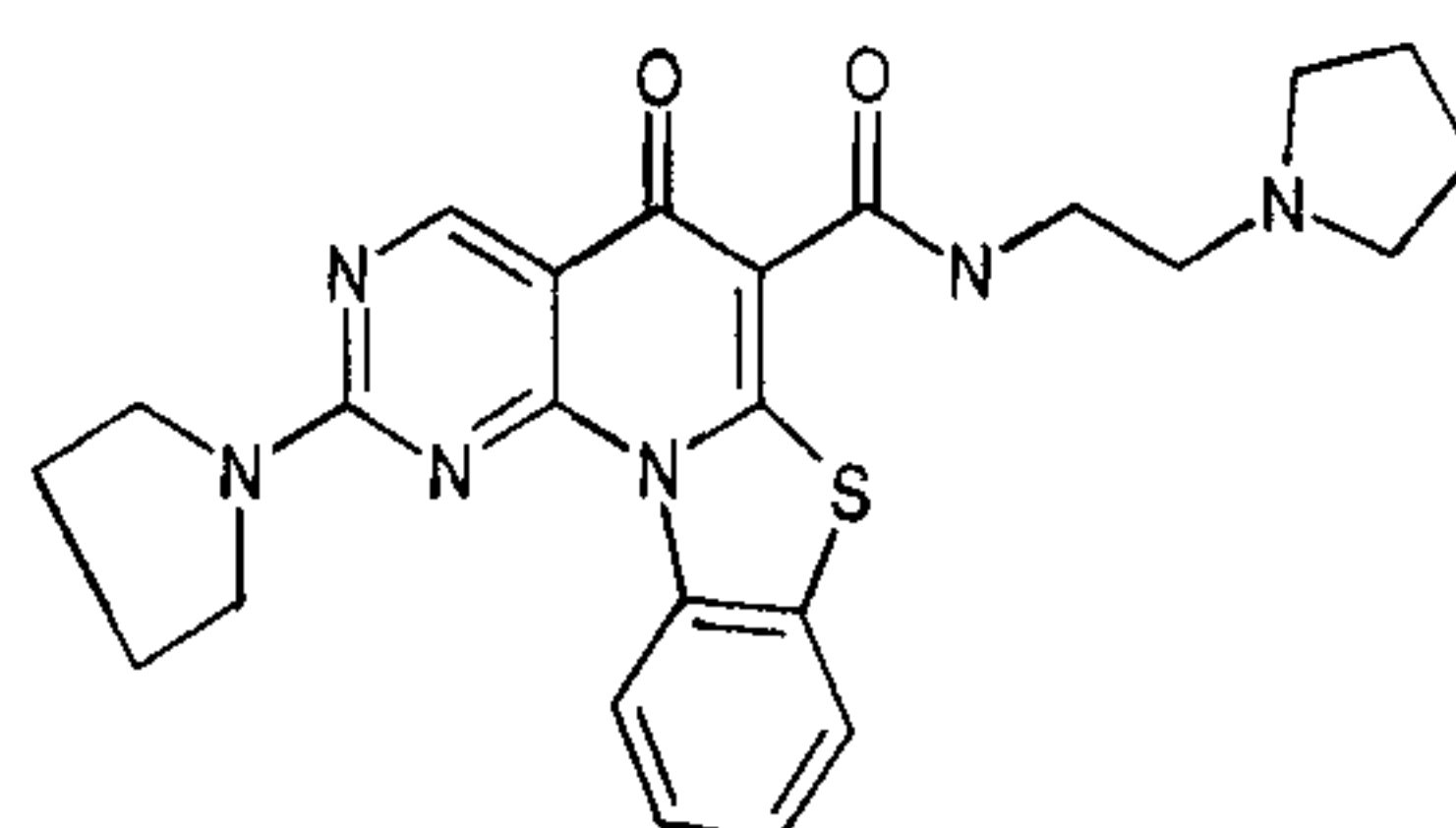
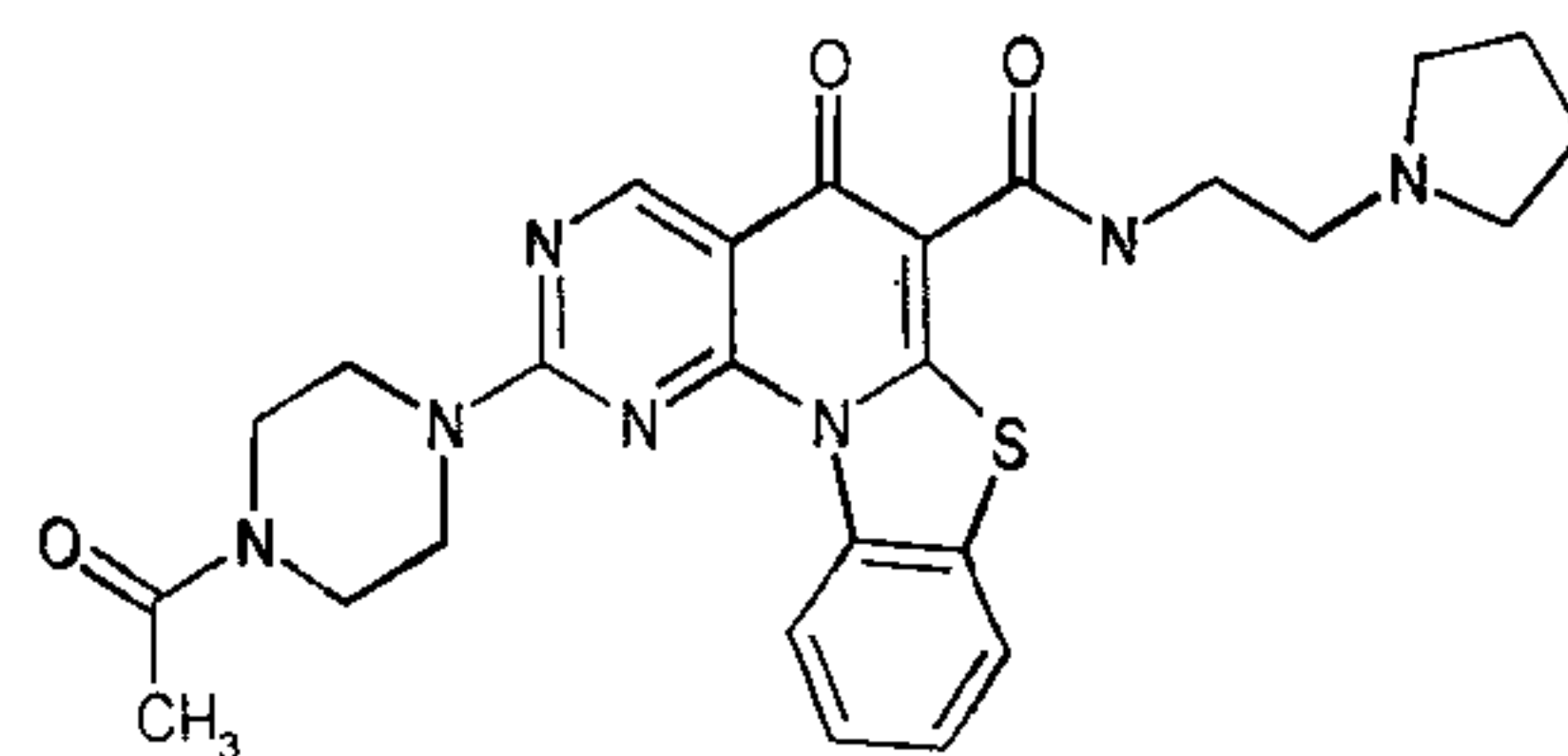
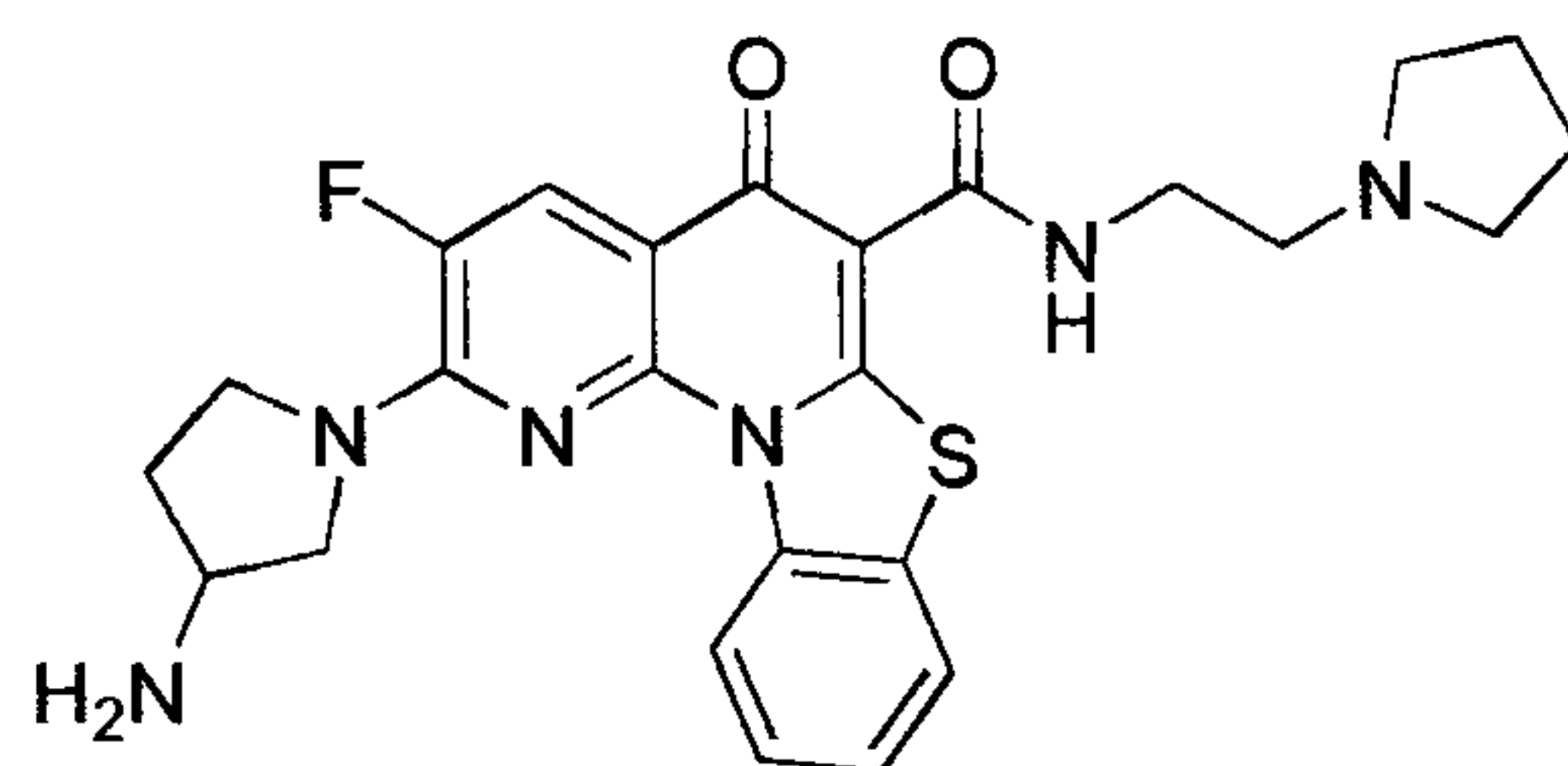
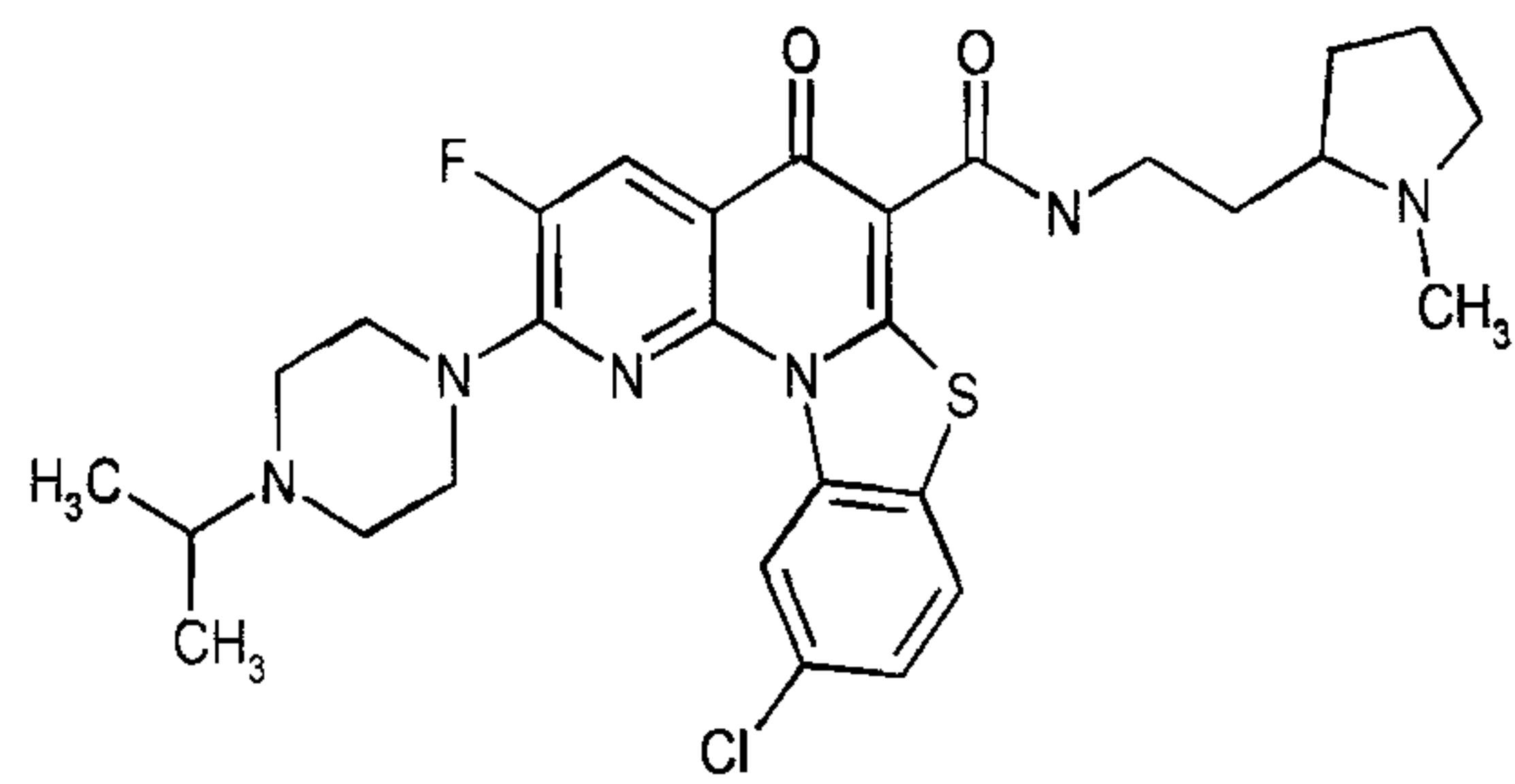
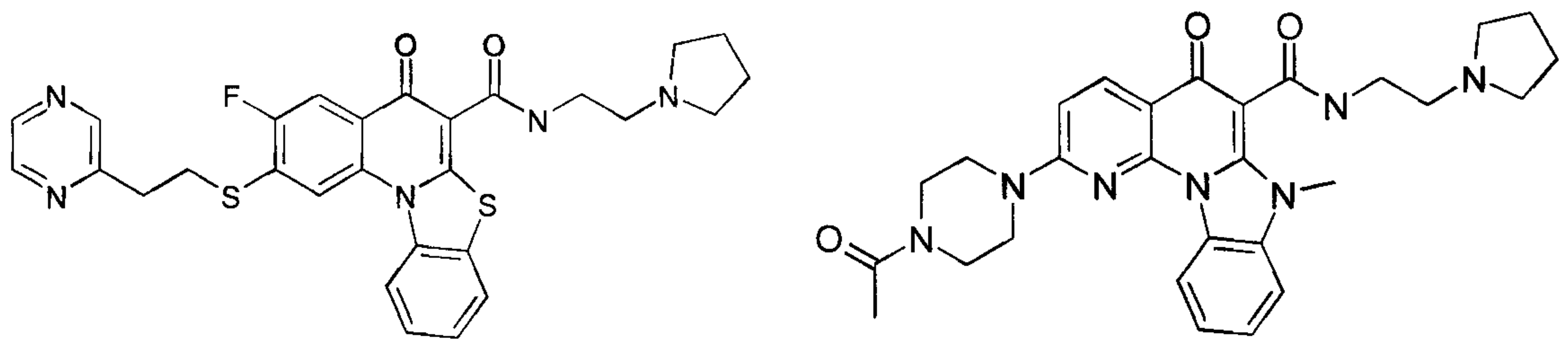
$R^2$  is  $NR^3R^4$ ;

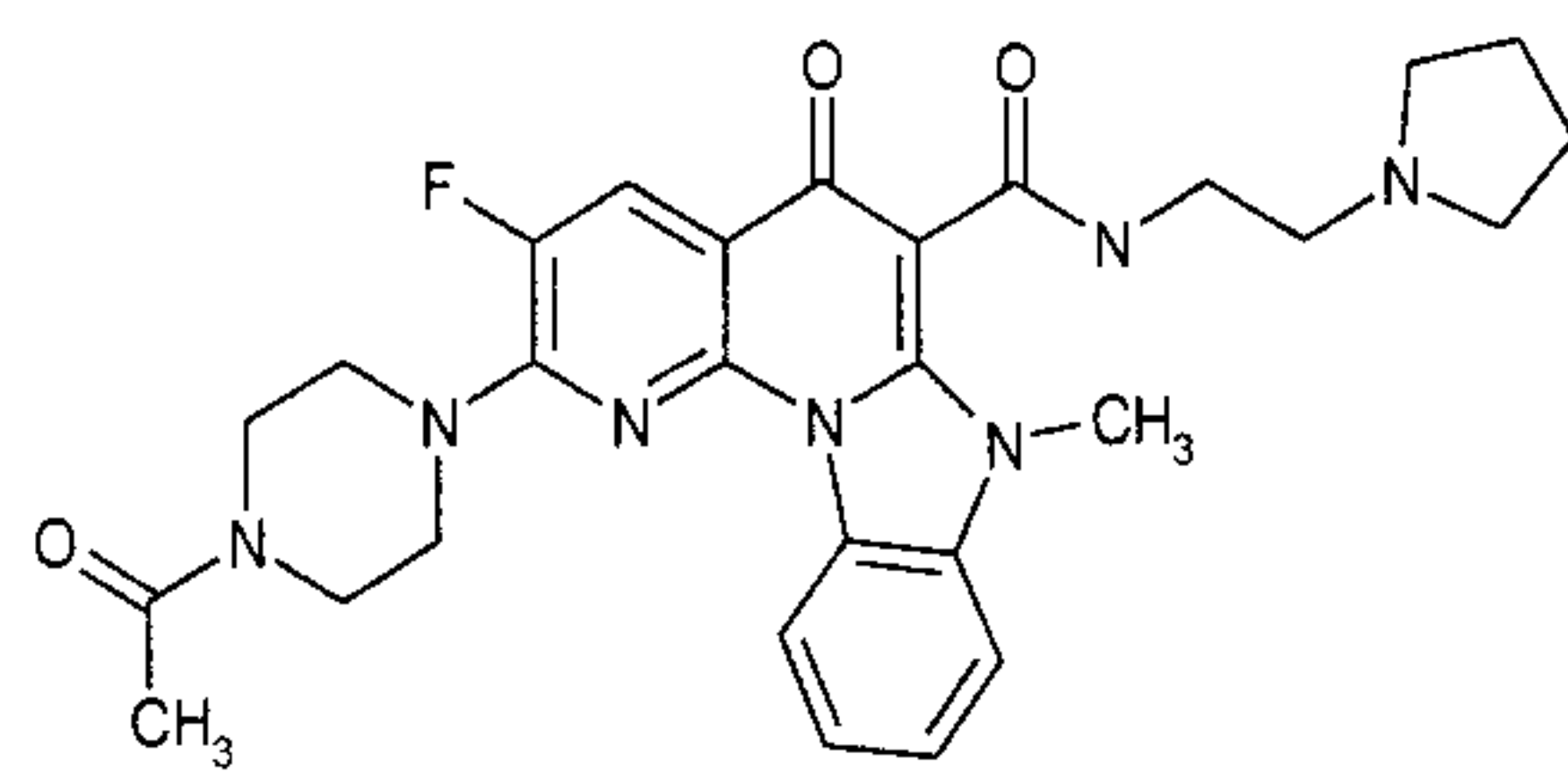
$R^3$  is H or  $C_{1-6}$  alkyl;

$n$  is 1-6; and

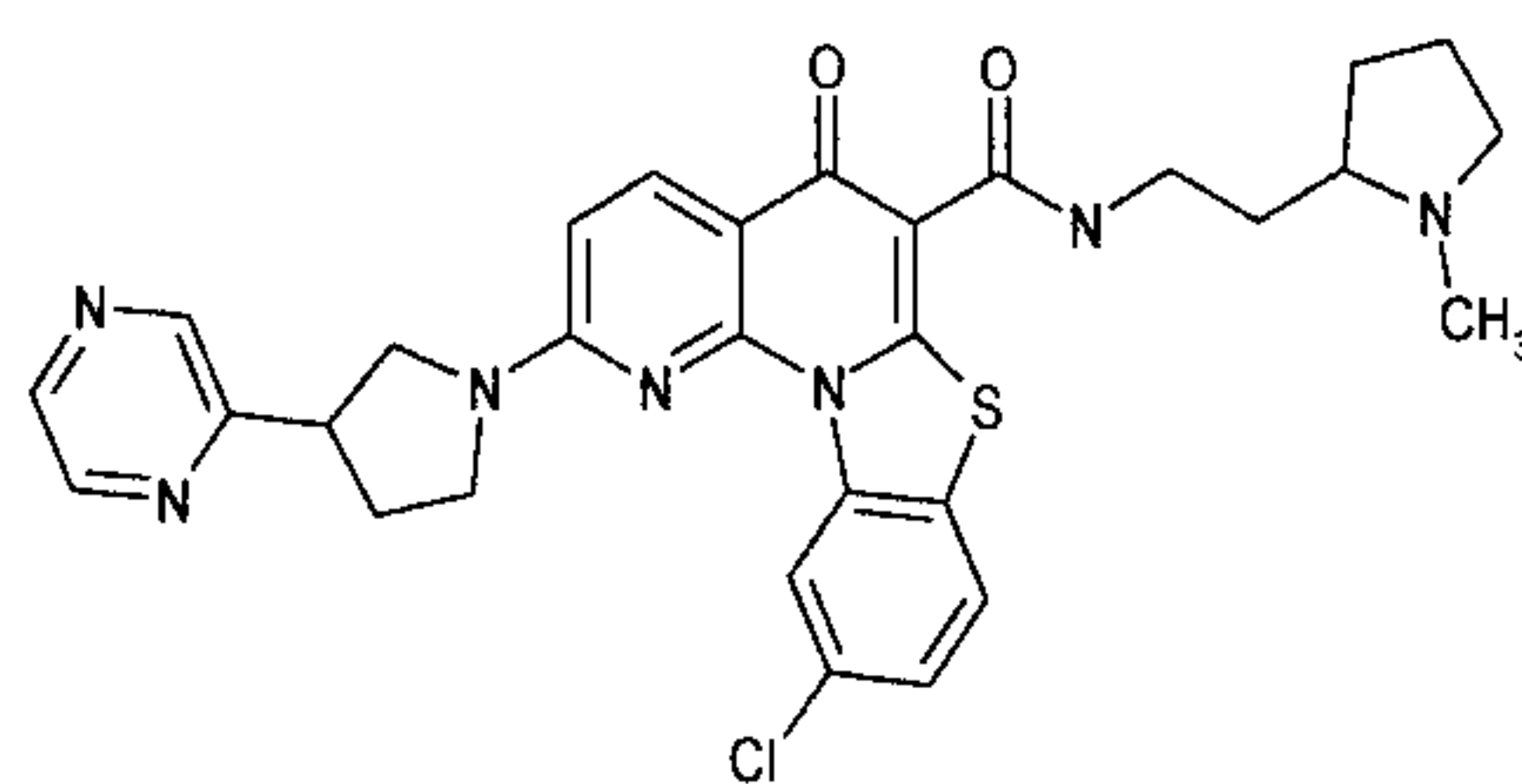
$R^4$  is H or a  $C_{1-10}$  alkyl or  $C_{2-10}$  alkenyl optionally containing one or more non-adjacent heteroatoms selected from N, O and S, and optionally substituted with a carbocyclic or heterocyclic ring; and wherein in  $NR^3R^4$ ,  $R^3$  and  $R^4$  may form an optionally substituted ring.

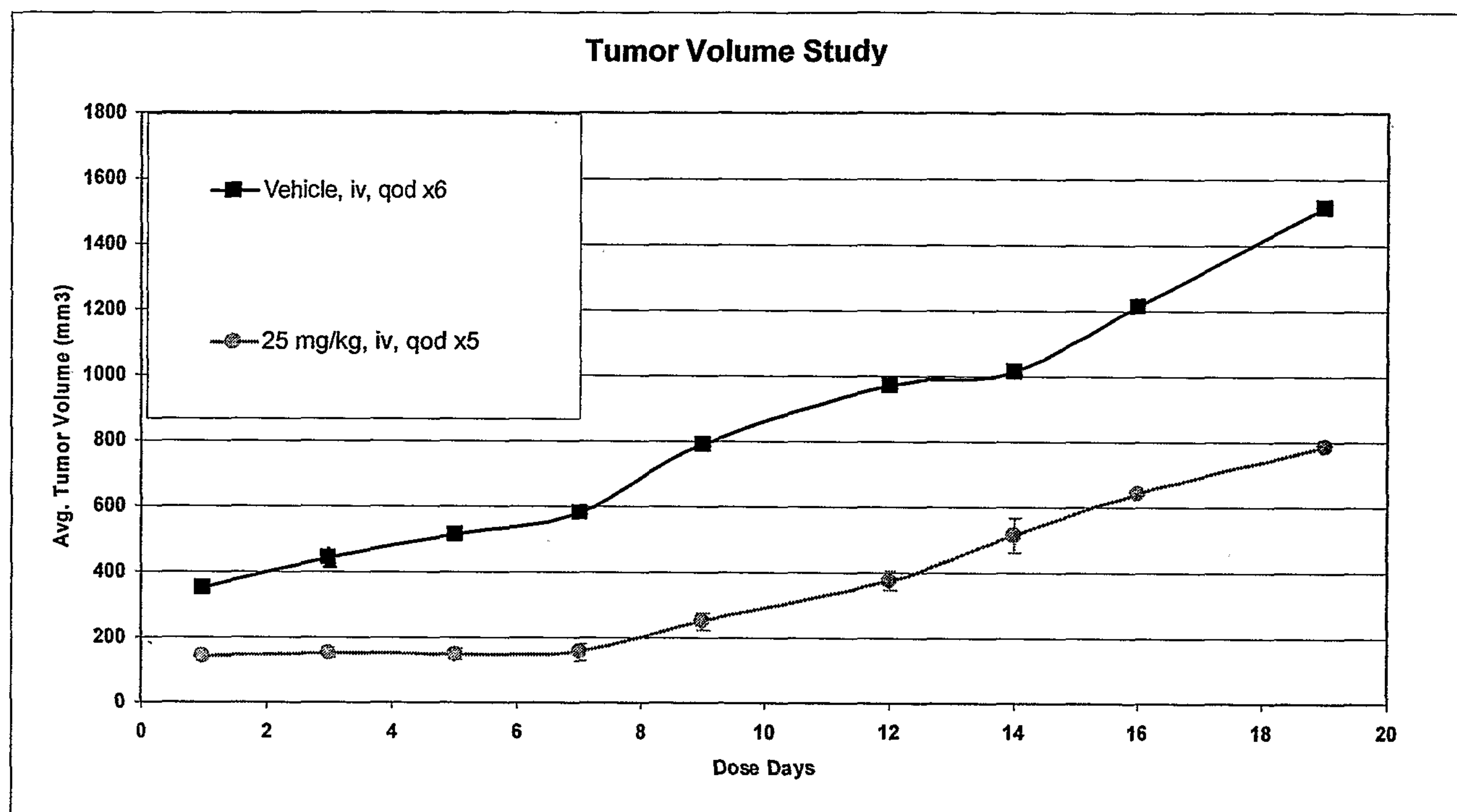
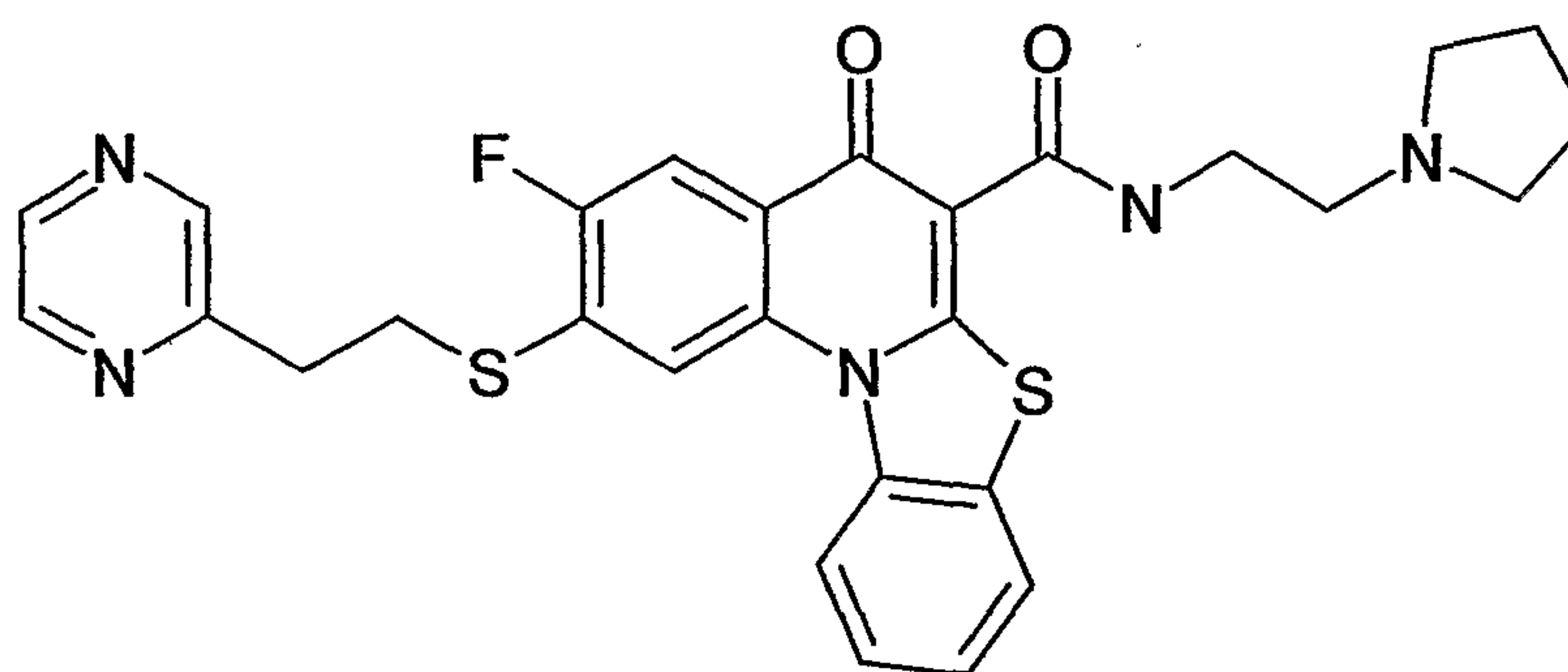
59. The method of any one of claims 46 to 57, for preparing a compound selected from the group consisting of:

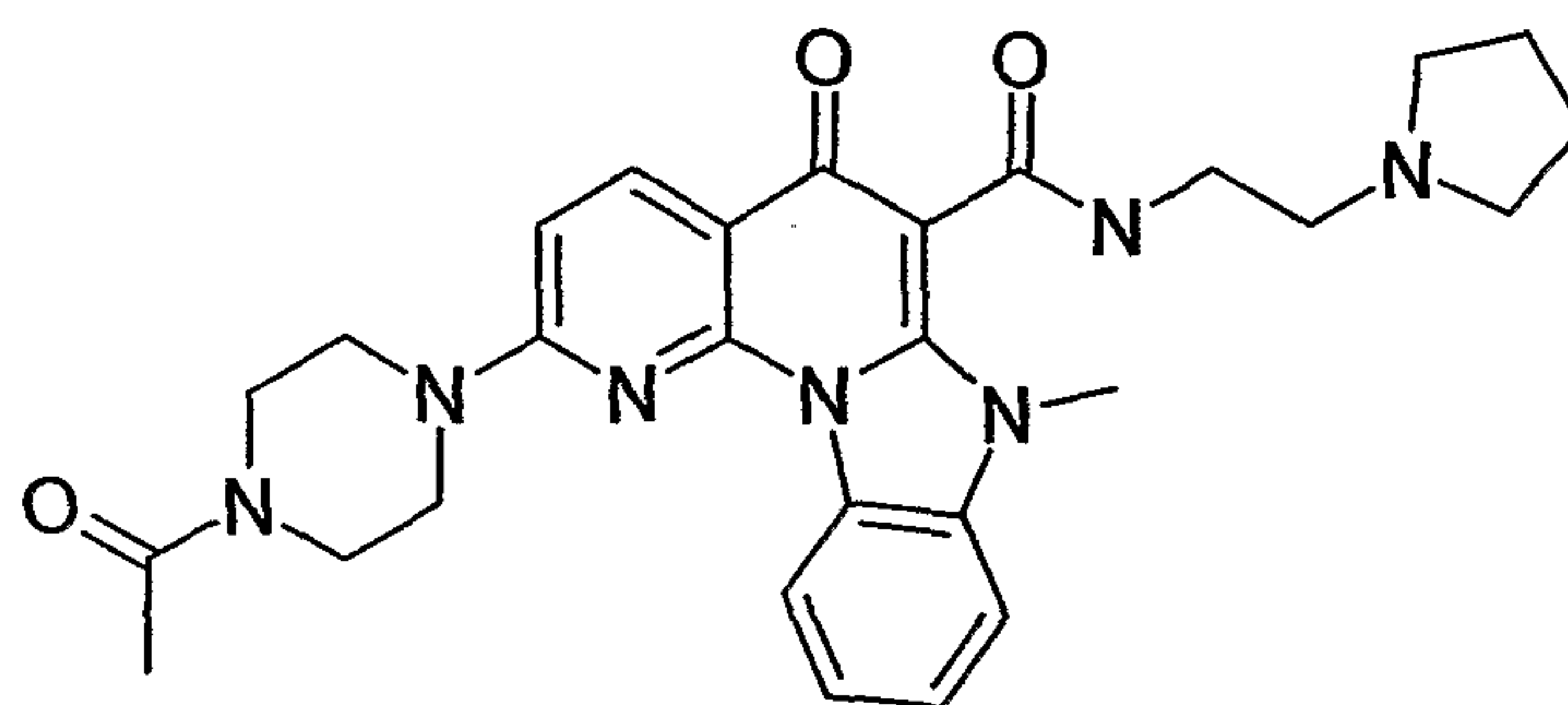
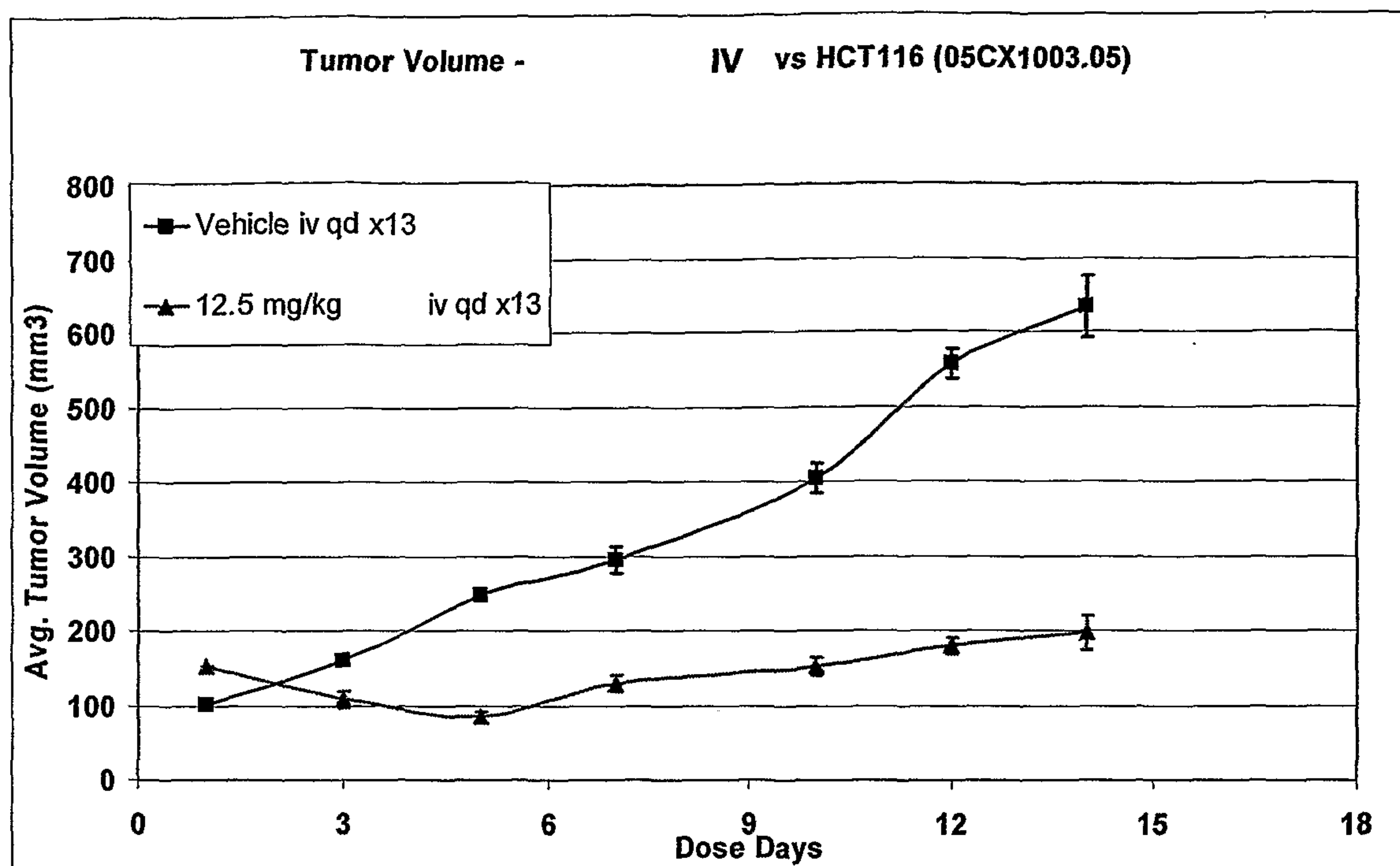


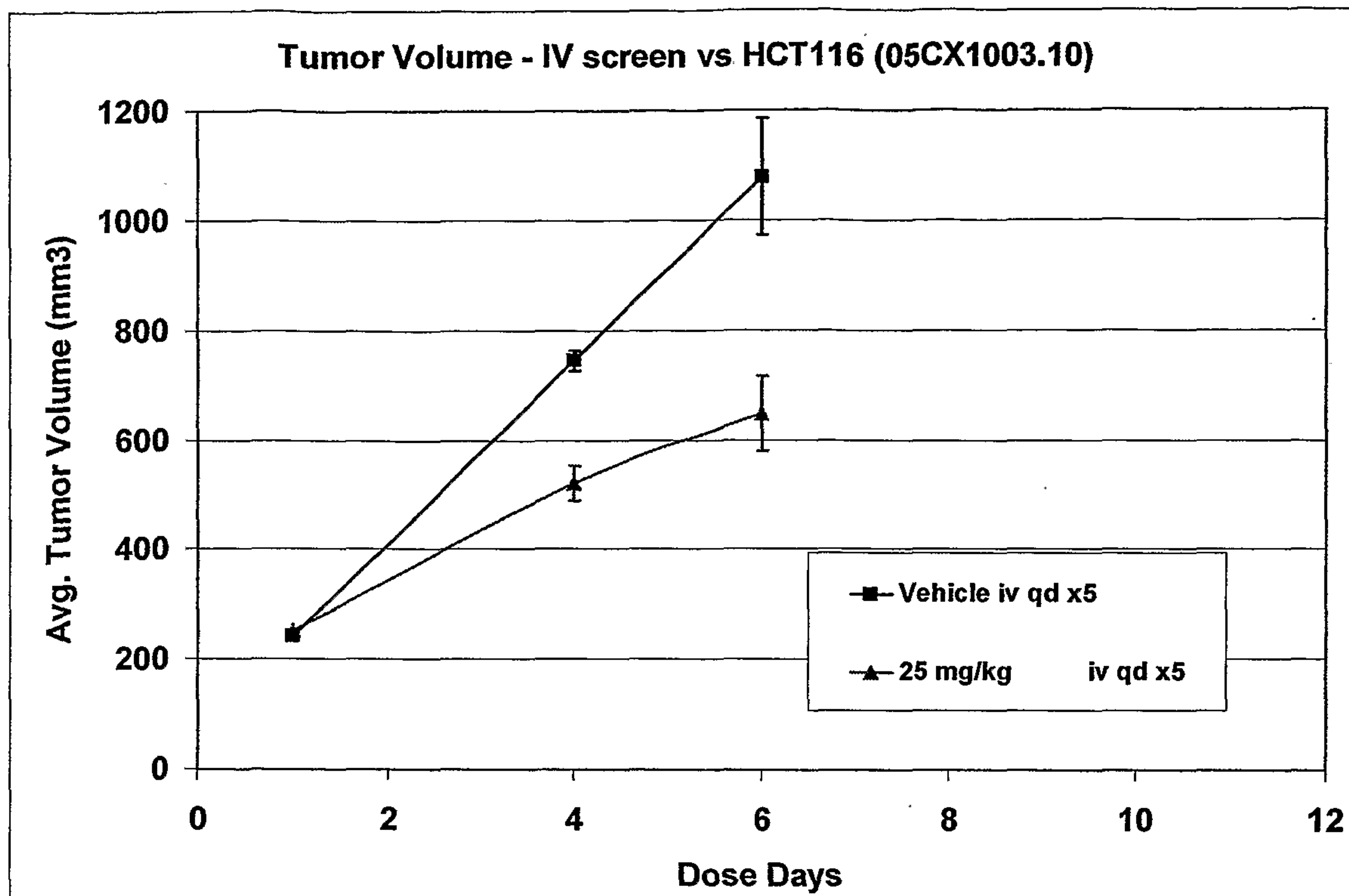
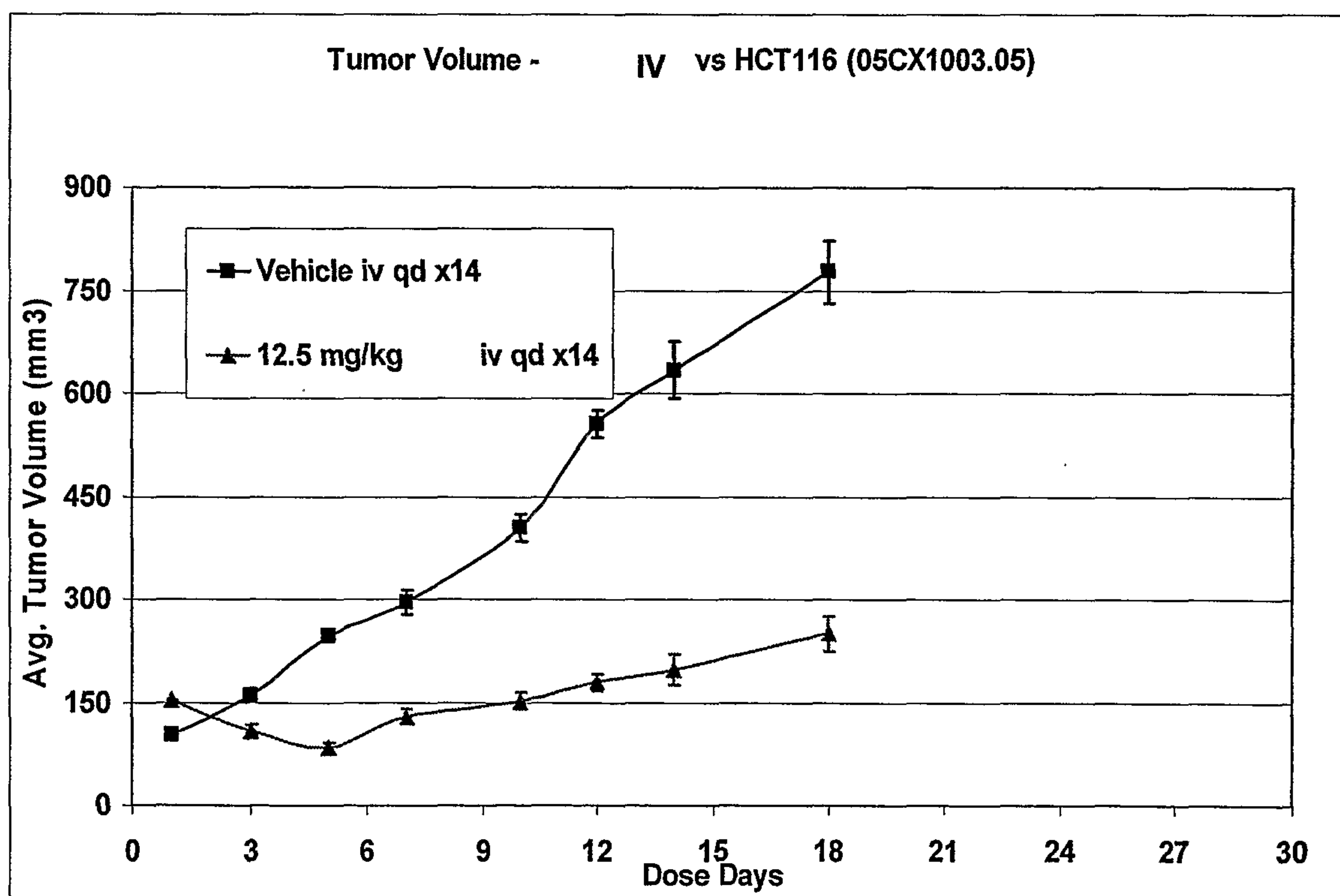


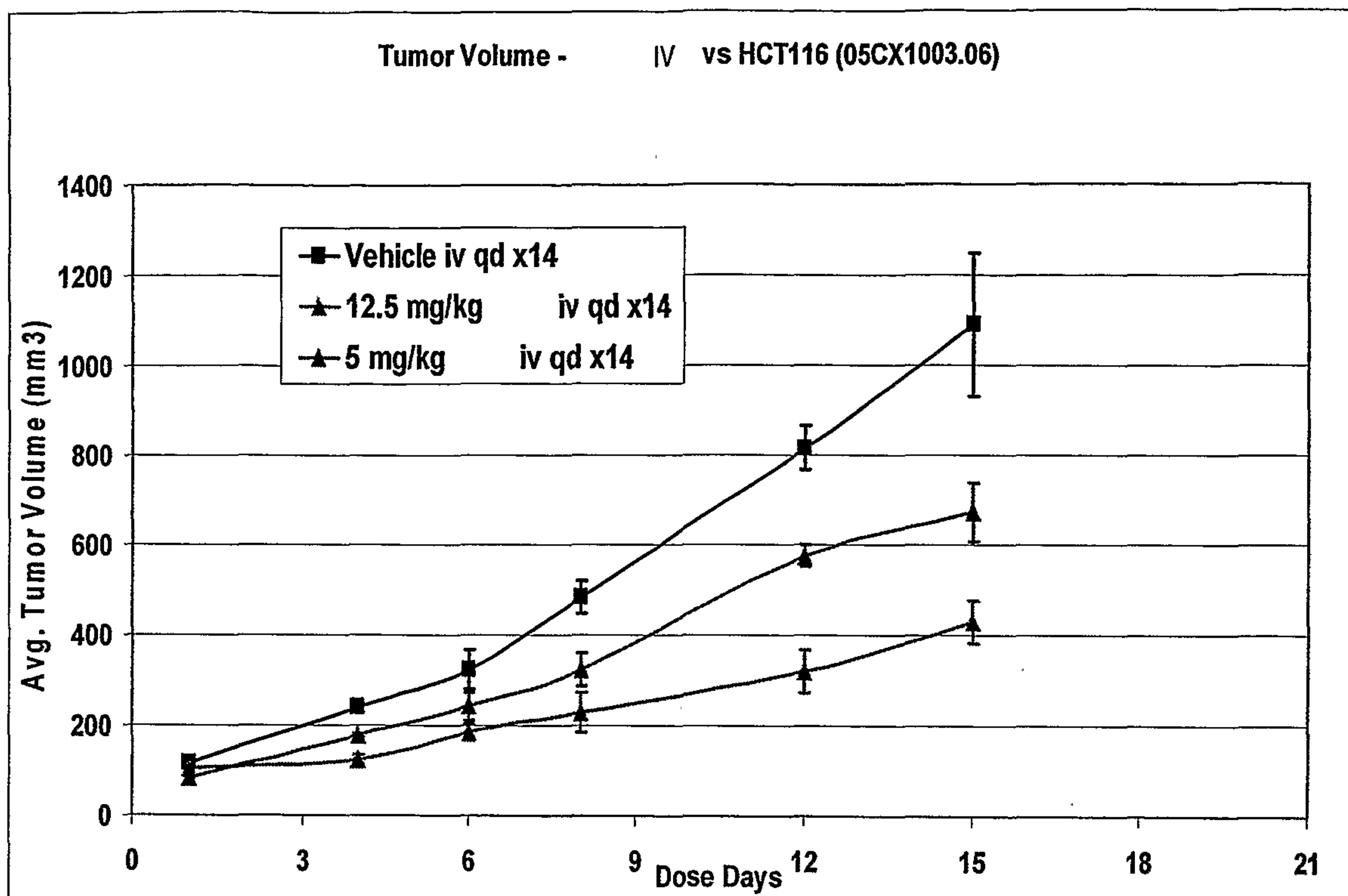
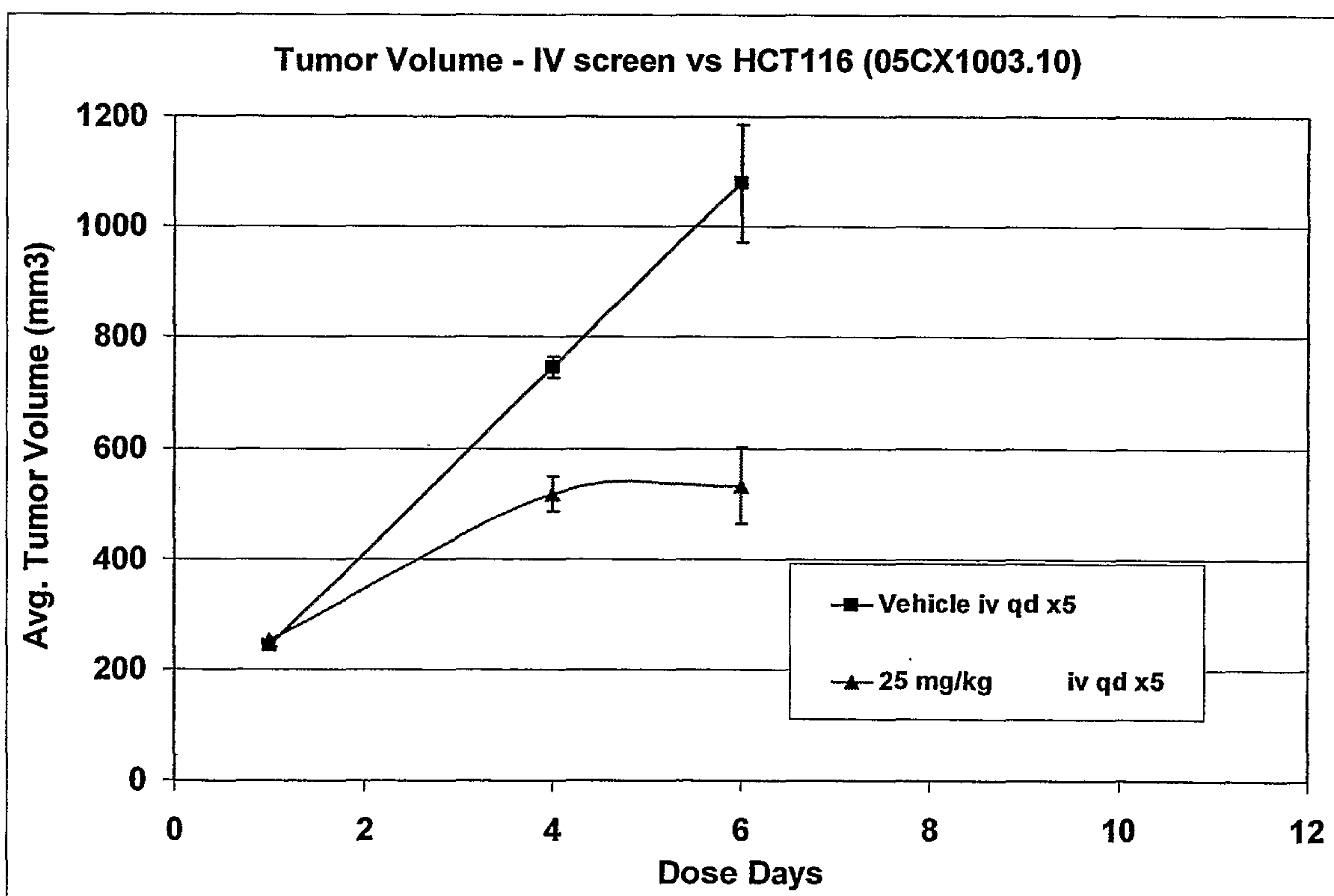
, and

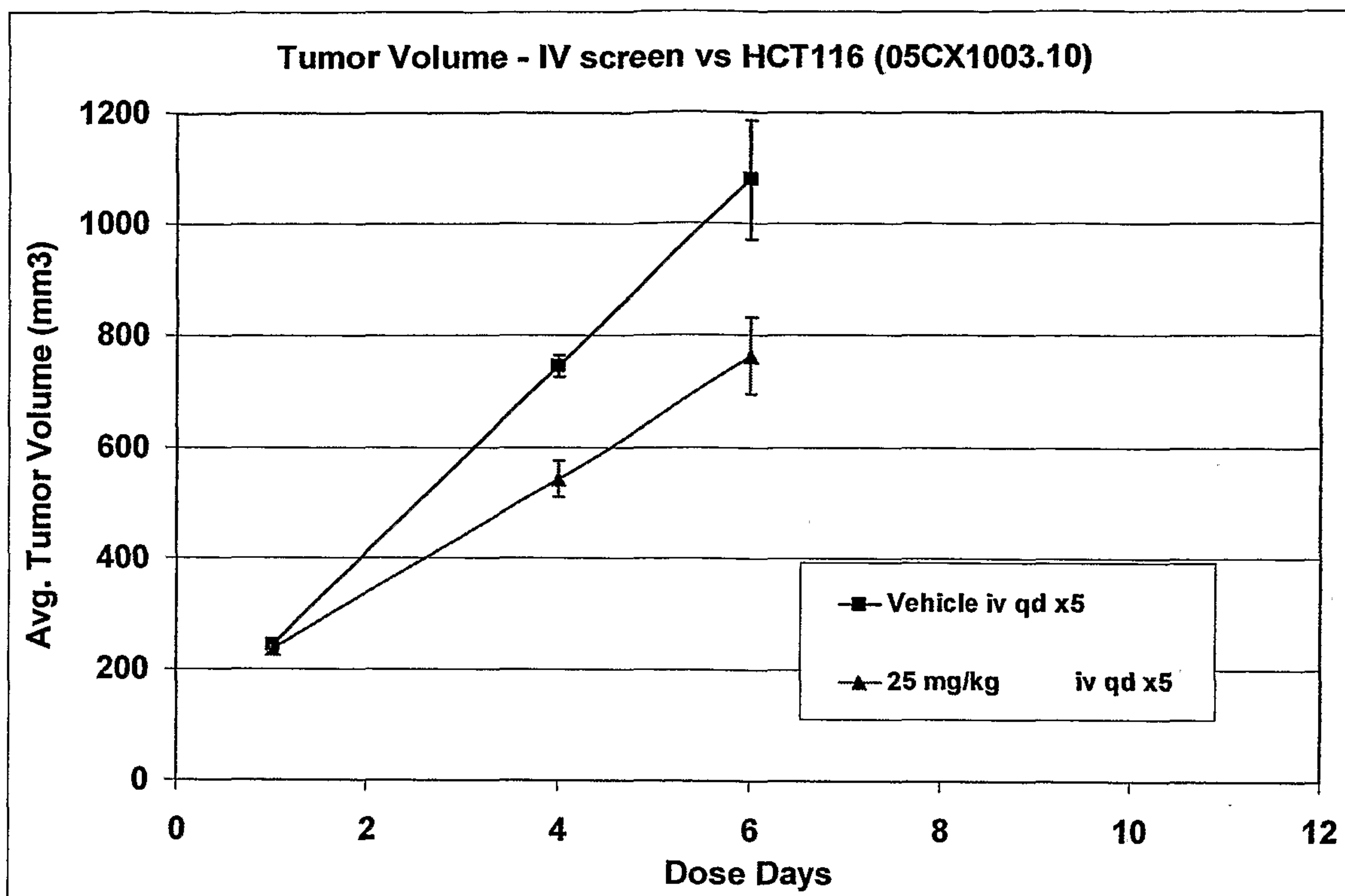
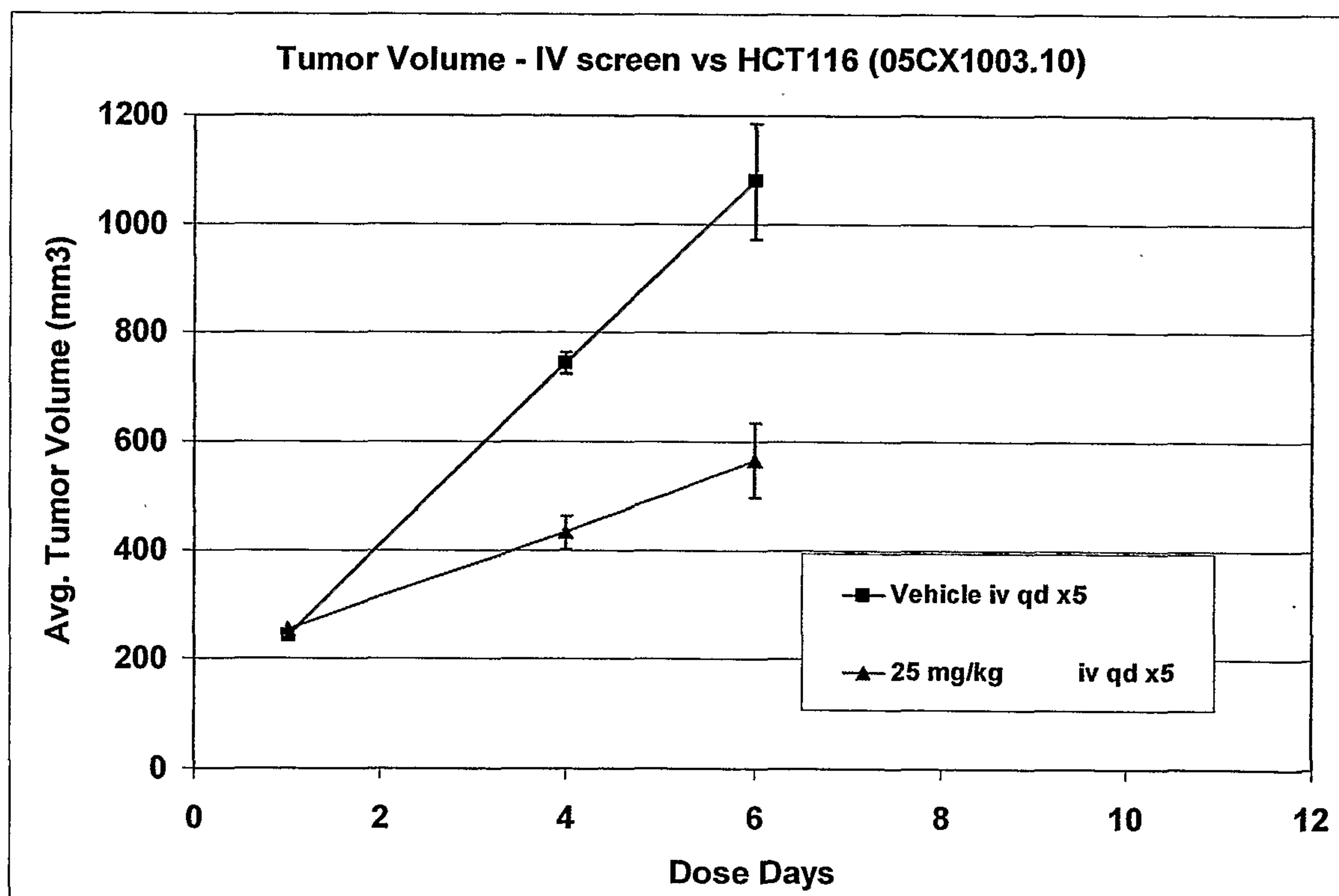


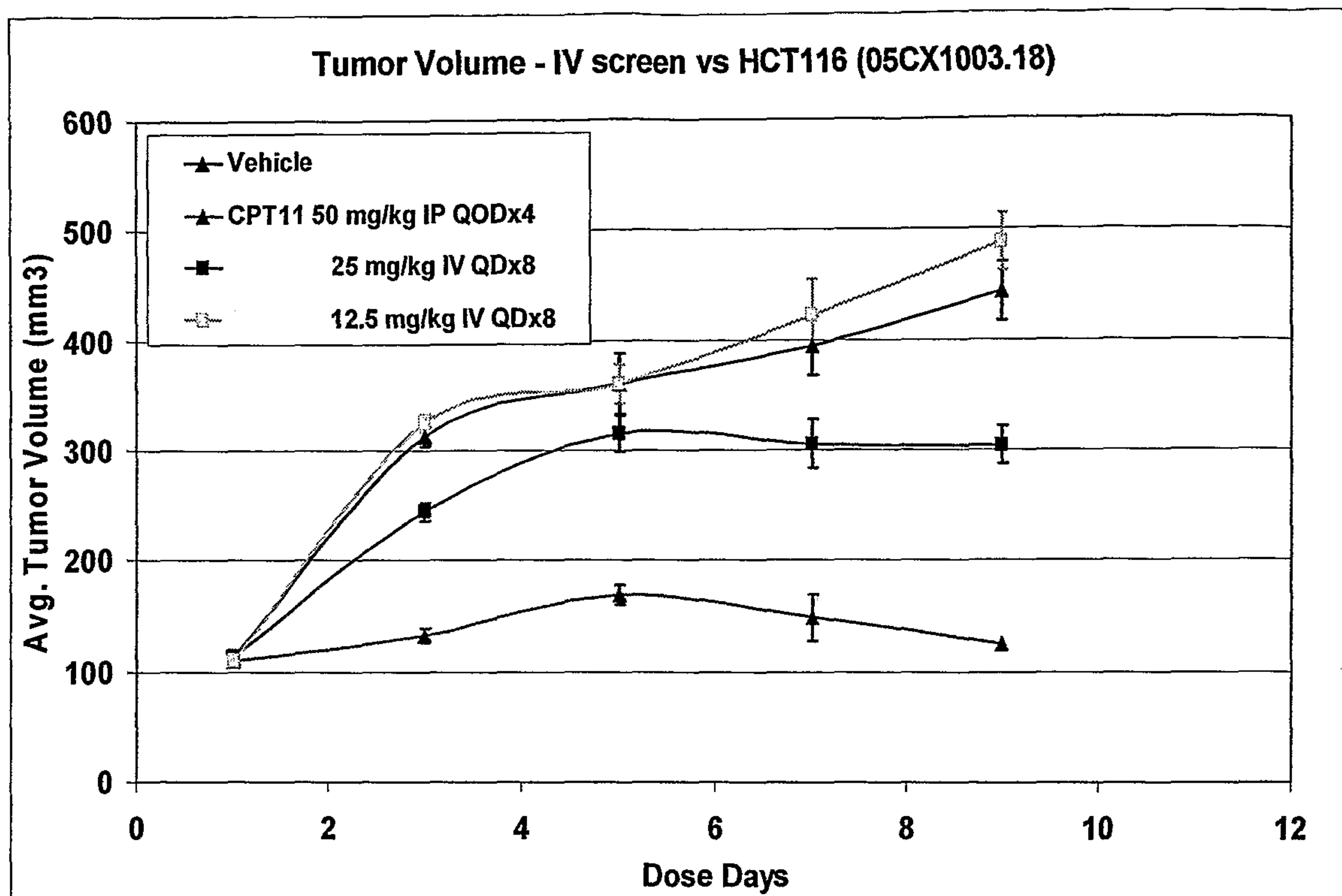
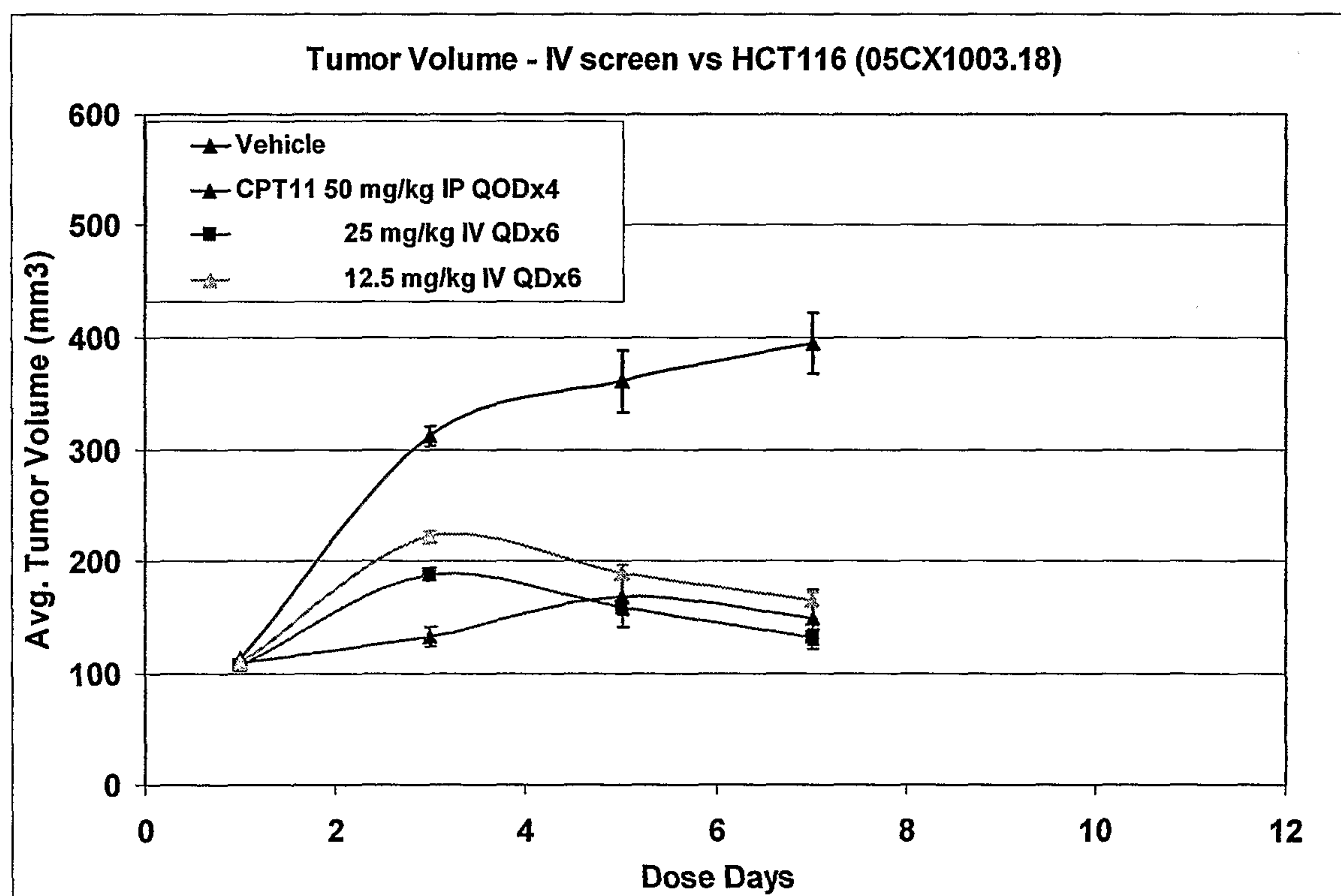
**Figure 1**

**Figure 2**

**Figure 3****Figure 4**

**Figure 5****Figure 6**

**Figure 7****Figure 8**

**Figure 9****Figure 10**

