New Applications of Imidazotetrazinone Prodrugs

Synthesis and mechanistic investigation of novel imidazotetrazinones as prodrugs of aziridines and as traceless carriers for drug delivery to the central nervous system.

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Submitted for the degree

of Doctor of Philosophy

Institute of Cancer Therapeutics

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BRADFORD UNIVERSITY

2010

Abstract

New imidazotetrazinones have been synthesised that possess features in their structures to release aziridinium ions upon ring opening. Unstable 2-aminoethylisocyanates were required in this preparation, which were synthesized with BOC-protection of the amino group to counteract the reactivity of the amine towards the isocyanate group in the case of aliphatic amines; in contrast, anilinoethylisocyanates were synthesized unprotected. Substituents with a range of electron-withdrawing and electron-releasing properties were introduced at the *p*-position of the aniline ring. A ^{13}C-labelled study confirmed the release of the aziridinium ion by these imidazotetrazinones in neutral pH buffer solution. Furthermore the kinetics of the hydrolysis in neutral aqueous solution of some these new tetrazines were similar to temozolomide, in addition to useful acid stability. Other imidazotetrazinones were synthesised for the purpose of releasing alcohols and phenols. Their synthesis was performed with a one-carbon linker between the imidazotetrazinone 3-position and the alcohols or phenols to be released. The release of alcohol and phenol through the hydrolysis of the intermediate diazonium ions to the unstable hemiacetals that decomposed to the alcohol and phenol was confirmed by ^{1}H NMR. The kinetics of the hydrolysis of these tetrazines in neutral aqueous solution showed a faster reaction rate compared with temozolomide ($t_{1/2}$ = 0.53 and 0.36 h compared with temozolomide 1.4 h).

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ABBREVIATIONS

AcOH	Acetic acid
AIC	5-Aminoimidazole-4-carboxamide
Arg	Arginine
A	Adenine
Å	Ångstrom
*O*6-BG	Benzylguanine
BCNU	N,N′-bis(2-chloroethyl)-N-nitrosourea
BOC_2O	Di-tert-butyl dicarbonate
BOC	*tert*-butoxycarbonyl
BBB	Blood brain barrier
BER	Base Excision Repair
b.p	Boiling point
Cys	Cysteine
C	Cytosine
ºC	Degree Celsius
DCM	Dichloromethane
DMAP	4-Dimethylaminopyridine
DPPA	Diphenylphosphorylazide
DMSO	Dimethylsulfoxide
DMF	Dimethylformamide
DTIC	5-(3,3-Dimethyltriazenyl)imidazole-4-carboxamide
Diazo-IC	5-Diazoimidazole-4-carboxamide
DNA	Deoxyribonucleic acid

EtOH	Ethanol
Et_3N	Triethylamine
EtOAc	Ethyl acetate
Et_2O	Diethyl ether
Et	Ethyl
g	gram
G	guanine
Glu	Glutamine
His	Histidine
h	Hours
IR	Infrared Spectroscopy
Me	Methyl
MeOH	Methanol
mmol	milli mole
M	Molar
m.p.	Melting point
MS	Mass Spectrometry
MTIC	5-(3-Methyltriazenyl)imidazole-4-carboxamide
MNU	*N*-methyl-*N*-nitrosourea
MCTIC	5-(3-(2-Chloroethyl)triazenyl)imidazole-4carboxamide
MMR	Mismatch Repair
MGMT	*O*6-Methylguaninemethyltransferase
μM	micromolar
M	Molar
min	minute

m	medium (IR)
MHz	megahertz
mg	milligram
NMR	Nuclear Magnetic Resonance
No.	Number
*O*6-MeG	*O*6-methylguanine
*O*6-BG	*O*6-benzylguanine
PrOH	*i*-propanol
PET	Positron Emission Tomography
PARP	Poly(ADP-ribose)polymerase
rt	Room temperature
R	Alkyl
s	strong (IR)
s	singlet (NMR)
T	thymine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
$t_{1/2}$	half-life
t	triplet (NMR)
Δ	Heating under reflux
μ	Micro

AKNOWLEDGEMENTS

This thesis would not have been possible unless I had close support and guidance from a lot of people. First, I am heartily thankful to my supervisor, Dr Richard Wheelhouse, whose encouragement, supervision and support from the preliminary start up to the stage of concluding my work and presenting it in a good way. He enabled me to develop an understanding the entire project.

I want to thank Dimitris who put me at the start point of my work with a great help.

My family especially my wife for the great support I had from them. Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

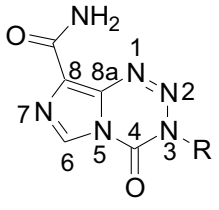
CHAPTER 1

INTRODUCTION

Chapter 1 Introduction

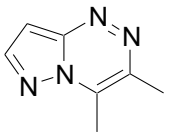
1.1 Imidazotetrazinone origin as a prodrug delivery system

Imidazotetrazinones **1** are compounds characterized by arrays of nitrogen atoms which confer unique chemical and biological properties. [1] Their structure is a variation of the commonly-known triazenes (acyclic system). They developed from pyrazolotriazine **2** and aryldimethyltriazines **3** in the early 1960s, to the imidazolyltriazene dacarbazine **4** (DTIC).



1

1 a: R = CH_3
b: CH_2CH_2Cl
c: CH_2CH_3
d: CH_2CH_2Br
e: CH_2CF_3



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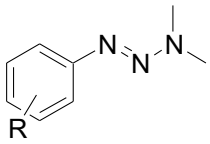

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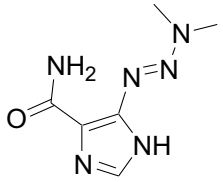
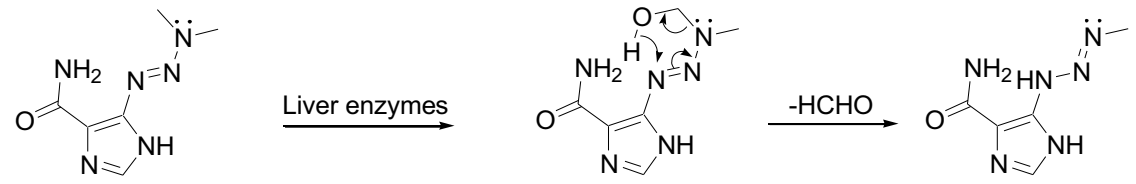


4

DTIC entered clinical practice in the 1970s, on the basis of its potent activity against a range of rodent tumours. It required metabolic activation in the liver, the metabolic activation is a P450-mediated metabolic oxidative demethylation through the formation of hydroxymethyl precursor **5** that loses a molecule of formaldehyde to form monomethyl triazene MTIC **6**,[2] which is the active agent of the prodrug Scheme 1. However MTIC possesses a poor tissue distribution that is responsible for the unassuming clinical performance of DTIC. This prompted the synthesis of imidazotetrazinone prodrug temozolomide **1a**, which liberates the same active agent (MTIC).[3]



Scheme 1. Metabolic Activation of Dacarbazine

Temozolomide **1a** is a good delivery system for MTIC to the site of action at DNA, with good tissue distribution. Its propensity to ring opening is enhanced by the bicyclic system with bridgehead nitrogen atom of the imidazotetrazinone ring, resulting in the activation of temozolomide under the chemical control of the pH rather than metabolic activation in the liver.[4]

In 1980 Robert Stone synthesized the first example of the new ring system imidazo [5,1-d]-1,2,3,5-tetrazine azolastone (mitozolomide **1b**) making use of a published new route, to fused 1,2,3,5-tetrazines from the interaction of diazoazoles and isocyanates by Ege & Gilbert in 1979.[5] Mitozolomide **1b**,[6] the bifuntional alkylating agent, entered the clinic with high hopes in 1983. Unfortunately, despite its curative activity in mice, mitozolomide was found to cause severe and unpredictable myelosuppression which limited its clinical usage.[7] At this stage temozolomide **1a** which is the *N*-methyl congener of mitozolomide, was selected as a less toxic, second generation analogue for clinical trial.

Temozolomide **1a** demonstrated a different toxicological profile, it is a mono-functional methylating agent,[3] characterized by an unusual stability in acidic pH which allows its excellent oral bioavailability,[8] it possesses a good tissue distribution and can penetrate

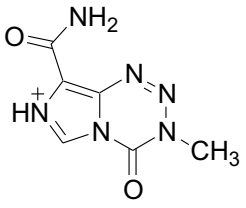
into the central nervous system, such penetration was not exhibited by other MTIC prodrugs.[9, 10]

Vaupel et al., 1989[11] showed that there are differences in pH between healthy brain tissue (pH 6.96-7.05) and glioma (7.15-7.22), these differences may contribute to the selective toxicity of temozolomide towards gliomas. The lower pH of healthy tissue would stabilise the prodrug and favour passage of the intact prodrug to the tumour cell. However, it is of great importance to consider other factors that affect temozolomide toxicity such as the activity of DNA repair enzymes MMR & MGMT which influence the susceptibility of tumor to temozolomide.[12]

1.2 Imidazotetrazinone chemistry

1.2.1 Imidazotetrazinone aqueous reaction

The hydrolysis of imidazotetrazinones is unusually pH dependent. These compounds are stable in 1 M sulphuric acid and can even be recovered unchanged from hot concentrated sulphuric acid, in addition to the photostability of their aqueous solution.[6] In the alkali 5 % sodium carbonate, they decomposed rapidly by the tetrazine ring-opening Figure 1.[13] This figure shows temozolomide stability at acidic pH and being labile above pH 7. The stability of temozolomide in acidic media allows its survival in the strong acid in the stomach and is important for its absorption intact, therefore it can be administered orally.[8] A ^{15}N NMR study has shown that this unusual stability in acid is due to the protonation on the temozolomide taking place at *N*-7 rather than *O*-4, which would be required to catalyze the ring-opening reaction, Structure **7**.[14]



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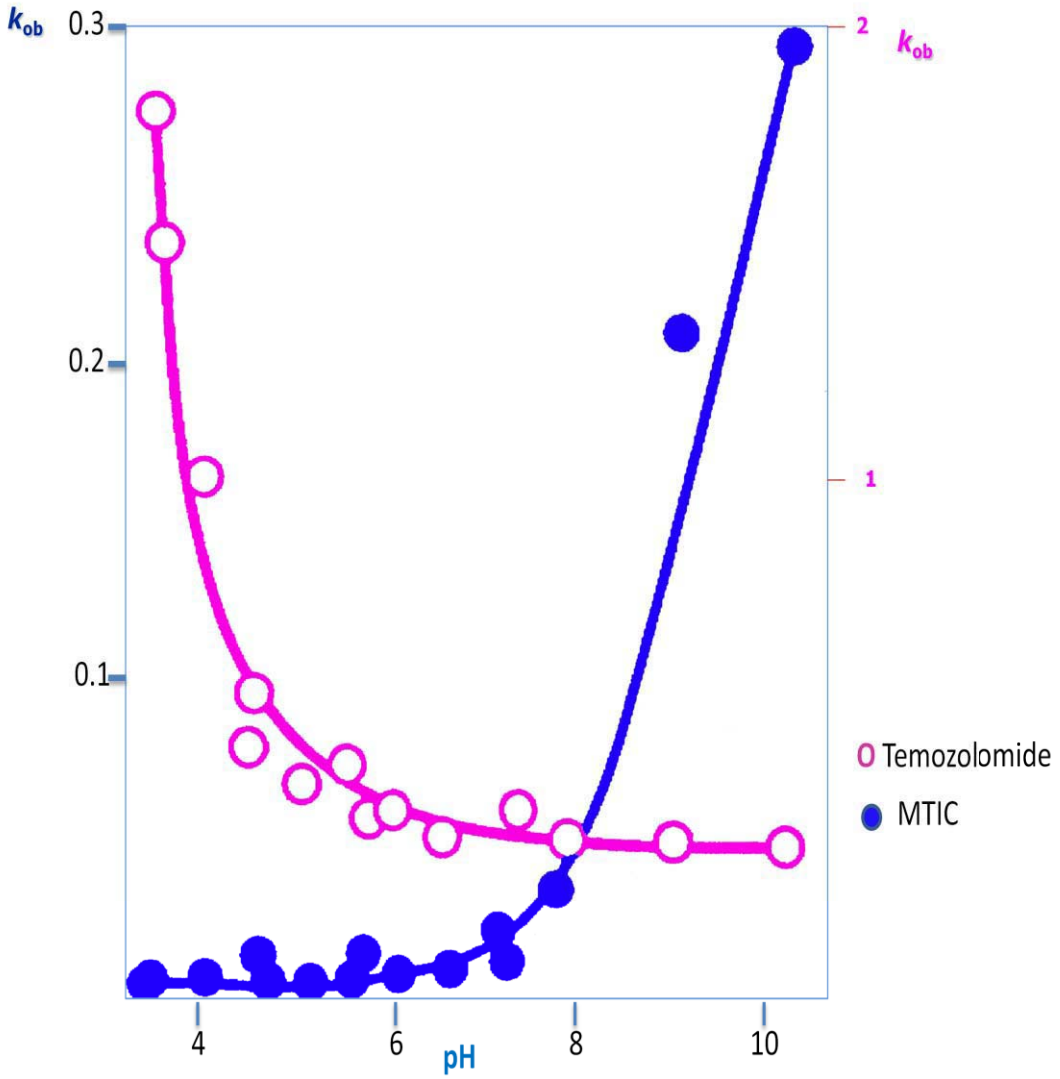
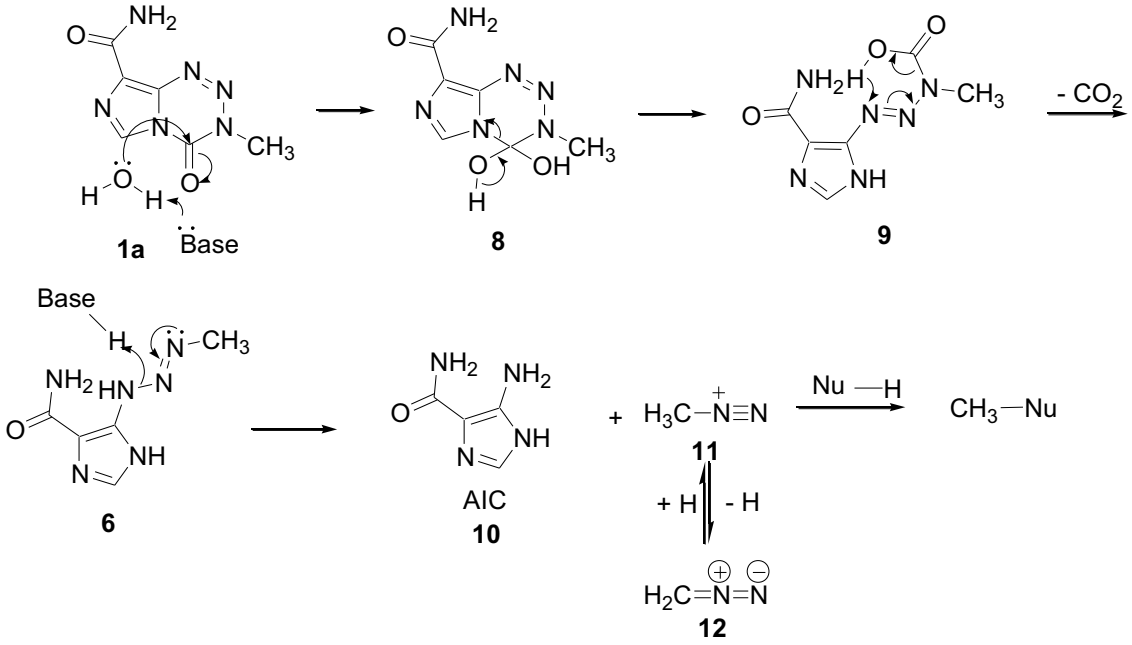


Figure 1. Plot of Stability Constant (k_{ob}) versus pH of Temozolomide and MTIC[15]

The temozolomide reaction in neutral aqueous medium provides an indication to its biological activity as a pro-drug.[3] In 0.1 M phosphate buffer pH 7.4 at 37 ºC, temozolomide undergoes ring-opening to the open-chain triazene MTIC **6** with half-life

of 1.83 h,[15] Scheme 2. MTIC is same metabolite formed upon the metabolic activation of the antitumor prodrug DTIC **4**.[16] MTIC on the other hand is unstable below pH 7 but more stable at the alkaline pH values, Figure 1.[15]



Scheme 2. Temozolomide Reaction in Aqueous Medium

Addition of water (**1a** → **8**) is the rate-limiting step in the aqueous decomposition of temozolomide **1a**, Scheme 2. Base-catalysed addition of water to C-4 forms tetrahedral adduct **8**, which undergoes ring-opening to give unstable carbamic acid **9**, spontaneous decarboxylation leads to MTIC **6**. This is then followed by the acid-catalysed fragmentation of MTIC to form AIC **10** and methyldiazonium ion **11**.[15]

The methyldiazonium ion **11** is the methylating agent, it is relatively stable and this stability comes as a result of the proton loss and gain equilibrium with diazomethane **12**. Moreover, the stability of the methyldiazonium ion was proved by deuterium

incorporation and it responsible for the life time of the ion, which allows it to encounter its DNA target in cells.[15]

Figure 2 shows the results of the NMR study of temozolomide decomposition in deuterated phosphate buffer solution (pD 7.8 at 37 ºC). The figure shows deuterium incorporation into CH_3OD, CH_2DOD, CHD_2OD in addition to the phosphate esters. This implies that methyldiazonium **11** is in equilibrium with diazomethane **12** and D_2O, Scheme 2.

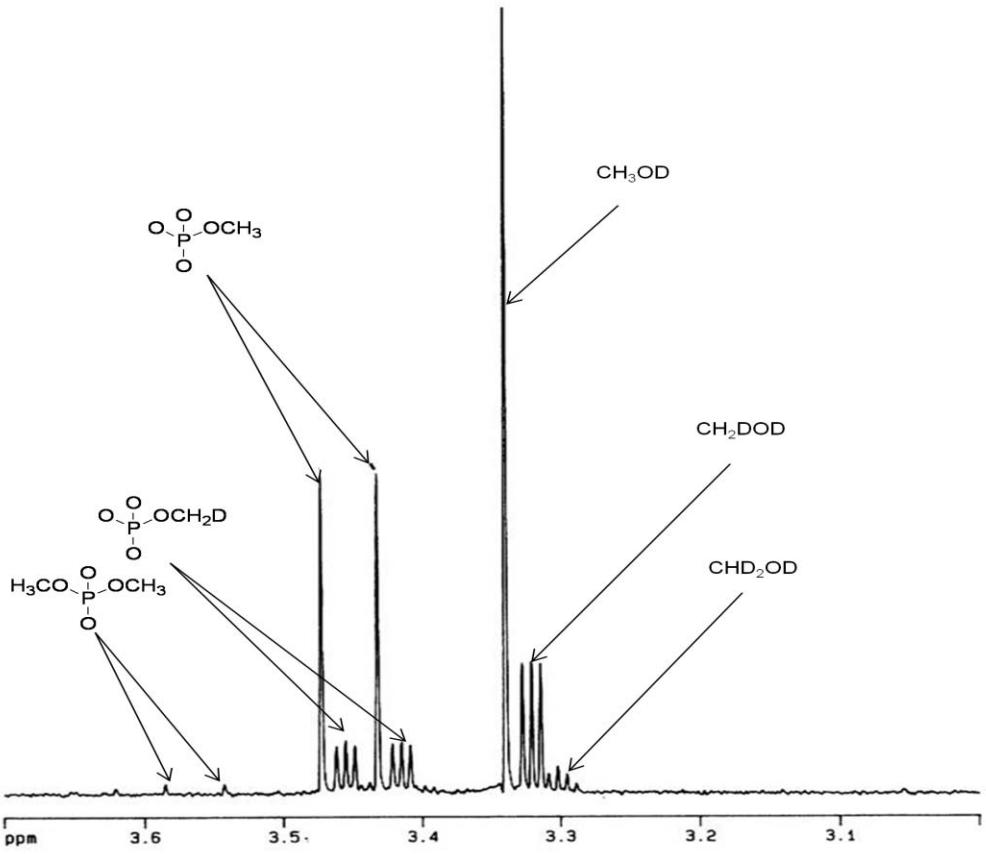
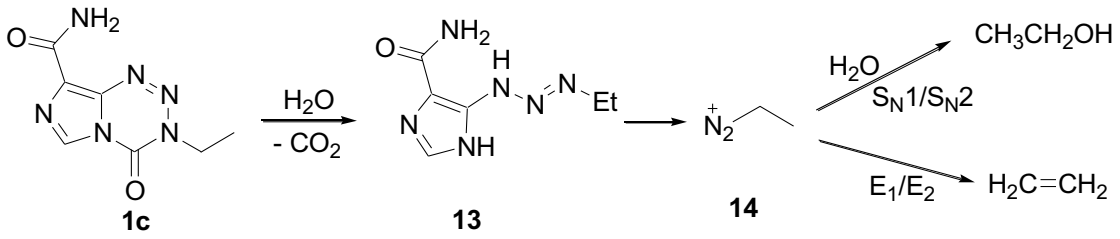


Figure 2. Methyl region of ^{1}H NMR spectrum of decomposition of temozolomide in deuterated phosphate buffer pD 7.8 at 37 ºC (Adapted from ref 15)[15]

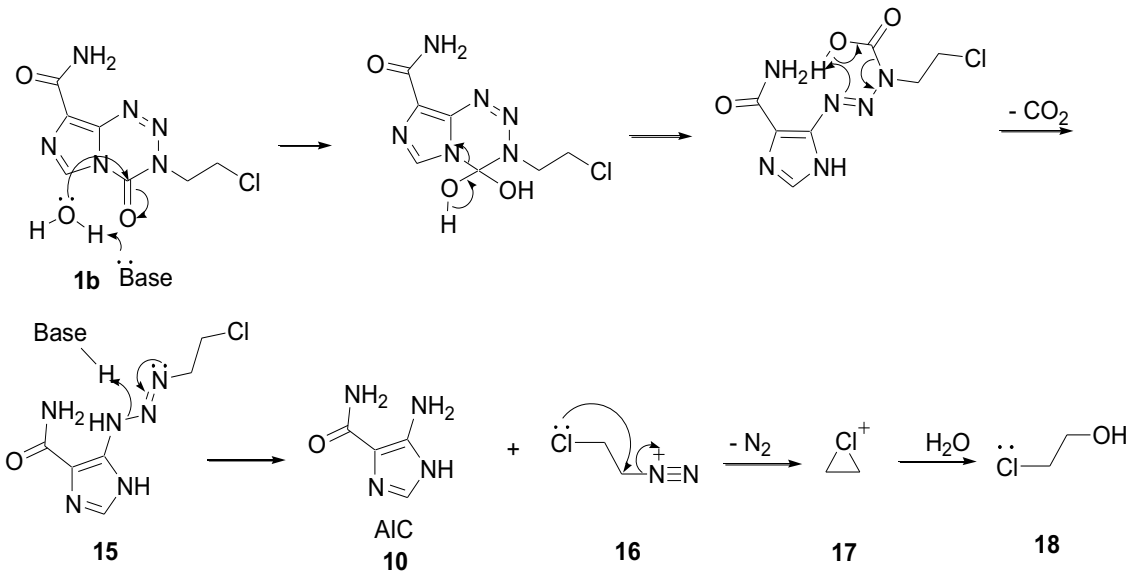
In contrast, 3-ethylimidazotetrazinone **1c** is devoid of antitumor activity,[4] it undergoes a ring opening in the phosphate buffer pH (7.4) to the ethyltriazine **13**, which upon

further fragmentation results in ethyldiazonium ion **14**, which characterized by a short lifetime for finding its biological target, compared with that of methyldiazonium ion **11**.[17] A ^{1}H NMR study of the decomposition in phosphate buffer showed a small peak of ethene with a peak for ethanol, this reflects the immediate reaction of the diazonium ion with water to alcohol or by elimination to ethene, Scheme 3.[17]



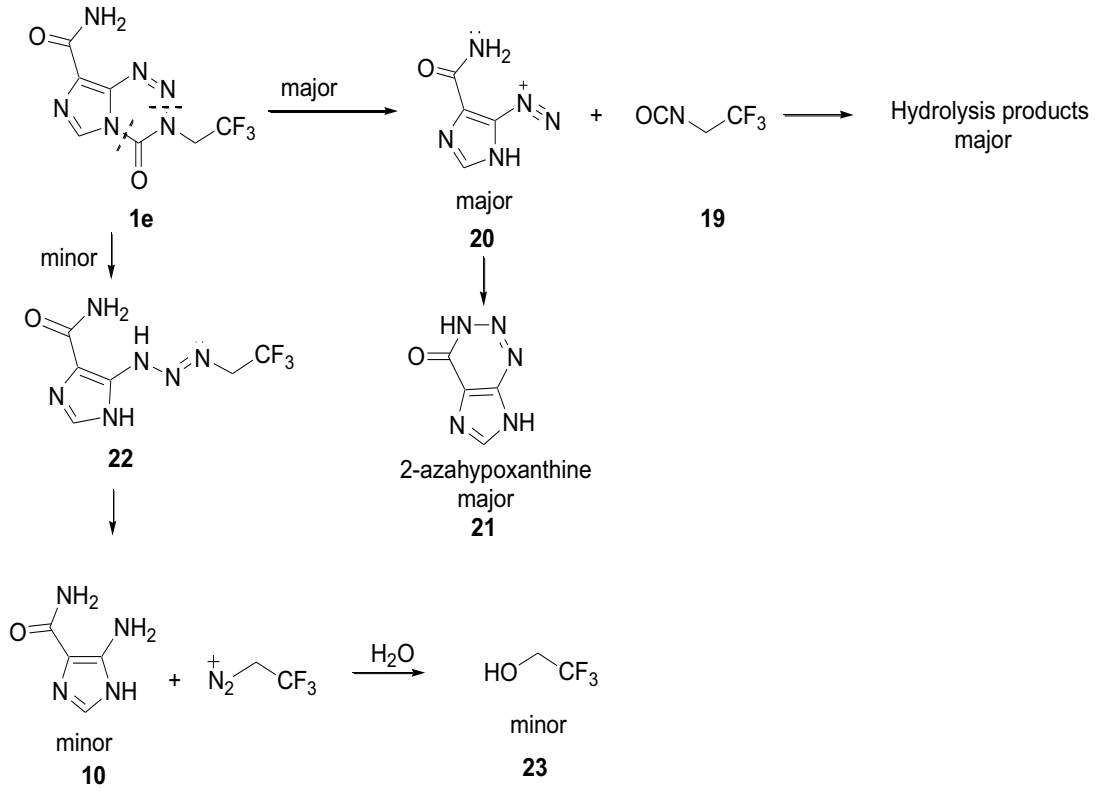
Scheme 3, Ethylimidazotetrazinone neutral aqueous decomposition

The 3-(2-chloroethyl)imidazotetrazinone mitozolomide **1b**, follows a similar aqueous decomposition to temozolomide. Its decomposition was studied in 5% aqueous sodium carbonate and resulted in the chloroethylating agent MCTIC **15**.[6] MCTIC acts as a bifunctional alkylating agent. Acid-catalysed fragmentation gives 2-chloroethyl-diazonium ion **16** and AIC **10**; participation of the chloro group with one of its unshared electron pairs gives cyclic chloronium ion **17**. This reacts with water in the medium to give 2-chloroethanol **18** and nitrogen,[6, 14] Scheme 4.



Scheme 4. Mitozolomide neutral aqueous hydrolysis

3-(2,2,2-Trifluoroethyl)imidazotetrazinone **1e,** which possesses more potent *in vitro* anitumour activity than both mitozolomide and temozolomide,[17] has a totally different pattern of decomposition in phosphate buffer pH 7.4 from that observed in the case of mitozolomide and temozolomide.[17] The strongly electron-withdrawing trifluoroethyl group of **1e** significantly affects the electronic character of the tetrazine ring, which results in weakening and thus breaking of the bonds 2/3 and 4/5,[17] Scheme 5. The major products detected were the result of hydrolysis of trifluoroethylisocyanate **19** and cyclization of diazo-IC **20** to 2-azahypoxanthine **21**. Minor products detected were AIC **10** and trifluoroethanol **22**, which were assumed to be produced by the usual tetrazine ring opening mechanism i.e analogous to that of temozolomide.[17]



Scheme 5. 3-(2,2,2-Trifluoroethyl)imidazotetrazinone **1e** decomposition in aqueous medium

1.2.2 Reaction with DNA

The reactions of imidazotetrazinones discussed in the previous section with the model nucleophiles OH_2, PO_4^{2-} will be extended to the consideration of reactions where the nucleophiles are sites on DNA. Guanine bases in DNA have a more negative molecular electrostatic potential than other bases.[15, 18] The atom with the most negative potential is the *N*7 of guanine which is also the most sterically accessible. This explains why this atom is most frequently alkylated by electrophilic drugs e.g. *N*-methyl-*N*-nitrosourea, cisplatin, nitrogen mustards, and bisulfan.[19]

Calculations of partial atomic charges in nucleic acid bases and the electrostatic contribution to DNA base-pairing,[20] show that a run of three consecutive guanines has a

higher dipole moment than other sequences of bases, this is consistent with the observed enhanced nucleophilicity and basicity of the major-groove microenvironment G-rich sequences and any associated water molecules, which may facilitate sequence-selective conversion of the prodrug temozolomide to MTIC.[21] The second factor that determines the selectivity for guanine alkylation is the steric ease of access to guanine in the major groove.[22] A study by Abraham & Smith[20] tested the accessibility of different potential electrophile-acceptor atoms in DNA, it confirmed that *N*7(G) accessibility is 10 Å^2, whereas *O*6(G) is 8 Å^2 and *N*7(A) is 8 Å^2 (Table 1), the study also showed that *N*7 guanine has the greatest negative potential.[20]

Upon treating DNA with ^{14}C-labelled temozolomide, 70% of the label transferred to DNA was associated with *N*7 guanine, 5% with *O*6 of guanine, and 25% with other sites.[23] Moreover, studies also showed that the favoured site of MTIC reaction is at G*N*7.[21] These data are consistent with both the steric accessibilities and electrostatic potentials shown in Table 1.

There was a proposal, supported by molecular modelling studies, that indicated temozolomide could make a productive, hydrogen-bonded association with DNA in which the role of carboxamide group may be vital in orienting the drug towards guanine-cytosine sites.[15] On the other hand, the fact that unstable species MTIC also favoured the methylation of *N*7 residue of the middle guanine in runs of guanines in isolated DNA confuses the picture stated by the molecular modelling study.[24]

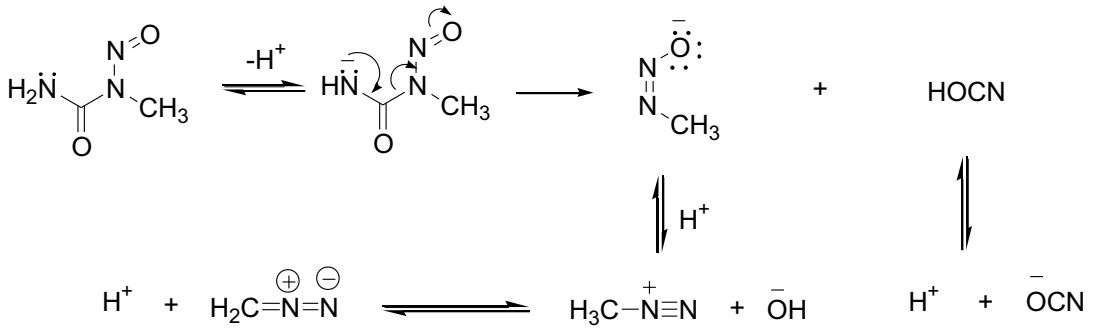
The study made by Clark also found no evidence of the weak association of temozolomide and mitozolomide with DNA at the major groove: the rate of reaction was not sequence dependent and they concluded that the conversion of imidazotetrazinone prodrug to drug occurs in free solution under influence of the local

pH, rather than in the major groove catalysed by a target DNA sequence as previously proposed.[24] This fact is in agreement with half-life (1.83 h) of temozolomide in phosphate buffer (pH 7.4 at 37 ºC) being comparable with its mean plasma half-life in patients (1.81 h).[15] Furthermore, the Clark study shows no rate enhancement observed by the presence of the guanine rich oligonucleotides in the buffer solution used for testing the rate of prodrug activation.[24]

Atom	Accessibility ($Å^2$)	Potential (kcal mol^{-1})	Groove
*N*7 (G)	10	-683	Major
*O*6 (G)	8	-654	Major
*N*7 (A)	8	-650	Major
*O*4(T)	3	-612	Major
*O*2 (T)	7	-663	Minor
*N*3 (A)	2	-668	Minor
*O*2 (C)	4	-645	Minor
N3 (G)	2	-670	Minor

Table 1. Steric surface area accessibilities and potentials of atoms in B-DNA

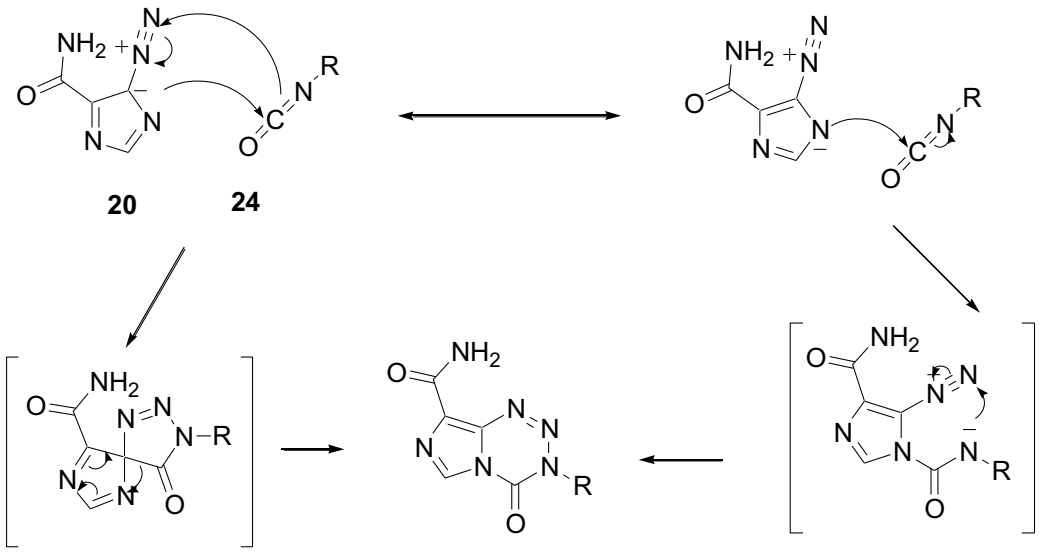
In several respects, temozolomide most closely resembles the promutagen *N*-methyl-*N*-nitrosourea (MNU), which also does not require metabolic activation but is chemically activated at pH 7.4 to generate methyldiazonium, Scheme 6.[25]



Scheme 6. Fragmentation mechanism for the base-induced decomposition of MNU in water

1.2.3. Imidazotetrazinone Synthesis

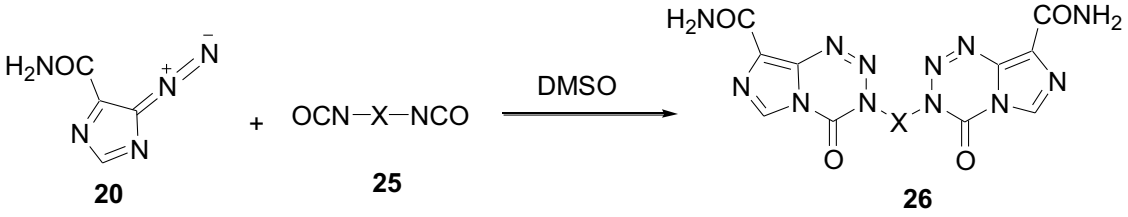
The synthesis of azolotetrazinones by Ege and Gilbert[5] from the interaction of diazoazoles and isocyanates provided the impetus for the synthesis of the first imidazotetrazinone mitozolomide **1b**, from the reaction of diazo-IC **20** with 2-chloroethylisocyanate **24b** in dichloromethane at 25 ºC in the dark.[6] This reaction was used for the synthesis of different imidazotetrazinones, the interaction of methyl, *n*-propyl isocyanate and aryl isocyanates with diazo-IC in dichloromethane or ethyl acetate shown by Scheme 7 led to the different imidazotetrazinones.[6]



1, 24 **a**: R = CH_3 **i**: C_6H_4-Cl-*p*
b: CH_2CH_2Cl **j**: C_6H_4-CN-*m*
c: CH_2CH_2 **k**: Naphth-1-yl
d: *n*-pr
e: CH_2CF_3
f: C_6H_4-Me-*p*
g: C_6H_4-OMe-*p*
h: C_6H_4-OEt-*p*

Scheme 7. Two possible attacks between the diazo-IC ring and isocyanates in the synthesis of different imidazotetrazinones

Temozolomide **1a** was synthesised from the reaction of methylisocyanate **24a** with diazo-IC **20**. This reaction took a long time in solvents such as dichloromethane and ethyl acetate but it is characterized by a high yield and high purity of the product. The reaction time was improved by using DMSO as the reaction solvent, this route of synthesis gives access to a wide range of analogues[26] and it can be used for the synthesis of bisimidazotetrazinones **26** when the diisocyanates **25** were reacted with diazo-IC **20** Scheme 8.[26]



25, 26 a: X = $(CH_2)_2$ **i**: $CH_2S(CH_2)_2SCH_2$
b: $(CH_2)_4$ **j**: $(CH_2)_2S(CH_2)_2$
c: $(CH_2)_6$ **k**: $(CH_2)_2SCH_2S(CH_2)_2$
d: $(CH_2)_8$ **l**: $(CH_2)_2SS(CH_2)_2$
e: $(CH_2)_{12}$
f: CH_2SCH_2
g: CH_2OCH_2
h: $CH_2O(CH_2)_2OCH_2$

Scheme 8. Synthesis of bisimidazotetrazinones

The original method can accommodate modifications that led to preparation of a variety of isotopically labelled forms of the drug.[27] Sites of temozolomide that have been prepared with ^{15}N (N-2, N-3) and ^{13}C (CH_3) for nuclear magnetic resonance (NMR) studies[27] and ^{11}C (CH_3) for positron emission tomography (PET) imaging are indicated on Figure 3.[28] Mitozolomide has also been radiolabelled with ^{14}C (C-6) for pharmacokinetic experiments.[29]

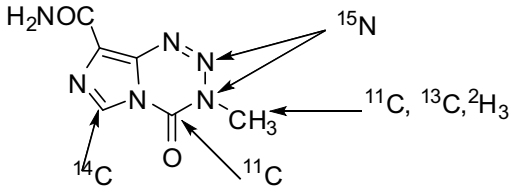
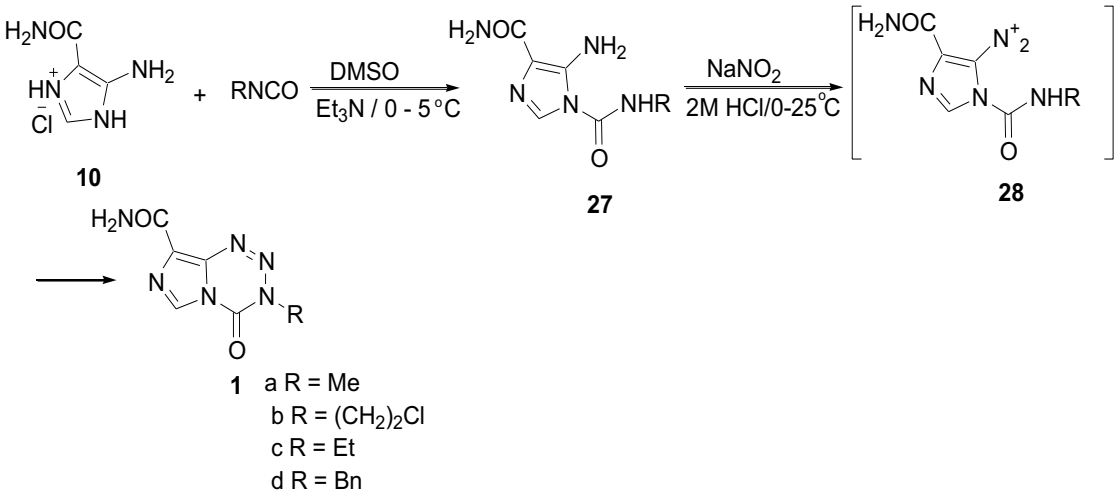


Figure 3. Isotopically-labelled forms of temozolomide

The original route of synthesis of the tetrazines, Scheme 7, faced some problems, the first of which was the low boiling point of methylisocyanate (39 ºC) and second one is the instability of the diazo-IC. The third is the failure to obtain some tetrazine products by using these reagents[30] e.g. 4-thiotemozolomide was not formed by reacting methylisothiocyanate with 5-diazoimidazole-4-carboxamide in DMSO.[30]

In 1997 Wang and Stevens[31] modified the method for synthesis of temozolomide and its analogues. In this method, 4-aminoimidazole-4-carboxamide hydrochloride (AIC **10** salt) instead of the potentially unstable diazoimidazole was used to react with the isocyanate to the urea **27**, diazotization of the urea resulted in the unstable diazo urea **28** which cyclised in situ to give the imidazotetrazinones **1**, Scheme 9.[30]

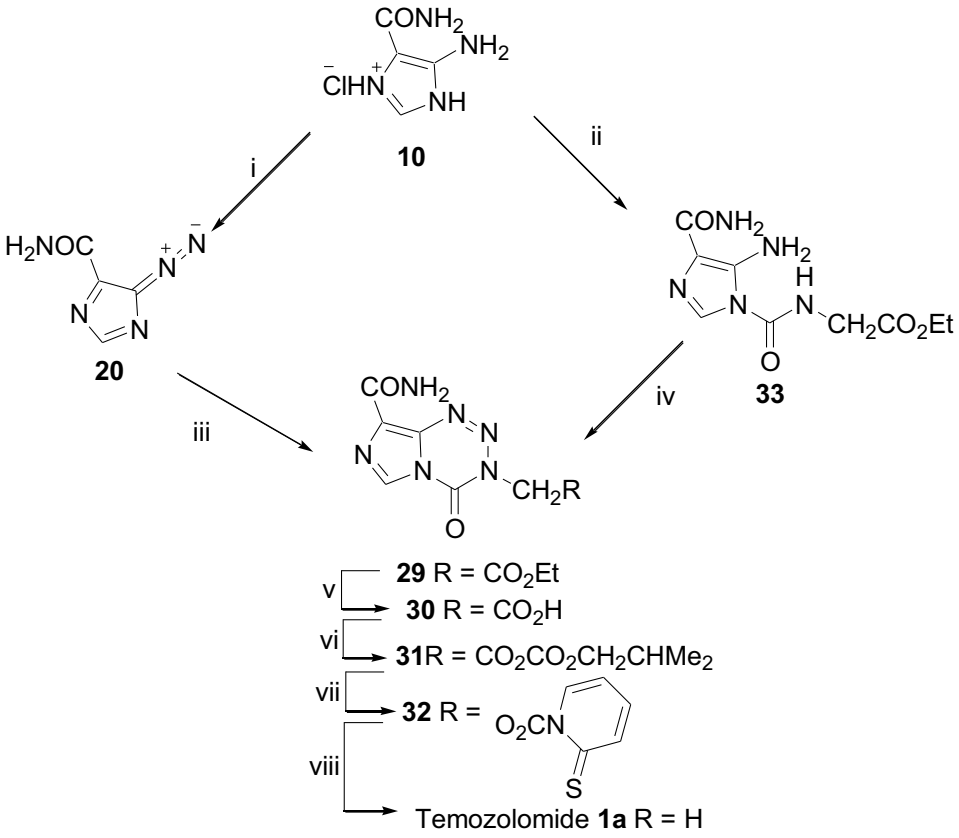


Scheme 9. Alternative tetrazines synthesis without using of diazo-IC

In an alternative synthesis, Scheme 10,[32] temozolomide was prepared by the reaction of commercially-available, non-volatile ethyl isocyanatoacetate and diazo-IC **20** to give

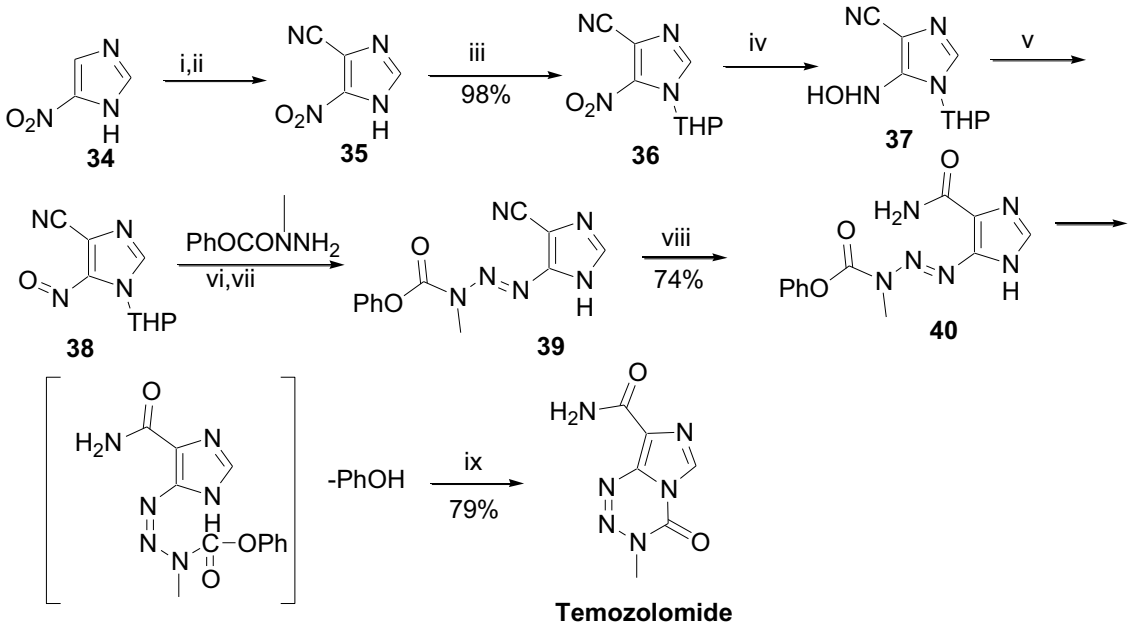
ester **29** in 80% yield. Hydrolysis to the acid **30** was accomplished in 80% yield. The direct conversion of ester **29** or the acid **30** to temozolomide failed, so the carboxylic acid was converted to reactive mixed anhydride **31** with isobutylchloroformate/ *N*-methylmorpholine and then to 1-substituted pyridine2(1H)-thione **32**. Homolytic cleavage of **32** with AIBN (initiator) and tributyltin hydride gave temozolomide, **1a** Scheme 10.

An alternative route to the ester **29** in Scheme 10, is by carbamoylation of the AIC **10** used in form of its hydrochloride salt to give the urea **33** which cyclised to the ester **29**.[32]



Scheme 10. *Reagents and conditions:* (i) $NaNO_2$ (excess), 2 M HCl 0 ºC; (ii) EtO_2CCH_2NCO, DMSO, pyridine, 20 ºC; (iii)EtO_2CCH_2NCO, DMSO, 25 ºC (iv)$NaNO_2$, 2 M HCl, 0 ºC; (v) 5 M aq. HCl, 45 ºC; (vi) Me_2CHCH_2OCOCl, *N*-methymorphine, DMF, –15 ºC; (vii) 2-mercaptopyridine-*N*-oxide, Et_3N, –15 ºC; (viii) Bu_3SnH, AlBN (catalyst), DMF, hν, 25 ºC

In addition temozolomide can be synthesised *via* a condensation reaction between nitrosoimidazole **38** and hydrazides and subsequent cyclization of the resulted triazene **39**, Scheme 11.[33] This gave temozolomide in an overall yield of 44%. In this method 4-nitroimidazole **34** was used as starting material and the carboxamide substituent present in temozolomide was introduced to the starting 4-nitroimidazole in the form of a nitrile group to give the 5-nitro-4-carbonitrile **35**. The 5-nitro-4-carbonitrile can also be synthesized by the cine substitution reaction of the 1,4-dinitroimidazole using potassium cyanate.[34] Protection of the NH group of the imidazole ring of **35** with dihydropyran was used to give THP-derivative **36** which improves the imidazole solubility. Controlled reduction of the nitro group of **36** resulted in the hydroxylamine **37**; oxidation of the hydroxylamine gave the nitroso compound **38**. Condensation of the nitroso compound with the hydrazide and the deprotection of THP resulted in the triazene **39**, hydrolysis of the cyano group of **39** in strong acidic conditions resulted in the triazene with a carboxamide group **40**. The cyclization of **40** was achieved by irradiation at 366 nm in acetone-MeOH to give temozolomide.



Scheme 11. *Reagents and conditions:* (i) HNO_3, Ac_2O, 85%; (ii) KCN, $NaHCO_3$, MeOH, H_2O; rt, 62%; (iii) dihydropyran, *p*-TsOH, EtOAc; (iv) H_2, 10% Pt/C, EtOAc; rt, 60 min; (v) 2 eq. $NaIO_4$ in H_2O, EtOAc; 0 ºC, 30 min; (vi) DCM-AcOH 5:2; rt, 2 h; (vii) TFA-AcOH-MeOH 3:6:10; rt, 5 h; (viii) conc. Aq. HCl-AcOH 2:1; 60-65 ºC, 25 min; (ix) acetone-MeOH 2:1, 366 nm; rt, 1 h.

Regardless of the development of alternative methods of synthesis of temozolomide, which do not required the use of methylisocyanate or the potentially unstable diazo-IC, the original synthesis remains the method of choice for large scale and laboratory production of imidazotetrazinones.

1.3 Clinical Effects Imidazotetrazinones

The antitumor activity of temozolomide is largely attributed to the methylation of DNA,[15] it is mediated *via* the formation of *O*6-methylguanine adducts. Although this accounts for only a small percentage of DNA adducts that are formed, it is potentially carcinogenic, mutagenic and cytotoxic.[35]

In addition to the inhibitory effect on DNA replication, DNA methylation by temozolomide may also disturb other DNA-dependent processes, such as regulation of

gene expression. A series of studies showed that temozolomide could induce differentiation in the K562 human erythroleukaemia cell line.[36]

It was also suggested that the formation of a carbamoylating isocyanate as in case of mitozolomide, may result in the inhibition of certain enzyme activities, such as the inhibition of cellular esterase activity observed in EMT6 mouse mammary tumour cells.[37]

Early pre-clinical experimentation with temozolomide indicated that it possessed good antitumour activity when administered intraperitoneally (i.p.) against both haematological (L1210 and P388 leukaemias) and solid (e.g. M5076 sarcoma, ADJ/PC6A plasmacytoma, B16 melanoma, Lewis lung carcinoma) murine tumour models.[3] This activity was found to be schedule-dependent,[3] with multiple administrations being more effective than a single bolus dose.

Like mitozolomide, temozolomide demonstrated good tissue distribution, including penetration across the blood-brain barrier, and was found to have a bioavailability of 0.98 when administered orally.[38] Antitumour activity was therefore maintained when temozolomide was administered orally to L1210-bearing mice.[3]

The plasma pharmacokinetics of temozolomide following oral administration to mice were characterized by a rapid absorption phase (the peak plasma concentration of temozolomide being achieved within 30 min of administration) and mono-exponential elimination (with elimination half-life of 1.29 h).[3]

The predominant route of temozolomide elimination is by renal excretion and occurs with 5 – 10% as unchanged drug, although an unidentified acidic urinary metabolite is also produced.[39] These observations were found to correlate with the subsequent finding

of phase I clinical studies, which indicated that rapid absorption of temozolomide also occurred in man (the peak plasma concentration occurred within 0.7 h of oral dosing) and the drug elimination could be best described by a one-compartment model with a half-life of 1.81 h.[8]

In addition to the unresolved metabolite, the urine of patients was also found to contain the 8-carboxylic acid metabolite of temozolomide, which has equivalent cytotoxicity to temozolomide and is thought to be generated extrahepatically.[39]

Temozolomide crosses the blood-brain barrier with 9 – 29% of the serum concentration detected in the cerebrospinal fluid.[40] Biodistribution has also been studied by PET scanning, by using $^{11}C4$ and $^{11}CH_3$ lablled temozolomide, to investigate the uptake of the drug by brain tumours. This study confirmed the capability of temozolomide to cross the blood-brain barrier.[41]

The pharmacokinetics of temozolomide were explored, by the analysis of plasma after the treatment with temozolomide or the ^{11}C-lablled temozolomide and for its metabolites and the breath analysis for the $^{11}CO_2$. The study showed the higher levels of $^{11}CO_2$ in the plasma and the breath and lower level of temozolomide, this provides evidence that temozolomide undergoes decarboxylation and ring opening in plasma. On other hand positron emission tomography (PET) imaging was used to confirm that ^{11}C- labelled temozolomide at the methyl group, achieved a selective methylation to brain tumors relative to the surrounding healthy brain tissue.[41]

Treatment with temozolomide is schedule-dependent,[3] a 5-day schedule repeated every 28 days was studied. The recommended dose of temozolomide is 200 mg/m^2 daily for 5 days repeated every 4 weeks. No clinical activity was seen with the single dose

schedule but activity was seen in some patients with melanoma and a patient with mycosis fungoides.[8]

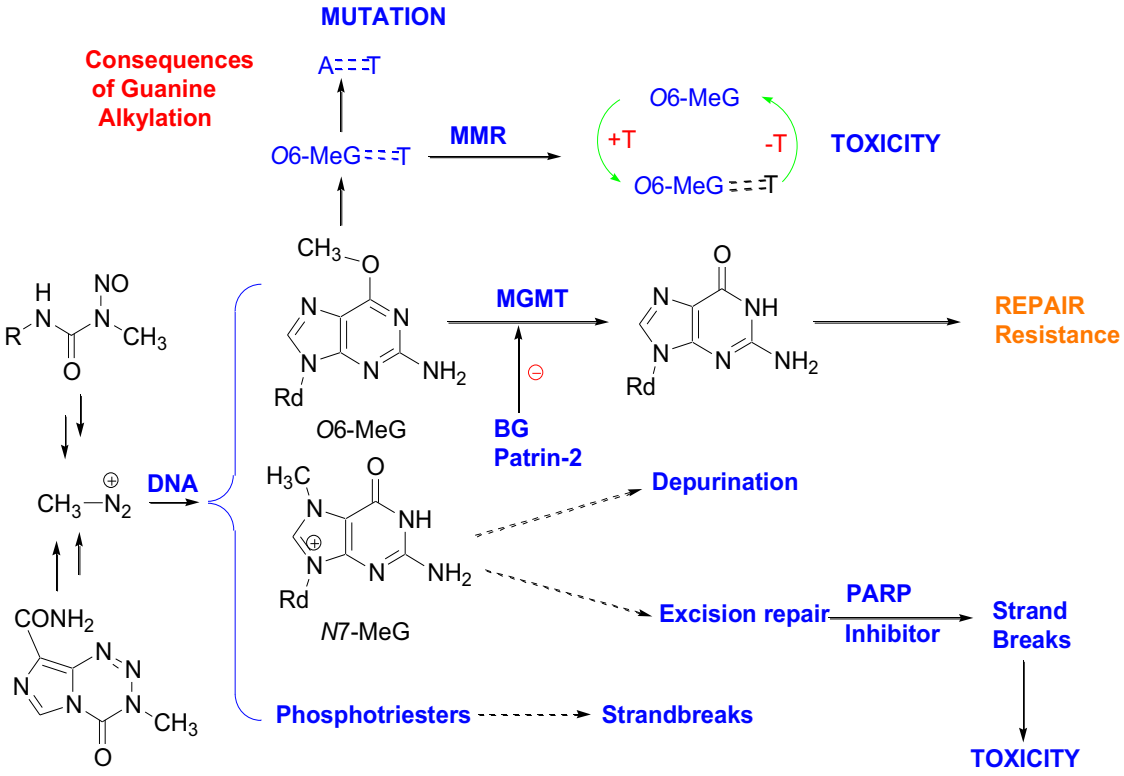
Temozolomide was used in a trial treatment of metastatic malignant melanoma and it showed activity comparable with other currently-used agents. In addition to its uses in the central nervous system tumours, temozolomide shows 8% response in soft tissue sarcoma. However no response was shown in renal cell cancer[42] and biopsies taken in this study demonstrated high levels of *O*6-methlguanine-DNA methyltransferase (MGMT) a protein responsible for repair of DNA damage caused by temozolomide in kidney tissue.[43]

The cytotoxicity of temozolomide is affected by different DNA-repair mechanisms. Scheme 12 shows some of these mechanisms and their relationship to the cyototoxicity of temozolomide:

1) *O*6-methylguanine-DNA methyltransferase (MGMT);

2) DNA – mismatch repair (MMR);

3) Base excision repair (BER).



Scheme 12. The role of the different DNA repair mechanisms in the cytotoxicity of temozolomide

1.4 Resistance to Temozolomide treatment

1.4.1 *O*6-Methylguanine-DNA methyltransferase MGMT

DNA damage by temozolomide is liable to repair by the protein ATG which also known as *O*6-methylguanine-DNA-methyltransferase (MGMT), which is a protein considered as one of the components of cellular resistance to alkylating drugs. It repairs many adducts at the *O*6-position of guanine including ethyl-, 2-chloroethyl- and other aliphatic groups and also benzyl and pyridyloxobutyl adducts. MGMT works by accepting the methyl group from the *O*6-Me guanine in DNA to an internal cysteine residue (Cys145) within its active site. It works through a Glu-His-Water-Cys hydrogen bonded network, in which His146 acts as a water-mediated base resulting in the formation of the thiolate anion at Cys145 to act as a nucleophile to cleave a methyl group from the *O*6-G, in an irreversible stoichiometric reaction. This restores guanine in

DNA but inactivates MGMT, Scheme 13.[44] The protein mechanism of action requires different roles to be played by the different domains that form its structure, some of the amino acids form the DNA binding site responsible for the conformation that leads to the flipping of the methylated guanine in to the active catalytic site in a suitable orientation to the Cys145 for methyl transfer. Arg128, located at the beginning of the recognition helix is an arginine finger that interacts through the minor groove *via* charged hydrogen bond with the orphaned cytosine (previously base paired to the flipped-out guanine) to stabilise the extrahelical DNA conformation, Figure 4. This is in addition to the role of the hydrophobic part of the active site which responsible for the selectivity of the protein to repair the *O*6-methylated guanine.[45]

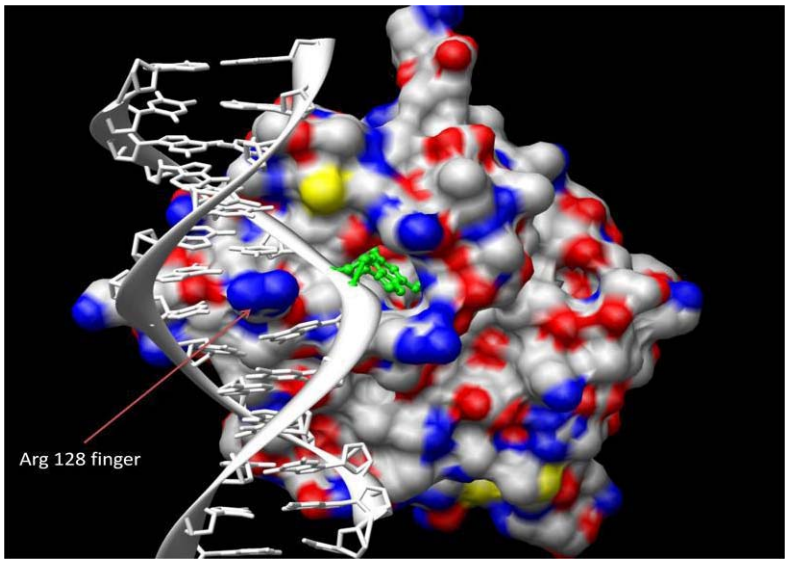
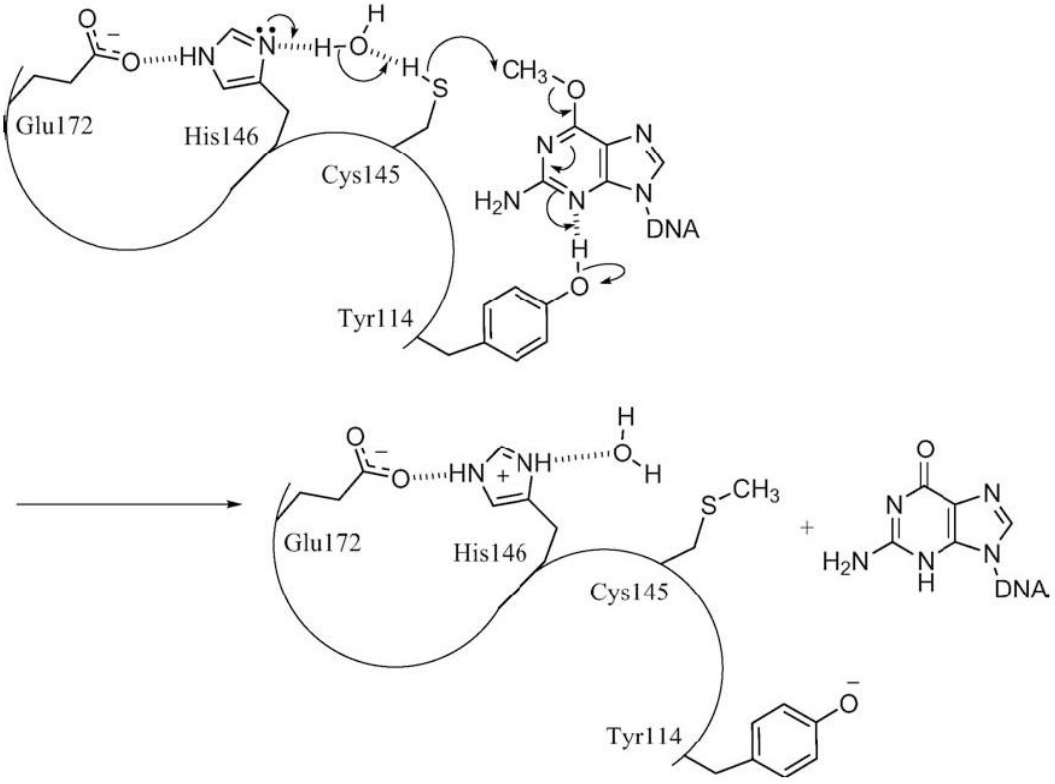


Figure 4. MGMT bond to DNA showing the flipped-out *O*6MeG in the active site of the protein and the Arg128 finger stabilizing the extrahelical structure of DAN by hydrogen bonded to the orphaned cytocine. Figure courtesy of Richard Wheelhouse using coordinates from refrence no 46.[46]

The protein, after accepting the methyl group, undergoes proteolysis, the restoration of activity and the protective mechanism require *de novo* protein synthesis which takes up to 72 h.[47]



Scheme 13. Proposed reaction mechanism for MGMT, His 146, by intervention of a bridging water molecule, deprotonates Cys145 to facilitate nucleophilc attack at the *O*6-alkylcarbon with simultaneous protonation of *N*3 by Tyr114[48]

Generally the level of MGMT in tumour cells is higher than normal cells especially in higher grade glioma and in advanced ovarian carcinoma.[49]

Studies have suggested a link between pre-treatment MGMT levels and clinical outcome.[50] Glioblastoma and melanoma, the tumours that are currently treated with temozolomide, show the lowest mean MGMT concentration.[51]

Inactivation of alkyltransferase

An important factor in developing anticancer therapies that target MGMT is that MGMT-mediated repair is stoichiometric. Therefore, since one MGMT molecule is needed for each offending alkyl-G molecule, the level of MGMT in the cell can be completely depleted by excess *O*6-alkylated guanines. Reaction with any *O*6-alkylguanine pseudosubstrate can inactivate it and, once removed, the restoration of activity and the protective mechanism require new protein synthesis which takes up to 72 h.[47] Therefore treating cell with non-toxic MGMT pseudosubstrates e.g *O*6-benzylguanine inactivates MGMT through the covalent transfer of the benzyl group to the active site cysteine and by doing so, sensitises tumor cells to temozolomide and other alkylating agents such as BCNU. Benzylguanine is rapidly converted to another equally potent MGMT inhibitor, 8-oxo-*O*6-benzylguanine in human by CYP1A2 and CYP3A4. Therefore, combination with DTIC is not appropriate because DTIC activation occurs using the same enzymes. The irreversible mechanism of MGMT has been used clinically in the pre-treatment of patients with agents that react with the protein and result in its inactivation, which then enhance the antitumour activity of the alkylating drug.[52]

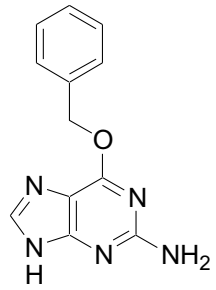
*O*6-Benzylguanine (*O*6-BG) **41** has been used since it is a good substrate for the enzyme; the benzyl group is a more efficiently displaced group than methyl group (methylguanine) in the bimolecular displacement reaction, the stability of the benzyl carbocation over that of the methyl one is behind this advantage of benzyl guanine.[53]

Near-complete inactivation (>99.5%) of MGMT activity was achieved with 5 μM *O*6-benzylguanine for 4 h, while 100 μM *O*6-methylguanine was needed to obtain a maximal effect of 80% reduction in the protein activity. [53]

Pretreating cells with MGMT activity of >15 fmol/mg protein with the MGMT inhibitor, *O*6-benzylguanine (*O*6-BG), potentiated chemosensitivity to temozolomide. In contrast, *O*6-BG did not sensitise a cell line (ZR-75-1) expressing very low levels of this protein. When BG pretreatment was combined with repeat doses of temozolomide a dramatic potentiation (300 fold) was seen in MAWI cells, which express high levels of MGMT, but not in a cell line (U373) expressing lower levels of MGMT.[12]

*O*6-(4-bromothienyl)guanine (PaTrin-2) **42** is another agent under development as a pre-treatment to enhance the activity of temozolomide and other alkylating agents. It is orally available and more potent in decreasing the maximum tolerated dose of the alkylating agent than *O*6-BG.[54] It was synthesized during the clinical trials of *O*6-BG which was started late in 1992 at Trinity College Dublin. The study showed that replacement of the benzyl group of *O*6-BG with a heterocyclic ring as in case of *O*6-furfurylguanine **43** and *O*6-thenylguanine (PaTrin-1) **42** led to improvement in the activity as an MGMT inhibitor. The IC_{50} of *O*6-BG, *O*6-furfurylguanine and *O*6-thenylguanine were 0.02, 0.03 and 0.018 μM respectively.[54]

The large size of the sulphur atom in the thiophene ring gives similar steric character as the six membered ring of the benzyl group in *O*6-BG.[55] The introduction of halogen substituents on the thiophene ring of PaTrin-1, confered the greatest activity, of which the bromo group at position 4 provided the most active *O*6-(4-bromothenyl) guanine (PaTrin-2) **42** (IC_{50} 0.0034μM).[54]



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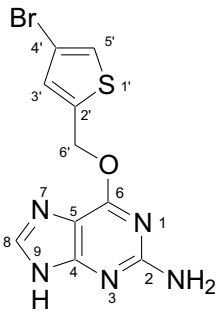

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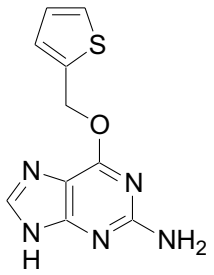

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1.4.2 DNA – mismatch repair

MMR is a system for recognizing and repairing erroneous insertions/deletions loops (IDLs), and mis-incorporation of bases that can arise during DNA replication and recombination, as well as repairing some forms of DNA damage. It primarily corrects single base-pair mismatches and small misalignment IDLs, which arise during replication. The MMR is strand-specific. As the newly synthesized (daughter) strand commonly includes errors, the MMR machinery distinguishes the newly-synthesized strand from the template, excising the mismatch nucleotide from the nascent strand, therefore providing the DNA polymerase with another chance to generate an error-free copy of the template sequence. In the absence of MMR base-base mismatches remain uncorrected, which results in a mutator phenotype that may turn into cancer. The MMR machinery has to satisfy two criteria:

1) it must efficiently recognise base-base mismatches and IDLs;

2) it must direct the repair machinery to the newly-synthesised DNA strand, which carried the erroneous genetic information.

The MMR system is also involved in the signalling and / or the appropriate processing of different types of DNA damage: MMR-deficient cell lines are more resistant to death

that is induced by several different types of chemical than matched MMR-proficient ones. Some cell lines that express low levels of MGMT still show resistance to temozolomide, which indicates that other mechanisms for resistance, may be involved. This is due to low levels of the mismatch repair mechanism (MMR).[56, 57]

There are two types of mismatch repair: long patch and short patch. Long patch can repair all types of mismatches (although it is primarily replication associated) and can excise tracts up to a few kilobases long. Short patch repair handles only specific mismatches caused by damage to the genome, and removes lengths of around 10 nucleotides. Successful mismatch repair requires the error-free execution of three events:[58]

1) detection of a single mismatch, of which there are eight kinds, in the newly-synthesized DNA;

2) determining which of the two bases in the pair is incorrect;

3) correcting the error by excision repair.

The mismatch repair machinery has a number of cues which distinguish the newly synthesized strand from the template (parental). In gram-negative bacteria transient hemimethylation distinguishes the strands (the parental is methylated and daughter is not). In other prokaryotes and eukaryotes the exact mechanism is not clear.[58]

The intact DNA-mismatch repair pathway is very important for temozolomide activity and any mutation affecting the correct functioning of the pathway results in loss of sensitivity to temozolomide. Figure 5 shows the mismatch pairing of *O*6-MeG to thymidine as a result of guanine methylation with temozolomide. The recognition of

mismatch pairing by the active MMR mechanism following DNA replication, leads to the futile cycle of deoxythymidine excision and replacement opposite the modified guanine site of the parent strand, that generates long lived-strand breaks, which end in apoptotic cell death.[59]

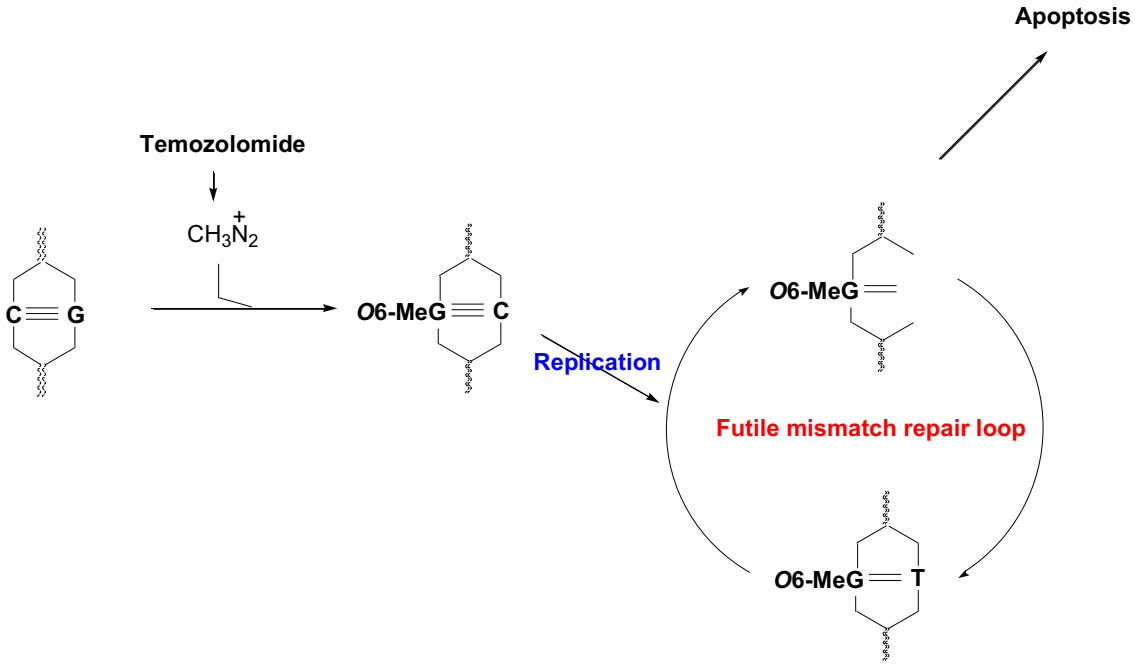


Figure 5. Mechanism of temozolomide cytotoxicity.[59]

1.4.3 Base excision repair and PARP

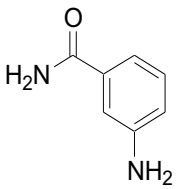
Base excision repair (BER) is a cellular mechanism that can repair damaged DNA during DNA replication. Repairing DNA sequence errors is necessary so that mutations are not induced during replication.

Single bases in DNA can be chemically mutated, for example by deamination or alkylation, resulting in incorrect base-pairing and consequently mutations in the DNA. Base excision repair is a family of enzymes that catalyze reactions collectively responsible for efficient repair of DNA.

The BER pathway begins with the excision of a damaged base by an enzyme called DNA glycosylase. DNA glycosylases bind to chemically-altered (damaged) bases and catalyzes the cleavage (hydrolysis) of the bond linking the modified base to its sugar, which result in the release of the modified base from the DNA chain. In order to fill the gap (replace the missing nucleotide), DNA polymerase inserts the correct nucleotide into the gap and links it to the normal nucleotide.

Temozolomide cytotoxicity correlates largely with the formation of *O*6-MeG. However this is one of several methylation sites in DNA, with 70% of lesions caused by the drug at *N*7-position on guanine. Repair of this lesion by BER results in lack of toxicity through this alkylation.[60]

Poly(ADP-ribose)polymerase (PARP) is involved in sensing and signalling of DNA damage generated by methylating agents at *N*7. Non-toxic PARP inhibitors such as 3-aminobenzamide **44**, benzamide **45**, 3,4-dihydro-5-methoxyisoquinolin-1(2H)-one **46** and 8-hydroxy-2-methylquinazolin-4(3H)-one **47** enhance the cytotoxicity of temozolomide-generated *N* 7-guanine methylation in tumour cell lines, Scheme 11.[61]



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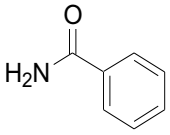

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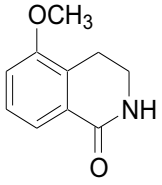

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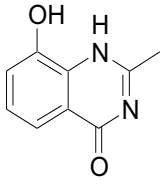

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1.5 Design and Rationale

The limitation of temozolomide treatment, represented in the repair mechanisms discussed in the previous section, promotes the design and synthesis of new imidazotetrazinones that may possess all the advantage features of the imidazotetrazinones that are shown by temozolomide:

i) good delivery system as a prodrug;

ii) acid stable then orally intended, in addition to 100% oral availability;

iii) crossing of the blood brain barrier;

iv) it is chemically activated, no need for metabolic activation;

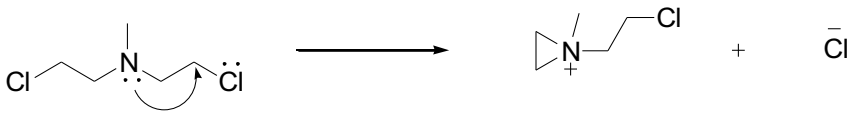
v) tumour localizing potential.

In addition, other new structure features may enable their overcoming the clinical limitations of temozolomide. In this study the imidazotetrazinone synthesis was planned for the release aziridinium ions upon the aqueous hydrolysis and ring opening at physiological pH 7.4. Aziridines are clinically-useful intermediates, sufficiently stable to find their target on DNA as shown by other alkylating agents e.g. the nitrogen mustard alkylating agents cyclophospamide, melphalan, chlorambucil and nitrosoureas, e.g. BCNU.[62]

1.5.1 Aziridinium ions of biological interest

Aziridinium ions have received much attention in biological studies, several aziridinium ions generated *in situ* from halogenated ethylamines are reagents for the alkylation of biological nucleophiles (nucleic acids, proteins); the formation of aziridinium ions from the antitumour nitrogen mustard 2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine, is

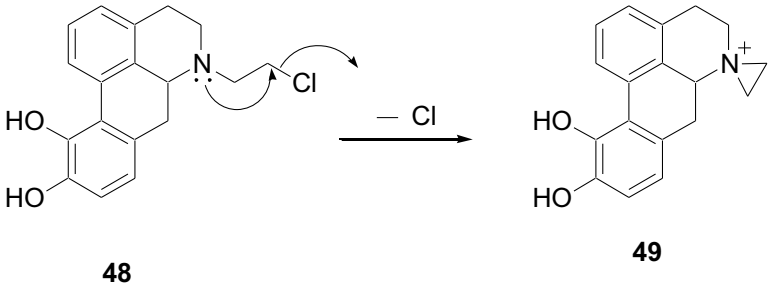
believed to be the first step in the cross linking of double stranded DNA and consequent antitumour action of this drug of the nitrogen mustard class, [62] Scheme 14.



Scheme14. Cyclisation to the aziridinium ion in nitrogen mustard

Moreover, the *in situ* generation of aziridinium ions from diethyl-2-chloroethylamine hydrochloride at pH 7 and 37 ºC, has been applied as a measurement technique to compare the alkylation reactivity of nucleic acid bases in double helical DNA, polynucleic acids and monomer nucleic acid bases. It was used to compare 1) the influence of the stacking of the bases in double helix DNA on enhancing such alkylation, 2) the effect of the negative phosphate of the backbone in increasing their nucleophilicity, and 3) the effect of the hydrogen bonds in blocking the reactive nucleophilic centres e.g adenine base compared with the accessible nucleophiles of guanine. The study reflects the helpful *in situ* generation of aziridinium ions in identifying bases of high potential for such alkylation. Guanine in double helical DNA was found to be of the highest potential compared with other bases.[63]

In another application, aziridinium ions **49** from *N*-(2-chloroethyl)norapomorphine **48**, Scheme 15 were used in a dopamine antagonist, for their irreversible alkylation of a membrane receptor. Another application for aziridinium ions, they were used based on the similarities that they possessed to parts of certain molecules or its affinity to interact irreversibly with certain reactive parts or interacting with a proteins carriers.[62]

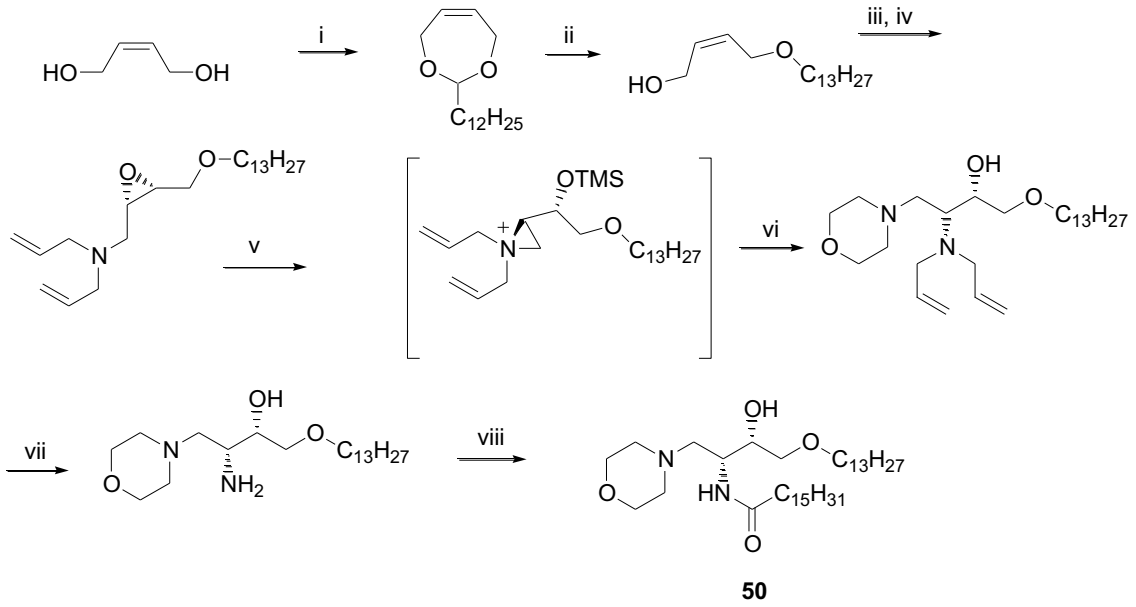


48 **49**

Scheme 15. Aziridinium ion from *N*-(2-chloroethyl)norapomorphine

Scheme 16 outlines another application for aziridinium ions in the synthesis of glucosylceramide synthase inhibitor **50**. Glucocerebroside, 1-*O*-β-D-glucopyranosylceramide is widely distributed in normal and pathologenic tissue, including normal human serum, plasma, erythrocytes, kidney and aortic tissue, as well as in the central nervous system. It is the major cerebroside found in the spleen of patients with Gaucher's disease. The biosynthesis of glucosylceramide relies on the coupling of UDPglucose to C-1 of an *N*-acylsphingosine (ceramide) mediated by glucosylceramide synthase, inhibition of this process is a promising target for cancer chemotherapy.[64]

In this study the new imidazotetrazinones were designed to combine the advantages of the imidazotetrazinone nucleus, with the possibility of formation of aziridinium ions using an ethylamine side chain, upon the ring opening the new imidazotetrazinones may result in a good delivery prodrug for aziridinium ions to their site of action.

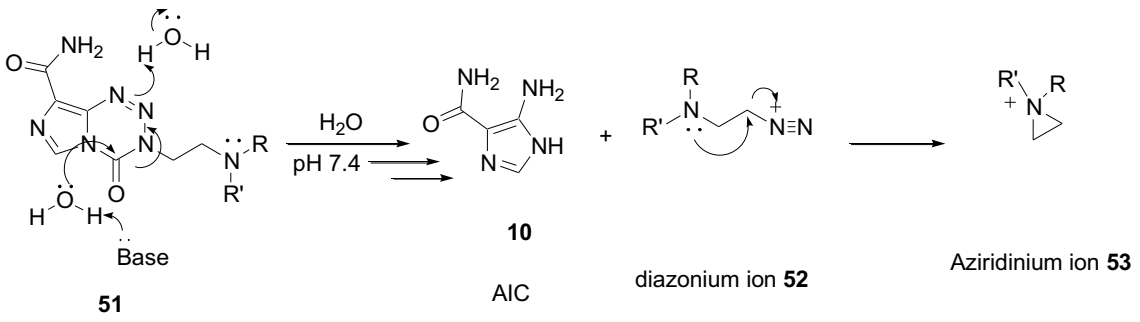


Scheme 16. Synthesis of glucosylceramide synthase inhibitor **50**. *Reagents and conditions:* (i) $C_{12}H_{25}CHO$, TsOH, C_6H_6, heat (98%); (ii) $AlCl_3$, $LiAlH_4$, Et_2O (91%); (iii) $Ti(OPri)_4$, (+)-DET, TBHP, CH_2Cl_2 (76%); (iv) TsCl, pyridine (70%); $(allyl)_2NH$, KI, DMF (81%); (v) TMSOTf, CH_2Cl_2, – 78 °C; (vi) morpholine, – 78 °C to rt; K_2CO_3, MeOH (61%); (vii) Pd/C, $MeSO_3H$, H_2O (82%); (viii) *p*-nitrophenyl palmitate, pyridine (63%)

1.5.2 Design of Aziridinium-ion-release imidazotetrazinones

Imidazotetrazinones, designed with an ethyl substituent at position 3 linked to an aliphatic or aromatic amino group contain the functional groups required to result in aziridinium ion formation. The design considers all the possible substitutions on the amine side chain and the aromatic ring that can result in different influences on the stability of the synthesized molecule and required for the optimum reactions in the aqueous conditions later. Accordingly, the pH-influenced ring opening of the new designed imidazotetrazinones should result in ethyldiazonium ion **52** with an internal nucleophilic nitrogen atom, which then reacts in a favoured 3-*exo*-*tet* intramolecular cyclization reaction in which the diazo group leaves as molecular nitrogen and results in the aziridinium ion. The process was design to resemble the release of the chloronium ion from mitozolomide **1b**, Scheme 4, and the aziridinium ion release by nitrogen mustards, Scheme 14.[62]

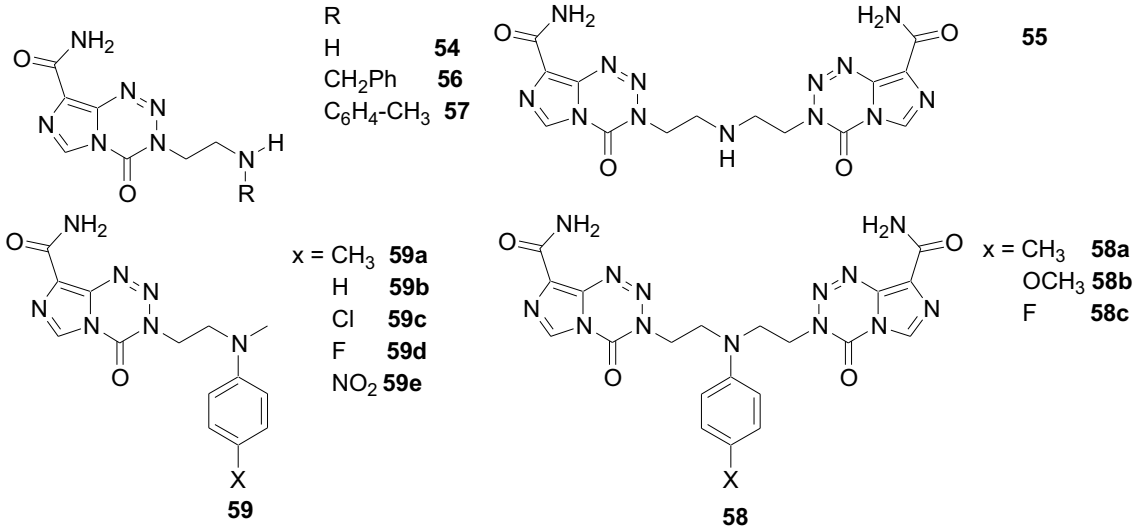
According to the above mentioned features, the new imidazotetrazinones showed in the general structure **51** are predicted to undergo aqueous hydrolysis at neutral pH according to Scheme 17 to AIC **10** and diazonium ion **52** that may cyclise subsequently to an aziridinium ion, **53**.



R, R' Aliphatic and / or Aromatic groups.

Scheme 17. The proposed mechanism of general imidazotetrazinone **51**

According to the designing scheme 17 the targeted molecules for synthesis are:



CHAPTER 2

SYNTHESIS

Chapter 2 Synthesis of Imidazotetrazinones for Aziridine Release

2.1 Introduction

The target molecules for synthesis were defined at the end of the previous chapter and are summarised in Figure 6

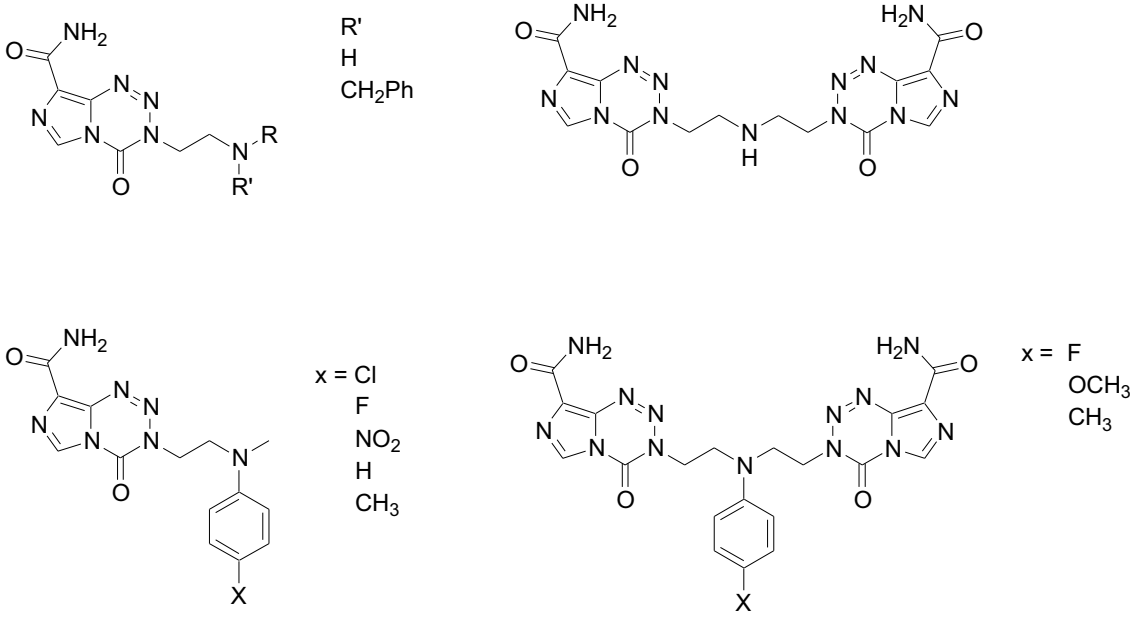
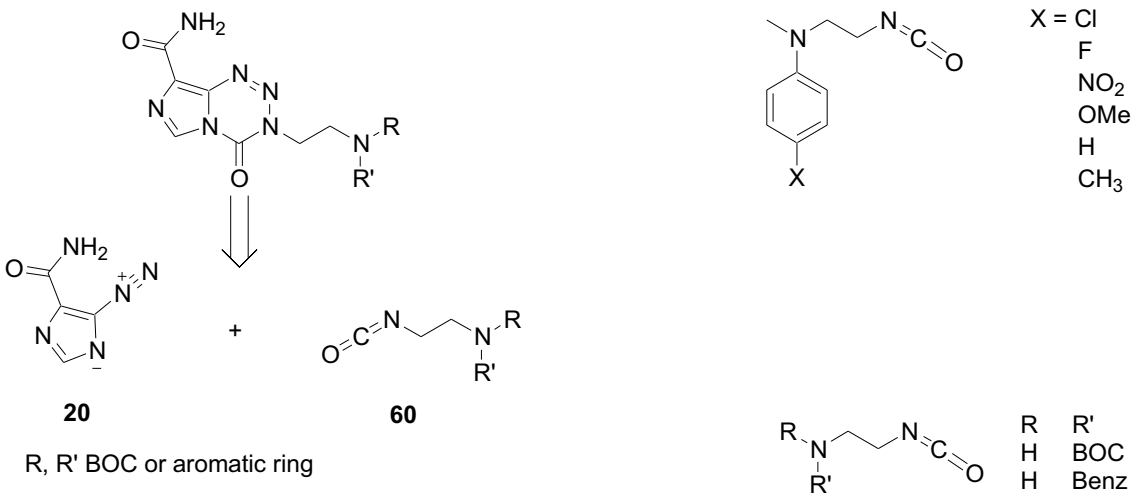


Figure 6. Target molecules for synthesis

2.2 Synthesis

The retrosynthetic analysis outlined in Scheme 18 was used to plan the route of the synthesis of the target imidazotetrazinones. The disconnection follows the original synthesis of imidazotetrazinones by Stevens *et al.*[6] The synthesis starts with the preparation of isocyanates which are then reacted with diazo-IC to from the imidazotetrazinones. This retrosynthetic analysis was used for all imidazotetrazinone compounds in this thesis.



Scheme 18. General retrosynthesis plan for imidazotetrazinones

β-Aminoisocyanates, **60**, are highly unstable compounds, able to undergo cyclization by nucleophilic attack of the amino group on the isocyanate group, this makes the protection of the amino group crucial for ensuring their stability against such cyclization. The substituents shown on the amino group nitrogen of the isocyanate in Scheme 18 were either a BOC protecting group or an aromatic ring, both were required to add a steric effect and an electronic effect to reduce the reactivity of the lone pair on the nitrogen, by doing so they prevent the favoured 5-*exo-trig* intramolecular reaction with the isocyanate group. A BOC group was used as a protecting group for the isocyanates synthesized from aliphatic amines, its bulk (hindrance effect) and electron-withdrawing effects greatly increased the stability of the isocyanates, Figure 7.

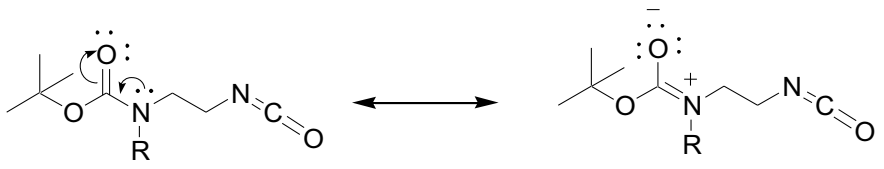


Figure 7. Carbamate resonance contribution to *β*-aminoisocyanate stability

In the case of the isocyanates synthesized from aromatic amines (anilines) the aromatic ring and the *N*-methyl group add steric hindrance around the nitrogen of the amine; delocalization of the unshared electrons of the nitrogen into the aromatic ring also reduced its nucleophilicity towards the isocyanate group, Figure 8.

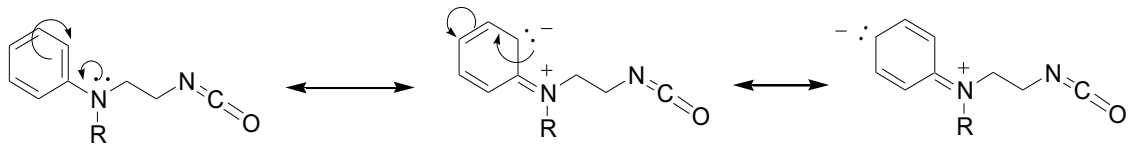
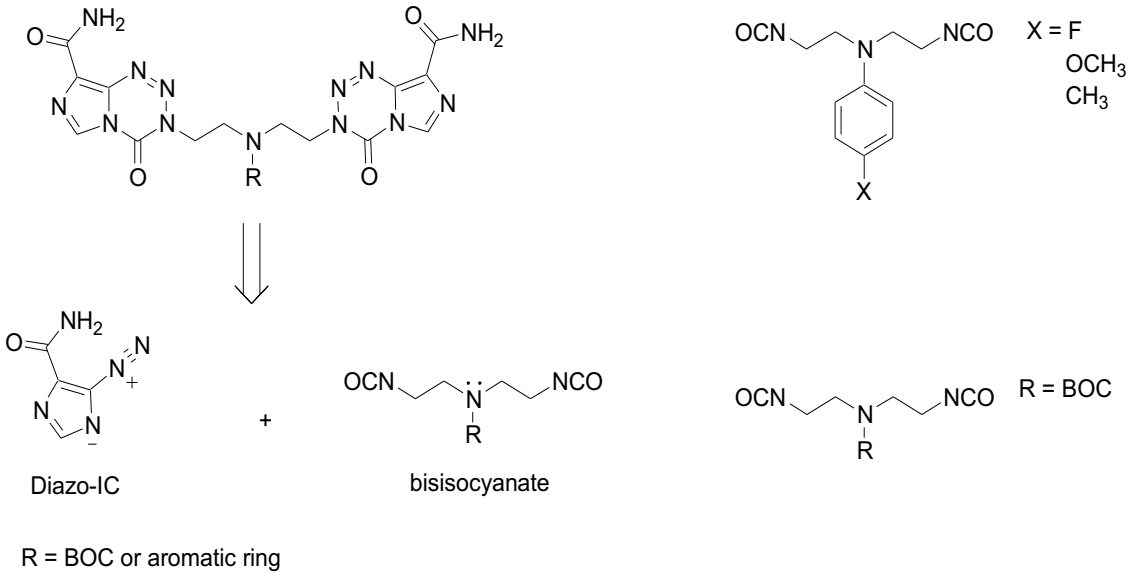


Figure 8. Aromatic ring contribution to isocyanate stability

The bisimidazotetrazinone synthesis was designed to react a suitable bisisocyanate with diazo-IC **20**. The retrosynthetic analysis outlined in Scheme 19 showed that the bisisocyanate was synthesized from an aliphatic or aromatic amine. BOC protection was again used in the case of the aliphatic amines; bisisocyanates synthesized from anilines were used without additional protection.



Scheme 19. Retrosynthetic plan for bisimidazotetrazinones

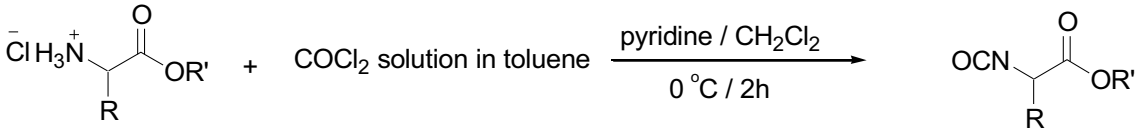
2.2.1 Synthesis of the Isocyanates

Routes to Isocyanates

There are many different routes by which isocyanates can be synthesized. They differ in their yields and the ease of isolation of the pure isocyanate. The following methods are examples that show different ways of isocyanate synthesis from industrial to research laboratory methods.

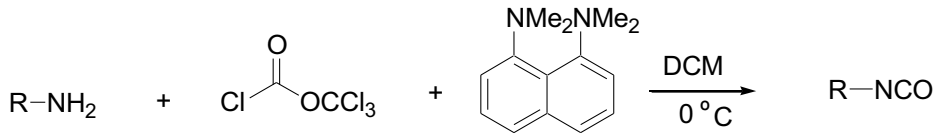
1) Isocyanates from amines and phosgene/triphosgene

   i) The generation of amino acid ester isocyanates has been achieved in yields of 72% – 95% by the addition of a commercially-available solution of phosgene in toluene to a mixture of an amino acid ester hydrochloride and pyridine,[65] Scheme 20.



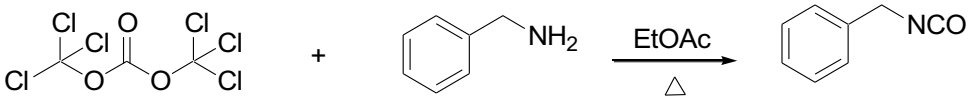
Scheme 20. Isocyanates from amines and phosgene

   ii) In a related preparation, reaction of aliphatic amines with trichloromethyl chloroformate (diphosgene) at 0 ºC in the presence of the non-nucleophilic base 1,8-bis(dimethylamino)naphthalene can afford isocyanates in yields of 78% , Scheme 21. [66]



Scheme 21. Isocyanates from aliphatic amine and diphosgene

iii) The synthesis of isocyanates by heating amines and triphosgene in ethyl acetate affords a 56% yield, Scheme 22.

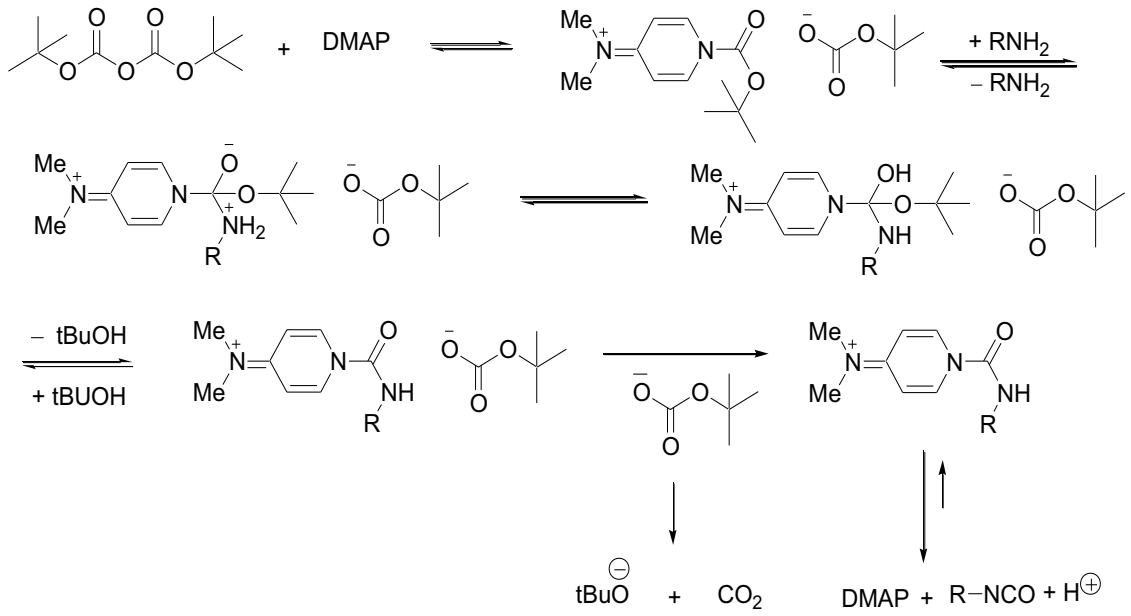


Scheme 22. Isocyanate from triphosgene and amines

The toxicity of the phosgene and quantity of HCl produced during the reaction limit the use of this method. The potential for salt formation made this method unsuitable for β-aminoisocyanate formation.

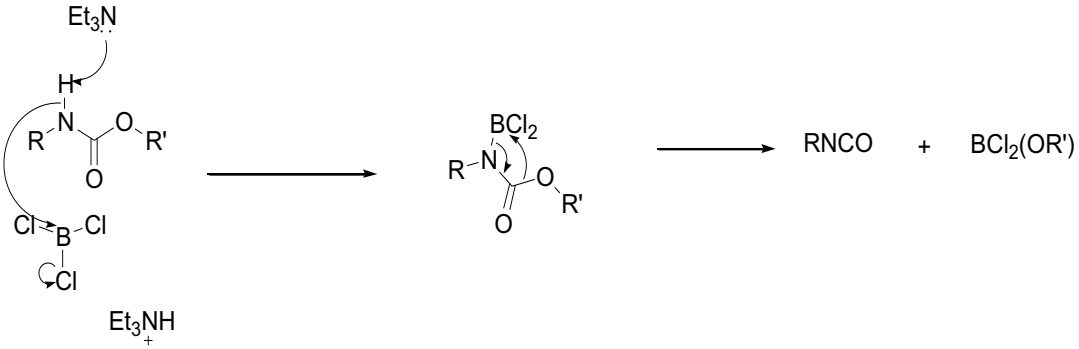
2) Thermolysis of ureas and carbamates

i) Isocyanates from the mild reaction of an active carbonate and an amine. Reaction of alkylamines and arylamines with the active carbonate di-*tert*-butyl dicarbonate (BOC_2O), in the presence of DMAP gives isocyanates in reported yields, which range according to the hindrance around the amine, from 49% – 97%,[67] Scheme 23. Although it is a quick reaction (10 min) the isolation methods used are not suitable for amino-isocyanates: in one method strong sulphuric acid was used which might drive the amino-isocyanate into the aqueous layer; another method used cold column chromatography for purification which might increase the loss of material in small scale production in the case of amino-isocyanates; moreover, recombination of the isocyanate product with *tert*-butanol is possible which greatly reduced the yields.



Scheme 23. Isocyanates from active carbonate and amines

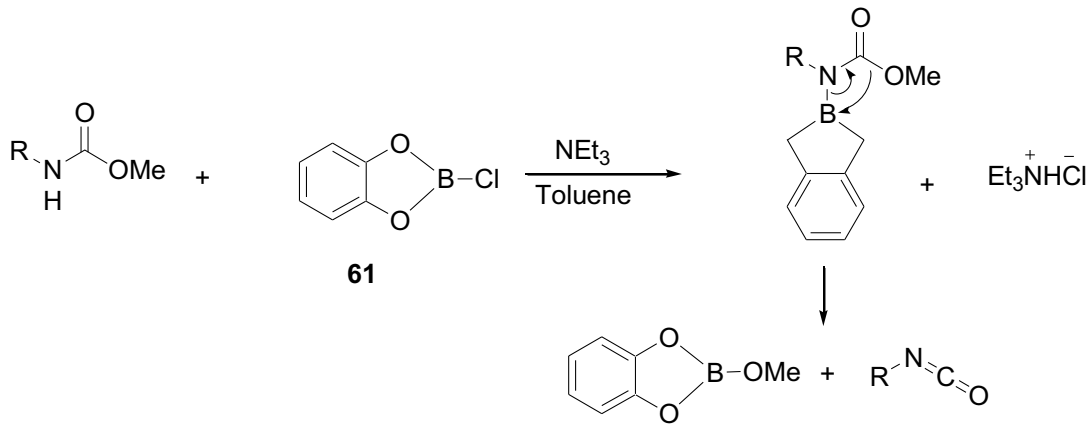
ii) The conversion of carbamate esters to isocyanates and diisocyanates of industrial importance is possible using BCl_3 in the presence of Et_3N; the reaction is simple in execution and work-up, occurring under mild conditions and affording isocyanates in excellent yields 70 – 79%,[68] Scheme 24. Although good yields are generated from this method which is mainly used in industry, usage of a Lewis acid limits its application for amino-isocyanates due to the basic nitrogen that could associate with it.



Scheme 24. Isocyanates from carbamates, employing boron trichloride

iii) Isocyanates synthesized by thermolysis of carbamate esters (urethanes).[69]

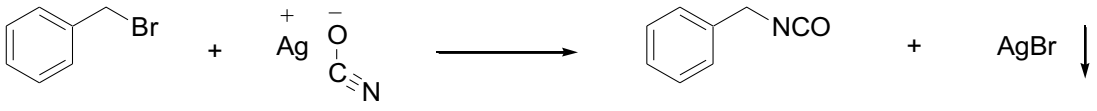
In this method an alcohol is eliminated from a carbamate ester in the presence of chlorocatecholborane **61** and triethylamine. The chlorocatecholborane combines with the alcohol that results from the decomposition of the urethane and avoids the back reaction of alcohol with the produced isocyanate. This method is used for large scale production of isocyanates in industry, the yields range from 45% – 70%, Scheme 25.



Scheme 25. Thermolysis of carbamate esters to isocyanates

3) Isocyanates from alkyl halides and silver cyanate [70]

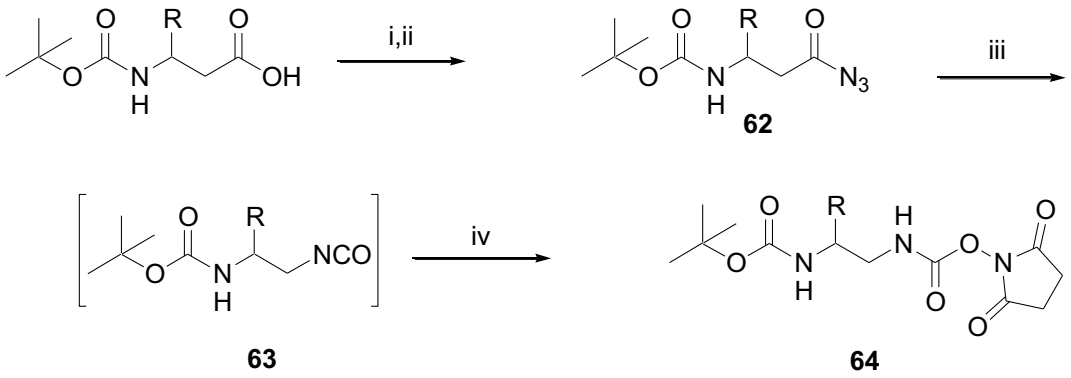
Benzyl isocyanate has been prepared by the reaction of silver cyanate with benzyl bromide or chloride, the product purity by this method was not good and the product was always contaminated with chlorine or bromine, Scheme 26.



Scheme 26. Isocyanates from alkyl halides and silver cyanate

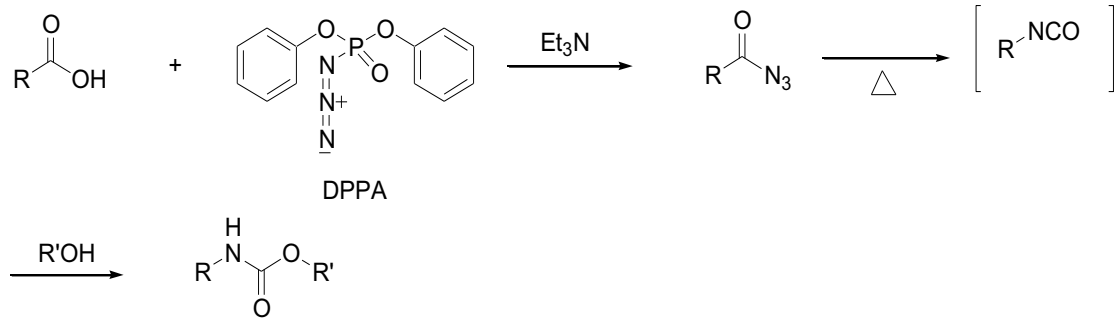
4) Curtius rearrangement

i) *In situ* generation of isocyanate **63** was achieved by heating of azide **62** in toluene and subsequently trapped by *N*-hydoxysuccinimide to form the carbamate 6**4** in a yield of 60%. In this method, BOC-protected amino acids were converted to acyl azides by reaction of their mixed anhydrides (formed with ethyl chloroformate / *N*-methylmorpholine [NMM] with NaN_3, Scheme 27.[71] This method is quite similar to the method selected in this study.



Scheme 27. *Reagents and conditions*: (i) EtOCOCl, NMM, THF, – 20 ºC; (ii) NaN_3, H_2O; (iii) toluene, 65 ºC; (iv) *N*-hydroxysuccinimide, pyridine.

ii) Direct conversion of a carboxylic acid to a urethane may be achieved by heating under reflux an equimolar mixture of the carboxylic acid, triethylamine and the thermally-stable diphenylphosphorylazide (DPPA) in the presence of an alcohol for 5 – 25 h. This reaction is a modified Curtius method, performed through the intermediate carboxylic acid azide which undergoes thermolysis to the isocyanate. It was then reacted with the alcohol to form a urethane 50 – 75% yields.[72] The method showed the *in situ* usage of the isocyanate so no isolation methods for the isocyanate were mentioned, Scheme 28.

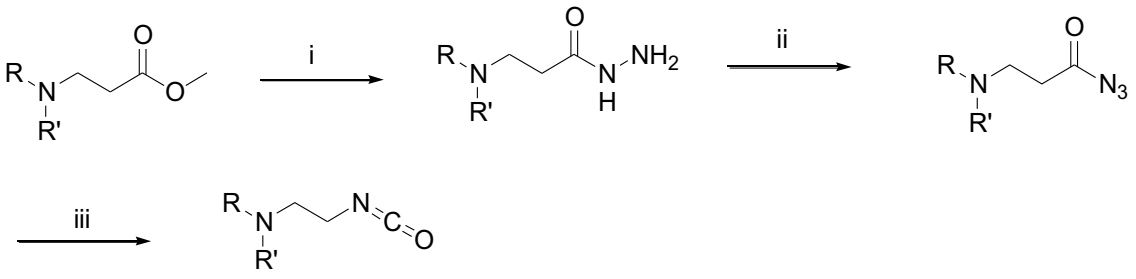


Scheme 28. *In situ* isocyanate generation in urethane synthesis

iii) Thermal sigmatropic rearrangement or Curtius rearrangement is the thermal decomposition of carboxylic azides to produce isocyanates, this method is the most convenient, the product purity is relatively high as only nitrogen is generated as a by-product from the thermolysis of the azide.[73]

Curtius rearrangement is a thermal rearrangement of carboxylic acid azides. There are different syntheses of azides available. Examples include reacting activated acid derivatives e.g. acid chlorides or acid anhydrides with NaN_3 or by heating a carboxylic acid and the thermally-stable organic azide DPPA in the presence of triethylamine. In the latter reaction, the carboxylate anion reacts in a nucleophilic substitution reaction with phosphyl azide to form the acylazide, Scheme 28. Diazotization of hydrazides is another method of azide synthesis. The hydrazides were synthesized from activated acid derivatives by their reaction with hydrazine.

In our laboratory the Curtius rearrangement has been frequently used for the synthesis of isocyanates: anhydrous solvents and dry conditions have been applied to generate material that needs little purification. Azides have been synthesized from the diazotization of hydrazides in a mixed aqueous/organic solvent system using $NaNO_2$ and a suitable acid, Scheme 29; the hydrazides were formed by reaction of esters with hydrazine monohydrate. This route consists of three steps through which the purity of the isocyanate can be achieved. At the ester stage, purification could be by solvent selectivity or distillation; the hydrazides were then pure. The hydrazide stage is a further purification step, especially as most were solids and could be readily recrystallised.



Scheme 29. *Reagents & conditions:* i) $NH_2NH_2 \cdot H_2O$, EtOH or *i*-PrOH; ii) $NaNO_2$ / Acid, 0 ºC; iii) anhydrous DCM, rt (R, R′ either BOC aliphatic or aromatic).

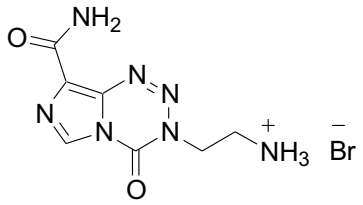
2.2.2 Synthesis of imidazotetrazinones for aziridinium ion release

Two types of imidazotetrazinone for the release of aziridinium ion were synthesised:

1) imidazotetrazinones bearing aliphatic amines;
2) imidazotetrazinones bearing anilines.

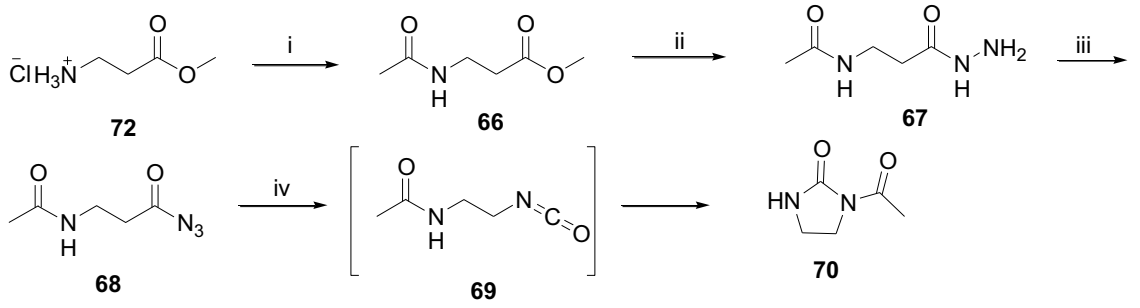
2.2.2.1 Imidazotetrazinones bearing aliphatic amines:

Synthesis of the 3-(2-aminoethyl)-imidazotetrazinone HBr salt 54



54

In this method, the BOC-protected aminoacid ester was used to avoid the reaction of the free amino group with the isocyanate group. In one trial reaction, acetyl protection was used, Scheme 30. Synthesis of the hydrazide **67** was successful but isocyanate **69** was not isolated. Analysis of the reaction mixture at the thermolysis stage by IR did not show the characteristic strong band at 2275 cm^{-1} of the isocyanate. Cyclic urea **70** formation was confirmed by ^{1}H NMR as a result of cyclization of *N*-acetylaminoethylisocyanate **69**; the mass spectrum showed cyclic urea $(M+H)^{\cdot+}$ m/z 129.3 and the IR showed bands at 1653 for NHCONH and 3261 for CONH instead of the isocyanate band. This result showed that the acetyl group is not sufficiently electron-withdrawing or bulky for such protection.

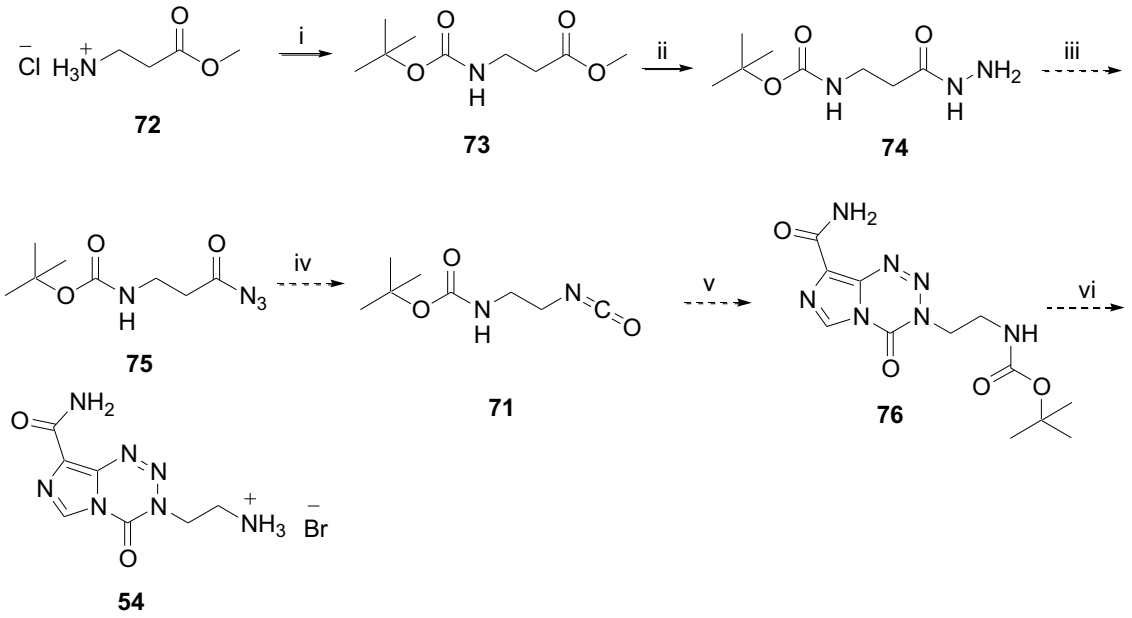


Scheme 30. Cyclization of acetyl-protected-β-aminoisocyanate **69**. *Reagents and conditions:* (i)acetic anhydride, Et_3N, DCM, 40 ºC, 95%; (ii) $NH_2NH_2 \cdot H_2O$, *i*-PrOH, 60%; (iii) $NaNO_2$ / HCl 37%, DCM/H_2O, 0 – 5 ºC; (iv) anhydrous DCM, rt.

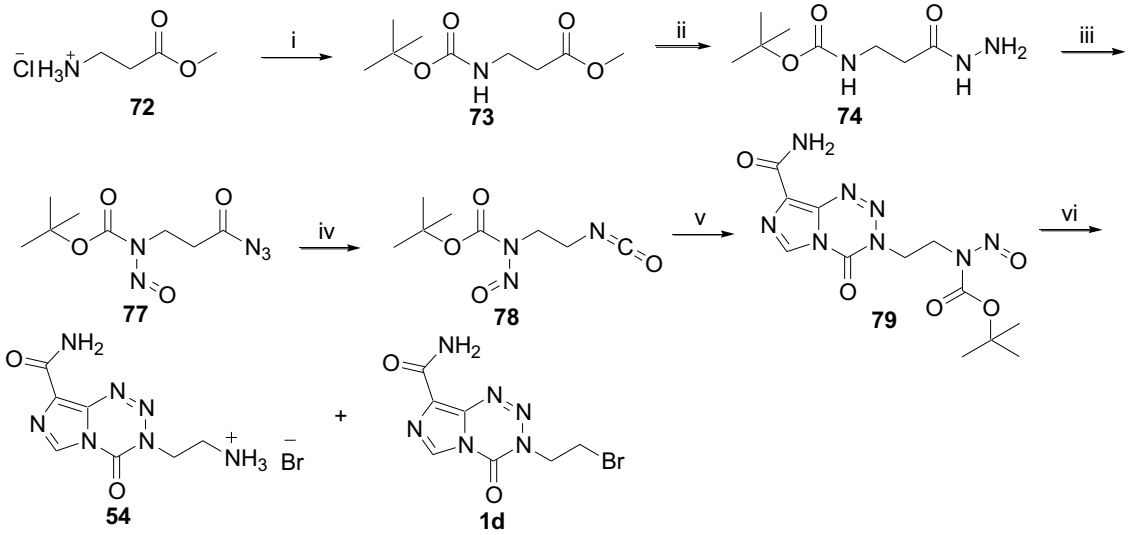
Changing to BOC protection[74] facilitated preparation of the isocyanate **71**, which was reacted further under nitrogen with diazo-IC **20** in anhydrous DMSO to give a BOC-protected imidazotetrazinone. The process of the synthesis started by converting β-alanine methylester **72** to the BOC-protected ester **73** then to hydrazide **74** by reaction with hydrazine monohydrate.[75] Diazotization of hydrazide **74** using aqueous $NaNO_2$/HCl gave a product presumed to be azide **75**,[76] Scheme 31. An isocyanate was prepared by Curtius rearrangement of the azide.[73] The isocyanate was reacted with diazo-IC to give an imidazotetrazinone.[77] Characterization (MS, CHN, ^{1}H NMR) of the compound presumed to be protected tetrazine **76**, showed that the isolated molecule differed from that expected.

The backward checking of the reaction process and the ^{1}H NMR of the intermediates, showed that at the stage of the azide preparation by hydrazide diazotization, a nitroso group had been introduced at the carbamate nitrogen i.e. *N*-nitrosoamide **79** was formed rather than BOC-protected amine 7**6**, Scheme 32. This was not surprising as these are the exact conditions used for the synthesis of *N*-nitrosoamides.[78] The ^{1}H NMR spectra of ester **73** and hydrazide **74** both show a peak due to the BOC-N<u>H</u>- at δ 5.18 whereas the same peak disappeared from the ^{1}H NMR of the azide **77**, the isocyanate **78** and the imidazotetrazinone **79**. Accurate mass measurement LSIMS (FAB) showed

$(M+H)^{\cdot +}$ m/z 353 and the elemental analysis showed molecular formula $C_{12}H_{16}N_8O_5$ which fits imidazotetrazinone **79** with the *N*-nitroso group, Scheme 32.



Scheme 31. Expected synthesis route to the imidazotetrazinone salt **54**. *Reagents and conditions:* i) BOC_2O, Et_3N, 100 ºC, 60%; ii) $NH_2NH_2{\cdot}H_2O$, *i*-PrOH; iii) $NaNO_2$, 10 M HCl, DCM/H_2O; iv) anhydrous DCM, 30 ºC, 48 h; v) **20**, DMSO, 30 ºC vi) HBr 48%, acetonitrile, rt.



Scheme 32. Route to the imidazotetrazinone salt **54**. *Reagents and conditions*: (i) BOC_2O, Et_3N, 100 ºC, 60%; (ii) $NH_2NH_2{\cdot}H_2O$, *i*-prOH, 94%; (iii) $NaNO_2$, 10 M HCl, DCM/H_2O, 68%; (iv) anhydrous DCM, 30 ºC, 48h, 60%; (v) **20**, DMSO, 30 ºC, 14%; (vi) HBr 48%, acetonitrile, rt, 88%.

Deprotection of the BOC group was achieved in acetonitrile using HBr, Scheme 32. This reaction resulted in both deprotecting the BOC group and reduction of the *N*-nitroso group which was removed in the form of ammonia to give the hydrobromide salt **54**, which precipitated from acetonitrile. Another solid was collected by evaporation of the acetonitrile layer. This solid was found to contain, in addition to salt **54**, another imidazotetrazinone. Spiking with authentic 3-(2-bromoethyl)imidazoterazine-4-one **1d** prepared from commercially-available 2-bromoethylisocyanate confirmed its identity, Figure 9.

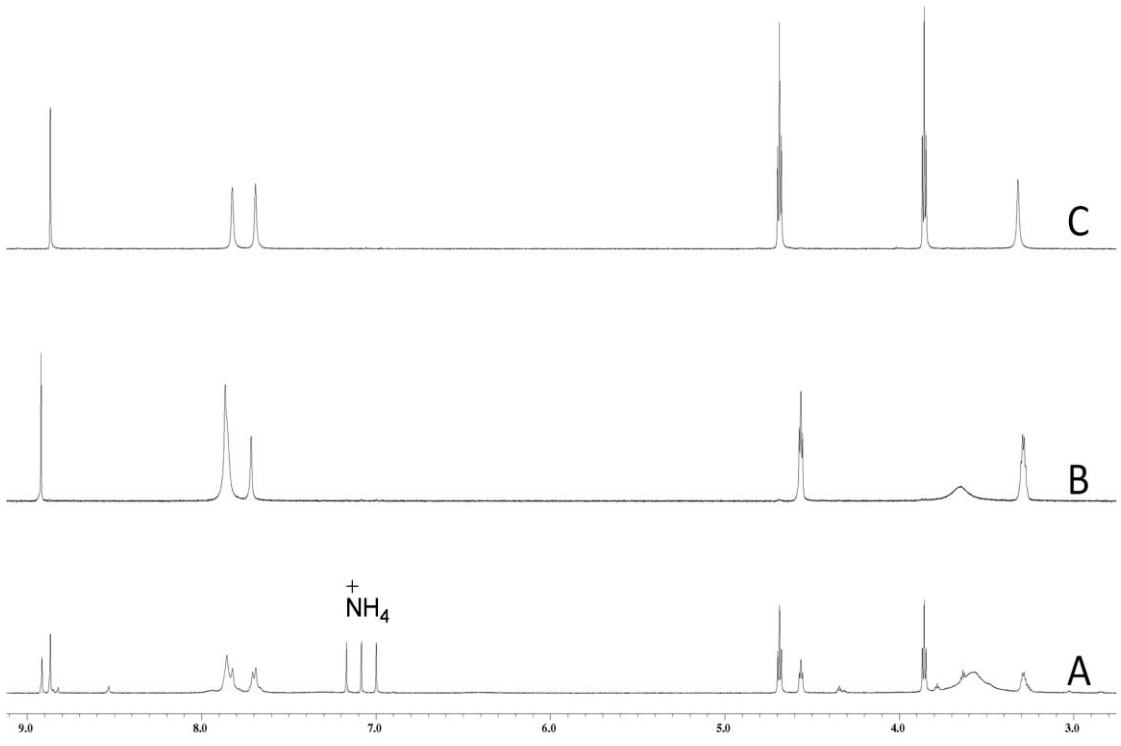
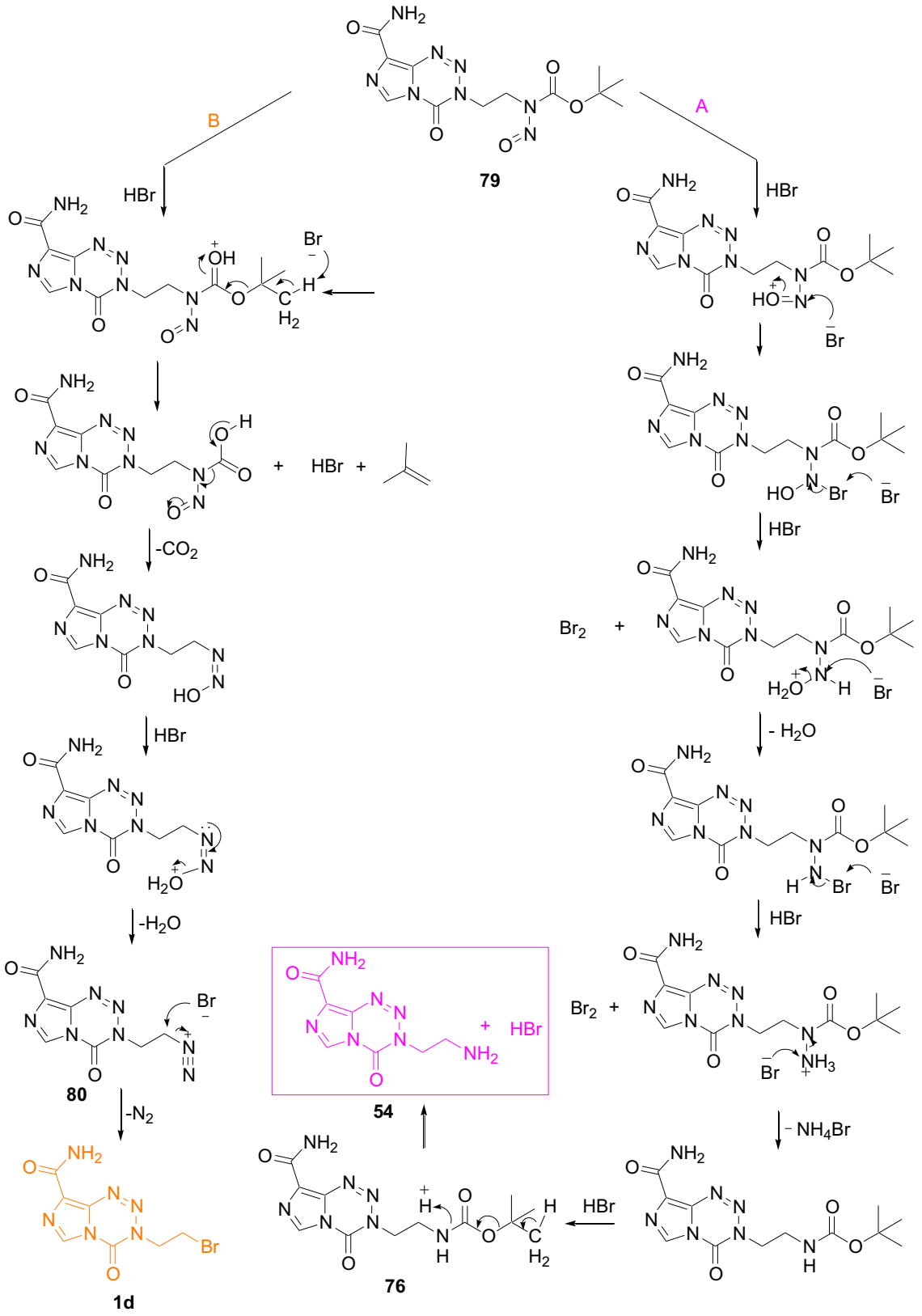


Figure 9 Confirmation of the identity of the by product as 3-(2-bromoethyl)imidazotetrazinone-4-one **1d**. Spectra of the mixture (A), tetrazine salt **54** (B), spiked with bromoethylimidazotetrazinone **1d (C)**.

A proposed mechanism for the acid-catalysed deprotection of the tetrazine **79** is shown in Scheme 33. In route A HBr reduction of the *N*-nitrosamide could lead to the BOC-

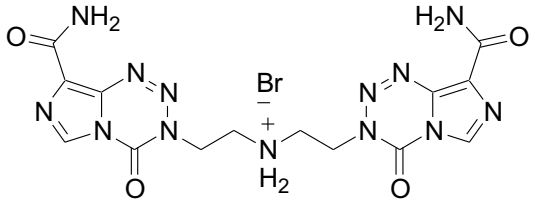
protected amine **76** with the loss of ammonia in form of ammonium bromide; subsequent acidolysis of the BOC group would give the amine hydrobromide salt **54**. Evidence that the *N*-nitroso may be cleaved by reduction route A appears on page 60, where its reduction proceeds with unexpected bromination of aniline ring. However, if the BOC group cleaved first, route B, reaction of the *N*-nitrosoamine could yield the alkyldiazonium intermediate **80**. Nucleophilic substitution by bromide ion would give the 3-(2-bromoethyl)imidazotetrazinone-4-one **1d**.

The *N*-nitroso group gave the impression that it acts as a separate protecting group, the ease of its removal resulted in the imidazotetrazinone hydrobromide salt **54** in higher yield compared with the byproduct 3-(2-bromoethyl)imidazotetrazinone-4-one **1d** (10:1). Consideration of likely reaction mechanisms suggests that the nitrosocarbamate competes with the BOC during acid deprotection such that the final product obtained is determined by the site of first protonation.



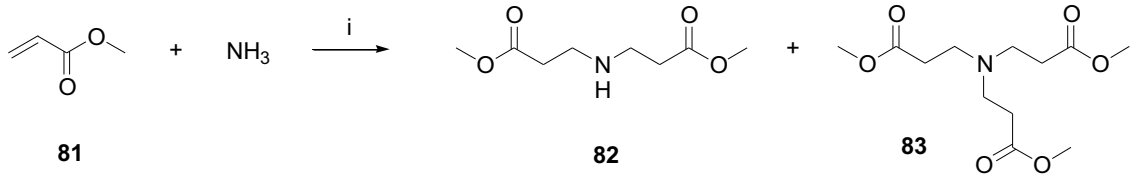
Scheme33. Mechanism for the formation of the amine salt **54** and bromoethylimidazotetrazinone **1d** from *N*-BOC-*N*-nitroso tetrazine **79**.

Synthesis of the aliphatic amine-linked-bisimidazotetrazinone 55



55

The route that led to bisimidazotetrazinone salt **55**, started with the synthesis of the dimeric ester **82,** Scheme 34, which was prepared by the aza-Michael addition of ammonia to methyl acrylate **81**. The reaction resulted in a mixture of two esters **82** and **83**, which were separated by fractional distillation (**82** b.p. 80 – 90 ºC, 0.05 mmHg; **83** left pure in distillation flask, identity confirmed by ^{1}H NMR). The major product was the trimer ester **83**. This might be attributed to the higher reactivity of the secondary amine compared with ammonia and the primary amine.

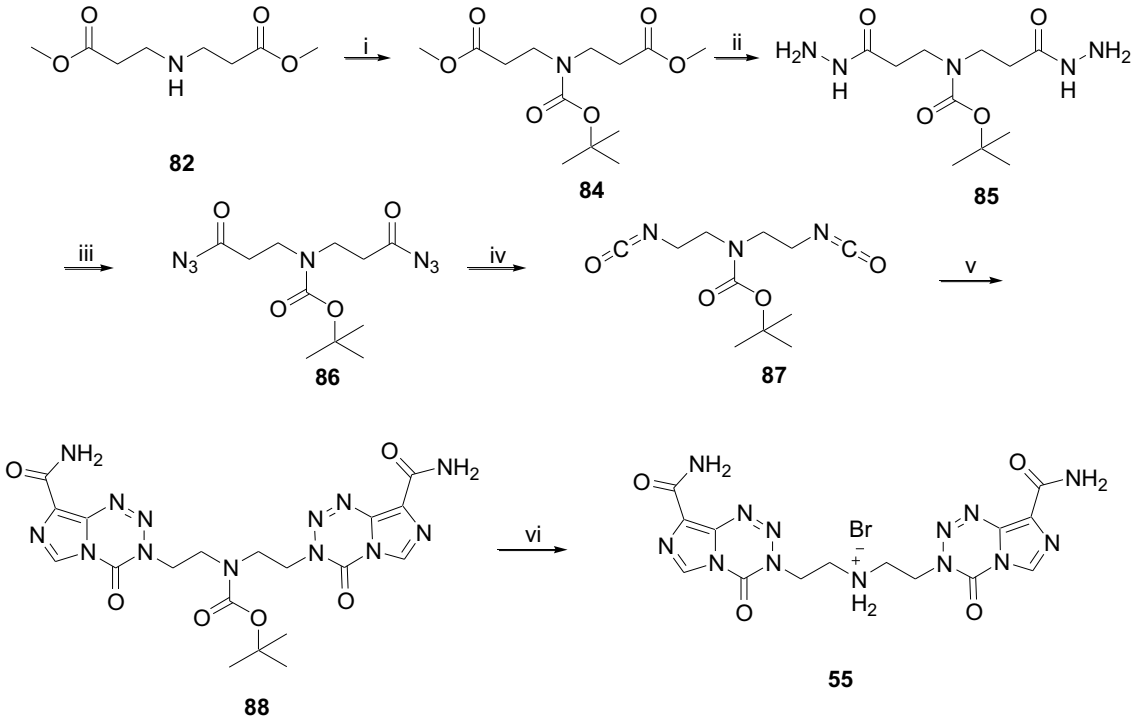


81 **82** **83**

Scheme 34. Dimeric ester **82** synthesis. *Reagents and conditions*: i) MeOH, rt, o/n, 25%.

The 2°-amino group of the dimeric ester **82** was BOC-protected; BOC-protected ester **84** was converted to hydrazide **85** in reaction with 10 equivalents of hydrazine monohydrate, the product was collected as a pure solid in good yield. Azide **86** was prepared by the diazotization reaction of the hydrazide using $NaNO_2$/HCl in a yield of

57%; the product was identified by IR, which showed the characteristic band at 2138 cm^{-1} and ^1H NMR also confirmed symmetrical dimeric azide formation, Scheme 35.



Scheme 35. Route to the bisimidazotetrazinone **55**. *Reagents and conditions*: i) BOC_2O, Et_3N, 100 ºC, 66%; ii) $NH_2NH_2{\cdot}H_2O$, *i*-PrOH, 70%; iii) $NaNO_2$, 10 M aq. HCl, DCM/H_2O, 57%; iv) anhydrous DCM, 30 ºC, 48 h, 52%; v) **20**, DMSO, rt; vi) aq. HBr 48%, acetonitile, rt, 57%.

Curtius rearrangement to bisisocyanate **87** was achieved by stirring an anhydrous DCM solution of azide **86** under nitrogen at room temperature without additional heating. Bisimidazotetrazinone synthesis from the reaction of bisisocyanate **87** and diazo-IC **20** in anhydrous DMSO gave a product that showed two non-equivalent imidazotetrazinones in the ^1H NMR spectrum, Figure 10A. The characterization by MS and CHN confirmed the BOC-bisimidazotetrazinone **88**. The magnetic non-equivalence of the imidazotetrazinone rings shown in the ^1H NMR spectrum may be attributed to

restricted rotation about the carbamate N–C(O) bond, Fig 11. HBr deprotection led to the symmetrical product the bromide salt **55**, Figure 10B.

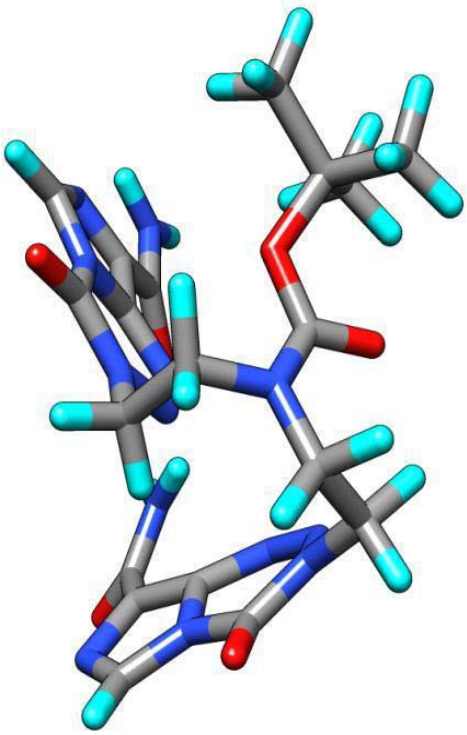


Figure 11. 3D minimised molecular model showing BOC-imidazotetrazinone-interaction in bisimidazotetrazinone **88** structure

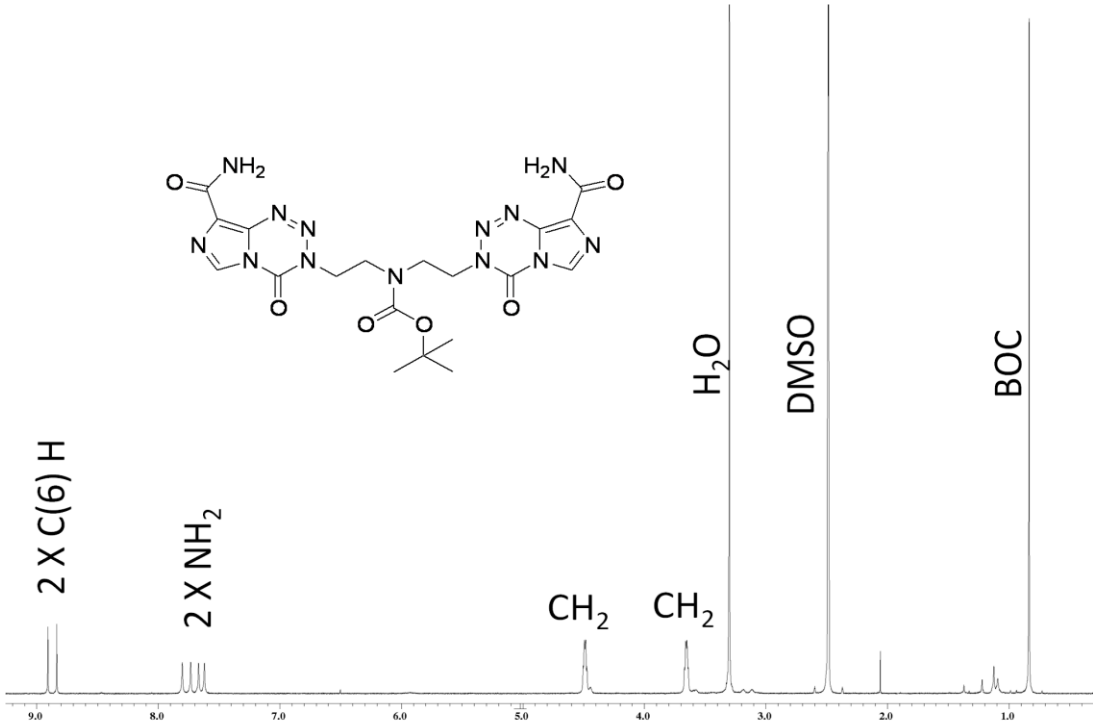


Figure 10A. ^{1}H NMR spectrum of BOC-bisimidazotetrazinone **88**

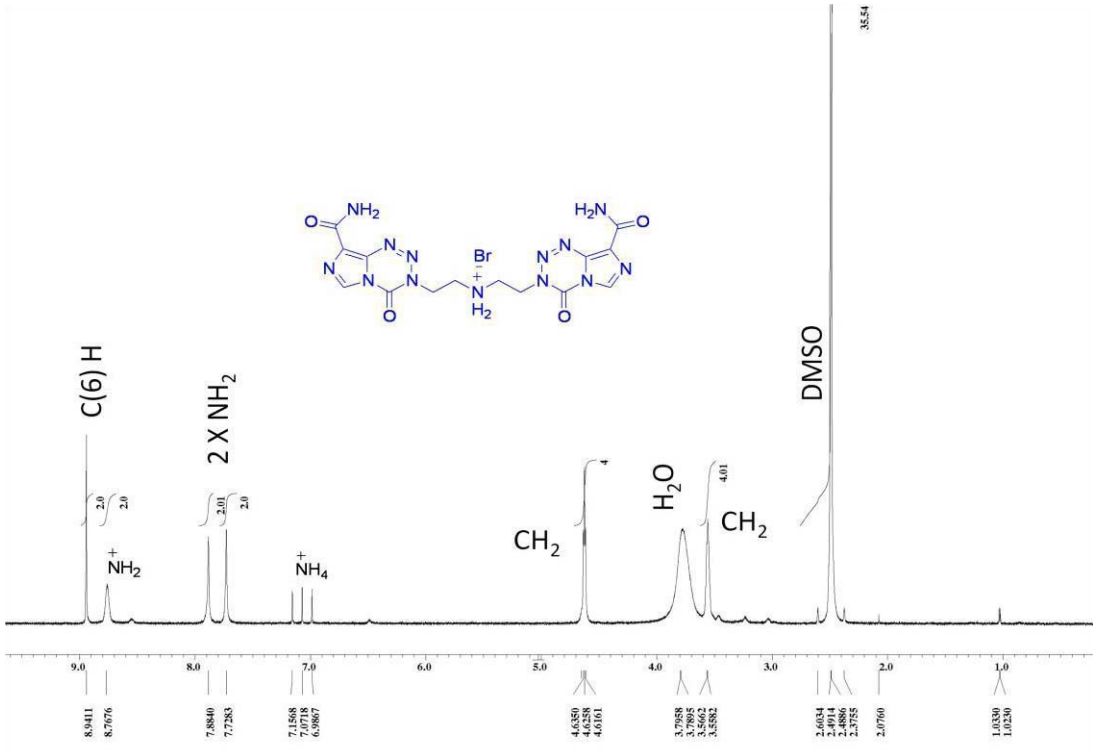
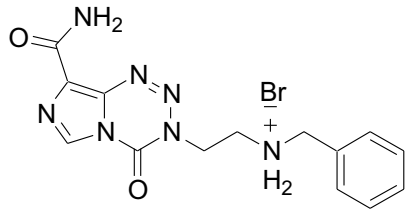


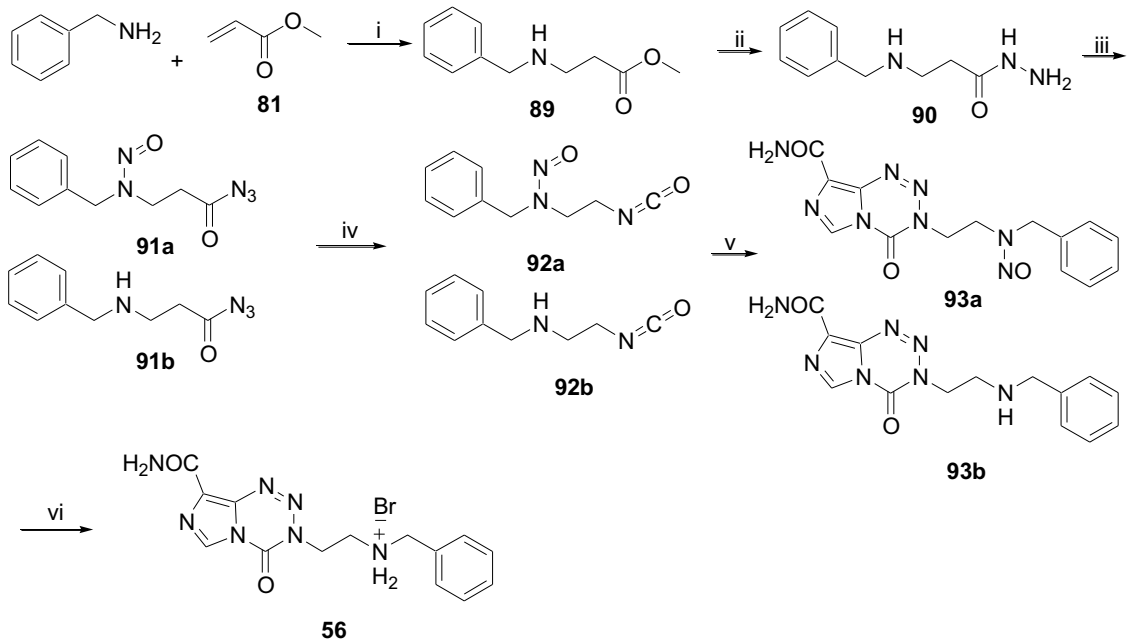
Figure 10B. ^{1}H NMR spectrum of bisimidazotetrazinone hydrobromide salt **55**

Synthesis of 3-(*N*-Benzylaminoethyl)imidazotetrazinone HBr salt 56



56

The synthesis of imidazotetrazinone salt **56** is outlined in Scheme 36. In this method benzylamine was reacted with methyl acrylate to give 3-benzylaminopropionic acid methyl ester **89**, which was carried through to imidazotetrazinone **56** without NH protection.



Scheme 36. Synthesis of 3-(*N*-benzylaminoethyl)imidazotetrazinone HBr salt **56**. *Reagents and conditions*: (i) MeOH, Δ, 60%; (ii) $NH_2NH_2{\cdot}H_2O$, EtOH, 88%; (iii) $NaNO_2$, 10 M aq. HCl, DCM/H_2O; (iv) anhydrous DCM, 30 ºC, 48 h, 40%; (v) Diazo-IC, DMSO, rt, 20%; (vi) aq. HBr 48%, acetonitile, rt, 48%.

Once again, this synthesis resulted in two imidazotetrazinones which showed different ^{1}H and ^{13}C NMR peak positions Figure 12, the mass spectrum showed the only the

molecular ion of the imidazotetrazinone **93a** with the *N*-nitroso group at the secondary amine. Backward checking of the reaction showed that at the stage of the azide formation, two different azides were formed and as a result, two different isocyanates. The story was understood clearly from the final imidazotetrazinone product, one of the imidazotetrazinones showed an *N*-nitroso group at the amine nitrogen, which was confirmed by its mass spectrum whereas the other one did not. Moreover, the evidence of having another imidazotetrazinone that resulted from a reaction of isocyanate **92b** with the diazo-IC, means that β-aminoisocyanate **92b** was relatively stable since no intramolecular cyclization took place. The ratio of the two products was 6:5 with the *N*-nitrosoaminoethylimidazotetrazinone slightly more, which might be due to the powerful electrophilicity of the NO^{+}.

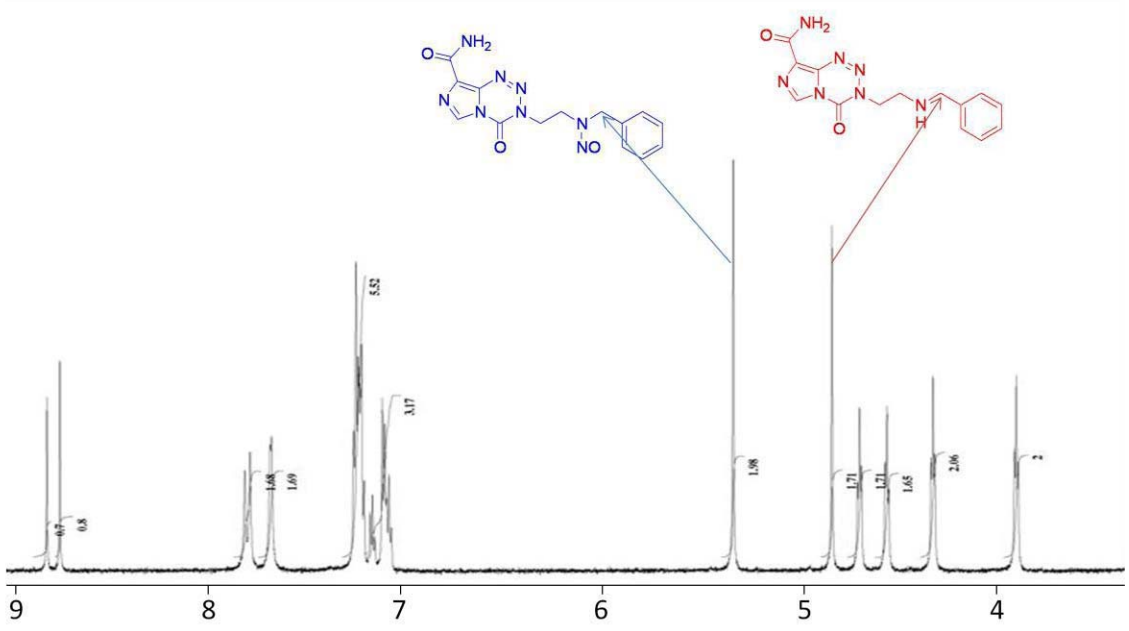
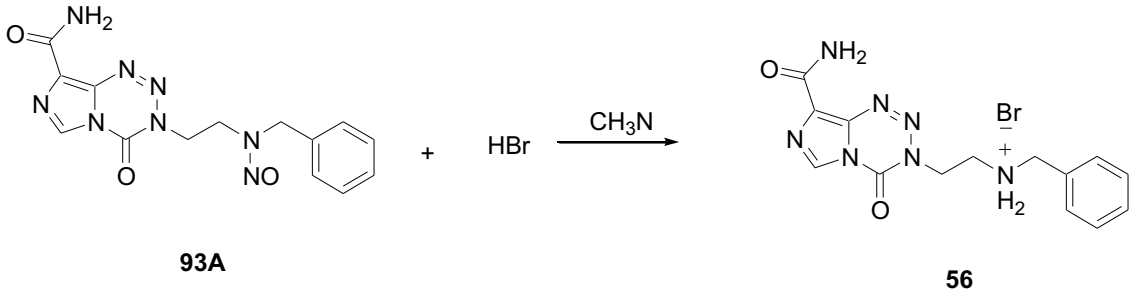


Figure 12. ^{1}H NMR of 3-(benzylamino)-ethylimidazotetrazinone that showed the formation of two tetrazines.

Upon the addition of hydrobromic acid to the tetrazine mixture, only one imidazotetrazinone HBr salt **56** was observed, Figure 13. Scheme 37 outlines a proposal

reaction to the reductive removal of the NO group by hydrobromic acid. In addition to the removal of the *N*-nitroso group HBr was expected to act as a debenzylating agent to give the tetrazine salt **54** but no debenzylation took place, only *N*-nitroso removal was observed. Performing other debenzylation methods is difficult in the presence of the imidazotetrazinone ring which is unlikely to survive catalytic hydrogenation.



93A **56**

Scheme 37. *N*-Nitroso removal from 3-(*N*-benzylaminoethyl)imidazotetrazinone **93a** to the hydrobromide salt **56** with aq. HBr 48%, CH_3CN

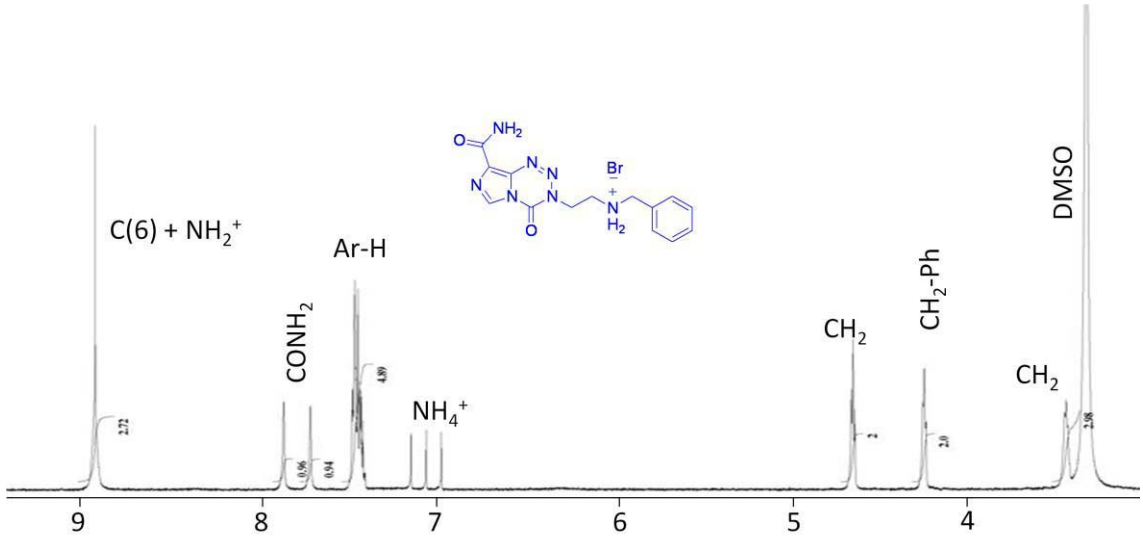
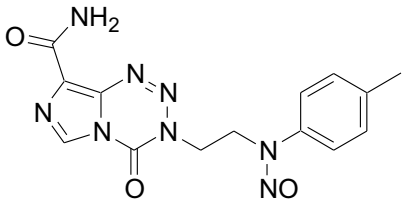


Figure 13. Imidazotetrazinone **56** HBr salt

2.2.2.2 Imidazotetrazinones bearing anilines

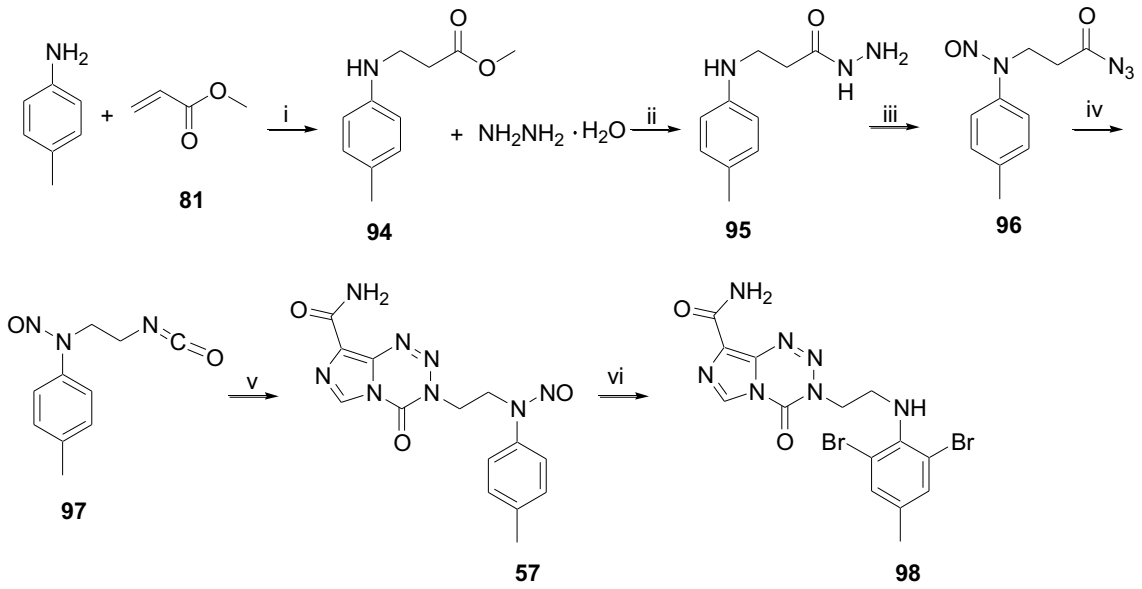
Both *p*-substituted primary anilines and ***N***-methylanilines were used for the synthesis of imidazotetrazinones, a range of substituents was used including electron-releasing and electron-withdrawing groups.

Imidazotetrazinones synthesized from *p*-substituted anilines



57

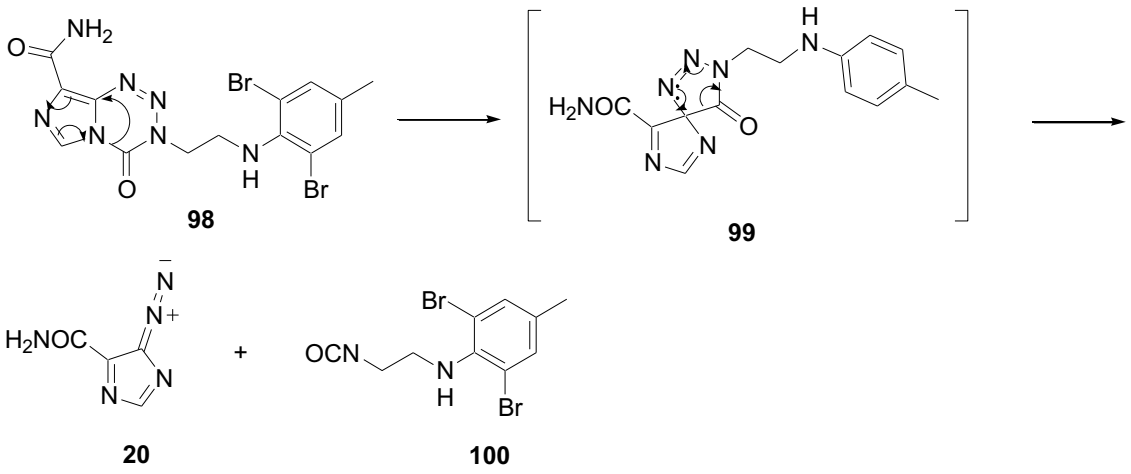
The synthesis of the monomeric imidazotetrazinone **57** is outlined in Scheme 38, the synthesis started with *p*-toluidine without protection of the primary aromatic amine. Ester **94**, synthesized by the reaction of *p*-toluidine and methyl acrylate was collected as a low melting point solid in a good yield and it was then converted to hydrazide **95**, diazotization of the hydrazide **95** gave azide **96**. The ^{1}H NMR spectrum of azide **96** showed the disappearance of the NH peak at δ 3.86 which had been observed in the spectra of ester **94** and hydrazide **95**. The formation of *N*-nitrosoimidazotetrazinone **57** was later confirmed by its mass spectrum & elemental analysis. Moreover, no intramolecular cyclization reaction was observed for the isocyanate **97**. This means the *N*-nitroso group acted as a protecting group to prevent the intramolecular cyclic urea formation. The isocyanate **97**, when reacted in anhydrous DMSO with diazo-IC **20** under N_2 resulted in the tetrazine **57** with an *N*-nitroso group at the aromatic amino group, MS showed its molecular ion m/z 342.31.



Scheme 38. Synthesis of 3-(*N*-(4-methylphenyl)-*N*-nitrosoamino)ethylimidazotetrazinone **57**. *Reagents and conditions*: (i) MeOH, Δ, 90%; (ii) $NH_2NH_2{\cdot}H_2O$, EtOH, 85%; (iii) $NaNO_2$, 10 M aq. HCl, DCM/H_2O; (iv) anhydrous DCM, rt , 48 h, 59%; (v) **20**, DMSO, rt. (vi) aq. HBr 48%, acetonitile, rt.

A trial *N*-nitroso group removal by 48% HBr in CH_3CN at room temperature was performed, a white precipitate was collected. ^{1}H NMR showed an imidazotetrazinone with only two protons on the aniline substituent (integral = 2H compared with 4H for tetrazine **57**). Furthermore, the coupling had disappeared so that the aniline ring of compound **98** showed only a simple singlet. The MS showed the $M^{\cdot+}$ ion of m/z 469.8, 471.8, and 473.8 in a ratio 1:2:1, which is characteristic of a compound bearing two bromine atoms. Furthermore, the mass spectrum showed fragments with m/z 332.8, 334.8 and 336.8 in a ratio 1:2:1 which is for isocyanate **100** with a dibrominated aniline ring and also showed the diazo-IC ion **20**, Figure 14. Fragmentation in the mass spectrometer led to generation of the moieties from which the imidazotetrazinone was synthesized; the mechanism of the thermal break down may involve a [1,5] sigmatropic shift *via* an unstable spirobicycle **99**,[13] Scheme 39.

The bromination that occurred on the aniline was referred to previously as evidence of the removal of the *N*-nitroso group from tetrazine **79** by a reduction reaction with HBr, Scheme 33.



Scheme 39. Fragmentation in hot non-nucleophilic solvent (acetonitrile) observed in the mass spectrum of tetrazine **98**

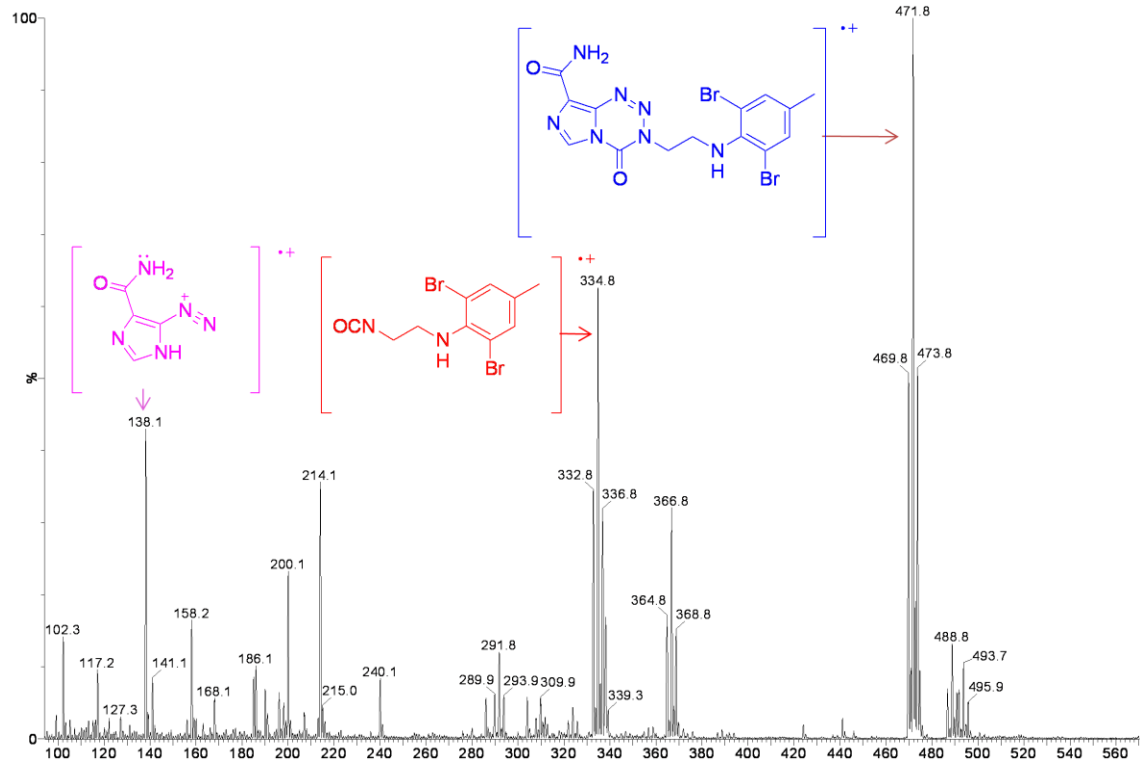
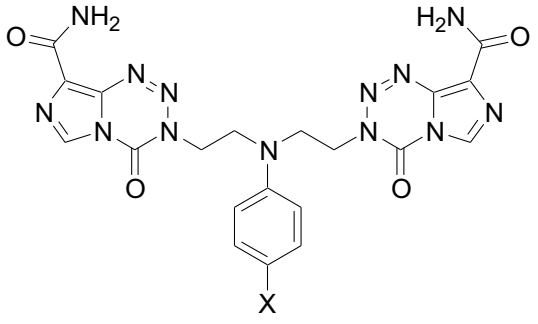


Figure 14. Mass spectrum showing bromination of the aniline ring of imidazotetrazinone **57** during HBr reduction *N*-nitroso group.

Synthesis of bisimidazotetrazinones 58a, b and c

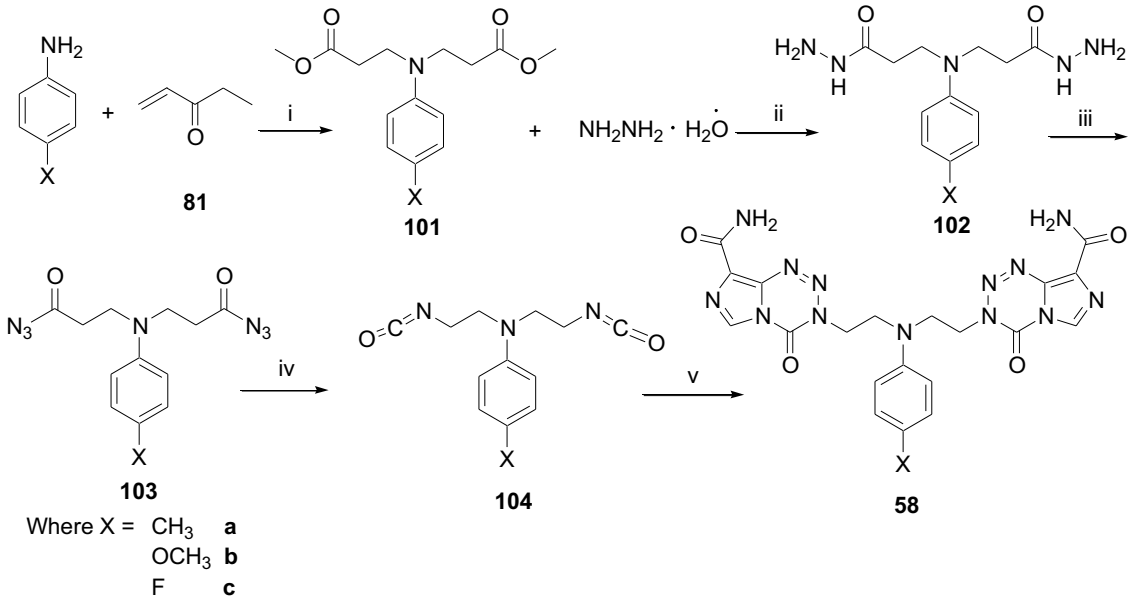


58

The synthesis of bisimidazotetrazinones **58a**, **b** and **c** started from *p*-substituted anilines, Scheme 40 outlines the general reaction sequence followed. In this synthesis the *p*-substituted anilines were pushed to react with excess methyl acrylate (10 equivalents) to give the dimeric esters, using CuCl as catalyst in acetic acid.[79] Esters were collected clean and in good yields, by fractional distillation as in case of ester **101a** and with solvent/solvent extraction ($CHCl_3$/aqueous Na_2CO_3) in case of **101b** and **c**. The hydrazides were synthesized from the reactions of esters with 10 equivalents of the hydrazine monohydrate in ethanol and were isolated as clean solids in high yields.

The azide synthesis was completed but with nitrated products as impurities as a result of aromatic ring nitration. The nitrosation occurred due to two factors: (i) the powerful electrophile (NO^+) and (ii) the highly activated aniline ring, Figure 15. The nitration occurred at the most activated and accessible positions on the aromatic ring, mainly the *o*-position to the amine as the *p*-position was blocked by another substituent. Such nitration was a major problem in the case of imidazotetrazinone **58b** due to the high reactivity of the aromatic ring, resulting from the electron releasing effect of both the amino and OCH_3 groups. Controlling of the temperature and the acid strength resulted in reduction of the incidence of nitration. The temperature was reduced to less than 5 ºC

and diluted acetic acid was used (0.16 – 0.64 M), this greatly reduced the nitrated azide impurities.



Scheme 40. Synthesis of dimeric tetrazines **58a**, **b** and **c**. *Reagents and conditions*: (i) AcOH, CuCl, Δ, 60 – 80%; (ii) $NH_2NH_2{\cdot}H_2O$, EtOH, 60 – 90%; (iii) $NaNO_2$, AcOH, DCM/H_2O; iv) anhydrous DCM, rt, 48 h, 55 – 70%; (v) **20**, DMSO, rt, 15 – 25%.

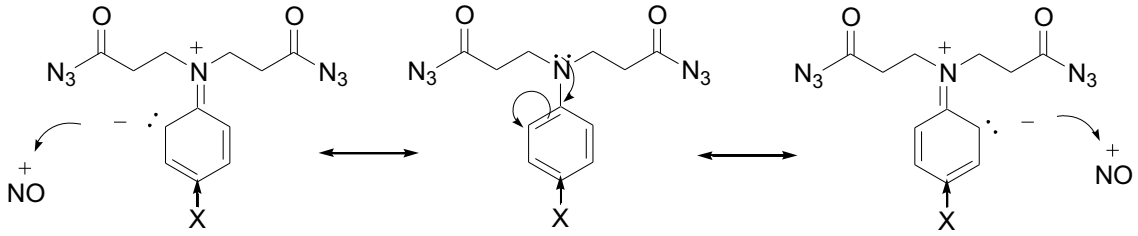
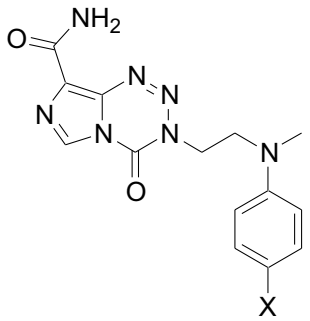


Figure 15. Aromatic ring *o*- nitration

The bisisocyanates were formed upon stirring the anhydrous DCM solutions of the crude azides at room temperature without further heating. It is quite important to keep the thermal situation to the lowest necessary for the Curtius rearrangement to proceed, in order to avoid any decomposition impurities which were observed to occur at temperatures higher than those required for the isocyanate formation. The isocyanate

was detected after 24 h, but the reaction only completed after 48 h, except in the case of the isocyanate with a *p*-fluoro group where the reaction finished in 24 h. The imidazotetrazinones were prepared from the bisisocyanates when mixed in anhydrous DMSO under N_2 with diazo-IC. Small amounts of imidazotetrazinone impurities were observed, mostly due to nitration of the aniline rings, which were separated by column chromatography purification. Imidazotetrazinones move on silica only when acetic acid is one of the solvent constituents, different amounts of acetic acid were used in chloroform for the purification of these imidazotetrazinones, fortunately imidazotetrazinones are stable in acidic media. After chromatography, the imidazotetrazinones showed some impurities that seemed to be washed out from the silica by acetic acid; these were removed by re-dissolving the isolated tetrazines in DMF from which the imidazotetrazinones were then precipitated by addition of water.

Imidazotetrazinones from *p*-substituted-*N*-methylaniline

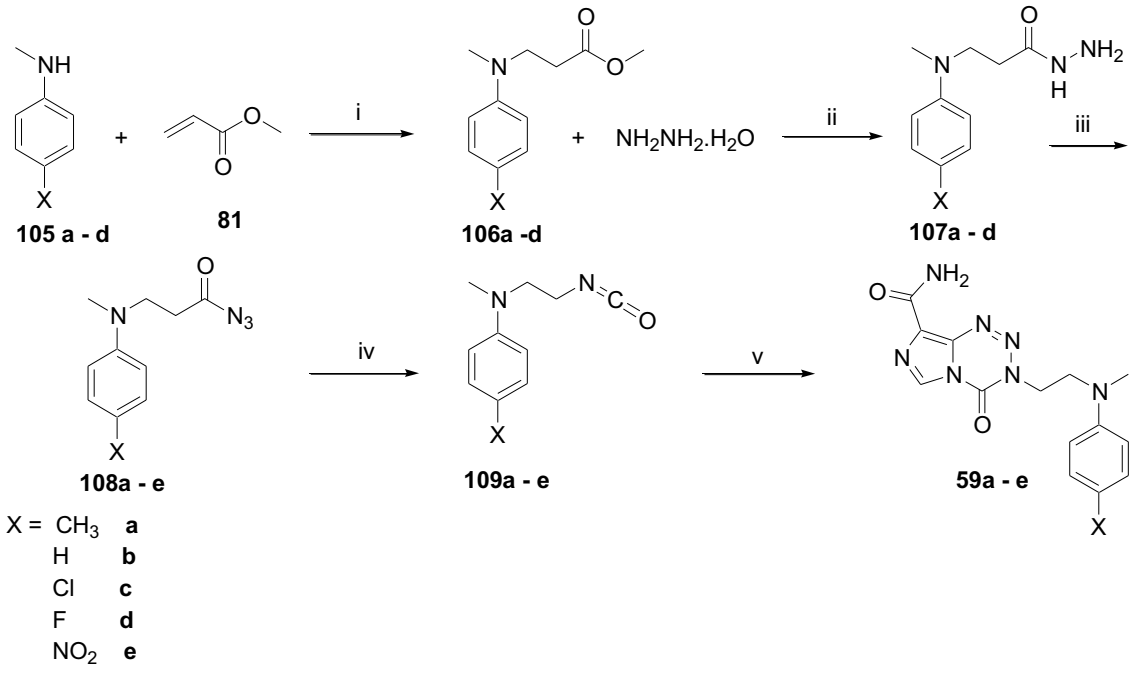


X = CH_3 **a**
H **b**
Cl **c**
F **d**
NO_2 **e**

59

N-methylanilines were used for the synthesis of five different imidazotetrazinones, Scheme 41, prepared following the general method. Unlike the monomeric ester synthesized from aniline, the esters synthesized from *N*-methylanilines, especially those substituted with electron-withdrawing groups (F, Cl), showed a low reactivity towards methyl acrylate, which required use of the catalyst CuCl and acetic acid in methanol.

Hydrazides were synthesized from the esters and six equivalents of hydrazine monohydrate in ethanol. Hydrazides were collected as clean solids in good yields by recrystallisation from ether. The azide synthesis was performed under very mildly acidic conditions, dilute acetic acid was used (0.16 M – 0.64 M), in addition to low temperature (< 5 °C) to avoid nitration on the aromatic rings.

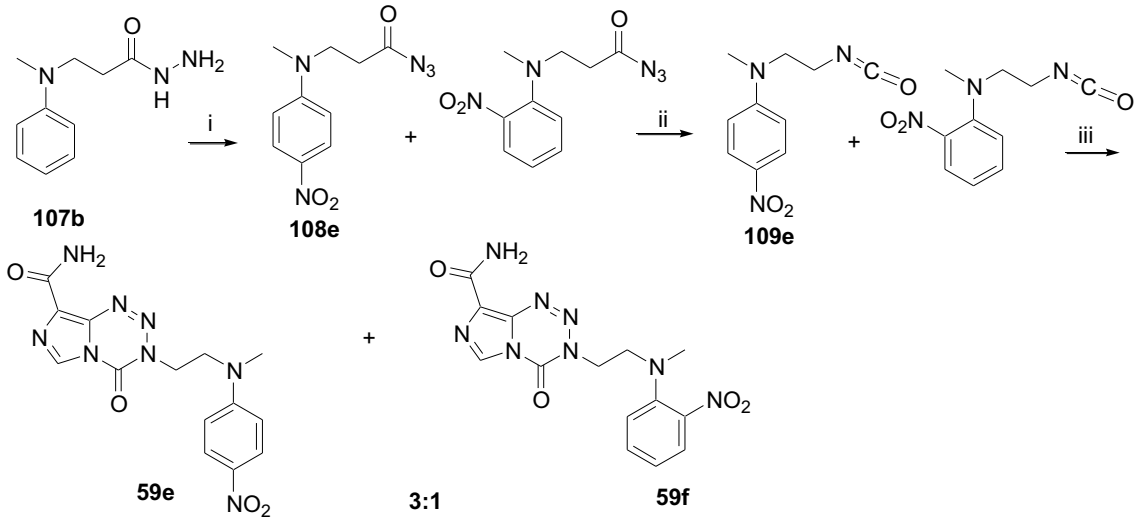


Scheme 41. Synthesis of monomeric imidazotetrazinones **59a-e**. *Reagents and conditions*: i) MeOH, CuCl, AcOH, Δ, 67 – 98%; (ii) $NH_2NH_2{\cdot}H_2O$, EtOH, 72 – 95%; (iii) $NaNO_2$, AcOH, DCM/H_2O; (iv) anhydrous DCM, rt , 48 h, 70 – 90%; (v) **20**, DMSO, rt, 6 – 22%.

In the case of the tetrazine **59e** the azide was prepared from the hydazide of unsubstituted *N*-methylaniline **107b** and in this case the diazotization was performed in relatively strongly acidic conditions (37% aq. HCl) and a higher temperature was used (5 ºC), this was required to push the reaction, in addition to diazotization of the hydrazide, to nitrate the aniline ring. The main nitrated products formed were at the *p*-position and *o*- position in a ratio 3:1 due to hindrance at the *o*- position, Scheme 42.

The lowest yield was obtained with tetrazine **59e**, the nitration reaction leads to a mixture of both the *p*-isomer and the *o*-isomer, after the chromatography separation only 6% was found from **59e**.

Anilinoethylimidazotetrazinones **59a-e** were separated by column chromatography from any nitrated impurities, different ratios of acetic acid in chloroform were used from 10 – 20%.



Scheme 42. Synthesis of monomeric imidazotetrazinone **59e**. *Reagents and conditions:* (i) $NaNO_2$, HCl 37%, DCM/H_2O; (ii) anhydrous DCM, rt , 48 h, 90%; (iii) **20**, DMSO, rt, 6 %.

Reflection on the Chemistry of the Anilinoethylimidazotetrazinones

Generally the synthesis of imidazotetrazinones having a secondary amine or BOC-protected primary amine in the ester intermediate used, proceeded with *N*-nitroso formation, in which the NH group played a very important role in trapping the powerful electrophile (NO^+) that was generated during the diazotization reaction. NO^+ replaced the proton and accordingly resulted in *N*-nitroso imidazotetrazinones Figure 16. *N*-Nitroso group removal was found to be easy by reduction with HBr to remove it in the form of ammonia, evident as the δ 7.1 triplet in the ^1H NMR spectra (Figures 9 and 13). *N*-Nitroso group removal from imidazotetrazinone **57** resulted in bromination on the

aniline ring which was not observed in case of tetrazine **93A**; this was attributed to the highly activated aromatic ring in case of imidazotetrazinone **57** and provides further evidence of the reductive nature of the reduction reaction by HBr that took place generating Br^+ and resulting in the formation of **1d** as a by-product as proposed in Scheme 33.

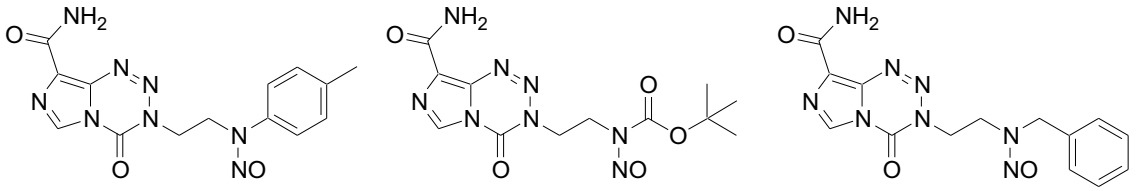


Figure 16. Imidazotetrazinones that showed an *N*-nitroso group

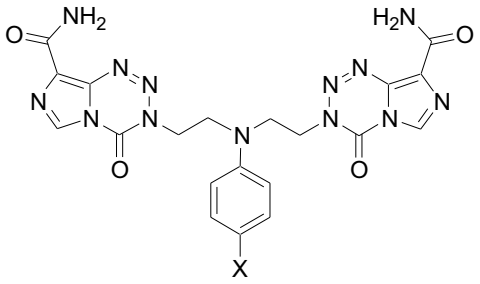
In contrast, for those anilinoethylimidazotetrazinones synthesised from esters having tertiary aromatic amines (**58a-c** and **59a-d**) Figure 17, which lacked an NH group, the NO^+ electrophile attacked the aniline ring in an electrophilic substitution reaction and resulted in nitrated by-products. Even when the reaction was closely monitored and controlled with temperature reduction and low acid strength, there was evidence of such nitration.

Purification Control

The impurities observed in the synthesis of the imidazotetrazinones (**57**, **79** and **93A**) were observed to be different from those observed in the case of anilinoethylimidazotetrazinones (**58a-c** and **59a-d**). In the first case, those synthesized from esters having secondary or BOC-protected-primary amines, impurities were observed to be from the previous synthetic intermediates i.e amine, ester or the

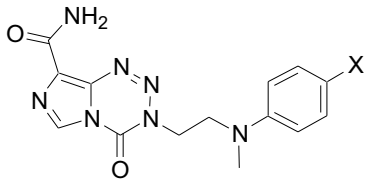
hydrazide. These were soluble in chloroform, whereas the tetrazines synthesized from them were only partially soluble in chloroform, accordingly the impurities could be easily washed out from the finished product with cold chloroform. Another possible impurity came from cyclization of diazo-IC to the triazine azahypoxanthine. This is insoluble in chloroform, whereas the imidazotetrazinones with the *N*-nitroso group showed partial solubility in large volumes of chloroform; in these cases the imidazotetrazinones were purified by filtration of their chloroform solutions after drying over anhydrous $MgSO_4$.

For the anilinoethylimidazotetrazinones derived from esters having tertiary anilines (**58a-c** and **59a-d**), the impurities were mainly the nitrated by-products, which showed very similar solubility properties to the desired tetrazines in most organic solvents; consequently their purification by washing or solvent/solvent extraction was limited and only column chromatography was useful.



58

X = CH_3 **a**
OCH_3 **b**
F **c**

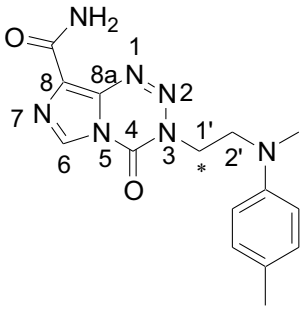


59

X = CH_3 **a**
H **b**
Cl **c**
F **d**

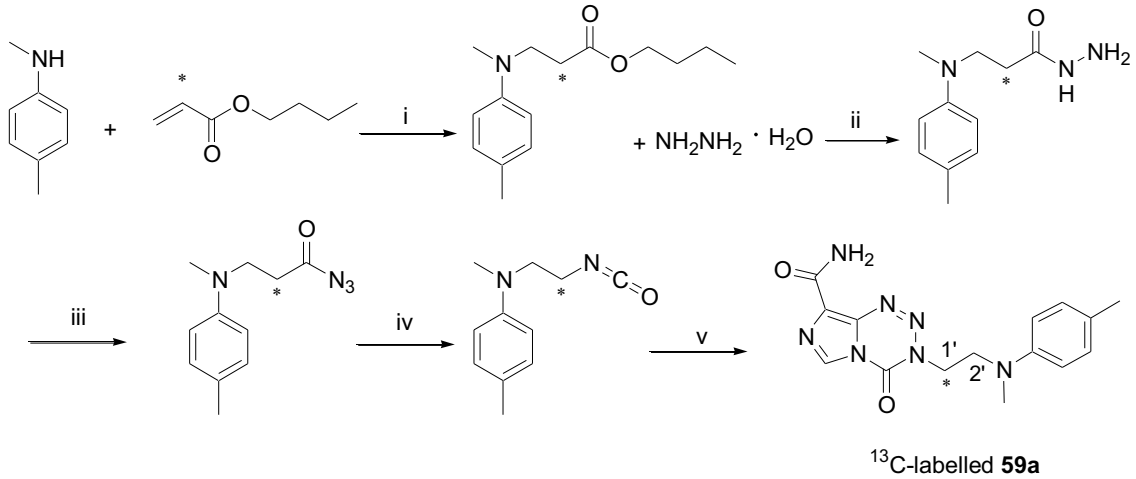
Figure 17. Imidazotetrazinones synthesized from esters lacking the NH group

The synthesis of ^{13}C-1'-labelled imidazotetrazinone



^{13}C-labelled **59a**

The imidazotetrazinone **59a** was synthesized with a ^{13}C-label at position 1' of the side chain, Scheme 43. In this synthesis, butyl [2-^{13}C]acrylate, instead of methylacrylate, was used (the only commercially-available, labelled acrylate). The butylacrylate showed less reactivity compared with methylacrylate, this might be due to the butyl group in both its electronic inductive and steric hindrance effects. The rest of the reaction was performed as in case of the unlabelled tetrazine **59a**. The labelled imidazotetrazinone was characterized by ^{1}H NMR, ^{13}C NMR (Fig 18 A & B) and MS, all confirmed the labelled imidazotetrazinone formation.



^{13}C-labelled **59a**

Scheme 43. Synthesis of ^{13}C-lablled imidazotetrazinone **59a**. *Reagents and conditions:* (i) CuCl, AcOH, Δ (ii) $NH_2NH_2{\cdot}H_2O$, EtOH (iii) $NaNO_2$, AcOH, DCM/H_2O (iv) anhydrous DCM, rt , 48 h (v) **20**, DMSO, rt

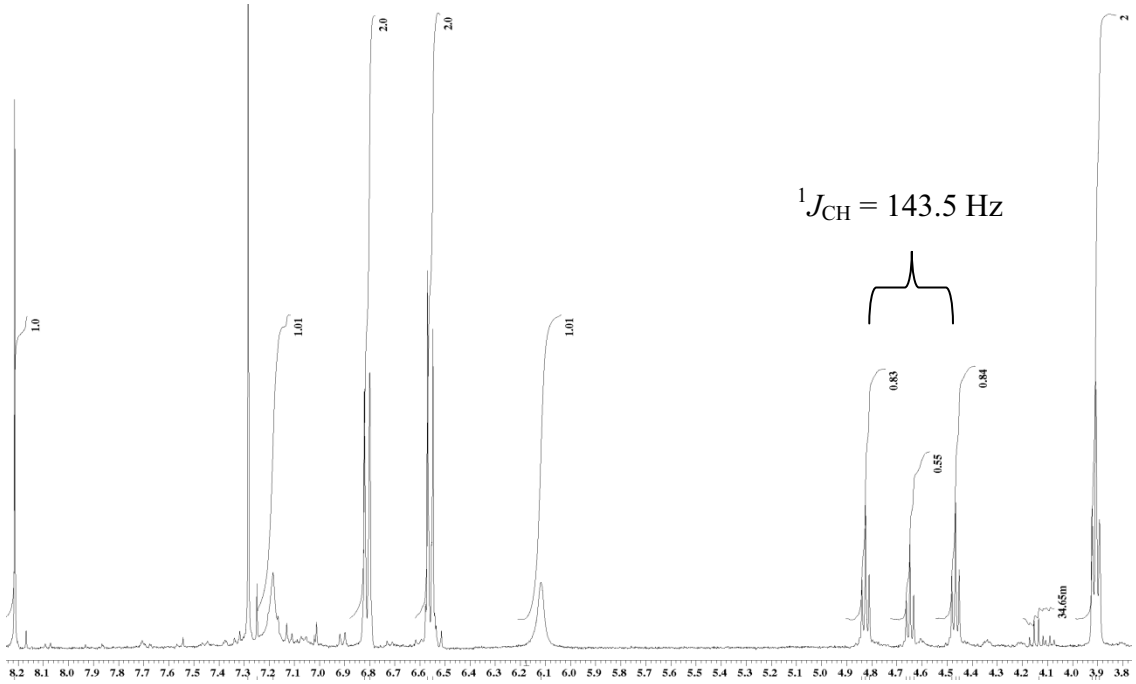


Figure 18A. ^{1}H NMR Spectrum of labelled tetrazine **59a.**

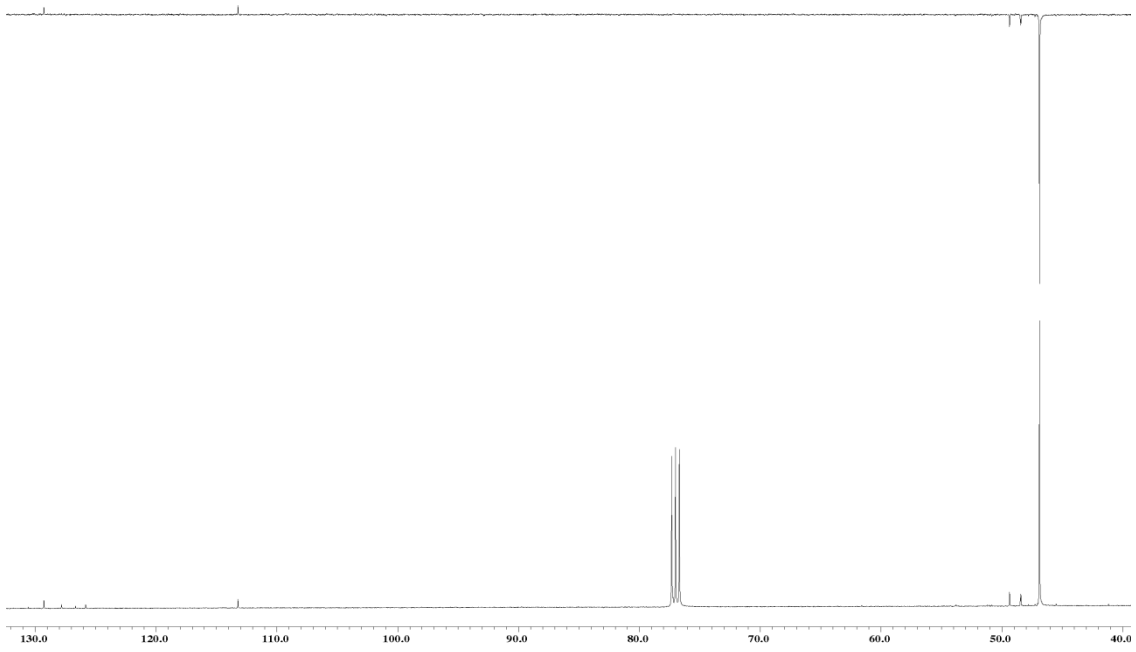
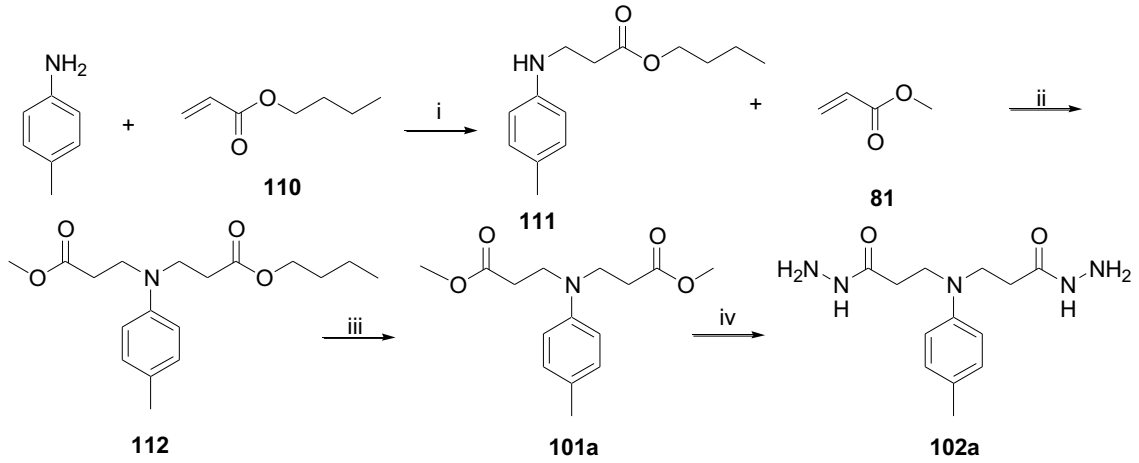


Figure 18B. ^{13}C-CPD and DEPT 135(top) NMR spectra of labelled tetrazine **59a.**

A trial for preparing bisimidazotetrazinone **58a** with a ^{13}C-label was started by the reaction of unlabelled butyl acrylate with *p*-toluidine for the ester formation. The trial considered two important issues i) the expensive labelled material to be handled and ii) the amount of labelled material (100 mg) to be carried over 5 steps to the final product. The dimer ester formation from butyl acrylate and *p*-toluidine was a slow reaction and showed the presence of the monomer ester even under reflux in the presence of the catalyst CuCl and acetic acid. To avoid the distribution of the labelled material between the monomer and dimer and to avoid need to handle tiny amounts of these esters, the trial moved to the formation of the dimer ester by another way.

First monomer butyl ester formation, in a reaction of butyl acrylate in methanol under reflux with excess amount of *p*-toluidine, led to the monomer ester together with unreacted *p*-toluidine. This mixture was then reacted with excess methyl acrylate; two dimer esters formed one with different arms (methyl and butyl) and the symmetrical dimethyl ester, Scheme 44. Transesterification (butyl to methyl) was achieved by heating the mixture of the two esters in methanol in the presence of H_2SO_4 under reflux. This avoided having esters with two different reactivities (butyl and methyl) in the subsequent step. The hydrazide was successfully made from the swapped ester, and as in the case of synthesis of tetrazine **58a** the rest of the steps proceeded smoothly.

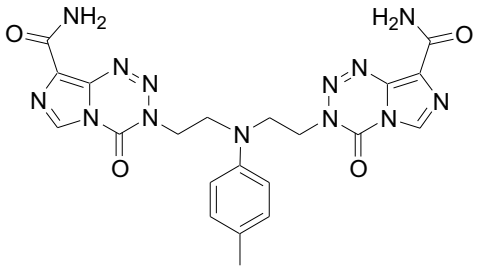
The conditions were then transferred to the labelled butyl acrylate. Preparation of the butyl monoester was successful, but the reaction of the monoester with excess methyl acrylate showed a complete loss of the labelled material. This result appears to demonstrate that the Michael addition is completely reversible so that, in the presence of an excess of un-labelled acrylate, the entire label was lost from the labelled mono ester **111**.



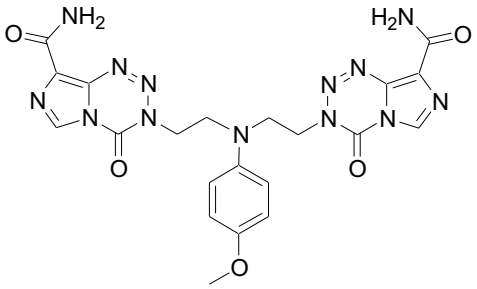
Scheme 44. Dimeric ester formation from butyl and methyl acrylate. *Reagents and conditions:* (i) MeOH, Reflux; (ii) CuCl, AcOH, Δ (iii) Conc H_2SO_4, MeOH, Δ; (iv) $NH_2NH_2{\cdot}H_2O$, *i*-PrOH

New Imidazotetrazinones Available for Biological Evaluation

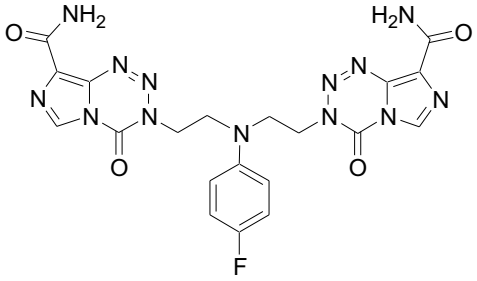
A. Bisimidazotetrazinones



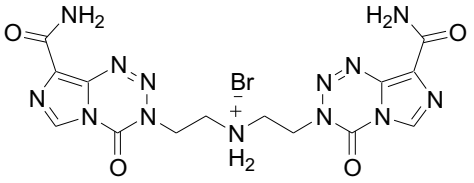
58a



58b

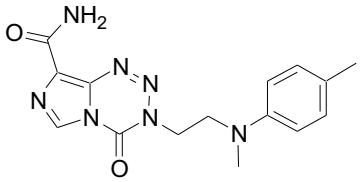


58c

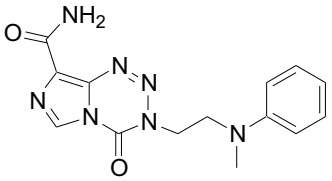


55

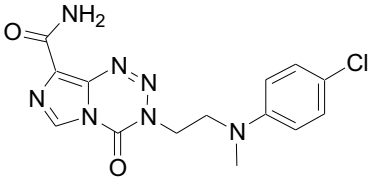
B. Monomer imidazotetrazinones



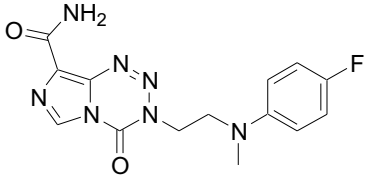
59a



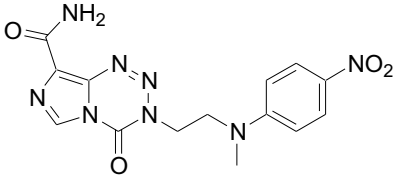
59b



59c



59d



59e

Table 2 shows selected chemical-shift (^{1}H and ^{13}C) data for imidazotetrazinones synthesized by the modified original method compared with the literature data for temozolomide synthesized by the original method.

Tetrazine	**H -6**	**C-4**	**C-6**	**C-8**	**C-8a**
Ethylimidazotetrazinone **1e**	8.80	139.3	129.1	131.0	135.1
Bromoethylimidazotetrazinone **1d**	8.97	139.6	129.6	131.7	134.6
Mitozolomide (lit) **1b**[80]	8.88	139.5	129.7	131.7	134.6
Temozolomide (lit) **1a**[80]	8.84	140.1	129.4	131.4	135.5
Imidazotetrazinone **79**	8.85	139.5	129.7	131.9	134.4
Imidazotetrazinone HBr **54**	8.92	140	129.5	131.7	134.8
BisimidazotetrazinoneHBr salt **55**	8.93	139.9	129.6	131.9	134.7
Imidazotetrazinone HBr salt **56**	8.96	140	129.6	131.8	134.7
Imidazotetrazinone **57**	8.78	139.4	129.5	131.7	134.4
Bisimidazotetrazinone **58a**	8.65	139.7	129.3	131.3	134.8
Bisimidazotetrazinone **58b**	8.76	139.6	129.2	131.3	134.8
Bisimidazotetrazinone **58c**	8.76	139.7	129.3	131.4	134.8
Imidazotetrazinone **59a**	8.72	139.5	129.1	131.2	134.7
Imidazotetrazinone **59b**	8.78	139.5	129.2	131.3	134.6
Imidazotetrazinone **59c**	8.78	139.6	129.2	131.4	134.7
Imidazotetrazinone **59d**	8.77	139.5	129.2	131.3	134.7
Imidazotetrazinone **59e**	8.81	139.7	129.5	131.6	134.7
Imidazotetrazinone **117a**	8.87	140	130.1	131.9	134.7
Imidazotetrazinone **117b**	8.87	139.7	130.4	132.4	134.4

Table 2. H-6 and ^{13}C Chemical-shift data (δ) for synthesized tetrazines compared with temozolomide literature data

CHAPTER 3

MECHANISTIC EVALUATION

Chapter 3 Mechanistic Evaluation of Imidazotetrazinones for Aziridinium Ion Release

3.1 Introduction

The aims of the mechanistic evaluation of imidazotetrazinones for the release aziridinium ions, summarised at the end of Chapter 2, are two-fold:

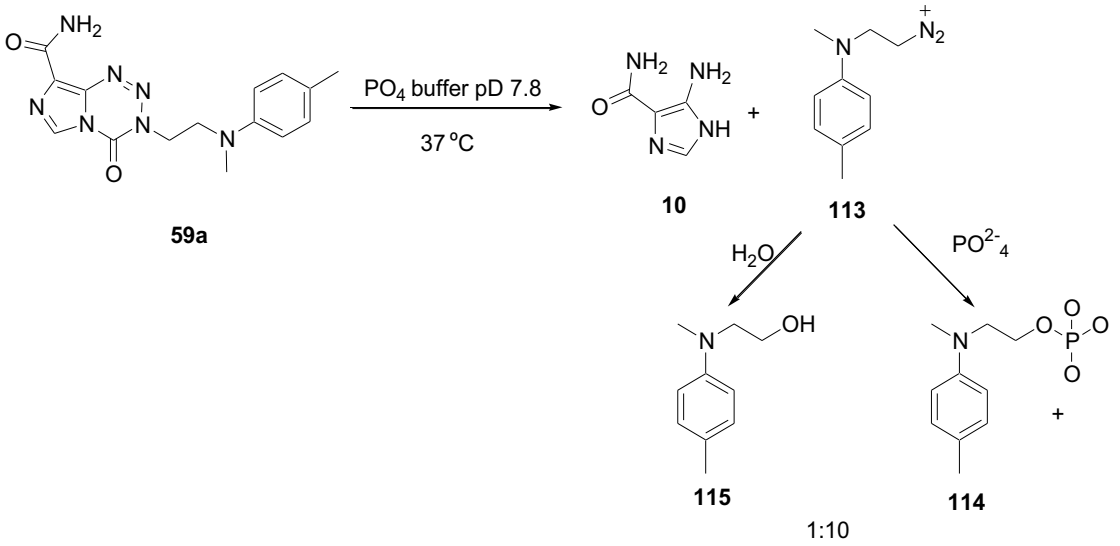
1) NMR studies of the imidazotetrazinone ring opening mechanism at neutral pH as evidence for aziridinium ion formation.
2) UV-Visible studies of the kinetics of decomposition of these tetrazines in acidic, neutral and alkaline pH, to compare the pH-dependence of the aqueous hydrolysis of these imidazotetrazinone with temozolomide.

3.2 NMR Studies of the ring opening reaction

The aim of the study was to establish evidence for the mechanism of breakdown of the imidazotetrazinone ring. In particular, for the release of aziridinium ions at neutral pH which may indicate a likely biological mechanism of action.

^{1}H NMR spectroscopy was used to investigate the imidazotetrazinones synthesized for release of aziridinium ions; ^{13}C-labelled and unlabelled imidazotetrazinone **59a** were selected for this study. Reaction of tetrazines with deuteriated phosphate buffer pD 7.8 (equivalent to pH 7.4) at 37 ºC was investigated by acquiring 1D ^{1}H NMR spectra every 30 min for 15 h, Figure 19A. The spectra showed the disappearance of imidazotetrazinone **59a** and appearance of its decomposition products with time. In the decomposition of the unlabelled imidazotetrazinone **59a**, the imidazole all reacted to AIC **10** and the 3-substituent group gave rise to another two products. Spiking with an authentic sample of alcohol **115** confirmed the identity of the minor product. The major product showed two different coupling patterns for the protons on the two aliphatic

carbons (quartet and triplet) unlike the minor product that showed only two triplets for both methylene groups, this coupling pattern is consistent with production of phosphomonoester **114**. Scheme 45 outlines the possible decomposition products of imidazotetrazinone **59a** based on the available nucleophiles in the system.



Scheme 45. Neutral pH hydrolysis of unlabelled imidazotetrazinone **59a**

The spiking of the product mixture at the end of the reaction with authentic AIC **10** and *N*-methyl-*p*-tolylaminoethanol **115** confirmed their identity, it showed an increase in the AIC singlet peak, whereas alcohol **115** addition resulted in the increase of the size of the minor two small triplet peaks at δ: 3.30 & 3.62 (compare spectrum B with spectrum A in Figure 19B). The quartet at 3.77 and triplet at 3.34 is for something else. The presence of the negatively charged phosphate of the buffer suggested these peaks to be the phosphate ester **114** and the quartet peak is due to the $^{3}J_{PH}$ coupling. The presence of an additional phosphorus-containing compound was confirmed by ^{31}P NMR Figure 19C.

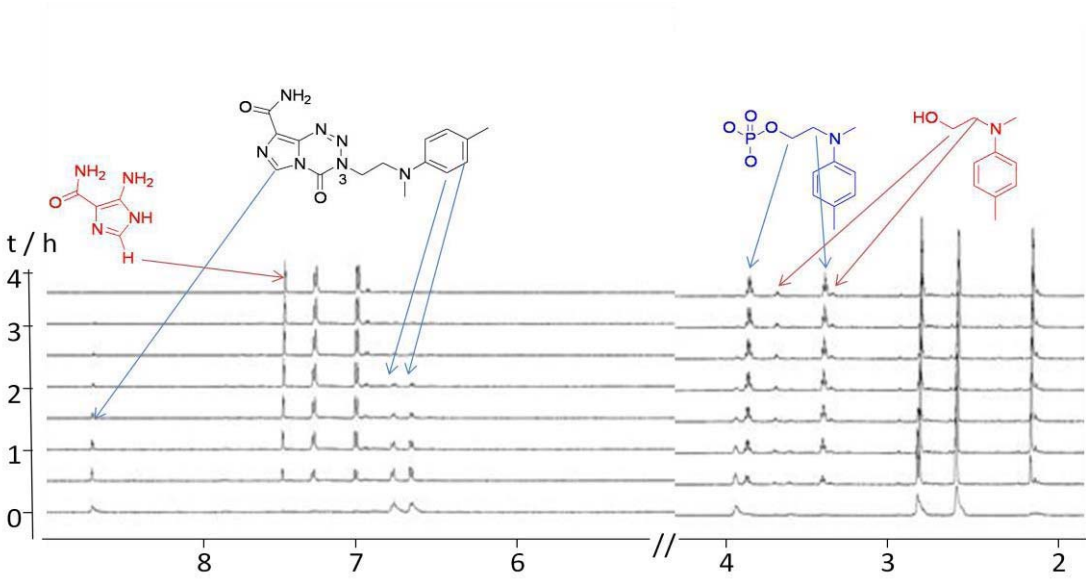


Figure 19A. Decomposition of imidazotetrazinone **59a** in phosphate buffer pH 7.4 at 37 ºC showing the 3-substituent giving rise to two products.

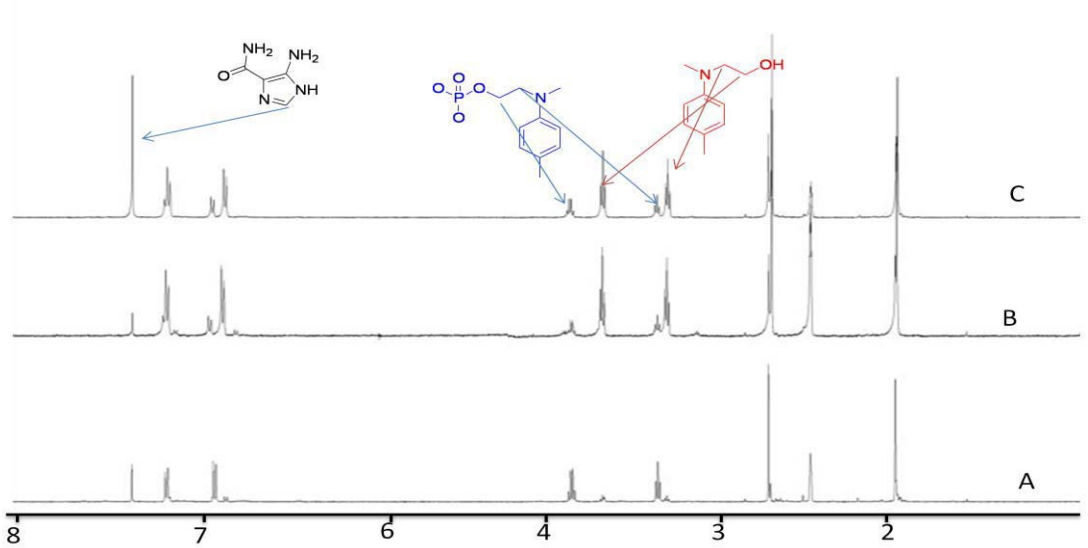


Figure 19B. ^{1}H NMR spectra of (A) decomposition products of imidazotetrazinone **59a;** (B) spiked with *N*-methyl-*N*-*p*-tolylaminoethanol, (C) spiked with AIC.

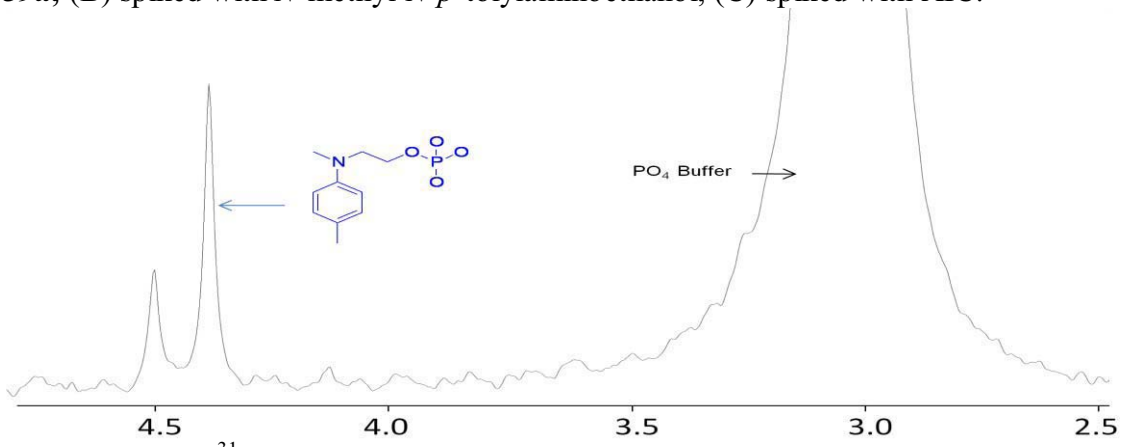
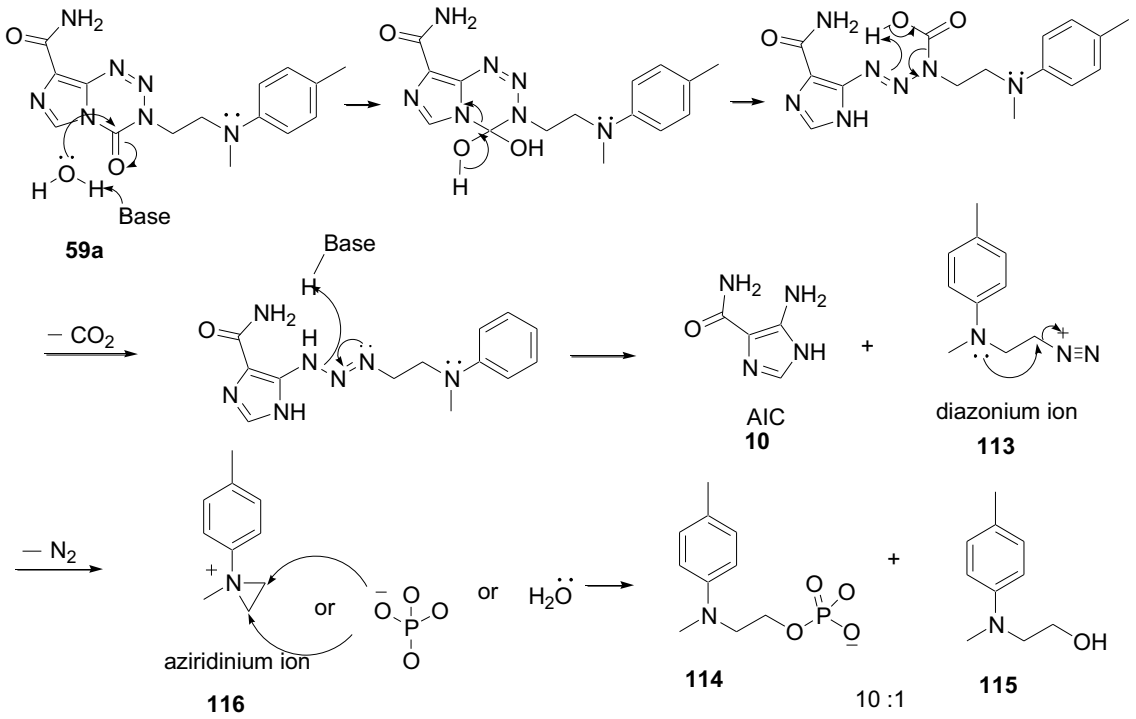


Figure 19C. ^{31}P NMR spectrum of the final decomposition products of imidazotetrazinone **59a**

Scheme 46 outlines a proposal for the decomposition mechanism of the unlabelled tetrazine **59a**. The proposal suggests the formation of a diazonium ion as the usual decomposition product of an imidazotetrazinone (AIC **10** + diazonium ion **113**) and due to the presence of the nitrogen of the aniline, which acts as a nucleophile, cyclization of the diazonium ion **113** to the aziridinium ion **116** took place. The scheme proposes the nucleophilic attack of a negatively charged phosphate at one of the two equivalent carbon sites of the aziridinium ion. Either would result in phosphomonoester **114**, which showed a quartet coupling for the protons attached to the carbon adjacent to the phosphate, this product was also confirmed by negative ion MS that showed m/z = 245.1.



Scheme 46. Proposed decomposition mechanism for unlabelled imidazotetrazinone **59a**

Evidence for the aziridinium ion intermediate **116** came from a study performed on the ^{13}C-labelled imidazotetrazinone **59a** under the same conditions. The ^{1}H NMR spectra that resulted from the study showed that the decomposition products are same as those mentioned above. Once again the $^{3}J_{PH}$ couplings were evident in the –P-O-CH_2-CH_2 unit, Figure 20.

The gated decoupled ^{13}C spectrum acquired for the products at the end of the reaction of the labelled-imidazotetrazinone **59a** (30000 scan over 99 h) showed that the reaction gave two products, the phosphomonoester **114** and the alcohol **115**, Figure 21. In the phosphomonoester the label was completely scrambled so it appeared equally at both CH_2 positions (44 %: 44 %) phosphomonoester **114a** & **b** Scheme 47. In the case of the alcohol product, the label was also scrambled but with a small excess of label in the position adjacent to the OH group (8%: 4 %). This result greatly helped in the confirmation of aziridinium ion formation; the scrambling of the labeled products, whether the phosphomonoester or alcohol, confirmed that these products resulted from the equal attacks of the system nucleophiles on the aziridinium ion sites, Figure 21, Scheme 47. The phosphomonoester **114a&b** shows the carbon-phosphorus coupling and it showed the ratio of phosphomonoesters **114** to alcohol **115** (10:1) which is due to the reactivity of the negatively charged phosphate over H_2O. The ratio shown for alcohols conclude that alcohol was produced as a result of two routes: (i) the direct hydrolysis of the ^{13}C-labelled-diazonium ion **113a** before cyclization and (ii) the equal H_2O attacks to the two sites of aziridinium ion, Scheme 47. Alcohol resulting from direct diazonium ion hydrolysis was shown to be half the amount of alcohol produced by H_2O attacks to the two sites of the aziridinium ion, this is an indication to how fast is the cyclization of the diazonium ion to the aziridinium ion.

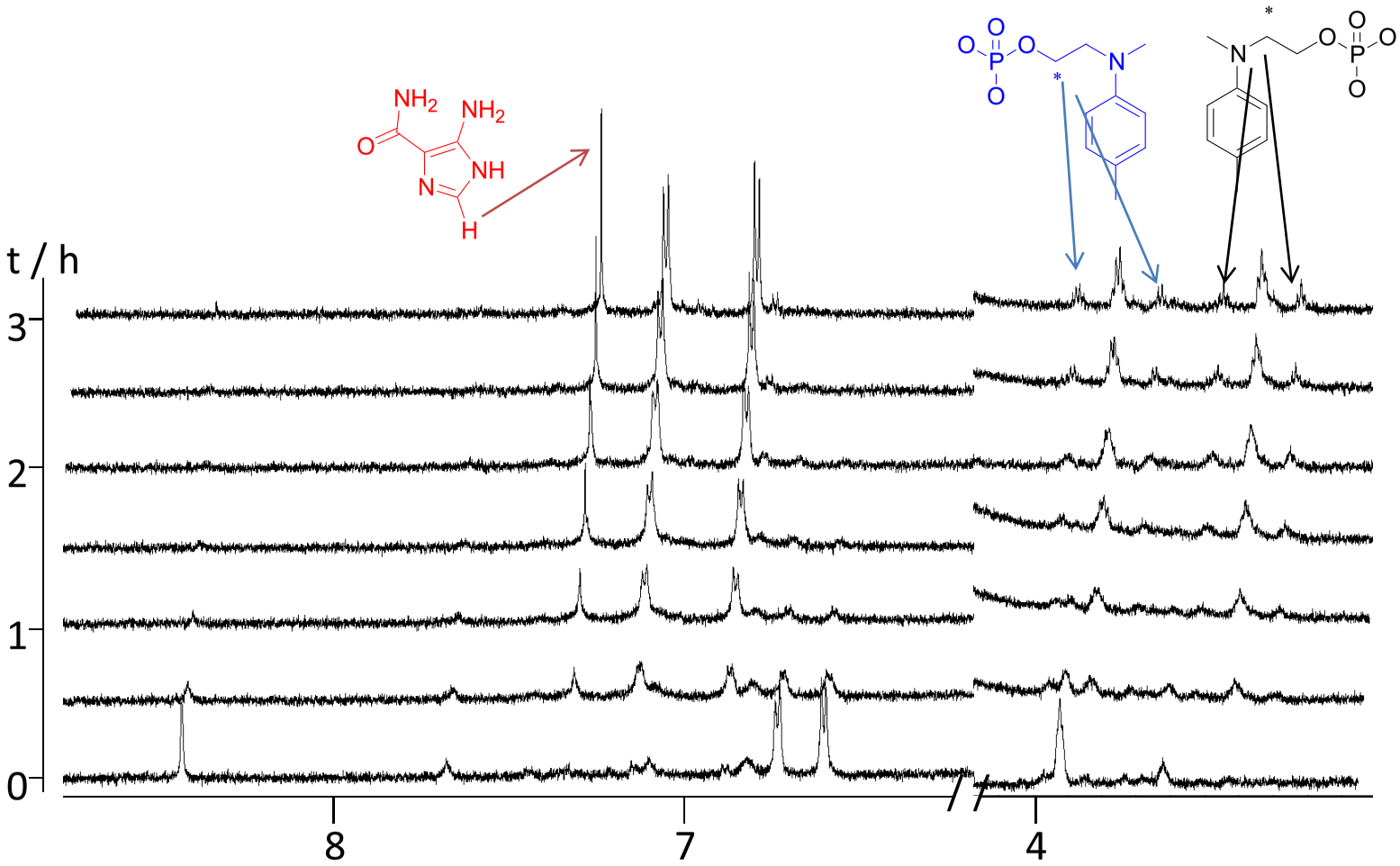


Figure 20. The decomposition of ^{13}C-labelled imidazotetrazine **59a** in phosphate buffer pH 7.4 at 37 ºC with time

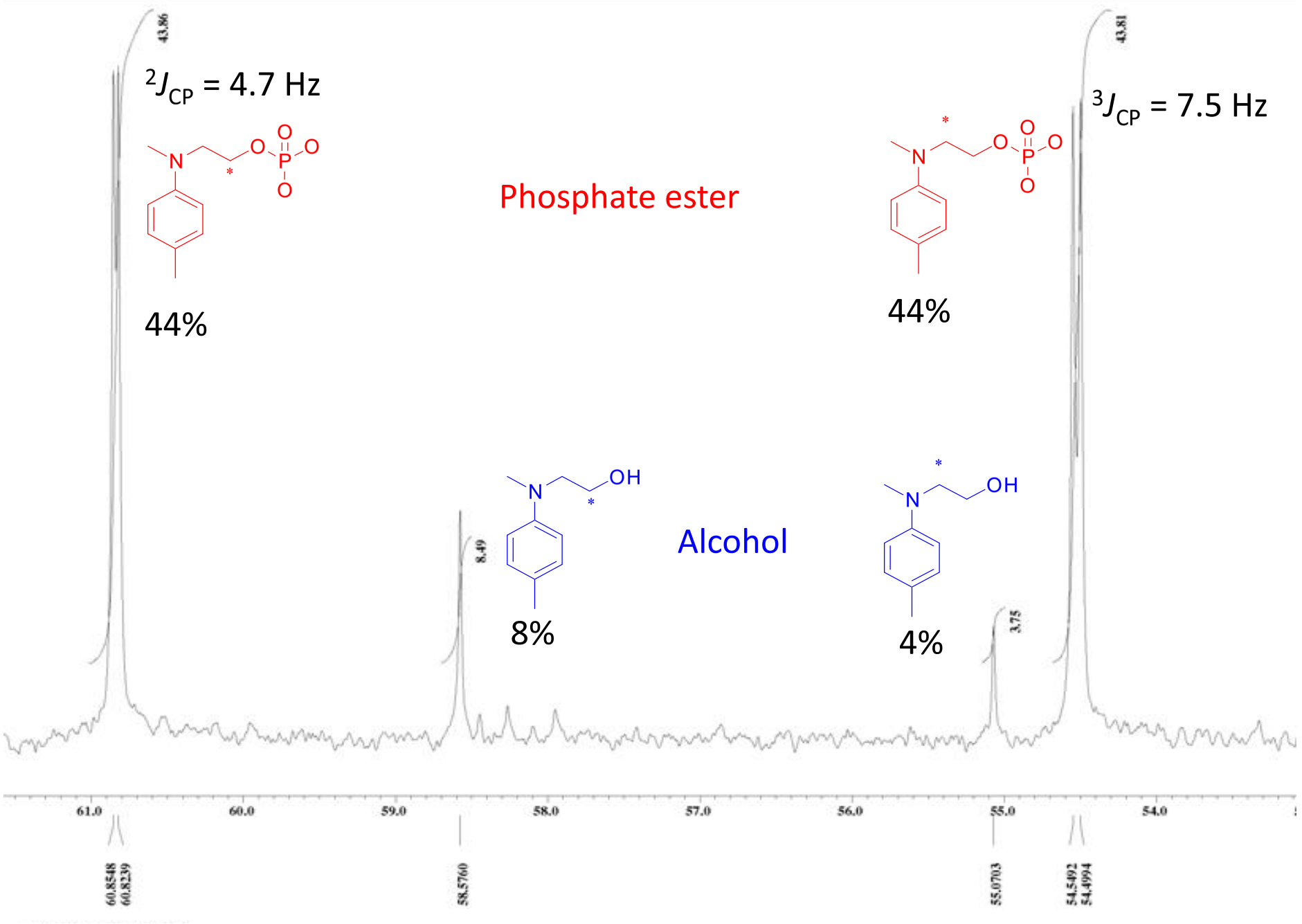
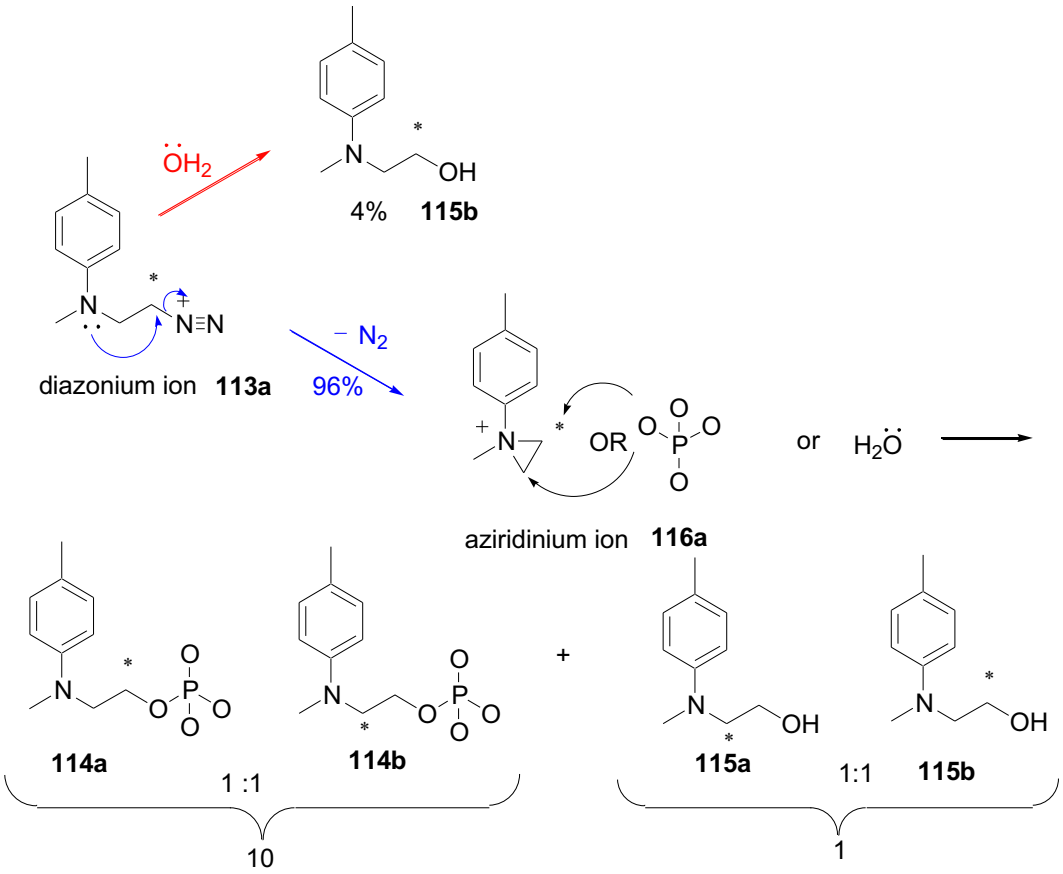


Figure 21. Gated decoupled ^{13}C spectrum of labelled imidazotetrazine **59a** decomposition product ratios



Scheme 47. ^{13}C-labelled diazonium ion cyclization to the aziridinium ion and products of system nucleophile attacks.

The NMR study on the aziridinium-ion-release-imidazotetrazinones exemplified by the tetrazine **59a** (^{13}C-labelled and unlabelled), showed the possibility of taming of the diazonium ion by the cyclization reaction that resulted in the aziridinium ion. This concluded that imidazotetrazinones synthesized for the release of aziridinium ion indeed can release aziridinium ions by aqueous hydrolysis at neutral pH. This reflects the possibility of chemical activation at the physiological pH for a future drug and the release aziridinium ions at their target of action. Moreover the study confirmed the similarity of the neutral aqueous hydrolysis of these imidazotetrazinones to that of temozolomide.

3.3 Hydrolysis Kinetics of Aziridinium-ion-release imidazotetrazinones

In the previous section, the NMR studies showed the ring opening mechanism and confirmed aziridinium ion release by imidazotetrazinone decomposition at neutral pH.

In this section, the kinetics of hydrolysis of aziridinium-ion-release imidazotetrazinones under acidic, neutral and alkaline conditions will be investigated using UV-Visible spectrophotometry. The study was designed to show the kinetics of the aqueous hydrolysis of these tetrazines at the neutral and alkaline pH and compare the results with temozolomide under the same condition. Pseudo 1st order rate constants (k') and half-lifes ($t_{1/2}$) at each studied condition were measured.

The expected reaction kinetics are pseudo first order reaction since the rate limiting step is addition of water (or hydroxide ion) to initiate the ring opening reaction. The concentration of water (or ^{-}OH) is effectively constant in the buffer solution. Accordingly the reaction rate is described by the integrated rate equation (1).

$$\ln[A] = -k' t + \ln[A]_o \quad \text{.....(1)}$$

Where [A] is the concentration of imidazotetrazinone at time (t) and $[A]_o$ is the initial concentration.

The plotting of ln[A] against time (t) will give a straight line with

gradient = $-k'$

The half life of the pseudo-first-order reaction is independent of the starting concentration and is given by equation 2.

$$t_{1/2} = \ln(2)/ k' \quad \text{.....(2)}$$

The study was performed on the 9 imidazotetrazinones summarised at the end of Chapter 2, in three different phosphate-citrate buffers of pH 4, 7.4 and 8 at 37 °C. The different pH values were chosen based on the known properties of temozolomide and their significance for acid stability in oral administration and the need for chemical activation at physiological pH.

3.3.1 Hydrolysis kinetics at pH 4

The pH 4 was selected to investigate the stability of aziridinium-ion-release-imidazotetrazinones under the acidic conditions. The gastric pH = 0.8,[81] so good stability at pH 4 will reflect a good stability under the gastric acid conditions so may facilitate oral bioavailability similar to temozolomide.

Time-dependent UV spectra of imidazotetrazinone **59d** are shown in Figure 22A. The imidazotetrazinone showed a strong absorbance at 327 nm which decreased with time. The absorbance bands of the aniline and AIC (the final u.v-active hydrolysis products of the imidazotetrazinone) overlapped in the 240 – 280 nm region so could not be resolved. The $\ln A_{327}$ vs time plot is shown in Figure 22B. This was straight line giving $k' = 0.5 \times 10^{-5}$ sec^{-1} and $t_{1/2} = 35.6$ h. Other tetrazines (**58a-b**, **59a-c** and **59e**) gave similar plots under these conditions.

The only exception was imidazotetrazinone **58c**, it is a bisimidazotetrazinone with a fluoro group at the *p*-position of the aniline ring. It showed non-linear kinetics, Figure 23A, the kinetics appeared fast at the beginning of the reaction with reaction rate 2.3×10^{-5} sec^{-1}, $t_{1/2}$ 8.3h, whereas at the end substantial curvature and slower reaction were seen, Figure 23B. This might be attributed to the non-equivalency in decomposition between the two imidazotetrazinone rings as a result of the electron-withdrawing effect

exerted by the fluoro group and one of the imidazotetrazinone rings on the second one, showing a fast decomposition rate of 1st ring at the beginning, then a slower rate for decomposition of the second ring.

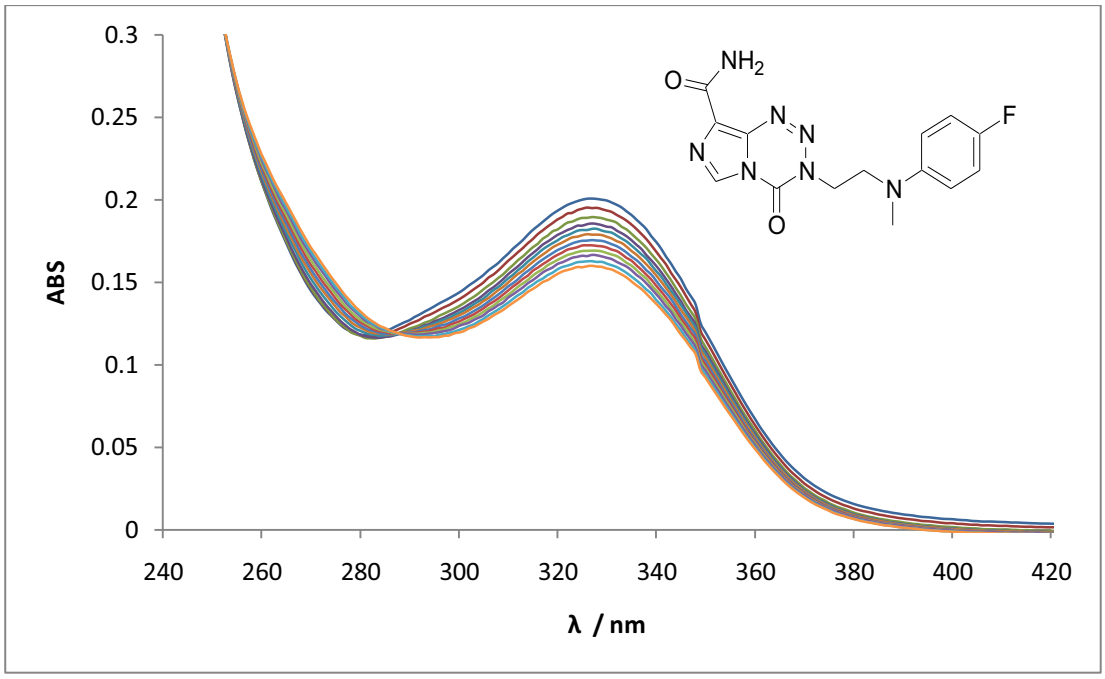


Figure 22A. U.V. spectra of imidazotetrazinone **59d** kinetics of decomposition pattern at pH4

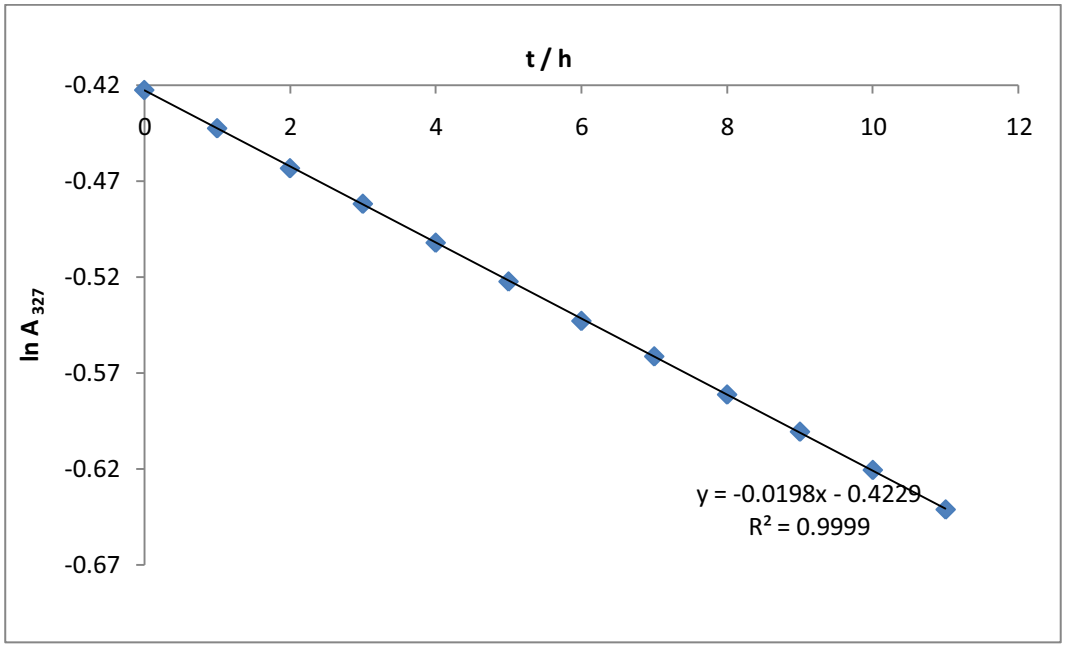


Figure 22B. Imidazotetrazinone **59d** hydrolysis kinetics at pH4

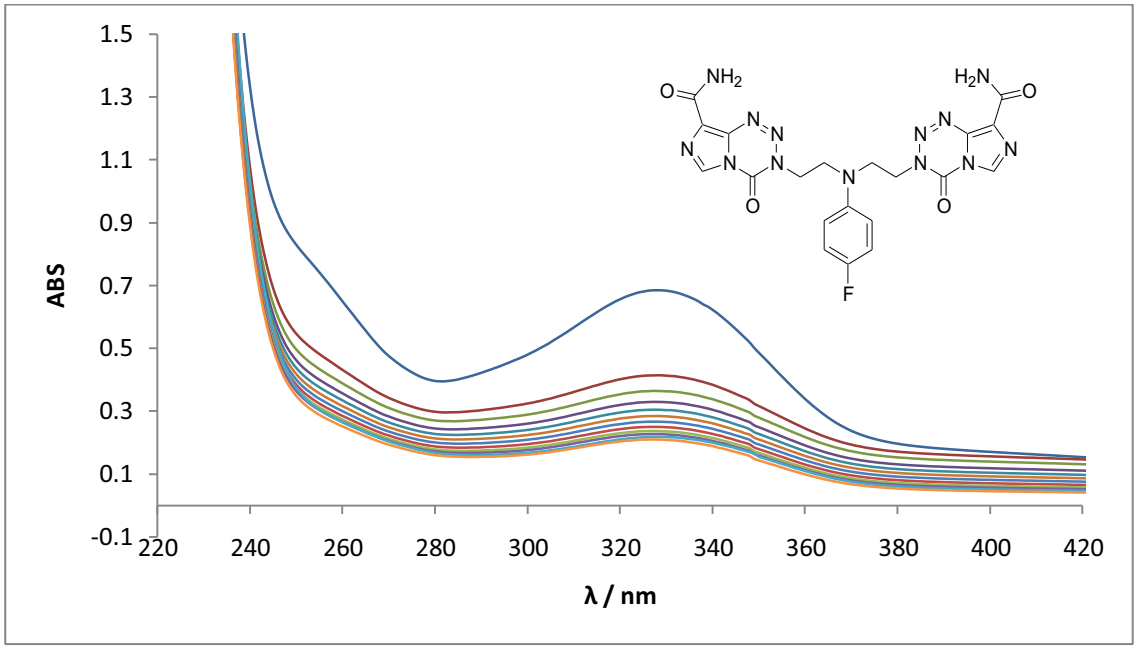


Figure 23A. U.V. spectra of imidazotetrazinone **58c** kinetics of decomposition pattern at pH4

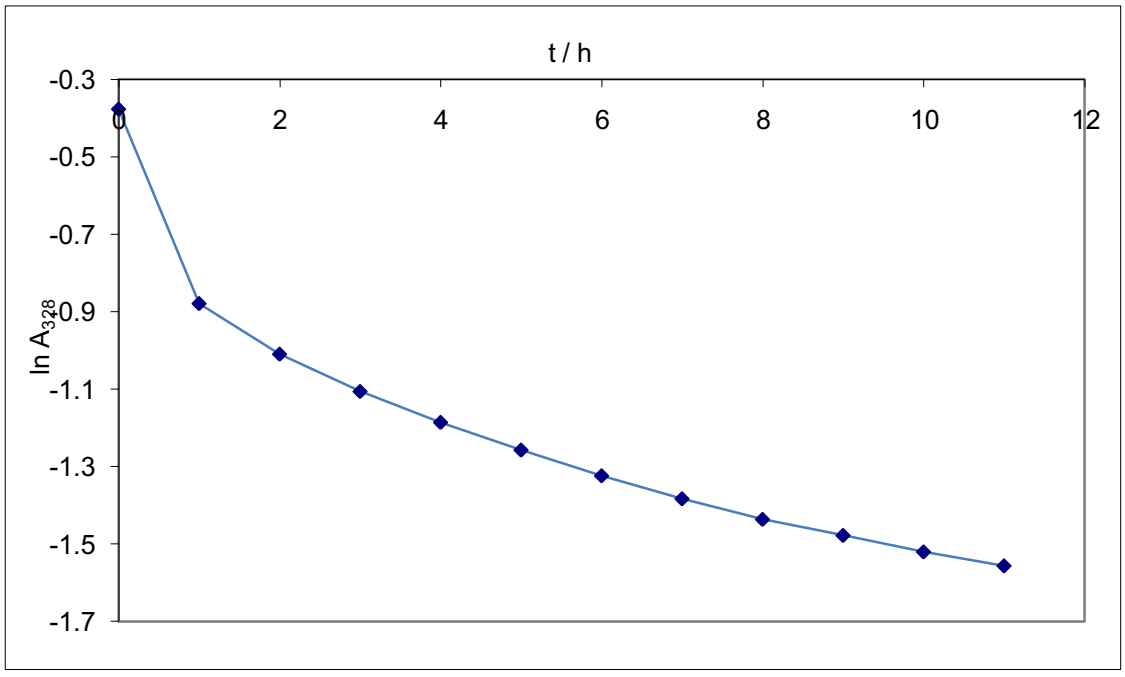


Figure 23B. Imidazotetrazinone **58c** hydrolysis kinetics at pH4

3.3.2 Hydrolysis kinetics at pH 7.4

The kinetics of decomposition at pH 7.4 were measured to show the aqueous hydrolysis at the neutral pH, to see whether behaviour of the new imidazotetrazinones was similar to temozolomide. Temozolomide showed a comparable half-life in phosphate buffer pH 7.4 at 37 °C (1.83 h) to the mean half-life measured in a group of patients (1.81 h).[15]

In this study aziridinium ion-release-imidazotetrazinones (**58a-c**, **59a-e** and **55**) solutions in buffer pH 7.4 were scanned at a time interval of 4 min for 1 h. The study showed that the decomposition of the tetrazines with time at this pH was a linear pseudo-first order reaction, for example compound **59b**, Figure 24A & B. The rate constants ranged between 14 – 33 × 10^{-5} sec^{-1}, compared with temozolomide that showed a $k' = 14 \times 10^{-5}$ sec^{-1} under the same conditions.

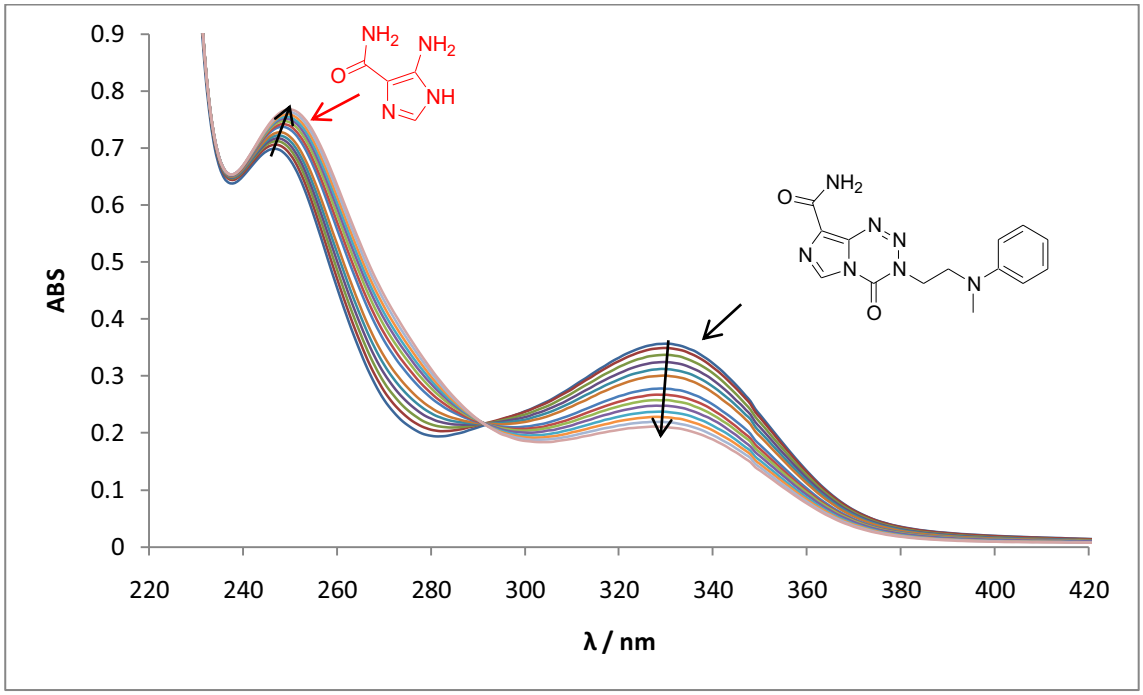


Figure 24A. U.V. spectra of imidazotetrazinone **59b** kinetics of decomposition pattern at pH 7.4

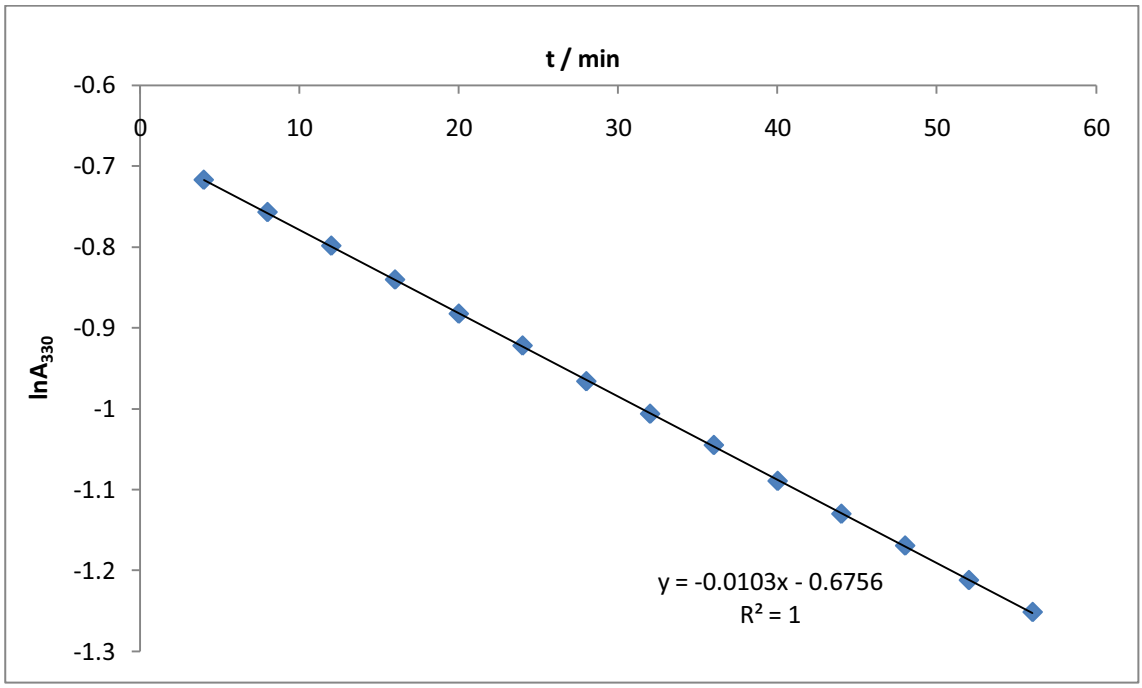


Figure 24B. Imidazotetrazinone **59b** hydrolysis kinetics at pH 7.4

Bisimidazotetrazinone **58c** showed a more complex decomposition pattern when the kinetics of its hydrolysis were performed at the same concentration used for other tetrazine but upon 10-fold dilution a usual pattern of tetrazine hydrolysis was observed with rate constant $k' = 10.3 \times 10^{-5}$ sec^{-1} which is unexpectedly less than that measured for temozolomide, Figure 25A & B. The complex pattern of decomposition observed at the relatively high concentration might be due to precipitation of the tetrazine when its DMSO solution was added to buffer solution.

The bisimidazotetrazinone HBr salt **55** showed shifting of the isosbestic point from 300 to 290 nm during the reaction and this appears to show rapid hydrolysis at the start of the reaction with rate constant $k'_1 = 61.2 \times 10^{-5}$ sec^{-1} and $t_{1/2} = 0.3$ h and then slowing once a substantial proportion of the tetrazine has been consumed, Figure 26A&B. This may be due to the combination of the positive nitrogen and imidazotetrazinone acting as powerful electron-withdrawing substituents. Once the first tetrazine ring has been converted to the alcohols the electron-withdrawing effect is less and hydrolysis of the second tetrazine is therefore slower with a rate constant $k'_2 = 20.8 \times 10^{-5}$ sec^{-1}, $t_{1/2} = 0.9$ h.

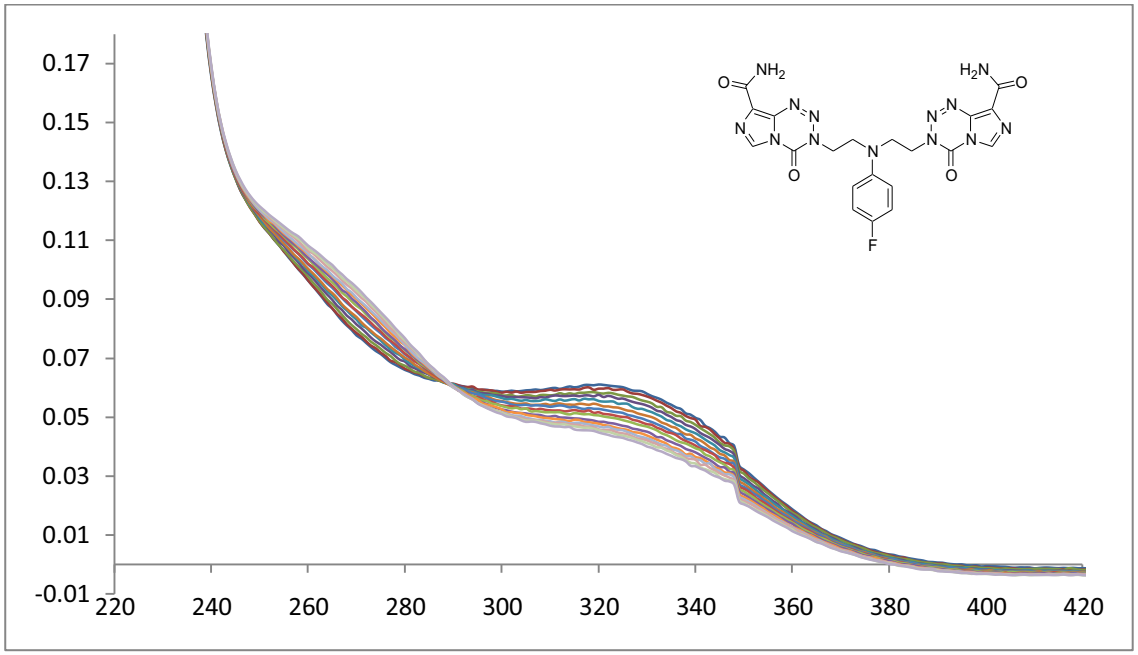


Figure 25A. U.V. spectra of imidazotetrazinone **58c** kinetics of decomposition pattern at pH 7.4

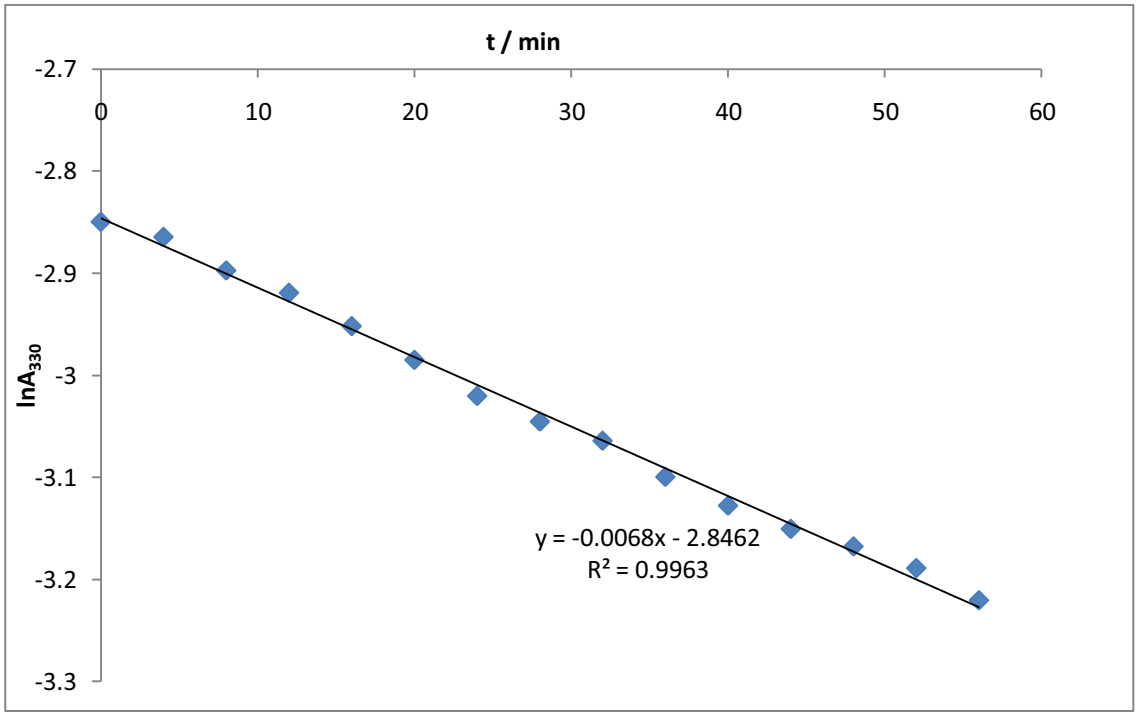


Figure 25B. Imidazotetrazinone **58c** hydrolysis kinetics at pH 7.4 and hydrolysis products AIC and aniline.

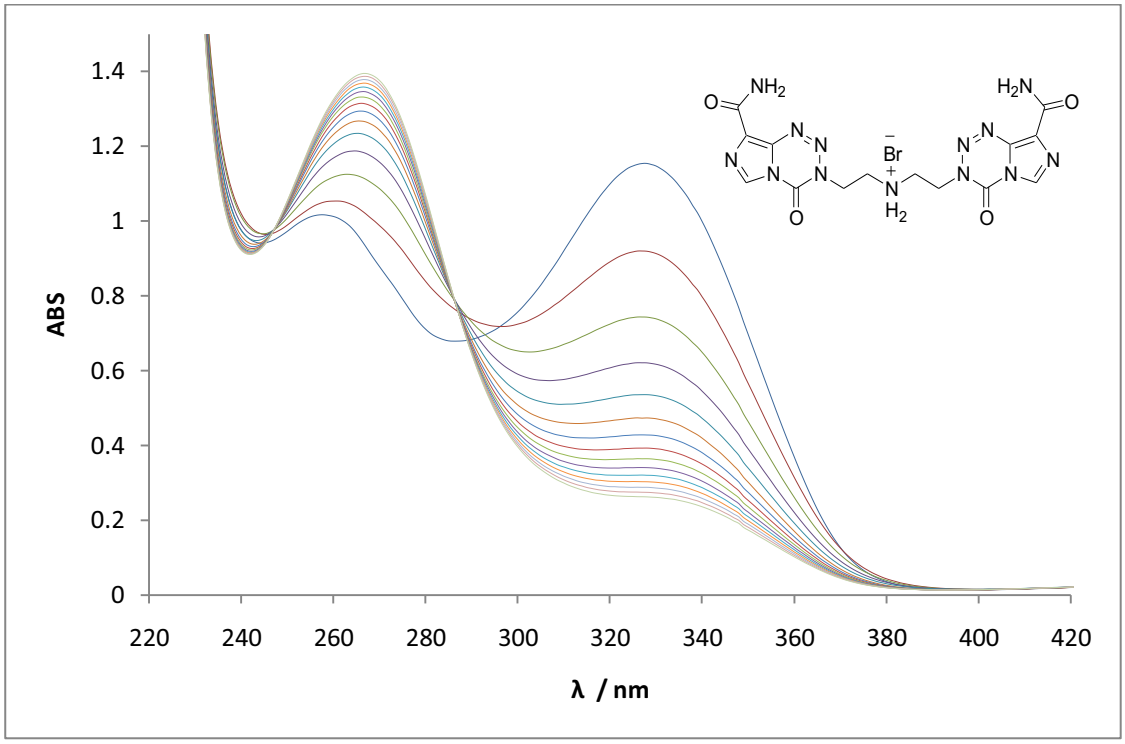


Figure 26A U.V. spectra of decomposition of bisimidazotetrazinone HBr **55** at pH 7.4

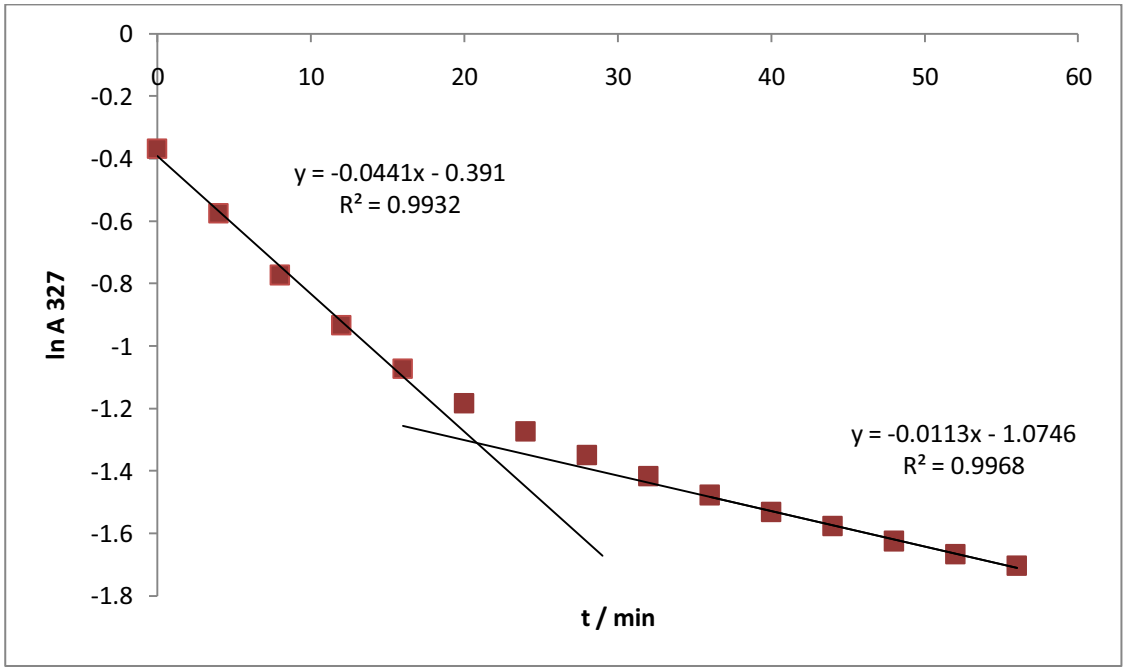


Figure 26B. The fast hydrolysis reaction of bisimidazotetrazinone **55** at pH 7.4

Imidazotetrazinone **59e**, the monomer with *p*-nitro group on the aniline ring, showed in addition to AIC at λ_{max} = 245 nm and the *p*-nitroaniline at λ_{max} = 412 nm as another decomposition product (in the visible region) Figure 27A & B. It showed a linear decomposition with rate $k'_2 = 33.3 \times 10^{-5}$ sec^{-1}, $t_{1/2}$ = 0.6 h.

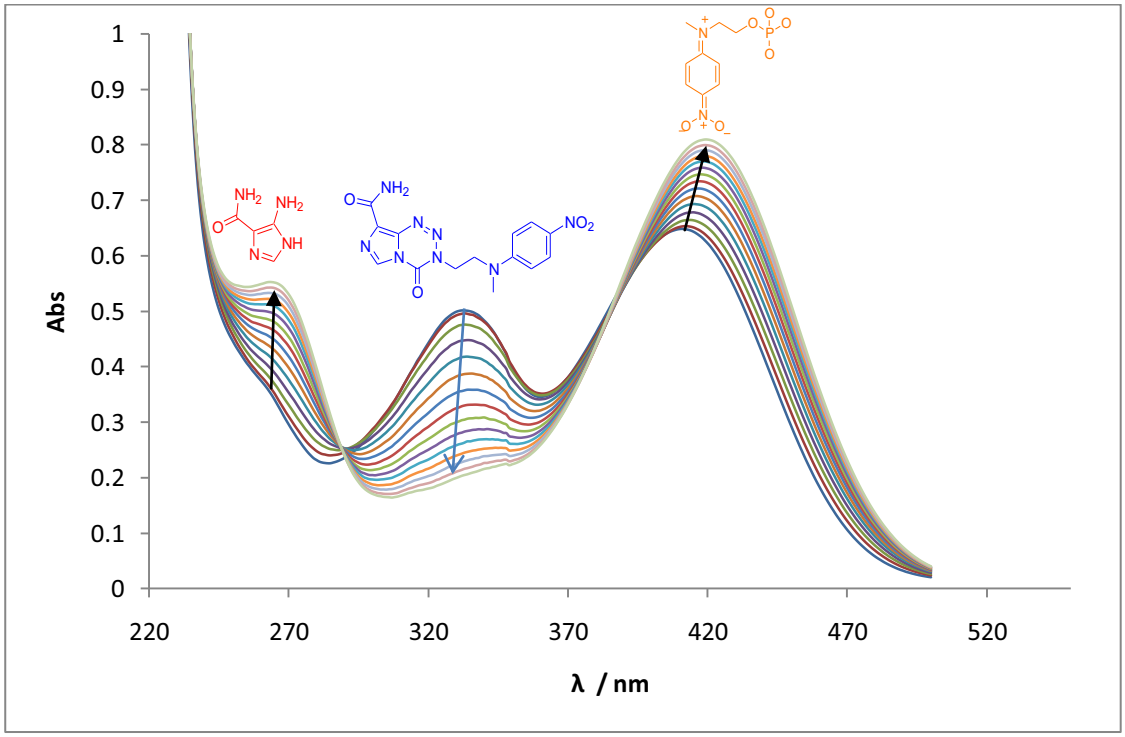


Figure 27A. U.V. spectra of decomposition of Imidazotetrazinone **59e** at pH 7.4

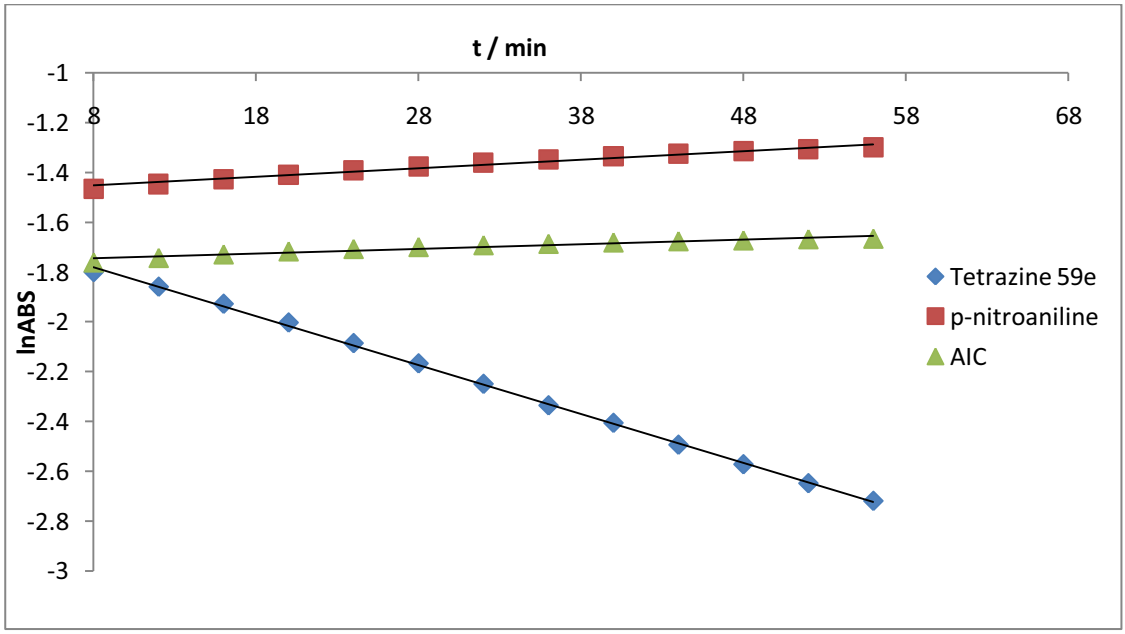


Figure 27B. U.V. spectra of decomposition of Imidazotetrazinone **59e** at pH 7.4

3.3.3 Hydrolysis kinetics at pH 8 and 37 °C

The kinetics of hydrolysis at pH 8 were investigated to find out the degree of the instability of the aziridinium-ion-release-imidazotetrazinones under alkaline conditions and the results were compared with the instability observed for temozolomide under the same conditions. pH 8 is six-times less acidic than pH 7.4 (1 pH unit is corresponds to a 10 fold change in $[H]^{+}$) and is the pH found at the beginning of the alkaline pH range, accordingly any instability or high rate of decomposition shown by the imidazotetrazinone at this pH will reveals it instability at the alkaline pH of the ileum and the small intestine (pH = 8.1 – 9.3). [81]

In common with other aziridinium-ion-release imidazotetrazinones, that showed linear decomposition at previous pH conditions and in this condition, the *p*-F bisimidazotetrazinone **58c** showed a linear pseudo first order kinetics but with relatively unexpected lower reaction rate and higher $t_{1/2}$ ($k' = 24.6 \times 10^{-5}$ sec^{-1}, $t_{1/2}$ = 0.8 h), Figure 28B. Figure 28A shows that bisimidazotetrazinone **58c** gave a simple pattern of decomposition of to AIC and aniline, unlike those observed at pH 4 and 7.4. This may be attributed to the strong nucleophiles present in this alkaline solution which result in equal attack to both tetrazine rings at the same time, so leads to their opening at the same rate.

Bisimidazotetrazinone HBr salt **55** showed the highest rate and shortest $t_{1/2}$ ($k' = 110.5 \times 10^{-5}$ sec^{-1}, $t_{1/2}$ = 0.2 h) in a biphasic linear-decomposition pattern, Fig 29A & B, it may be attributed to the strength of electron withdrawing effect exerted by the aliphatic amine nitrogen and the effect of one of tetrazine rings on each other, which leads to the higher instability of this tetrazine at this alkaline condition.

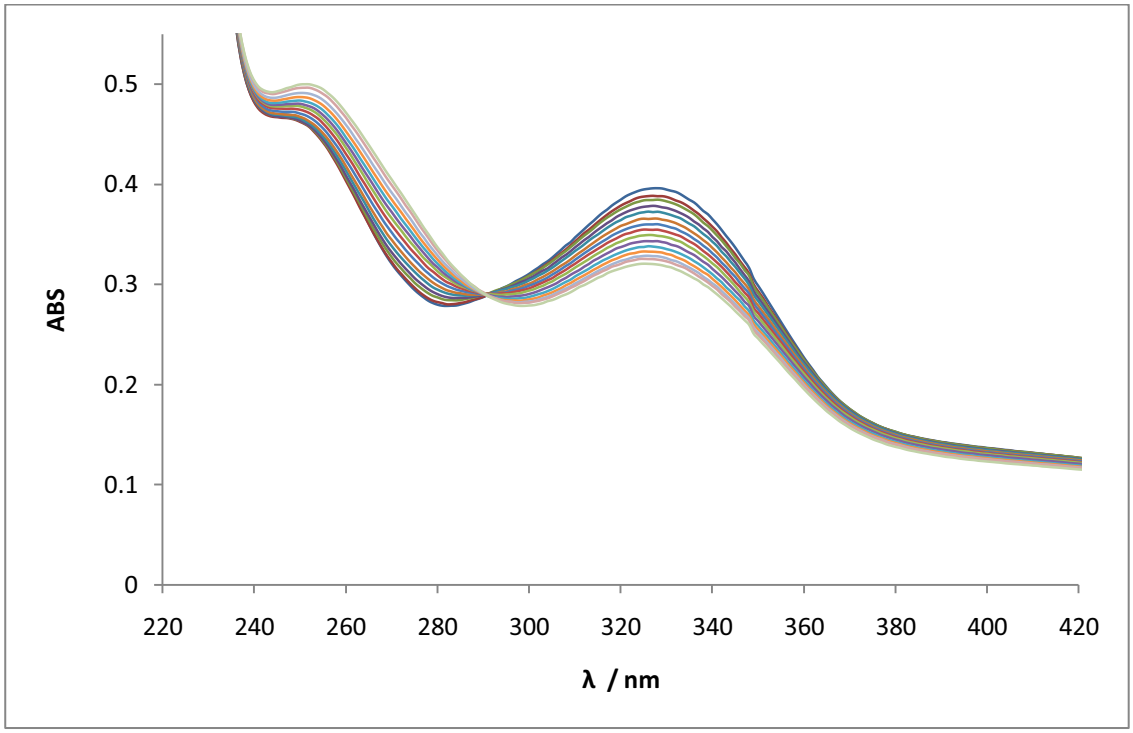


Figure 28A. U.V. spectra of decomposition of bisimidazotetrazinone **58c** at pH 8 and 37 °C

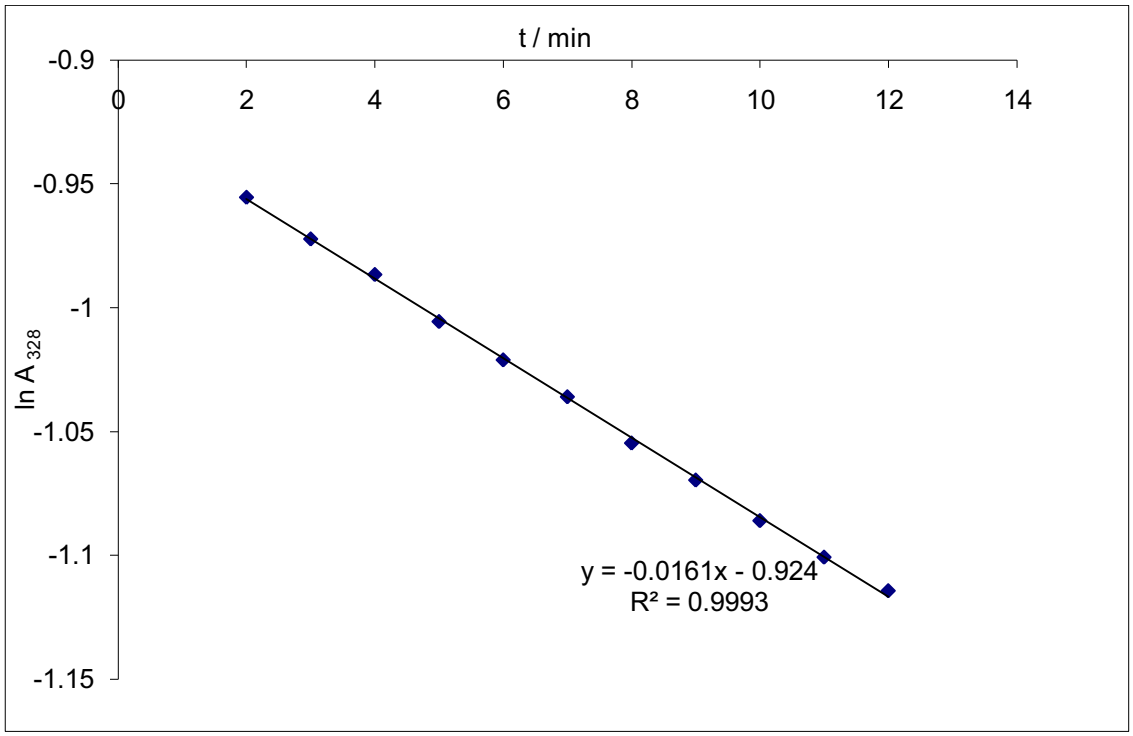


Figure 28B. Linear pseudo first order hydrolysis of bisimidazotetrazinone **58c** at pH 8 and 37 °C

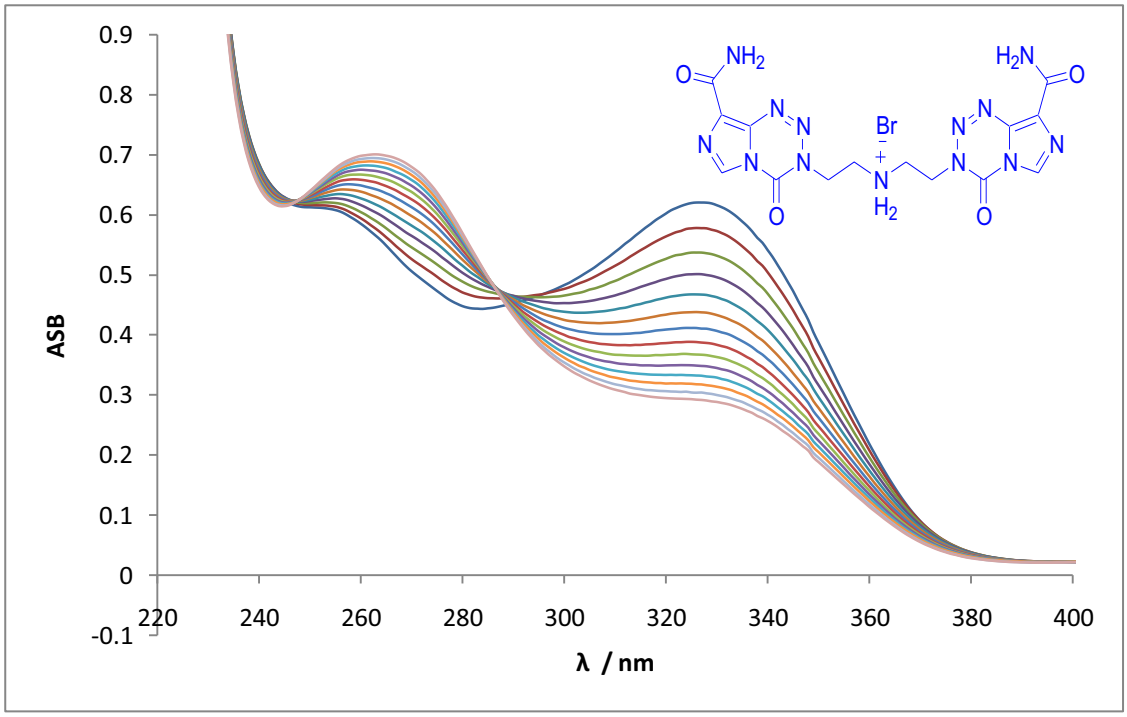


Figure 29A. U.V. spectra of decomposition of bisimidazotetrazinone HBr salt **55** at pH 8 at 37 °C

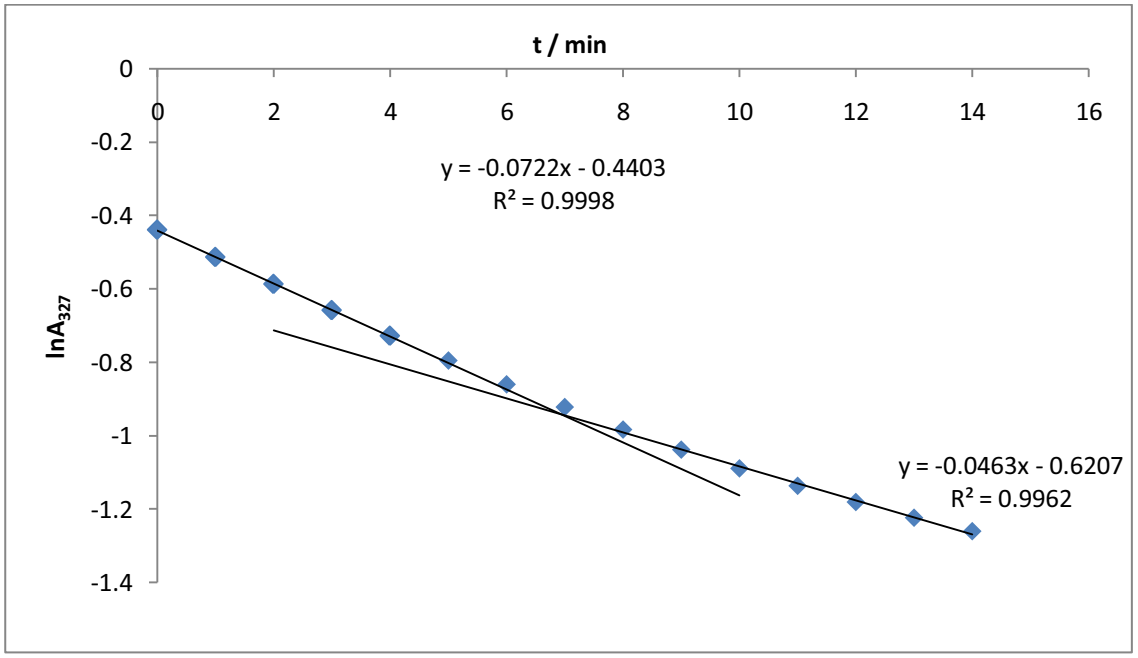


Figure 29B. Linear kinetics of bisimidazotetrazinone HBr salt **55** hydrolysis in buffer pH 8 at 37 °C

3.4 Conclusions

NMR study conclusion

The NMR study for the ring-opening reaction of aziridinium-ion-release-imidazotetrazinones concludes the following:

1) The tetrazine aqueous hydrolysis at neutral pH studied by NMR confirmed that aziridinium-ion-release imidazotetrazinones decomposed according to the usual ring opening of the imidazotetrazinones to AIC and the diazonium ion and it is comparable to temozolomide.
2) All the imidazotetrazinones studies by NMR showed the reaction mechanism of the decomposition at pH 7.4 that results in the release of the aziridinium ion, the aqueous neutral hydrolysis observed can be a good indicator of the chemical activation and aziridinium ion release at physiological pH for any future drug.
3) The NMR study demonstrated the fast diazonium ion intramolecular cyclization to the aziridinium ion formation (96% cyclized).

U.V. kinetics study conclusion

The rate of the decomposition (k' sec^{-1}) and the half-life ($t_{1/2}$) for aziridinium ion-release imidazotetrazinones and temozolomide studied at pH 4, 7.4, and 8 are presented in table 3.

The study showed that electron-withdrawing groups increase the rate of the decomposition and shorten the $t_{1/2}$ of the imidazotetrazinone ring. For example, the linear decomposition at pH 7.4 shown by monomer anilinoimidazotetrazinones **59c**, **d** and **e,** those having chloro, fluoro and nitro groups at *p*-position to aniline respectively, in contrast electron-releasing substituents which decrease the rate of hydrolysis are

represented by imidazotetrazinone **59a**. Imidazotetrazinone **59b** the one with hydrogen substituent at the *p*-position of the aniline ring (H), was taken as reference.

Imidazotetrazinone	pH 4.0		pH 7.4		pH 8.0	
	k' [a]	$t_{1/2}$ [b]	k' [a]	$t_{1/2}$ [b]	k' [a]	$t_{1/2}$ [b]
58a	0.4 ± 0.006	48.5	14.4 ± 0.7	1.3	45.1±1.5	0.4
58b	0.2 ± 0.001	99.7	15.8±0.42	1.2	49.4±0.77	0.4
58c	2.3 ± 0.06	8.3	10.3±0.8	1.9	24.6±1.08	0.8
59a	0.5 ± 0.003	41.5	15.2 ± 0.3	1.3	49.5±0.36	0.4
59b	0.4 ± 0.001	54.8	17 ± 0.48	1.1	55.0±0.85	0.4
59c	0.2 ± 0.001	90.6	19.3 ± 0.9	1.0	61.8±1.0	0.3
59d	0.5 ± 0.008	35.6	21.5 ± 0.5	0.9	70.0±0.42	0.3
59e	0.4 ± 0.013	53.2	33.3 ± 1.2	0.6	61.5±0.89	0.3
55	1.4 ± 0.007	13.5	61.2 ± 2.3	0.3	110.5±3.0	0.2
Temozolomide**1a**	0.1 ± 0.001	235.0	14.0	1.4	46.2±0.2	0.42

[a] k' = pseudo first order rate constant, × 10^{-5} sec^{-1}, data are mean ± SD of 3 independent determinations.
[b] $t_{1/2}$ = half life in hours.

Table 3. Kinetic data for the hydrolysis of imidazotetrazinones at pH 4.0, 7.4, 8.0

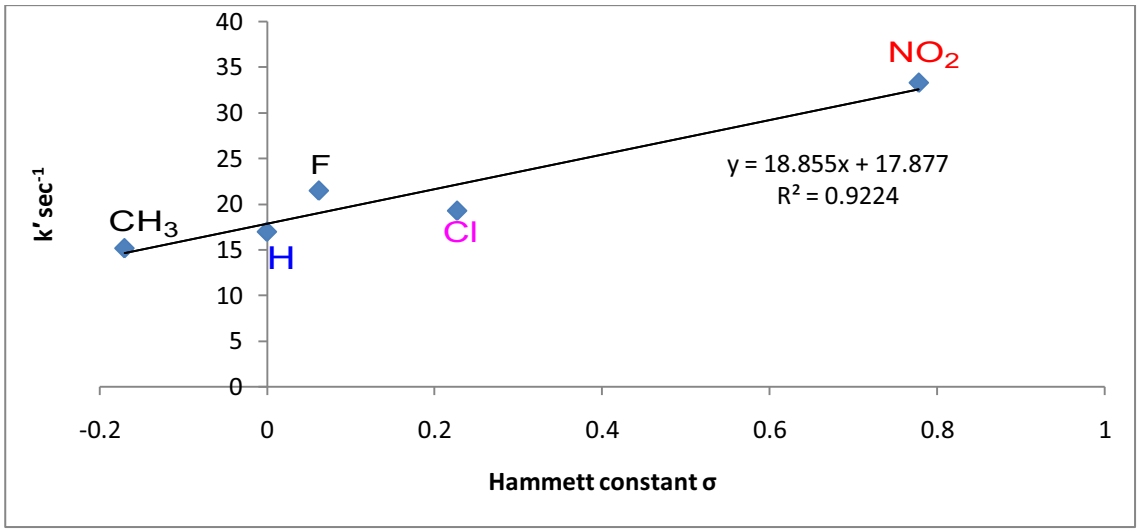


Figure 30. Tetrazine hydrolysis reaction rate constants versus Hammett constant (σ)

Figure 30 shows the linear correlation of the electronic effect (Hammett constant) against reaction rate at pH 7.4, *p*-fluoro tetrazine **59d** k' 21.5 × 10^{-5} sec^{-1} showed nearly similar effect to **59c** (*p*-chloro, k' = 19.3 × 10^{-5} sec^{-1}).

Moreover, at pH 7.4 the bisimidazotetrazinones **58a** (*p*-CH_3-aniline) showed reaction rate and $t_{1/2}$ (k' = 14.4 × 10^{-5} sec^{-1}, $t_{1/2}$ 1.3 h), **58b** (*p*-OCH_3-aniline) showed reaction rate and $t_{1/2}$ (k' = 15.8 × 10^{-5} sec^{-1}, $t_{1/2}$ 1.2 h), and the monomer tetrazine **59a** (*p*-CH_3-aniline) showed reaction rate and $t_{1/2}$ (k' = 15.2 × 10^{-5} sec^{-1}, $t_{1/2}$ 1.3h) were observed to a great extent to be comparable in their rate of decomposition and $t_{1/2}$ to temozolomide at the same conditions (14 × 10^{-5} sec^{-1}, $t_{1/2}$ 1.4 h).

In the kinetics of hydrolysis evaluation in buffer pH 8 at 37 °C, bisimidazotetrazinone **58a** showed k' = 45.1 × 10^{-5} sec^{-1}, $t_{1/2}$ = 0.4 h, **58b** k' = 49.4 × 10^{-5} sec^{-1}, $t_{1/2}$ = 0.4 h and the monomer tetrazine **59a** k' = 49.5 × 10^{-5} sec^{-1}, $t_{1/2}$ = 0.4 h, once again observed to a great extent to be comparable with temozolomide k' = 46.2 × 10^{-5} sec^{-1}, $t_{1/2}$ = 0.42 h which reflects the same instability under these conditions.

The kinetics study of hydrolysis performed at the three different pH on the aziridinium-ion-release imidazotetrazinones concludes the following:

1) The aqueous UV-Visible kinetic study showed that electron releasing groups optimized the pattern of decomposition and gave results of decomposition and stability quite similar to the lead drug temozolomide. In contrast, electron withdrawing groups quicken the rate of decomposition and sometimes lead to odd chemical behaviour e.g is the decomposition pattern observed from bisimidaotetrazine **58c** in pH 7.4 when relatively high concentration used, the

expected rate should be higher due to the withdrawing group but the reverse happened, it showed hydrolysis at two separate rates may be due to precipitation of either the tetrazine or the decomposition products specially AIC, upon 10-folds dilution to the first concentration a usual decomposition pattern of imidazotetrazinones was been observed.

2) The neutral aqueous hydrolysis study showed to a great extent, equivalent chemical behaviour to temozolomide by bisimidazotetrazinones **58a**, **58b** and the monomer imidazotetrazinone **59a,** whereas their stability in the acidic condition was observed to be less than temozolomide, although it looks reasonable stability when compared with the time required for oral absorption of any future drug.
3) The bisimidazotetrazinone HBr salt **55** showed a relatively lower stability in all pH studies compared with the other imidazotetrazinones, it shows neither optimum rate at the neutral aqueous hydrolysis nor reasonable acid stability. In addition to the biphasic kinetics of hydrolysis with two different rates, higher rate at the starting of the reaction, which slow down at the end of the reaction, this can conclude the independent hydrolysis of the two imidazotetrazinone rings of this imidazotetrazinone HBr salt.

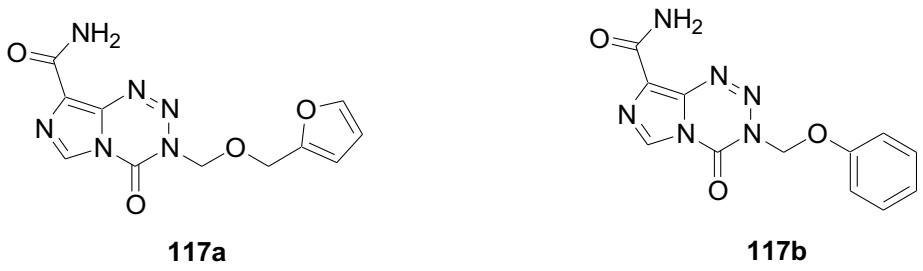
CHAPTER 4

IMIDAZOTETRAZINES FOR ALCOHOL AND PHENOL RELEASE

Chapter 4 Imidazotetrazinones for Alcohol or Phenol Release

4.1 Introduction

This is a study of the synthesis and hydrolysis of alcohol- or phenol-release imidazotetrazinones that may be used for improving the delivery of highly polar alcoholic or phenolic drugs. It is proposed that the favourable uptake and distribution properties of the imidazotetrazinone may be conferred on the linked drug and that hydrolysis of the imidazotetrazinone at the site of action may liberate the free alcohol or phenol. The target molecules for synthesis are summarised in Figure 31



117a **117b**

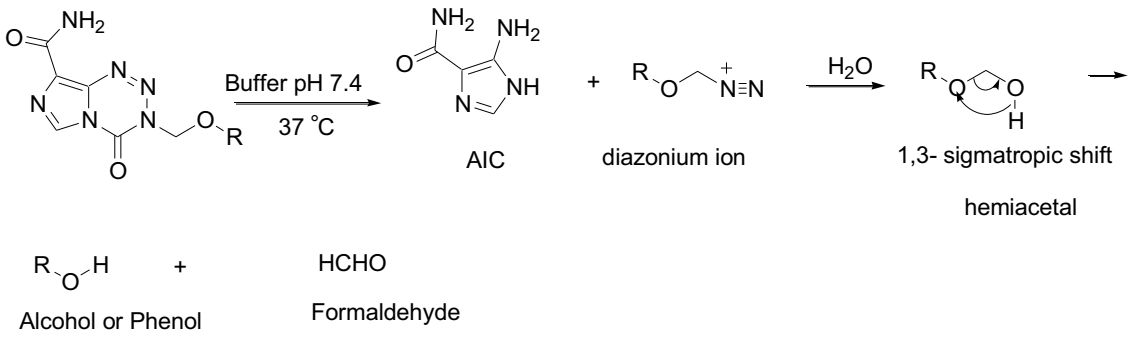
Figure 31. Alcohol and phenol target molecules for synthesis

4.2 Design

The usual aqueous hydrolysis at neutral pH of the imidazotetrazinone ring to AIC and diazonium ion, in addition to the potential hydrolysis reaction of the diazonium ion to alcohol, promoted the idea for the synthesis of alcohol or phenol-release imidazotetrazinones. The hypothesis behind such synthesis was based on the following criteria:

1) imidazotetrazinone ring opening should result in diazonium ion release at pH 7.4.
2) the hydrolysis product of the diazonium ion should be the unstable hemiacetal.

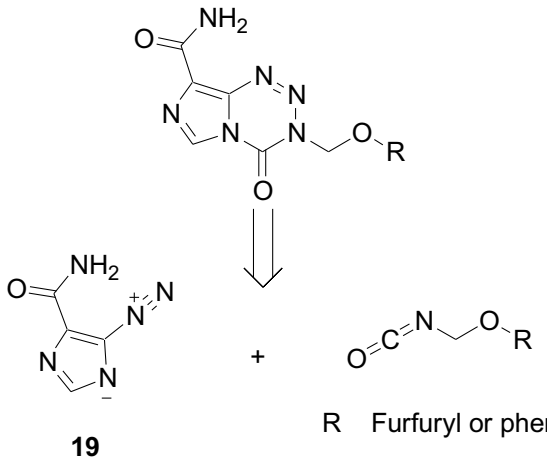
Scheme 48, outlines the proposed imidazotetrazinone prodrug and the mechanism of alcohol or phenol release.



Scheme 48. The proposed mechanism of alcohol or phenol release

4.3 Synthesis

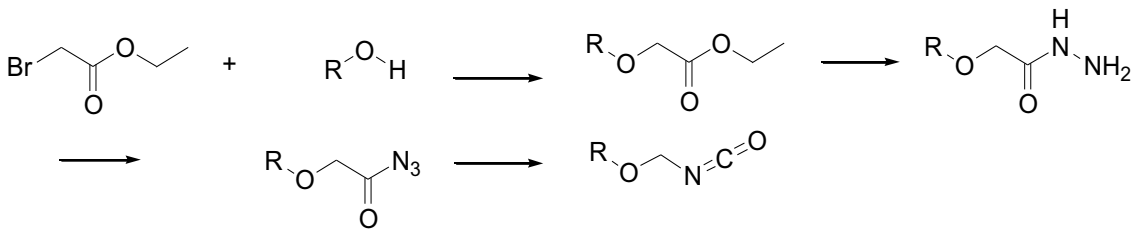
The retrosynthetic analysis outlined in Scheme 18 (Section 2.2) was adapted to plan the route of synthesis of the target alcohol and phenol-release-imidazotetrazinones, Scheme 49. The synthesis starts with the preparation of isocyanates which were then reacted with diazo-IC **19** to give the imidazotetrazinones.



Scheme 49. Retrosynthesis plan for alcohol-and phenol-release imidazotetrazinones.

4.3.1 Synthesis of the Isocyanates

The Curtius thermal rearrangement of azides was used for the synthesis of the isocyanates. The azides were synthesized by the diazotization of the hydrazides as discussed in Chapter 2 previously; hydrazides were synthesized from esters by reaction with hydrazine monohydrate. The esters were synthesized from the reaction of ethyl bromoacetate with the alcohol or phenol, Scheme 50 outlines the general route used for isocyanate synthesis.

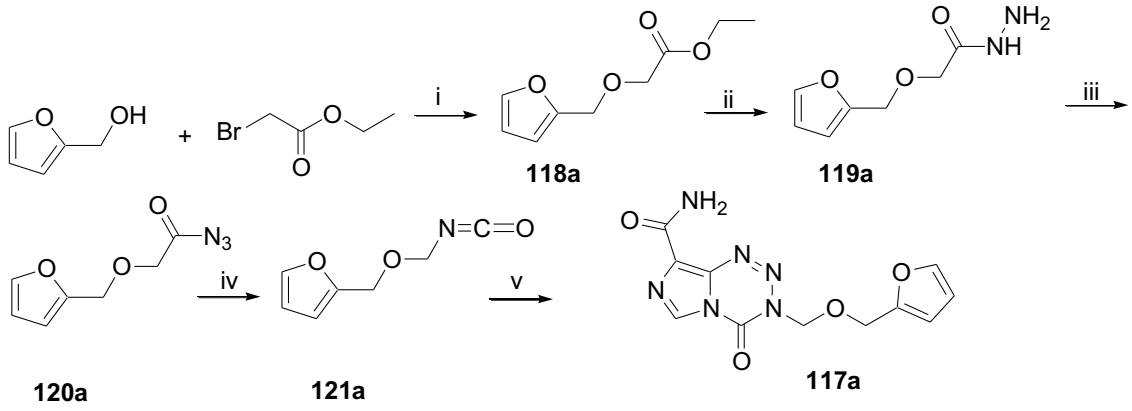


Scheme 50. General scheme for isocyanate preparation used in alcohol- and phenol-release imidazotetrazinone synthesis.

4.3.2 Synthesis of an imidazotetrazinone for the release of furfuryl alcohol

Scheme 51 outlines the synthesis of imidazotetrazinone **117a** for furfuryl alcohol release. In this synthesis, ester **118a** was formed upon the stirring of the furfurylalcohol in THF with NaH at room temperature to generate the reactive alkoxide (powerful nucleophile), which then reacted under reflux with the ethyl bromoacetate. The ester **118a** was collected by solvent/ solvent extraction after the solvent evaporation clean and in a good yield. The hydrazide **119a** was formed by the reaction of ester **118a** with six equivalents of hydrazine monohydrate and was collected clean and in good yield as a thick brown oil. The azide **120a** was formed with the diazotization mixture $NaNO_2$/HCl in DCM/H_2O solvent mixture. The anhydrous DCM solution of the azide was stirred under nitrogen directly at room temperature to give the isocyanate **121a** without further heating. The isocyanate was collected by the evaporation of DCM under reduced

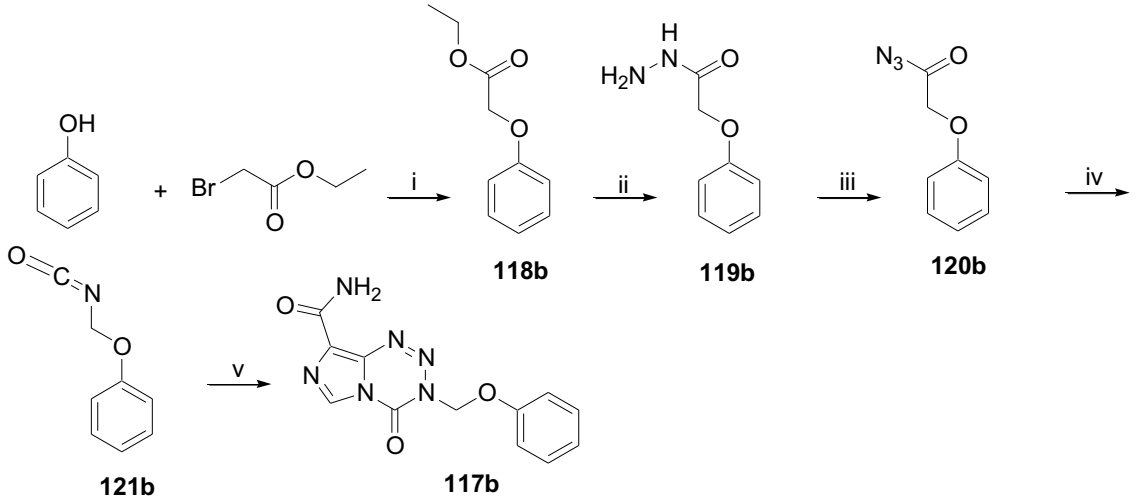
pressure under cold conditions (ice bath) to avoid isocyanate loss by evaporation. The tetrazine **117a** formed by the reaction of the isocyanate **121a** and diazo-IC **20** in anhydrous DMSO at 35 °C protected from light. The tetrazine was extracted into $CHCl_3$ from an H_2O suspension of the reaction mixture. Further purification by column chromatography gave pure, white solid, **117a**.



Scheme 51 Synthesis of the imidazotetrazinone **117a**. *Reagents and conditions:* (i) NaH, THF, Δ, 97%; (ii) $NH_2NH_2 \cdot H_2O$, EtOH, 93%; (iii) $NaNO_2$,10 M aq. HCl, DCM/H_2O; (iv) anhydrous DCM, rt , 48 h, 58%; (v) Diazo-IC, DMSO, 35 °C, 48 h, 13%.

4.3.3 Synthesis of an imidazotetrazinone for the release of phenol

Scheme 52 outlines the synthesis of the imidazotetrazinone **117b**, the scheme showed similar reaction steps to those used in the synthesis of imidazotetrazinone **117a**, The only difference is the base used for generation of the phenoxide ion at the step of the ester formation, a mild base K_2CO_3 was used rather than NaH, and this is due to the higher acidity of phenolic proton compared with furfurylalcohol. The imidazotetrazinone **117b** was collected pure after column chromatography.



Scheme 52 Synthesis of the imidazotetrazinone **117b**. *Reagents and conditions:* i) K_2CO_3, THF, 70 °C, 50%; (ii) $NH_2NH_2 \cdot H_2O$, EtOH, 54%; (iii) $NaNO_2$,10 M aq. HCl, DCM/H_2O; iv) anhydrous DCM, rt , 24 h, 73%; (v) Diazo-IC, DMSO, 35 °C, 48 h, 32%.

4.4 Mechanistic Evaluation of the Imidazotetrazinones for Alcohol and Phenol release

Imidazotetrazinones **117a** & **117b** were evaluated for their release of alcohol or phenol respectively, by the studying of their ring-opening mechanism at neutral pH at 37°C by ^{1}H NMR. The kinetics of release of alcohol or phenol were studied at acidic, neutral and alkaline pH by UV-Visible spectrophotometry.

4.4.1 NMR Evaluation of alcohol and phenol-release-imidazotetrazinones

For the confirmation of the release of furfuryl alcohol by imidazotetrazinone **117a**, the tetrazine ring opening mechanism under neutral aqueous condition, was studied in deuteriated phosphate buffer pD 7.8 at 37 °C by ^{1}H NMR. The ^{1}H NMR spectra showed the disappearance of the imidazotetrazinone and the appearance of the decomposition products with time, Figure 32A. Spiking of the final hydrolysis products of imidazotetrazinone **117a** with authentic samples of furfurylalcohol, AIC and formaldehyde confirmed their identities, Figure 32B.

The study of phenol-release imidazotetrazinone **117b** hydrolysis reaction in phosphate buffer pH 7.4 at 37 °C was performed by the same method used for the imidazotetrazinone **117a** except that the total collection time for ^{1}H NMR spectra was reduced to 12 h. Figure 33A shows the disappearance of the tetrazine with time and the appearance of the decomposition products; it was a faster decomposition than that observed for all the previously studied imidazotetrazinones under the same conditions. Spiking of the final decomposition products with authentic samples of phenol and AIC confirmed their identities as a decomposition products of tetrazine **117b**, Figure 33B. This study confirmed the release of phenol according to the proposal Scheme 48.

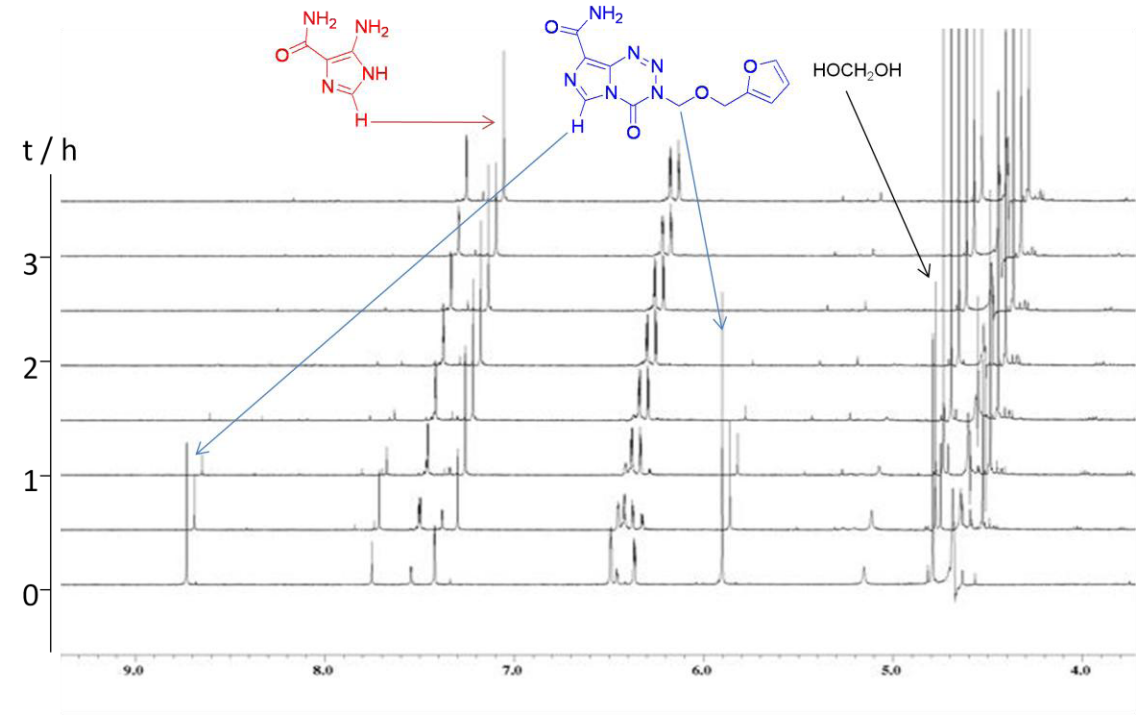


Figure 32A. Kinetics of decomposition of furfuryl-release imidazotetrazinone **117a** ^{1}H NMR

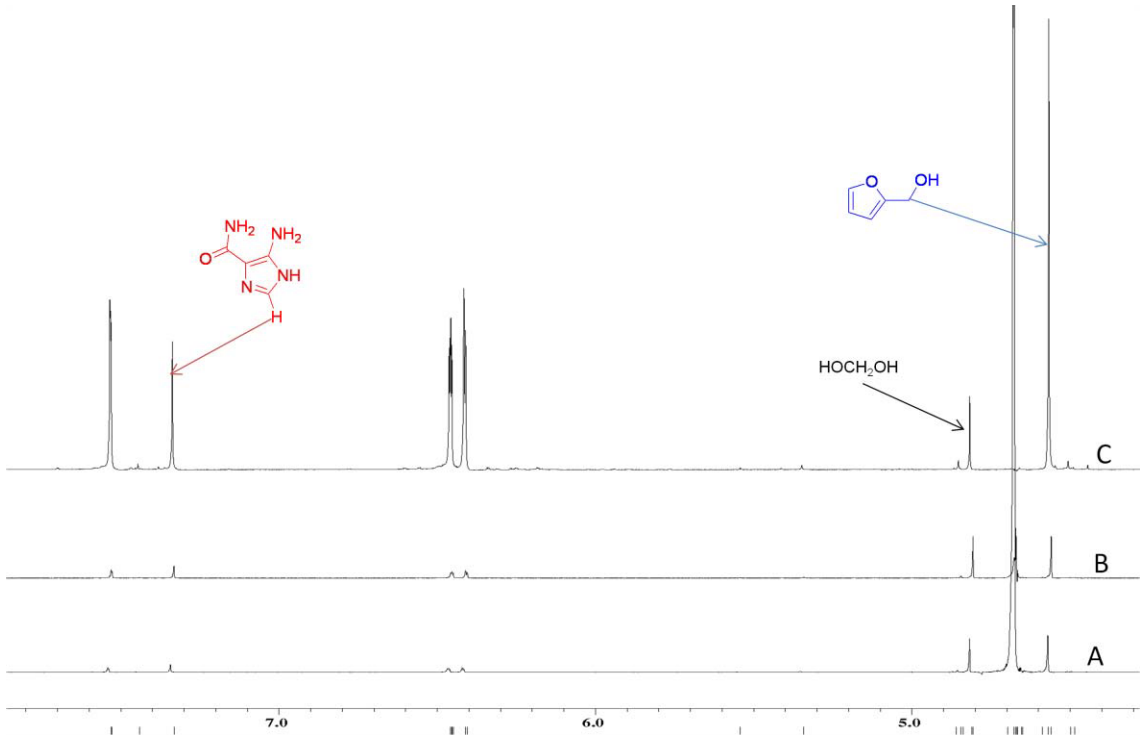


Figure 32B. Reaction products of hydrolysis of imidazotetrazinone **117a** (A), spiked with authentic furfuryl alcohol (B) and AIC + HCHO (C).

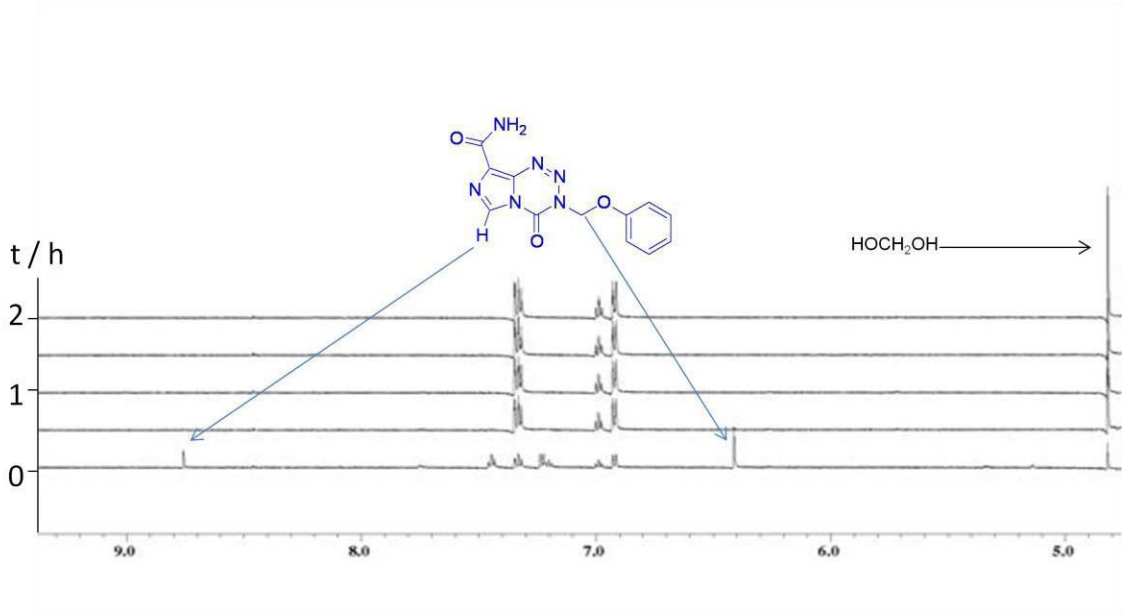


Figure 33A. Kinetic of decomposition of phenol-release-imidazotetrazinone **117b**

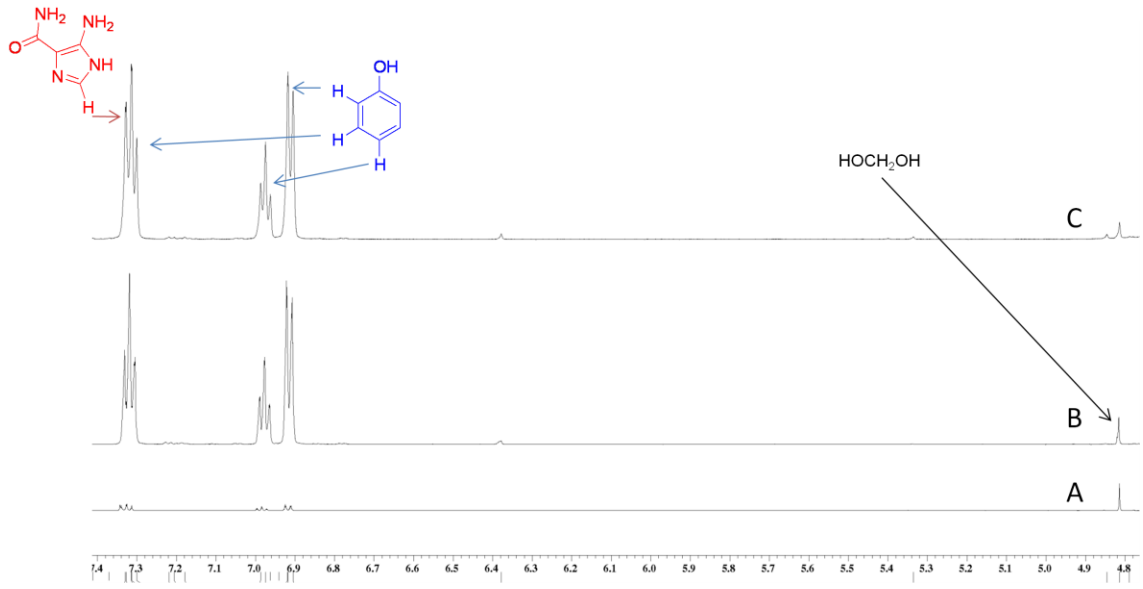


Figure 33B. Final products of hydrolysis of imidazotetrazinone **117b** (A), spiked with phenol (B) and AIC (C).

4.4.Kinetics of alcohol- and phenol-release imidazotetrazinone hydrolysis

The kinetics of hydrolysis for the release of alcohol and phenol by imidazotetrazinones **117a** & **117b** were measured in phosphate-citrate buffer pH 4, 7.4 and 8 at 37 °C (as in Chapter 3, Section 3.3). This study was performed to investigate the neutral aqueous hydrolysis of imidazotetrazinones **117a** & **117b** for the release of alcohols and phenols, and to assess how their stability under acidic conditions and instability under alkaline conditions compared with temozolomide. Kinetic data (k' and $t_{1/2}$) are summarised in Table 4.

The U.V. spectra of the kinetic studies performed at pH 4 for both imidazotetrazinones **117a** and **117b** showed a relatively high rate of decomposition. Imidazotetrazinone **117b** observed the highest rate and the shortest $t_{1/2}$ (1.3×10^{-5} sec^{-1}, $t_{1/2}$ 15.1 h) in this buffer and both of these tetrazines showed a linear pseudo-first-order decomposition, Figure 34A & B, accordingly both tetrazines showed far lower acid stability than that observed for temozolomide under the same conditions.

The kinetic study of these tetrazines under neutral conditions, showed much higher rates than that observed for temozolomide. Once again, imidazotetrazinone **117b** for the phenol release showed the highest rate of decomposition and the shortest $t_{1/2}$ (53.8×10^{-5} sec^{-1}, $t_{1/2}$ 0.36 h) with a linear decomposition to the AIC with a normal imidazotetrazinone pattern of decomposition, Figure 35A & B. Figure 35A showed the bands of phenol (λ = 268 nm) quite separate and resolved. The much higher rate of decomposition for these tetrazines was also observed at pH 8, also following pseudo 1st order kinetics.

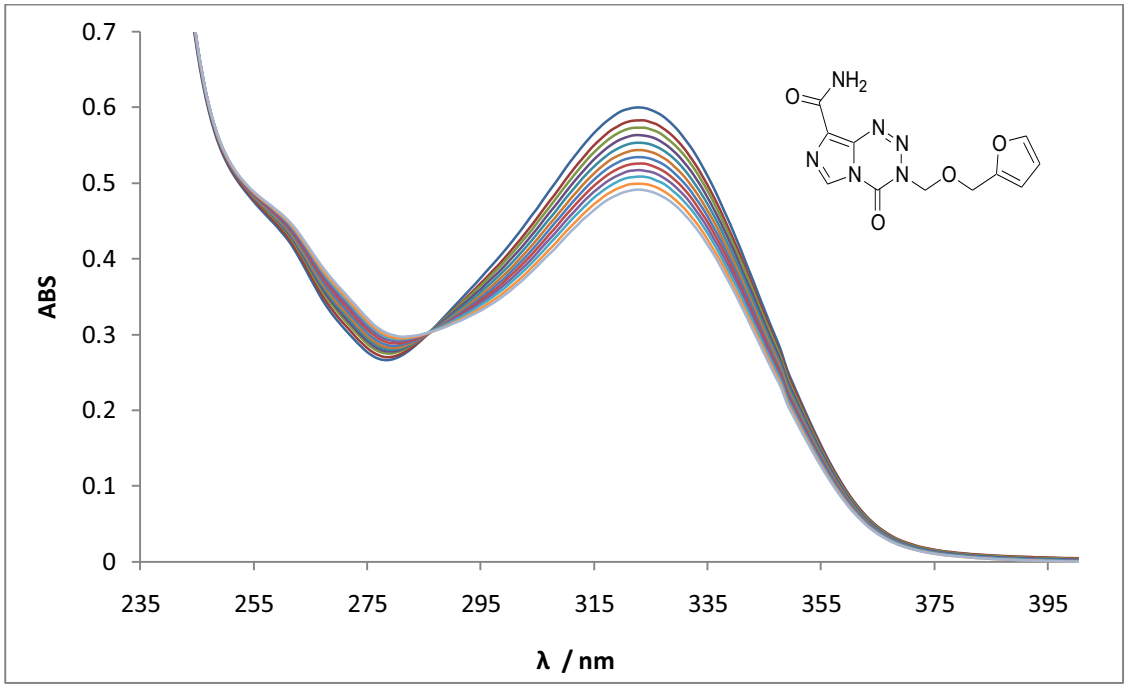


Fig 34A Decomposition pattern of decomposition of furfurylacohol-release-imidazotetrazinone **117a** in buffer pH 4 at 37 °C

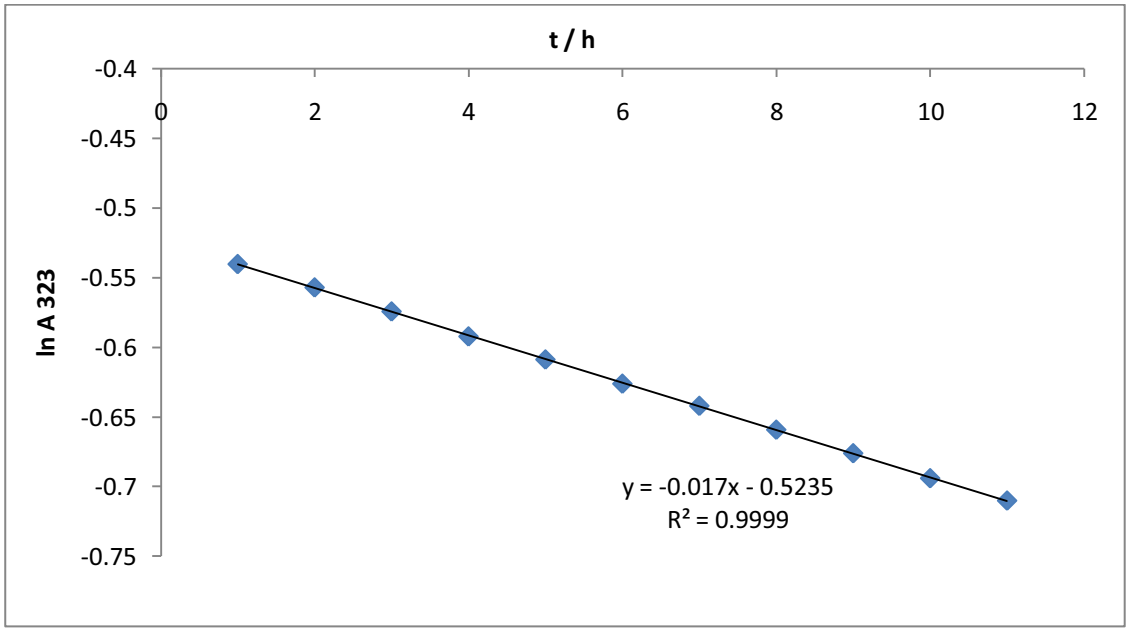


Fig 34B Linear kinetic of decomposition of furfurylacohol-release-imidazotetrazinone **117a** in buffer pH 4 at 37 °C

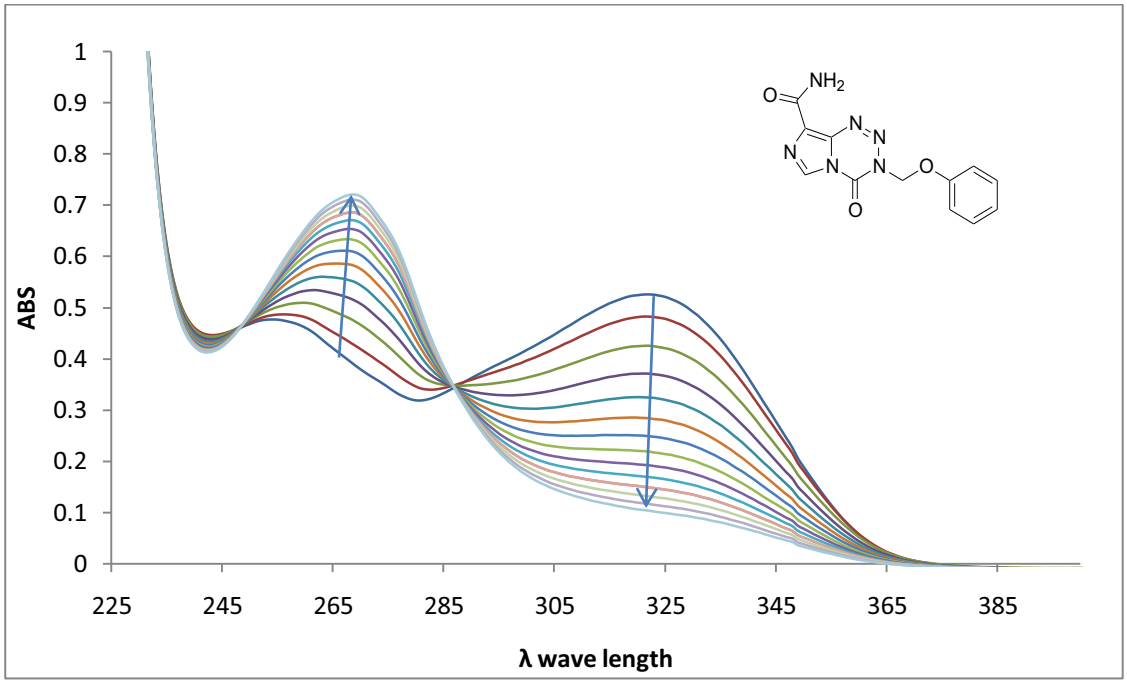


Figure 35A. Decomposition pattern of phenol-release-imidazotetrazinone **117b** in buffer pH 7.4 at 37 °C

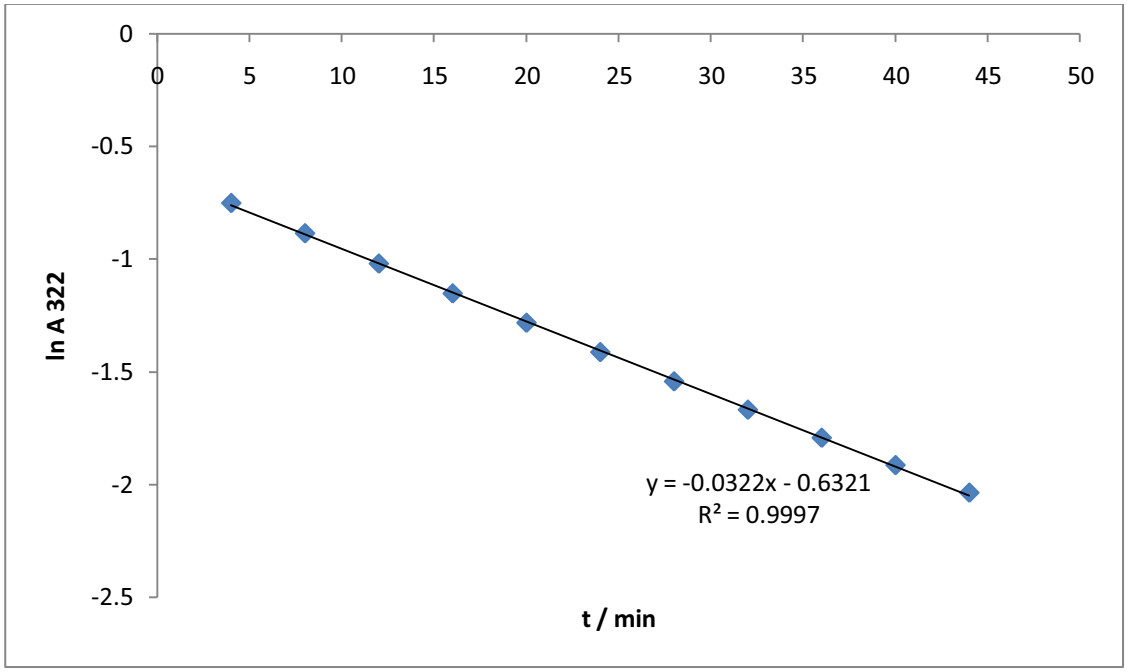
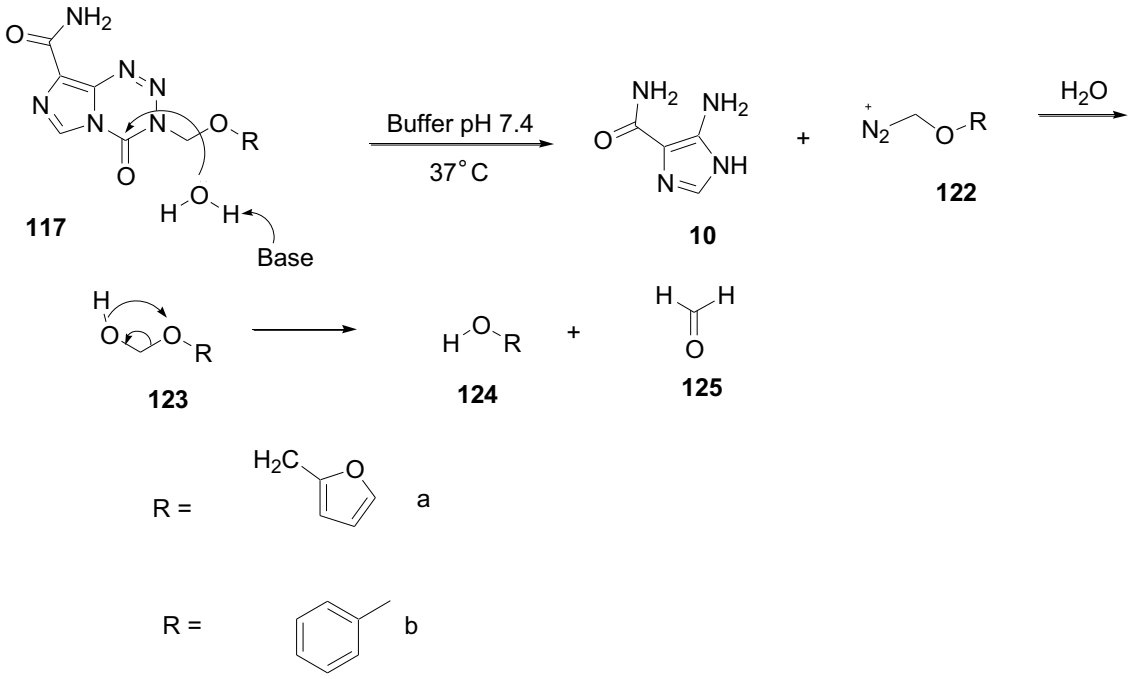


Figure 35B. Linear kinetic of decomposition of phenol-release-imidazotetrazinone **117b** in buffer pH 7.4 at 37 °C

4.4.2 Conclusion

Imidazotetrazinones synthesized for the purpose of alcohol and phenol release were synthesized successfully with a one-carbon linker between the imidazotetrazinone and the alcohol or phenol to be released. The mechanistic evaluation for their release of alcohol and phenol by NMR showed that they followed the usual pattern of decomposition of imidazotetrazinone (AIC + diazonium ion) upon aqueous hydrolysis; the diazonium ion **122** then hydrolyzed to the unstable hemiacetal **123** that spontaneously decomposed to the alcohol or phenol and formaldehyde **125**, Scheme 53.



Scheme 53. The aqueous hydrolysis of alcohol-phenol-release imidazotetrazinone **117a** & **b**

The ^{1}H NMR spectra of the final product mixtures showed the formaldehyde peak δ = 4.82. This study confirmed the release of furfurylalcohol and phenol by imidazotetrazinones **117a** and **b**.

Phenol-release imidazotetrazinone **117b** demonstrated a rapid decomposition rate; the imidazotetrazinone disappears in a very short time compared with that observed by furfuryl alcohol-release imidazotetrazinone **117a** and temozolomide. This may be attributed to the strong electron-withdrawing effect created by the phenolic oxygen which releases its unshared electrons, by resonance, into the aromatic ring, Figure 36. The ^{1}H NMR of the imidazotetrazinone **117a** showed the singlet of the two protons of the CH_2 attached to the imidazotetrazinone ring at δ 5.69, whereas imidazotetrazinone **117b** CH_2 protons were at δ 6.22, Figure 37.

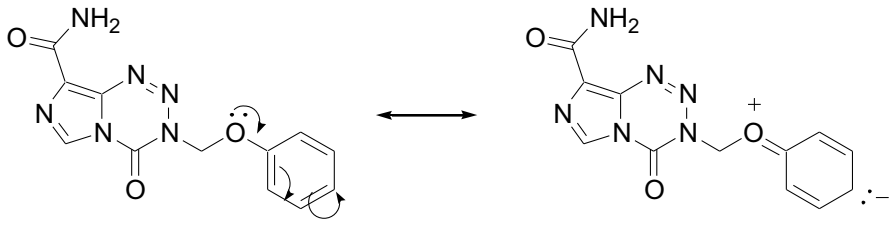


Figure 36 Phenoxy oxygen electron delocalization into the aromatic ring

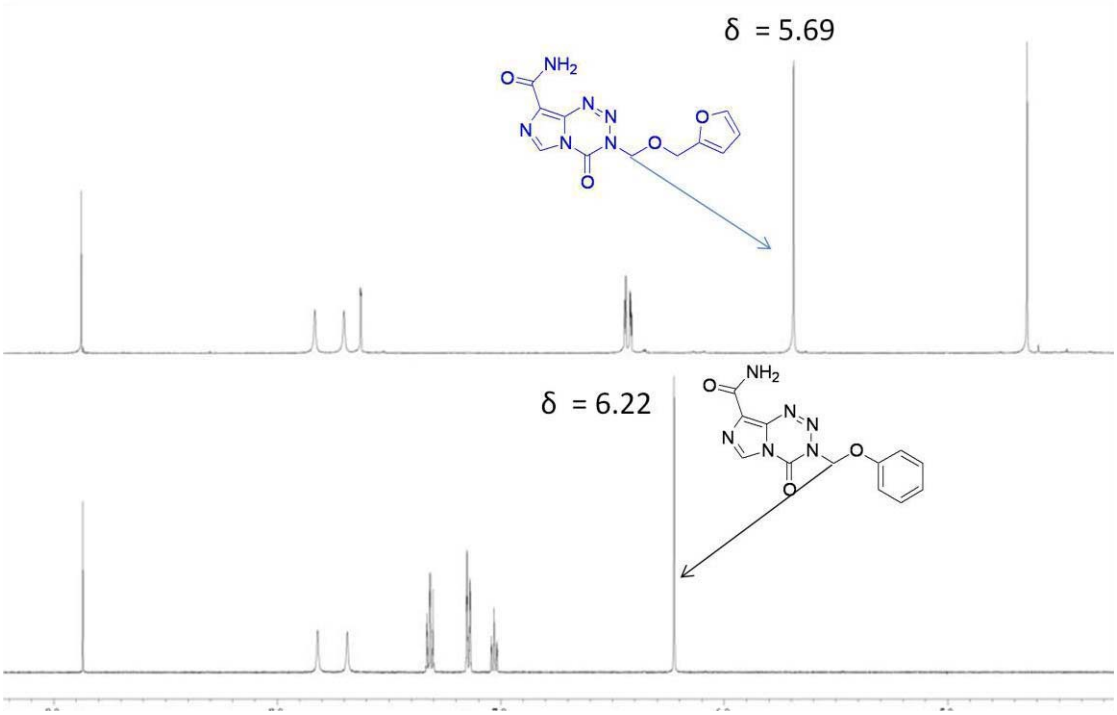


Figure 37. ^{1}H NMR spectra of imidazotetrazinones **117a** and **b** showing the positions of the "formaldehyde" methylene group

The kinetics study of the hydrolysis of imidazotetrazinones **117a** and **b** in buffer solutions of pH 4, 7.4 and 8, rates constants (k') and $t_{1/2}$ were summarised in table 4.

Imidazotetrazinone	pH 4.0		pH 7.4		pH 8.0	
	k' [a]	$t_{1/2}$ [b]	k' [a]	$t_{1/2}$ [b]	k' [a]	$t_{1/2}$ [b]
117a	0.48 ± 0.01	40.1	36.1 ± 0.8	0.53	55.6 ± 0.7	0.35
117b	1.3 ± 0.1	15.1	53.8 ±0.2	0.36	122.3±0.8	0.16
Temozolomide **1a**	0.1±0.001	235.0	14.0	1.4	46.2±0.2	0.42

[a] k' = pseudo first order rate constant, × 10^{-5} sec^{-1};
[b] $t_{1/2}$ = half life in hours.

Table 4. Alcohol-phenol-release-imidazotetrazinones rate of decomposition and $t_{1/2}$

This study showed the fast release of alcohol and phenol at the neutral pH, imidazotetrazinone **117a** release furfurylalcohol with a rate constant more than double (36.1×10^{-5} sec^{-1}) that observed by temozolomide (14×10^{-5} sec^{-1}), whereas the release of phenol by imidazotetrazinones **117b** had nearly 4 times the rate of temozolomide decomposition at the same condition. This was due to the strong electron-withdrawing effect of the alcoholic and phenolic oxygen atoms, found to exert more effect in the case of tetrazine **117b** due to the direct attachment of the oxygen to the aromatic ring, which resulted in the release of the lone pair electrons into the aromatic ring by resonance, therefore conferring a positive charge that increased its electron-withdrawing effect.

The UV-Visible kinetic study showed the highest hydrolysis rates of any tetrazine at all three pH conditions studied. Although this is the case, they still showed a promising and reasonable acidic stability compared with the time required for absorption from the acidic stomach pH (0.8 h) for any future delivered alcoholic or phenolic drug.
At all pH's, phenol release was faster than alcohol release and both faster than temozolomide hydrolysis.

These imidazotetrazinone can be used as a delivery system for alcoholic or phenolic drugs to the systemic circulation and the faster-released molecules i.e alcohol or phenols, may undergo normal distribution in the body on their own. This may achieve oral availability for non-orally-intended drugs or a faster onset of action for polar drugs that are slowly absorbed.

CHAPTER 5

CONCLUSION AND FUTURE WORK

Chapter 5 Conclusion

In this study, new imidazotetrazinones were synthesized that release aziridinium ions at physiological pH. Aziridinium ions have received considerable attention and interest in biological studies and are clinically-useful intermediates, sufficiently stable to find their targets on DNA, as shown by alkylating drugs, e.g. nitrogen mustards and nitrosoureas.[62]

The imidazotetrazinones were synthesized with either an aliphatic ethylamine side chain or with an anilinoethyl side chain at position three of the imidazotetrazinone ring. The anilinoethylimidazotetrazinones were synthesized with different substituents on the *p*-position of the aniline ring (electron withdrawing and electron releasing) to determine the different impacts on the mechanism of activation at neutral pH and the relative stability of the imidazotetrazinone under acidic conditions. Monomeric and bisimidazotetrazinones (bifunctional) tetrazines were synthesized. The synthesis of the new molecules proceeded successfully with high purity and all characterizations were performed to accepted publication standards, Figure 38.

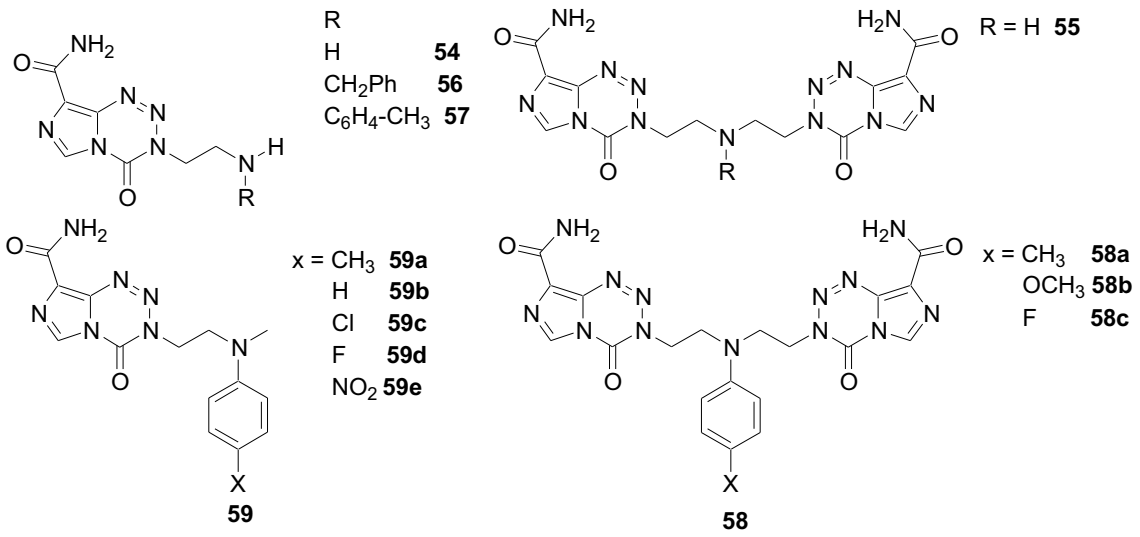
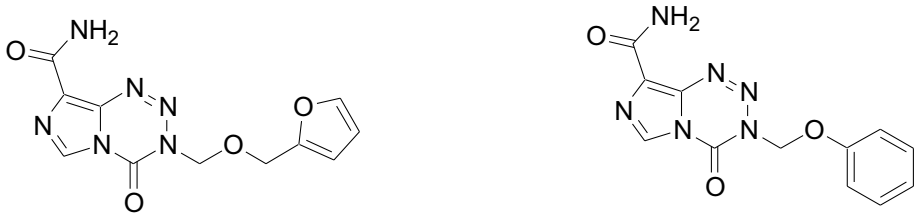


Figure 38. New imidazotetrazinones for aziridinium ion release.

A trial of another application for the imidazotetrazinone ring as a delivery system was also undertaken. The synthesis of imidazotetrazinones for the release of alcohols and phenols was performed. The aim behind such synthesis was the enhancement of the delivery of poorly-distributed polar drugs, through the imidazotetrazinone having advantages as a good oral delivery system, Figure 39.



117a **117b**

Figure 39. New imidazotetrazinones for alcohols and phenols release.

The NMR evaluation study of the reaction mechanism of imidazotetrazinone ring opening for the release of aziridinium ions used imidazotetrazinone **59a** ^{13}C-labelled and unlabelled as a representative of all of the imidazotetrazinones synthesized for the aziridinium ion release. The study showed the ring opening to AIC and a diazonium ion intermediate that cyclised (96%) to the aziridinium ion at neutral pH, which can be considered as an indication of the possibility of the ring opening under physiological conditions to release the aziridinium ion. The kinetics of hydrolysis performed on the aziridinium-ion-release imidazotetrazinones at neutral and acidic pH showed that anilinoethylimidazotetrazinones with an electron releasing group at the *p*-position of the aniline ring, were quite similar to temozolomide under the same conditions, which is an indication to expect the same chemical activation at physiological pH and oral availability for any future drug.

A biological assay was performed previously (in collaboration with Dr Roger Phillips Institute of Cancer Therapeutics, University of Bradford), the bifunctionl aziridinium-ion-release imidazotetrazinones **58a**, **b** and the monomer **59a** were tested, the cell lines A2780 with MMR^+ expression and A2780-Cp70 which lack functional MMR were used, an equal number of cells were seeded, PaTrin-2 was used to deplete the activity of MGMT on both cell lines. Temozolomide was used as control, Table 5, the result showed that tetrazine **58a** and **b** are independent on both MGMT and MMR in their activity, whereas the monomer **59a** showed slight dependency but still not as much as temozolomide. Based on these findings, the possible conclusion is that the alkylation by the aziridinium-ion-release tetrazine might take place exactly as in the case of nitrogen mustards at the *N*7-position of the guanine base of DNA. Accordingly, cytotoxic alkylation produced by these tetrazines may be through this alkylation rather than the cytotoxic *O*6-Me-guanine in the case of temozolomide which is repaired by MGMT and dependent on MMR. Referring to this study, the anticipated biological results for the monomeric aziridinium-ion-release tetrazines will be the similar especially for anilinoethyimidazotetrazinones carrying an electron releasing group at the *p*-position which showed an optimum effect (**58a**, **b** and **59a**) compared with compounds bearing a withdrawing group.

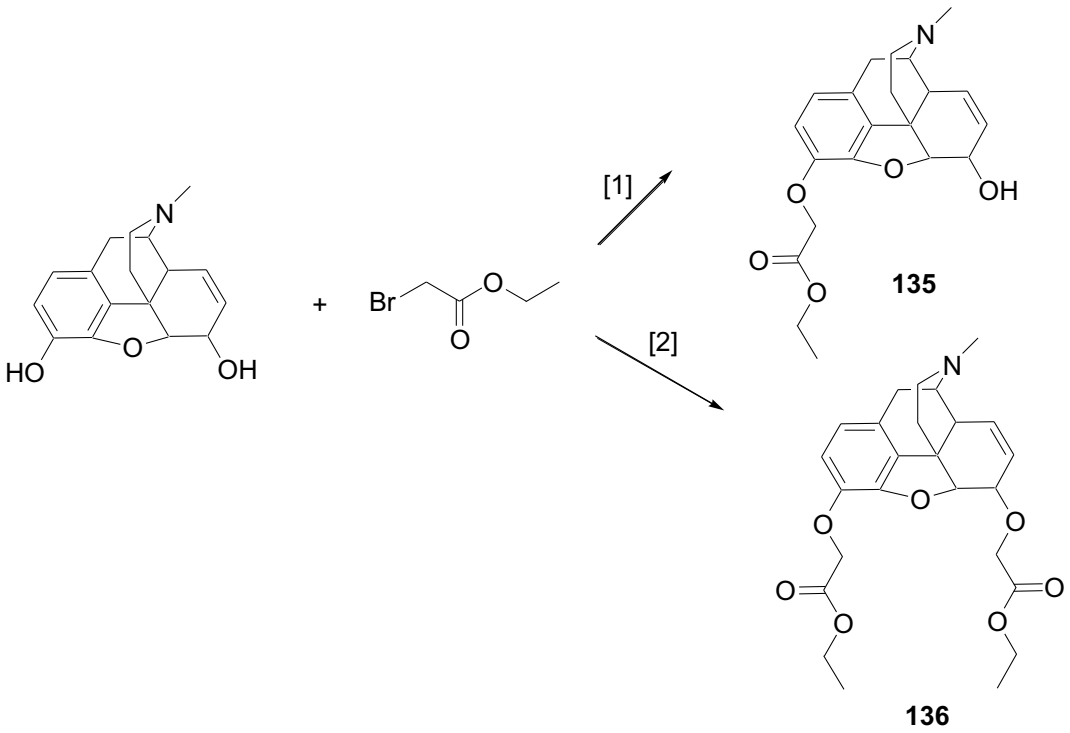
	IC_{50} / μM				IC_{50} ratios	
	A2780 (MMR^+)		A2780-Cp70 (MMR^-)		MMR EFFECT A2780/Cp70 +PaTrin-2	MGMT EFFECT -/+ PaTrin-2 A2780
	-PaTrin-2 Mean ± SD	+ PaTrin-2 Mean ± SD	- PaTrin-2 Mean ± SD	+PaTrin-2 Mean ± SD		
TMZ	>257	8.6 ± 0.32	> 257	>231	>27	>30
58a	0.7 ± 0.23	1.3 ± 0.07	6.5± 1.0	7.0 ± 0.10	5.8	0.58
58b	1.4 ± 0.36	2.08 ± 0.03	11± 1.5	11 ± 0.85	5.4	0.66
59a	54 ± 11	34 ± 3.1	62± 10	94 ± 3.3	2.8	1.6
54	30	11 ± 0.71	116	133 ± 1.0	13	2.9

Table 5. *In vitro* chemosensitivity data for bifunctional and monofuctional tetrazine compared to temozolomide.

The imidazotetrazinones carrying electron-releasing groups in the pendant phenyl rings (**58a**,**b** and **59a**) showed kinetics of hydrolysis, at neutral pH, similar to temozolomide, which may be the optimum at physiological pH for a good therapeutic activity. The strength of electron releasing varies from group to another, the OCH_3 is a stronger electron releasing group than CH_3, resulting in a slight decrease in the activity, compare the chemosensitivity of **58a** with **58b**. This shows that the electron-releasing substituent is required to optimise the ring opening but without increasing of the basicity of the aniline, which may delay formation of the aziridinium ion. Overall, it is the electropositivity of C4 of the tetrazine ring that controls the rate of ring-opening. Imidazotetrazinones carrying electron-withdrawing groups in the pendant phenyl rings,

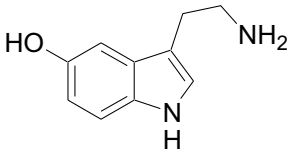
showed relatively higher rates of hydrolysis, that reflect fast release of aziridinium ions with relatively less stability.

Imidazotetrazinones for alcohol and phenol release is a promising study for the delivery of polar drugs to their targets, especially into the CNS. As part of the application, a trial study of linking morphine to the imidazotetrazinone ring was performed, the study was successful up to the stage of azide synthesis but isocyanate formation was not observed. For the linking of morphine to the imidazotetrazinone ring, the monomeric ester **135** was formed by the reaction of the phenolic hydroxy group of morphine with bromoethylacetate, Scheme 54[1] and the dimeric ester **136** was formed by the reaction of both alcoholic and phenolic hydroxyl groups, Scheme 54[2]. Both esters converted to hydrazides and then azides but no isocyanates formed which may be due to the reactivity of the neighbouring tertiary amine.



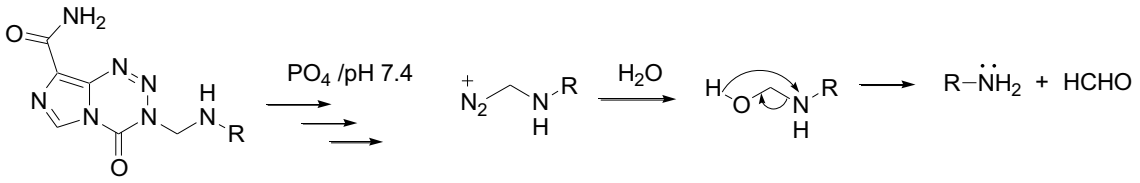
Scheme 54. Formation of morphine ethylacetate esters.

Serotonin or 5-Hydroxytryptamine (5-HT) **137** is a monoamine neurotransmitter that is primarily found in the gastrointestinal (GI) tract, platelets, and central nervous system (CNS) of humans and animals. Low serotonin levels are believed to be the cause of many cases of mild to severe depression which can lead to symptoms such as anxiety, apathy, fear, feelings of worthlessness, insomnia and fatigue. The most concrete evidence for the connection between serotonin and depression is the decreased concentrations of serotonin metabolites in the cerebrospinal fluid and brain tissues of depressed people. If depression arises as a result of a serotonin deficiency then pharmaceutical agents that increase the amount of serotonin in the brain should be helpful in treating depressed patients. Anti-depressant medications increase serotonin levels at the synapse by blocking the reuptake of serotonin into the presynaptic cell. Anti-depressants are one of the most highly prescribed medications despite the serious side-effects they can cause. The phenolic hydroxyl group of the serotonin can be used in a trial of linking it to the tetrazine as a vehicle to target its delivery to the brain.



137

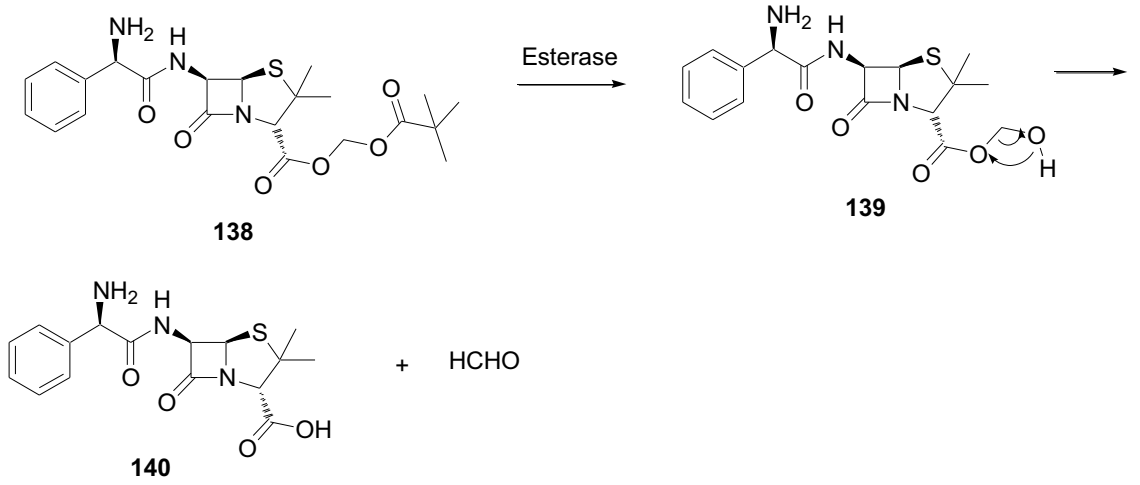
It may also be possible to apply the tetrazine modification, to tetrazine hydrolysis to release a hemiaminal (hemiacetal analogue) after hydrolysis of the diazonium ion intermediate. The hemiaminal may undergo decomposition similar to the hemiacetal resulting in the release of the amine containing drug, Scheme 55.



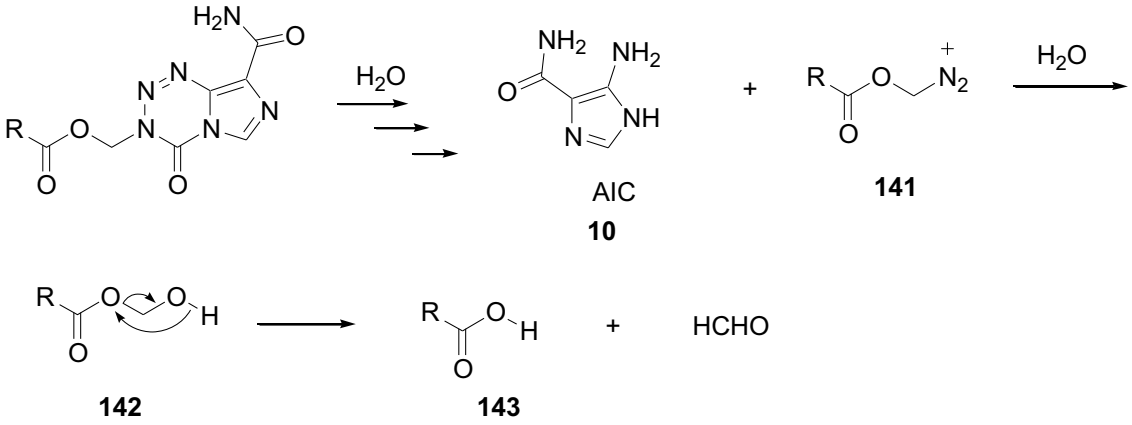
Scheme 55. Future application to imidazotetrazinone as a delivery system to primary amine containing drugs through unstable hemiaminal formation.

This study has shown that the imidazotetrazinones have enormous potential as an orally-available drug delivery system. It is possible that, as with temozolomide, these prodrugs could cross the BBB to release drugs in the CNS under the control of local pH.

Pivampicillin **138** is a prodrug that releases ampicillin when activated in the body by esterase enzymes;[82] it is a double ester prodrug hydrolysed enzymatically to unstable hydroxymethylester **139,** which undergoes spontaneous elimination to release ampicillin **140** and formaldehyde, Scheme 56. This may be another application for the imidazotetrazinone prodrug delivery system; this example it is a delivery for carboxylic acid drugs Scheme 57 oulines the proposed scheme of the release of carboxylic acids linked to an imidazotetrazinone ring.



Scheme 56. Pivampicillin prodrug enzymatical activation



Scheme 57. Proposal of imidazotetrazinones aqueous hydrolysis to carboxylic acid release

Experimental

Experimental

E.1 Reagents

Reagents were purchased from Sigma-Aldrich, Alfa Aesar and Fluka, solvents from Fisher Scientific.

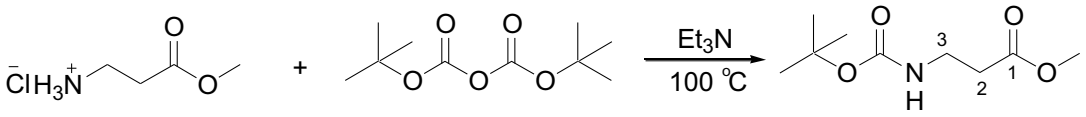
E.2 Instrumentation and general methods

TLC was performed on highly-purified silica gel plates with UV indicator (silica gel F_{245}), manufactured by Merck and visualized under UV light (366 or 254 nm) or stained with iodine. Melting points were determined with an Electrothermal IA9200 digital melting point apparatus. Infrared data were obtained using a Perkin Elmer (Paragon 1000) FT-IR Spectrophotometer.

NMR spectra were acquired on a JEOL ECA600 spectrometer observing ^{1}H at 600.17 MHz and ^{13}C at 150.91 MHz unless otherwise stated ^{13}C assignments made with the aid of the DEPT 135 experiment. Other NMR spectra were recorded on a Bruker-spectrospin AC400 observing ^{1}H at 400.13 MHz and ^{13}C at 100.62 MHz. Mass spectra were obtained from the EPSRC Mass Spectrometry Service centre at the University of Swansea and the Analytical Centre at the University of Bradford using a Micromass Quattro Ultima mass spectrometer. Elemental analyses were obtained from the Advanced Chemical and Material Analysis Unit at the University of Newcastle upon Tyne. A Varian-Cary 400Bio UV-Visible spectrophotometer equipped with a Peltier temperature controller was used for the kinetic studies.

E.3 Synthesis of imidazotetrazinones for aziridinium ion release in Chapter 2 section 2.2.2

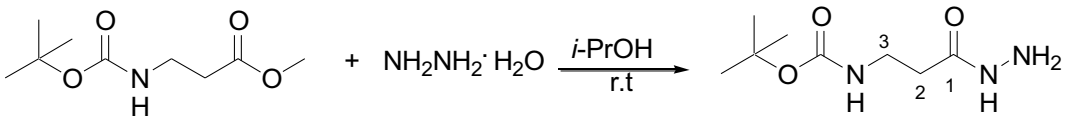
Methyl 3-(*t*-butoxycarbonylamino)propionate 73[83]



72 **73**

A mixture of β-alanine methyl ester hydrochloride salt **72** (2 g, 14.34 mmol) and di-*t*-butyl dicarbonate (5.63 g, 25.8 mmol) was heated under N_2 at 100 ºC for 2 h, triethylamine (1.45g, 14.34 mmol) was then added to the melt gradually with stirring and the mixture stirred at 100 ºC overnight. A white solid was observed in an oil vehicle. The reaction mixture was suspended in ethyl acetate (40 ml) and the solid was removed by filtration. The ethyl acetate filtrate was washed with water, dried over anhydrous $MgSO_4$, filtered and then evaporated to give a pale yellow oil. Distillation under reduced pressure gave BOC-protected aminoester **73**, a colourless oil b.p. 65 – 66 ºC, 0.1 mmHg (lit. 84 – 85 ºC at 0.4 mmHg),[83] (1.7 g, 60%). ^{1}H NMR ($CDCl_3$) δ: 5.18 (br s, 1H, NHCO), 3.47 (s, 3H, OCH_3), 3.17 (q, J = 5.9 Hz, 2H, 3-H), 2.31 (t, J = 5.9 Hz, 2H, 2-H), 1.39 (s, 9H, $C(CH_3)_3$); ^{13}C NMR ($CDCl_3$) δ: 172.6 (C-1), 155.7(OCONH), 78.9 ($OC(CH_3)_3$), 51.5 (OCH_3), 36.04 (C-3), 34.3 (C-2), 28.2 ($C(CH_3)_3$); MS (ES) m/z 204.2 (50%) $(M+H)^+$, 146.2(85%) $(C_7H_{15}NO_2+H)^+$, 105.2 (85%) $(C_4H_{10}NO_2)^+$, 83.3 (100%).

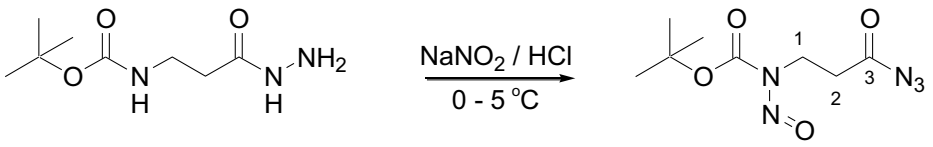
3-(*t*-Butoxycarbonylamino)propanoyl hydrazide 74[84]



73 **74**

BOC-protected ester **73** (2.0 g, 984 mmol) was dissolved in *i*-propanol (10 ml). Hydrazine monohydrate (2.95 g, 59.04 mmol) was added and the mixture stirred at r.t over night. Volatile compounds were removed by evaporation under reduced pressure. The resultant white solid was re-suspended in diethyl ether (30 ml) and collected by filtration; it was then dissolved in hot ethyl acetate (50 ml); cold hexane (50 ml) was added to the mixture gradually until a white solid precipitated. After cooling on ice, the solid hydrazide **74** was collected by filtration and dried (1.87g, 94%) m.p. 108 – 110 ºC (lit. 110 – 112 ºC).[84] ^{1}H NMR ($CDCl_3$) δ: 7.31 (br s, 1H, $NH_2N\underline{H}$), 5.18 (br s, 1H, N<u>H</u>BOC), 3.91 (br s, 2H, NH_2), 3.4 (q, *J* = 6.0 Hz, 2H, 3-H), 2.38 (t, *J* = 6.0 Hz, 2H, 2-H), 1.42 (s, 9H, CH_3); ^{13}C NMR ($CDCl_3$) δ: 172.2 (C-1), 156.2 (OCONH), 77.3 (<u>C</u>$(CH_3)_3$), 36.7 (C-3), 34.5 (C-2), 28.5 <u>C</u>H_3; MS (ES) m/z 204.2 (100%) $(M+H)^+$, 148.2 (98%) $(C_4H_9N_3O_3+H)^+$.

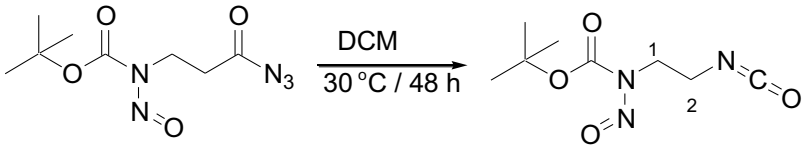
3-(*t*-Butoxycarbonyl-*N*-nitrosoamino)propanoyl azide77



74 **77**

The hydrazide **74** (1.8 g, 8.86 mmol) was suspended in a mixture of water (10 ml) and DCM (10 ml) and left to stir in an ice bath at 0 ºC. aq. HCl (5 ml, 10 M) was added gradually followed by $NaNO_2$ (3.67 g, 53.13 mmol) solution in water (10 ml) added dropwise, keeping the exothermic reaction at 0 – 5 ºC. DCM (10 ml) was added and the organic layer was separated, washed with water, dried over anhydrous $MgSO_4$, filtered and evaporated to give azide **77** as a yellow oil (1.22 g, 68%). Formation of the azide was confirmed by IR (strong band at 2145 cm^{-1}). ^1H NMR ($CDCl_3$) δ: 3.98 (t, 2H, *J* = 7.2 Hz, 1-H), 2.40 (t, 2H, *J* = 7.2 Hz, 2-H), 1.62 (s, 9H, CH_3); ^{13}C NMR 150.1 MHz ($CDCl_3$) δ: 177.5 (C-3), 151.7(OCON), 86.01 ($\underline{C}(CH_3)_3$, 35.8 (C-1), 33.8 (C-2), 28.1 ($\underline{C}H_3$); IR (liq film) 2986w (CH), 2145s (N_3), 1750s ($\underline{CO}NNO$), 1713s ($\underline{CO}N_3$), 1513m (NO) cm^{-1}.

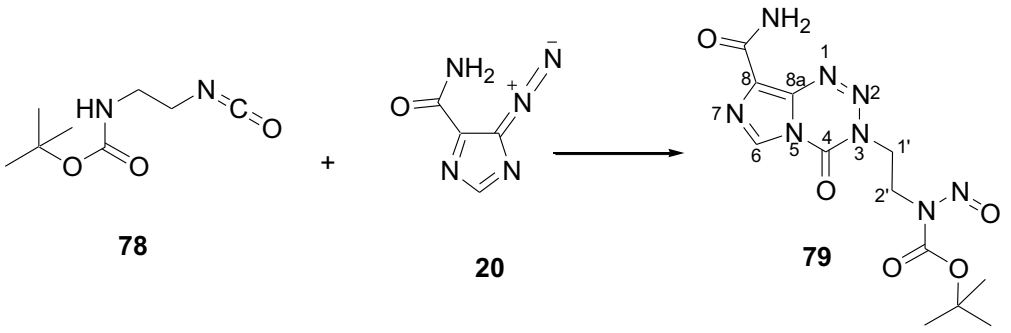
t-Butyl N-(2-isocyanatoethyl) *N*-Nitroso carbamate 78



77 **78**

The azide **77 (**1.22g, 5.7 mmol) was dissolved in anhydrous DCM (15 ml) and stirred under nitrogen at 30 ºC for 48 h. Isocyanate formation was confirmed by IR, which showed strong band at 2280 cm^{-1}. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature and the isocyanate **78** collected as a yellow oil (0.8 g, 60 %). ^{1}H NMR ($CDCl_3$), δ: 3.9 (t, 2H, *J* = 6.0 Hz, 1-H), 3.32 (t, 2H, *J* = 6.0 Hz, 2-H), 1.64 (s, 9H, CH_3); ^{13}C NMR ($CDCl_3$) δ: 151.85 (OCON), 149.51 (NCO), 86.2 ($\underline{C}(CH_3)_3$), 40.5 (C-1), 39.97 (2), 28.03 (CH_3); IR (KBr) 2280s (NCO), 1750s (CO), 1513m (NO) cm^{-1}.

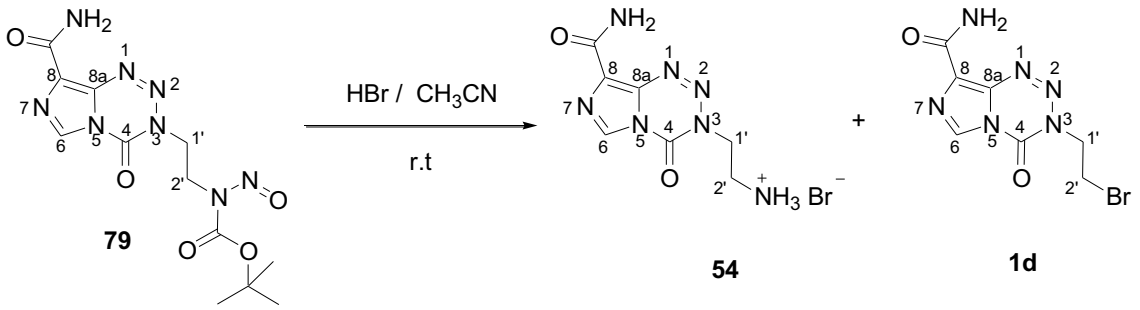
8-Carbamoyl-3-((*N*-t-butoxycarbonyl-*N*-nitroso)-2-aminoethyl)imidazo[5,1-d][1,2,3,5] tetrazin-4(3H)-one 79



78 **20** **79**

The isocyanate **78** (0.80 g, 4.37 mmol) was dissolved in DMSO (0.9 ml) and added to a suspension of diazo-IC **20** (0.6 g, 4.37 mmol) in DMSO (0.9 ml), the mixture was stirred at 30 ºC protected from light. The reaction was complete after 36 h as monitored by ^{1}H NMR. Water (10 ml) was added and the mixture filtered, then the residue washed with copious amounts of water until the washings came through colourless. The solid was washed with *i*-propanol and left to dry on the filter. The solid was suspended in chloroform (20 ml), the solution filtered, and the chloroform filtrate evaporated to dryness to give the imidazotetrazinone **79** as a pale yellow solid (0.11 g, 14 % yield), m.p. 154 – 157 ºC; ^{1}H NMR (DMSO-d_6) δ: 8.85 (s, 1H, 6-H), 7.70 & 7.58 (2 × br s, 2H, $CONH_2$), 4.36 (t, 2H, J = 6.1 Hz, 1'-H), 4.14 (t, 2H, J = 6.1, 2'-H), 1.42 (s, 9H, ($C\underline{H}_3$); ^{13}C NMR (DMSO-d_6) δ: 161.7 ($CONH_2$), 151.9 ($(CH_3)_3CO\underline{C}O$), 139.5 (C-4), 134.4 (C-8a), 131.9 (C-8), 129.7 (C-6), 86.1 ($(CH_3)_3\underline{C}$), 46.3 (C-1'), 39.8 (C-2'), 27.8 ($\underline{C}H_3$); IR (KBr) 3445m (NH), 3137m (NH), 2986m (CH_2), 1753s (C(4)O), 1681s ($CONH_2$), 1454s (NO) cm^{-1}; MS(FAB): m/z 353 (100%)$(M+H)^+$, 307(70%), 288.9(35%), 705(10%)$(2M+H)^+$; CHN Found: C, 36.54; H, 3.78; N, 26.96. $C_{12}H_{16}N_8O_5 \cdot 0.5CHCl_3$, requires C, 36.44; H, 4.04; N, 27.2%.

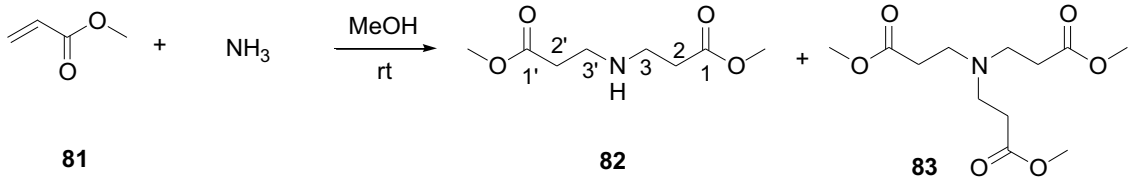
3-(2-Aminoethyl)imidazo[5,1-d][1,2,3,5]tetrazin-4(3H)-one hydrobromide salt 54



HBr (0.65 g, 8.03mmol) was added over 30 min to a stirred solution of BOC-protected imidazotetrazinone **79** (0.1 g, 0.31 mmol) in acetonitrile (6 ml). The mixture was stirred overnight and a precipitate formed. The solid was collected by filtration and dried under vacuum, ^{1}H NMR showed some impurities. The solid was re-suspended in CH_3CN, filtered and then dried to give the imidazotetrazinone hydrobromide salt **54 (**0.088 g, 88 %). The CH_3CN layer was evaporated to dryness, an orange solid was collected as a second crop. This was re-suspended into two portions of CH_3CN (10 ml) and the acetonitrile was evaporated to remove excess HBr. The solid residue was collected after complete evaporation of the acid and the solvent. ^{1}H NMR showed the imidazotetrazinone salt second crop with a significant impurity. Spiking of the solid sample in DMSO-d with authentic 3-(2-bromoethyl)imidazotetrazinone **1d** showed the impurity to be this compound as by-product. Compound **54** m.p. 145 – 148 º C; ^{1}H NMR (DMSO-d) δ: 8.92 (s, 1H, 6-H), 7.74 & 7.89 (2 br s, 2H, $N\underline{H_2}CO$), 7.83 (br s, 3H, NH_3^+), 4.56 (t, 2H, *J* = 5.8 Hz, 1'-H), 3.30 (hex, 2H, *J* = 5.8 Hz, 2'-H); ^{13}C NMR (DMSO-d), δ: 161.9 ($CONH_2$), 140 (C-4), 134.8 (C-8a), 131.7 (C-8), 129.5 (C-6), 47.0 (C-2'), 38.2 (C-1'); IR (KBr) 3410s (NH), 3137w (NH), 1750s (C(4)O), 1670 ($CONH_2$), 1603m, 1460m (C=C) cm^{-1}; MS (ES): m/z 527.1(18%) $(2M+ Br)^+$, 224.1(M^+) (23%),

138.2(38%), 119.2 (100%), 102.2 (58%), 87.2(10%); CHN Found: C, 23.24; H, 2.94; N, 25.95. $C_7H_9N_7O_2 \cdot 2HBr \cdot 0.3\ C_2H_3N$, requires C, 23.21; H, 3.04; N, 26.00%.

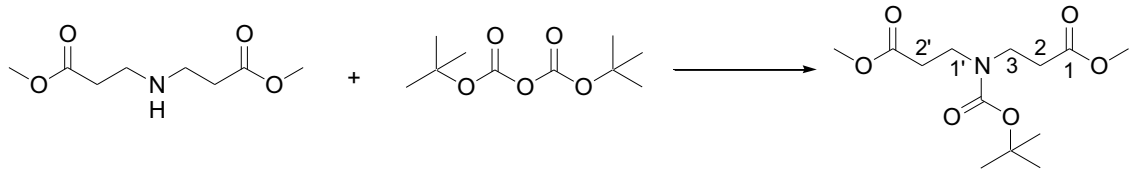
Dimethyl 3,3′-iminodipropanoate 82 and the trimethyl3,3′,3′′-iminotripropanoate 83[85]



Ammonia (30 g, 1.764 mol) was condensed into methanol (200 ml) using a dry ice and acetone trap. Methyl acrylate **81** (303.8 g, 3.53 mol) was added over 30 min and the reaction mixture was then stirred at rt, over night. The methanol was evaporated to give a colourless viscous oil. ^{1}H NMR showed a mixture of esters **82** and **83**. The ester **82** was collected by fractional distillation at 80 – 90 ºC, 0.05 mmHg (lit. 75 ºC, 0.05 mmHg).[85] ^{1}HNMR ($CDCl_3$) δ: 3.53 (s, 6H, 2×OCH_3), 2.75 (t, 4H, *J* = 6.3 Hz, H-3,3'), 2.35 (t, 4H, *J* = 6.3 Hz, H-2,2'), 1.47 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$) 173.0 (C-1,1'), 51.74 (OCH_3), 44.83 (C-3&3'), 34.48 (C-2&2'); IR (Liq film) 3323w (NH), 2955m (CH st), 1736s (CO), 1173m (C-O) cm^{-1}; MS (ES): m/z 190.2 (100%) $(M+H)^+$.

The ester **83** was left behind in the distillation flask with 100 % purity as shown by ^{1}H NMR and was of 3 fold yield more than the ester **82**. ^{1}H NMR ($CDCl_3$) δ: 3.62 (s, 9H, 3×OCH_3), 2.69 (t, 6H, *J* = 7.1 Hz, NCH_2), 2.38 (t, 6H, *J* = 7.1 Hz, $COCH_2$); ^{13}C NMR ($CDCl_3$) δ: 173.04 (CO), 51.6 (OCH_3), 49.2 (N CH_2), 32.7 (CH_2CO).

t-Butyl-*N*,*N*-bis(3-methoxy-3-oxopropyl)carbamate **84** [86]

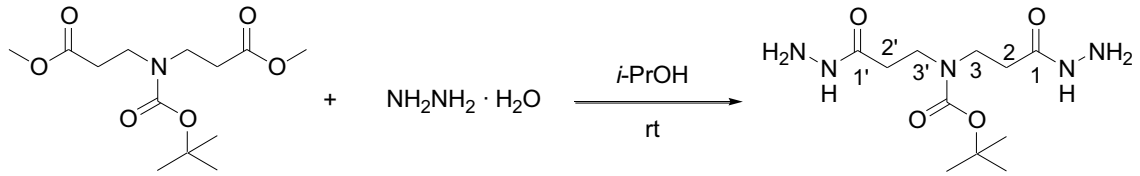


82 **84**

The aminodiester **82** (11.3 g, 59.8 mmol) was mixed with di-*t*-butyl dicarbonate (23.5 g, 107.6 mmol) and heated under N_2 to 100 ºC without solvent. Et_3N (6.05 g, 59.8 mmol) was added slowly to the mixture, the reaction mixture was then stirred at 100 ºC, overnight. The reaction mixture was partitioned between equal volumes of water / chloroform (25 ml); the chloroform layer was separated, washed with H_2O, dried with anhydrous $MgSO_4$ and then filtered. The chloroform layer was evaporated to yield a yellow oil, **84** (11.00 g, 66 %).

^{1}H NMR ($CDCl_3$) δ: 3.61 (s, 6H, 2 × OCH_3), 3.45 (t, 4H, *J* = 6.3 Hz, H-3,1'), 2.51(t, 4H, *J* = 6.3 Hz, H-2,2'), 1.38 (s, 9H, $C(CH_3)_3$), ^{13}C NMR ($CDCl_3$) 172.4 ($CH_2\underline{C}OOCH_3$), 155.1 (NCOO), 80.1 ($O\underline{C}(CH_3)_3$), 51.7 (OCH_3), 44.2 (C-3&3'), 33.9 (C-2&2'), 28.4 ($C\underline{C}H_3)_3$; IR (liq film) 2977m (CH st), 1739 ($\underline{CO}CH_3$), 1696 (BOC-$\underline{CO}$), 1164s (C-O) cm^{-1}; MS(ES): m/z 290.1(100%) $(M+H)^+$.[86]

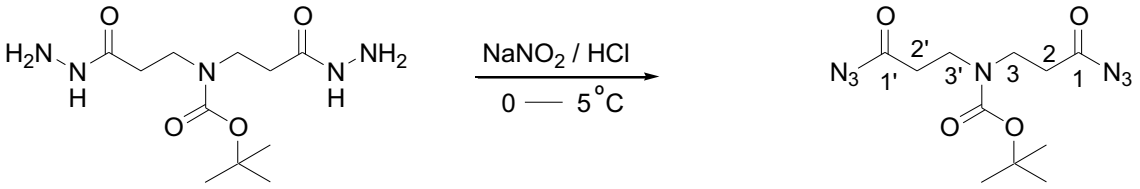
t-Butyl-*N*,*N*-bis(3-hydrazino-3-oxopropyl)carbamate 85



84 **85**

The BOC-protected aminodiester **84** (11.4 g, 39.5 mmol) was dissolved in *i*-propanol (40 ml), hydrazine monohydrate (19.8 g, 395 mmol) was added and the mixture stirred at rt, overnight. Volatile compounds were removed by evaporation to give a viscous yellow oil. ^{1}H NMR confirmed the product **85** with impurities. The oil was then dissolved in hot ethylacetate (30 ml), cold hexane (30 ml) was added and a white solid precipitated on cooling, the solid hydrazide **85** was collected by filtration, washed with chloroform and then dried. The solid was soluble in DMSO, methanol and water, insoluble in chloroform, dichloromethane, acetonitrile and diethyl ether. (8.10 g, 70%) m.p. 112 – 113.3ºC, ^{1}H NMR (DMSO-d_6), δ: 9.03 (br s, 2H, 2 × CONH), 4.19 (br s, 4H, 2 × NH_2), 3.30 (t, 4H, *J* = 6.5 Hz, H-3,3'), 2.24 (t, 4H, *J* = 6.5 Hz, H-2,2'), 1.39 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (DMSO-d_6) 170.2 (C-1,1'), 154.8 (NCOO), 79.2 ($\underline{C}(CH_3)_3$), 44.6 (C-3,3'), 33.7 (C-2,2'), 28.5 (CH_3); IR (KBr) 3267s (NH), 2977m (CH st), 1688s (BOC-$\underline{CO}$), 1649s ($\underline{CO}$NH), 1621m (NH), 1178s (C-O) cm^{-1}; MS (ES): m/z 290.2 (100%) $(M+H)^+$, 312.2 (70%) $(M+Na)^+$, 190.2 (25%).

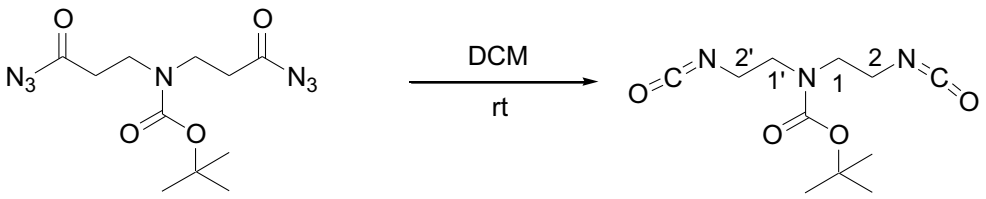
t-Butyl-*N*,*N*-bis(3-azido-3-oxopropyl)carbamate 86



85 **86**

Hydrazide **85** (1 g, 3.46 mmol) was suspended in a mixture of H_2O (10 ml) and DCM (10 ml) and was then stirred on an ice bath at 0 ºC. aq. HCl (4 ml 10 M) was added slowly to the mixture, followed by $NaNO_2$ (2.38 g, 34.6 mmol) solution in water (20 ml) drop-wise, keeping the exothermic reaction at 0 – 5 ºC. Further DCM (20 ml) was added and the DCM layer separated, washed with water, dried over $MgSO_4$ and then filtered. IR confirmed formation of the azide **86** (2138 cm^{-1}); the DCM was evaporated to yield a pale yellow oil (0.57 g, 57%).^{1}H NMR ($CDCl_3$) δ: 3.54 (t, 4H, *J* = 6.6 Hz, 3,3'-H), 2.63 (t, 4H, *J* = 6.6 Hz, 2,2'-H), 1.48 (s, 9H, CH_3); ^{13}C NMR ($CDCl_3$) δ: 174.0 (C-1,1'), 155.0(NCOO), 80.6 ($\underline{C}(CH_3)_3$, 36.0 (C-3,3'), 31.0 (C-2,2'), 28.3 (CH_3); IR (liq film) 2979m (CH_2 st), 2138s (CON_3), 1740s (CO), 1697s ($\underline{CO}C(CH_3)_3$) cm^{-1}.

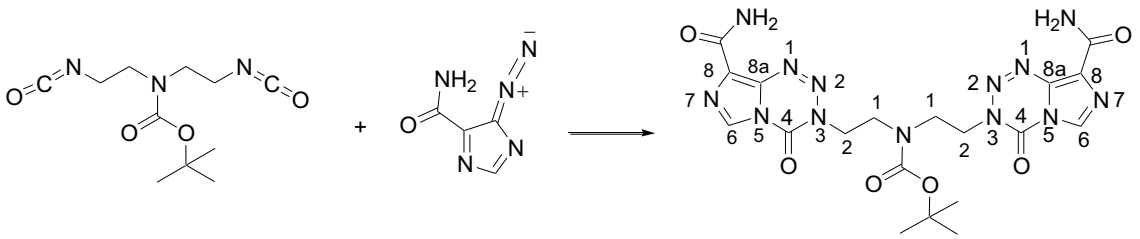
t-Butyl-*N*,*N*-bis(2-isocyanatoethyl)carbamate 87



86 **87**

The azide **86** (0.57 g, 1.83 mmol) was dissolved in anhydrous DCM (20 ml) and the solution stirred under nitrogen at rt for 48 h. The IR and ^{1}H NMR spectra confirmed the formation of isocyanate **87**. The DCM was evaporated and the isocyanate was re-suspended in anhydrous petroleum ether and decanted from an oily residue, evaporation of the petroleum ether gave brown oil as pure isocyanate **87** (0.2 g, 52.3%).^{1}H NMR ($CDCl_3$), δ: 3.46 (t, 4H, *J* = 6.7 Hz, 1,1'-H), 3.38 (t, 4H, *J* = 6.7 Hz, 2,2'-H), 1.42 (s, 9H, ($C\underline{H}_3)_3$); ^{13}C NMR ($CDCl_3$) δ: 155.2 (NCOO), 151.0 (NCO), 81.2 ($\underline{C}(CH_3)_3$), 49.1 (C-1,1'), 41.6 (C-2,2'), 28.4 (CH_3); IR (liq film) 2978w (CH_2 st), 2272s (NCO), 1695s ($COC(CH_3)_3$) cm^{-1}.

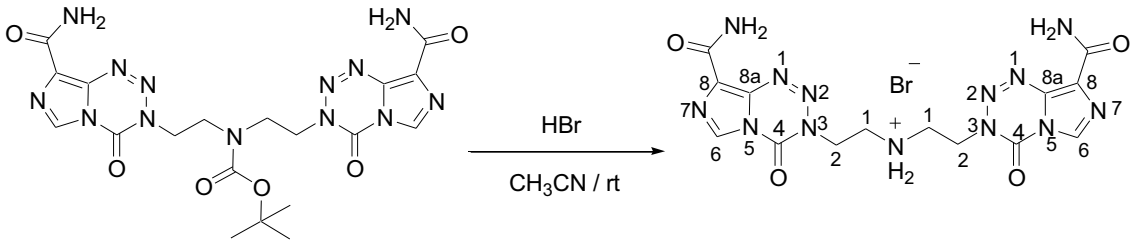
t-Butyl-*N*,*N*-bis(2-(8-carbamoyl-4-oxo-3H,4H-imidazotetrazin-3-yl)ethyl)carbamate 88



87 20 88

Isocyanate **87** (0.2 g, 0.783 mmol) dissolved in DMSO (300 μl) was transferred to suspension of diazo-IC **20** (0.24 g, 1.7 mmol) in DMSO (600 μl); the mixture was then stirred, protected from light, at rt. ^{1}H NMR was used to monitor the reaction; it showed two imidazotetrazinone 6-H peaks. ^{1}H NMR (DMSO-d_6), δ: 8.94 & 8.88 (s, 1H, 6-H), 7.85, 7.78 (2 × br s, 2H, $CONH_2$), 7.66, 7.62 (2 × br s, 2H, $CONH_2$), 4.62 (m, 4H, 2,2-H), 3.67 (m, 4H, 1,1-H), 0.84 (s, 9H, $C(CH_3)_3$); ^{13}C NMR (DMSO-d_6) δ: 161.8 ($CONH_2$), 155.4 ($(CH_3)_3CO\underline{C}O$), 139.6, 139.4 (C-4), 134.6 (C-8a), 131.5, 131.2 (C-8), 129.4, 129.2 (C-6), 79.8 ($(CH_3)_3\underline{C}$), 47.7, 47.4 (C-2,2), 45.0, 44.1 (C-1,1), 27.7 (CH_3); IR (KBr) 3420m (NH), 3137.4m (NH), 2955m (CH_2 st), 1750s (C(4)O), 1668s ($CONH_2$), 1598 m (C=C), 1462 m cm^{-1}; MS(ES): m/z 530 (25 %) $(M+H)^+$, 552(40%) $(M+Na)^+$, 144 (100 %); CHN Found: C, 42.38; H, 4.49, N, 33.82 $C_{19}H_{23}N_{13}O_6 \cdot 5\ H_2O$, requires C, 41.78, H, 4.65, N, 33.08%.

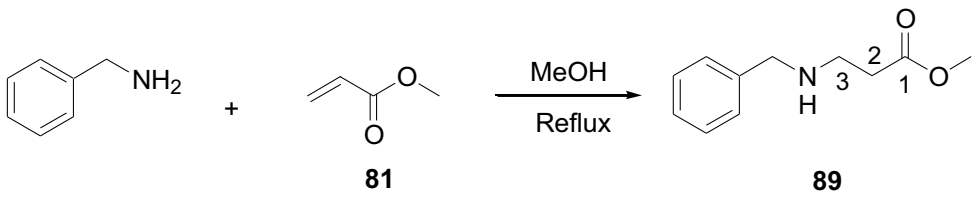
N,*N*-bis(2-(8-carbamoyl-4-oxo-3H,4H-imidazotetrazin-3-yl)ethyl)ammonium bromide 55



88 55

Aq. HBr (0.6 g, 6.8 mmol) was added slowly to a stirred solution of BOC-protected bisimidazotetrazinone **88** (0.36 g, 0.68 mmol) in CH_3CN (3 ml). The mixture was stirred overnight at rt and a precipitated formed. The solid was collected by filtration washed with cold acetonitrile (20 ml) and dried under vacuum, to give product **55 (**0.17 g, 57 %), m.p.175 – 176 ºC. ^{1}H NMR (DMSO-d_6) δ: 8.93 (s, 2H, 6-H), 8.80 (br s, 2H, $N\underline{H_2}^+$ exch. D_2O), 7.88 & 7.72 (2 × br s, 4H, 2 × $CON\underline{H_2}$ exch D_2O), 4.63 (t, 4H, *J* = 5.8 Hz, 2, 2-H), 3.55 (quin, 4H, *J* = 5.8 Hz, 1, 1-H); ^{13}C NMR (DMSO-d), δ: 161.9 ($CONH_2$), 139.9 (C-4), 134.7 (C-8a), 131.9 (C-8), 129.6 (C-6), 45.7 (C-2, 2), 45.5 (C-1, 1); IR (KBr) 3420m (NH), 2955m (CH st), 2792 (NH_2^+), 1750s (C(4)O), 1668 ($CONH_2$), 1598m, 1462m cm^{-1}; MS (ES): m/z 452 (13%) $(M + Na)^+$, 430 (100%) $(M)^+$, 127 (37%) $(AIC + H)^+$; CHN Found: C, 26.80; H, 3.50; N, 29.13. $C_{14}H_{15}N_{13}O_4 \cdot 2$ HBr·2 H_2O, requires C, 26.81; H, 3.37; N, 29.03%.

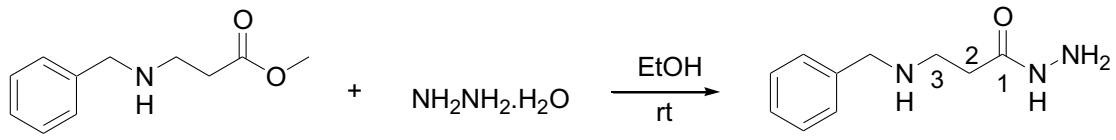
Methyl 3-benzylaminopropanoate 89 [87]



81 **89**

Benzylamine (10 g, 93.3 mmol) was dissolved in methanol (50 ml) and the solution stirred on an ice bath at 0 ºC; methyl acrylate (16.1 g, 186.6 mmol) was added over 3 min, the mixture was then stirred under reflux for 48 h, the reaction was monitored by ^{1}H NMR. The ^{1}H NMR spectrum showed ester **89** and some of the dimeric ester. Volatile compounds were removed by evaporation under vacuum to give a pale yellow oil. Fractional distillation gave the methyl 3-benzylaminopropanoate **89**, b.p. 77 ºC – 80 ºC at 0.05 mmHg (lit. 145 – 147 ºC, 7 mmHg)[87] (60 % yield); ^{1}H NMR ($CDCl_3$), δ: 7.29 (m, 5H, Ar-H), 3.78 (s, 2H, $PhCH_2$), 3.65 (s, 3H, OCH_3) 2.9 (t, 2H, J = 6.4 Hz, H-3), 2.51 (t, 2H, J = 6.4 Hz, H-2), 1.71 (s, 1H, $NHCH_2$); ^{13}C NMR ($CDCl_3$), δ: 173.2 (C-1), 140.3 ($ArCCH_2$), 128.6 (ArCH-*o*), 128.1 (ArCH-*m*), 127.0 (ArCH-*p*), 53.8 ($PhCH_2$), 53.6 (CH_3), 44.5 (C-3), 34.6 (C-2); IR (liq film) 3340m (NH), 2952w (CH st), 1736s (CO), 1643w, 1450m (C=C), 1173m (C-O) cm^{-1}; MS (ES): m/z 194.3 (65%) $(M + H)^+$, 120 (100%) $(C_8H_{10}N)^+$, 91.3 (100%)$(C_7H_7)^+$.

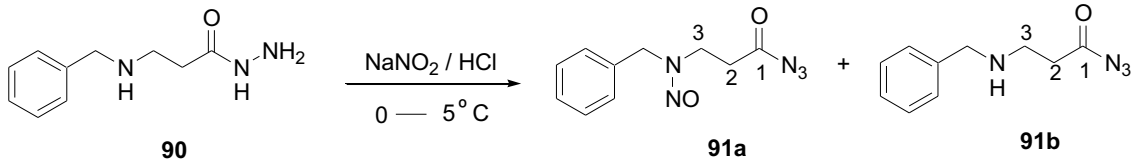
3-benzylaminopropanoyl hydrazide 90 [88]



89 **90**

Methyl 3-benzylaminopropanoate **89** (0.9 g, 4.7 mmol) was dissolved in ethanol (10 ml), hydrazine monohydrate (2.6 g, 28.2 mmol) was added and the mixture stirred at rt for 24 h, ^{1}H NMR showed completion of the reaction. The volatile compounds were removed by evaporation under reduced pressure to yield a viscous oil **90** (0.89 g, 98.8 %).^{1}H NMR ($CDCl_3$), δ: 8.93 (br s, 1H, CONH), 7.21 (m, 5H, Ar-H), 4.67 (br s, 1H, $PhCH_2NH$), 3.79 (br s, 2H, $CONHNH_2$), 3.68 (s, 2H, $PhCH_2$), 2.79 (t, 2H, J = 6.1 Hz, H-3), 2.26 (t, 2H, J = 6.1 Hz, H-2); ^{13}C NMR ($CDCl_3$), δ: 173.1 (C-1), 139.6 ($ArCCH_2$), 128.6 (2 × ArCH-*o*), 128.2 (2 × ArCH-*m*), 127.23 (ArCH-*p*), 53.7 ($PhCH_2$), 44.8(C-3), 34.3 (C-2); IR (liq film) 3292s br (NH), 3029w (Ar-H st), 2952w (CH st), 2361&2340 (NH st), 1648s (CONH), 1541m (C=C), 737 & 699 (mono-substituted aromatic ring) cm^{-1}; MS (ES): m/z 194.2 (100%) $(M+H)^+$.

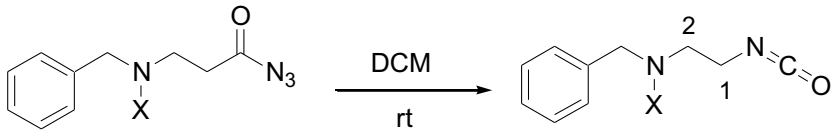
3-(*N*-Benzyl-N-nitrosoamino)propanoyl azide 91a and 3-benzylaminopropanoyl azide 91b



90 **91a** **91b**

Hydrazide **90** (1.5 g, 7.8 mmol) was dissolved in a mixture of H_2O (10 ml) and DCM (10 ml). The mixture was stirred on an ice bath at 0 ºC, HCl (5 ml, 10 M) was added slowly, then $NaNO_2$ (3.2 g, 46.6 mmol) solution in H_2O (10 ml) was added drop-wise keeping the exothermic reaction at 0 – 5 ºC. Further DCM (10 ml) was added and the DCM layer was then separated, washed with water, dried over $MgSO_4$. IR confirmed the formation of an azide, ^{1}H NMR showed two azides were present. The mixture of azides was used directly in the next step. ^{1}H NMR ($CDCl_3$), δ: 7.37 (m, 10 H, Ar-H), 7.1 (d, 2H, *J* = 8.1 Hz Ar-H), 5.36 (s, 2H, $PhCH_2$), 4.82 (s, 2H, $PhCH_2$), 4.31 (t, 2H, *J* = 6.6 Hz H-3), 3.64 (t, 2H, *J* = 6.6 Hz, H-3), 2.82 (t, 2H, *J* = 6.6 Hz H-2), 2.47 (t, 2H, *J* = 6.6 Hz H-2) ; ^{13}C NMR (CDCl3), δ: 178.4 & 177.7 (C-1), 134.6 & 133.9 (Ar<u>C</u>CH_2), 129.2 & 129.1 (2 × ArCH-*o*), 128.9 & 128.5 (2 × ArCH-*m*), 128.3 & 128.2 (ArCH-*p*), 57.1 & 47.2 ($PhCH_2$), 46.9 & 39.3(C-3), 35.9 & 33.2 (C-2); IR (liq film) 2924w (CH st), 2142s (CON_3), 1715s (CO), 1454s (N-NO), 737&699 (mono-substituted aromatic ring) cm^{-1}.

2-(*N*-benzyl-*N*-Nitrosoamino)ethyl isocyanate 92a and 2-Benzylaminoethylisocyanate 92b



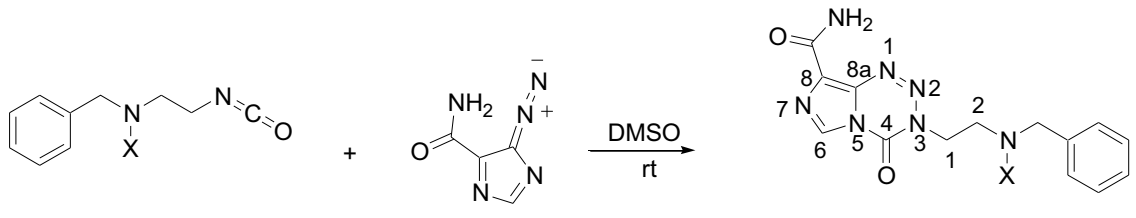
91 **92**

X = NO **a**
= H **b**

The azides **91a** & **b** solution in anhydrous DCM was stirred under nitrogen at rt for 24 h. Isocyanate formation was confirmed by IR. The ^{1}H NMR showed the formation of two isocyanates. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature). The isocyanates were collected as a crude pale brown oil (0.6 g).^{1}H NMR ($CDCl_3$), δ: 7.39 (m, 10H, Ar-H), 5.39 (s, 2H, $PhCH_2$), 4.85 (s, ≈ 2H, $PhCH_2$), 4.22 (t, 2H, J = 6.1 Hz, H-2), 3.62 (t, 2H, J = 6.1 Hz, H-1), 3.59 (t, 2H, J = 6.1 Hz, H-2), 3.37 (t, 2H, J = 6.1 Hz, H-1); ^{13}C NMR ($CDCl_3$), δ: 156.7 & 150.4 (NCO), 134.4 & 133.9 (ArCCH$_2$), 129.3 & 129.2 (2 × ArCH-*o*), 129.0 & 128.5 (2 × ArCH-*m*), 128.4 & 128.3 (ArCH-*p*), 57.3 & 51.8 ($PhCH_2$), 46.9 & 43.8(C-2), 41.8 & 39.5 (C-1); IR (liq film) 2918w (CH st), 2274s (NCO), 1454s (N-NO), 737&699 (mono-substituted aromatic ring) cm^{-1}.

3-{2-[Benzyl(nitroso)amino]ethyl}-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 93a &

3-[2-(Benzylamino)ethyl]-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 93b

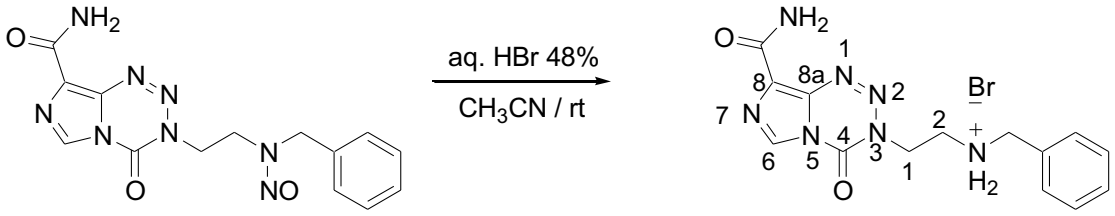


92 **20** **93**

X = NO **a**
X = H **b**

Isocyanates **92a, b** (0.6 g, 3.53 mmol) were dissolved in DMSO (0.7 ml) then the solution was transferred under nitrogen to a suspension of diazo-IC **20** (0.48 g, 3.5 mmol) in DMSO (1.4 ml), the mixture was then stirred protected from light at rt, overnight. The reaction was monitored using ^{1}H NMR, after 24 h it showed the formation of two imidazotetrazinones but the reaction was not yet complete. After 48 h the reaction mixture was suspended in water, filtered, the residue washed with copious amounts of water until the washings came through colourless, then dried. Small samples of the solid were tested for its solubility in different solvents, acetonitrile, *i*-propanol and chloroform. Isopropanol was found to dissolve only the impurities. The solid was then washed with *i*-propanol, filtered, and dried (0.2 g, 20%). The solid consisted of two imidazotetrazinones. ^{1}H NMR (DMSO-d_6) δ: 8.83 (s, 1H, 6-H), 8.76 (s, 1H, 6-H), 7.83, 7.70 (2 × br s, 2H, $CONH_2$), 7.80, 7.69 (2 × br s, 2H, $CONH_2$), 7.26 (m, 5H, Ar-H), 7.24 (m, 5H, Ar-H), 5.36 (s, 2H, $PhCH_2$), 4.86 (s, 2H, $PhCH_2$), 4.72 (t, 2H, *J* = 5.8 Hz, H-1), 4.58 (t, 2H, *J* = 5.8 Hz, H-2), 4.32 (t, 2H, *J* = 5.7 Hz, H-1), 3.89 (t, 2H, *J* = 5.7 Hz, H-2); ^{13}C NMR (DMSO-d_6) δ: 161.9, 161.4 ($CONH_2$), 139.2 (C-4), 135.4, 135.0 ($ArCCH_2$), 134.6, 134.5 (C-8a), 131.9, 131.6 (C-8), 129.6, 129.3 (C-6), 129.1 (2×

Ar<u>C</u>H-*o*), 128.9 (Ar<u>C</u>H-*p*), 128.5 (2 × Ar<u>C</u>H-*m*), 128.0, 56.4, 50.2 ($PhCH_2$), 47.5, 45.6 (C-1), 47.0, 43.2 (C-2); MS (ES): m/z 707.1 (100%)$(2M+Na)^+$, 365.2 (82%)$(M+Na)^+$, 343.2 (24%)$(M+H)^+$, 138.1(11%)$(C_4H_3N_5O+H)^+$. To remove the nitroso group, the solid mixture of tetrazines (0.083 g, 0.24 mmol) was dissolved in acetonitrile (3 ml) then hydrobromic acid (0.04 g, 0.48 mmol 48%) was added drop-wise to the solution, the mixture stirred at rt for 2 h, a solid was precipitated. The solid was collected by filtration, washed with copious amounts of acetonitrile and then dried. ^{1}H NMR showed a single compound, the tetrazine bromide salt **56**.

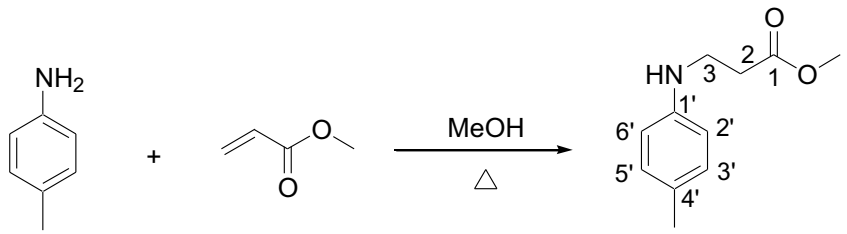


56

m.p. 143 – 144 ºC ^{1}H NMR (DMSO-d_6) δ: 8.96 (br s, 2H, NH_2^+), 8.93 (s, 1H, 6-H), 7.86 & 7.71 (2 × br s, 2H, $CONH_2$), 4.67 (t, 2H, *J* = 5.7 Hz, H-1), 4.27(t, 2H, *J* = 5.7 Hz, $PhCH_2$), 3.47 (quin, 2H, *J* = 5.7 Hz, H-2); ^{13}C NMR (DMSO-d_6) δ: 162.6 ($CONH_2$), 140 (C-4), 134.7 (C-8a), 132.2 (Ar<u>C</u>CH_2), 131.8 (C-8), 130.5 (2 × ArCH-*o*), 129.8 (ArCH-*p*), 129.6 (C-6), 129.4 (2 × Ar<u>C</u>H-*m*), 50.6 ($PhCH_2$), 45.5 (C-1), 45.3 (C-2); IR (KBr) 3441, 3337 m (NH_2^+), 3407m (Ar-H st), 3107 (NH), 2791w (CH st), 1758 (C(4)O), 1666 (CONH), 1606 (C=C), 721 & 702 cm^{-1}; MS (ES): m/z 473, 475 & 477 (100%) ($C_{14}H_{17}Br_2N_7O_2$)(1:2:1), 393&395(1:1) (100%) ($C_{14}H_{16}BrN_7O_2$), 313.9 (100%) $(C_{14}H_{16}N_7O_2)^+$; CHN Found: C, 31.62; H, 3.17; N, 19.08. $C_{14}H_{15}N_7O_2 \cdot 2.6HBr \cdot 0.1H_2O$, requires C, 32.0; H, 3.41; N, 18.66%.

E.3.2 Synthesis of imidazotetrazinones bearing anilines in Chapter 2 section 2.2.2.2

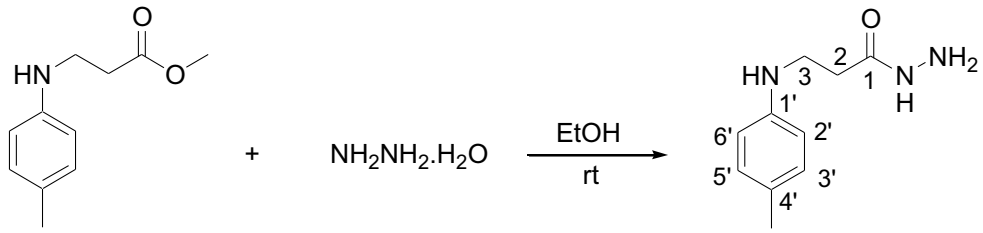
Methyl 3-(4-methylphenylamino)propanoate 94[89]



81 **94**

p-Toluidine (5 g, 46.7 mmol) was dissolved in methanol (15 ml), methyl acrylate (12 g, 140 mmol) was added and the reaction mixture was stirred at reflux, overnight. TLC showed product formation with unreacted *p*-toluidine. The methanol was evaporated to give a solid in an oil vehicle. The mixture was re-suspended in diethyl ether (10 ml); a solid of **94** was collected by filtration after cooling, washed with cold ether and then dried (4.5 g, 90%). m.p 59 – 62 ºC (lit. 60 – 61 ºC);[90] ^{1}H NMR ($CDCl_3$) δ: 6.98 (½AB, 2H, *J* = 8.4Hz, 3',5'-H), 6.54 (½AB, 2H, *J* = 8.4 Hz, 2',6'-H), 3.86 (br s, 1H, HN), 3.70 (s, 3H, OCH_3), 3.42 (t, 2H, *J* = 6.4 Hz, H-3), 2.60 (t, 2H, *J* = 6.4 Hz, H-2), 2.22 (s, 3H, CH_3-Ar); ^{13}C NMR ($CDCl_3$) 173.0 (C-1), 145.4 (C-1'), 129.9 (C-2',6'), 127.2 (C-4'), 113.4 (C-3',5'), 51.8 (OCH_3), 39.9 (C-3), 33.9 (C-2), 20.5 (CH_3-Ar); IR (KBr) 3391m (NH), 2910m (CH st), 1725s (CO), 1616s, 1524s (C=C) 803m (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES): m/z 194 (100%) $(M + H)^+$.

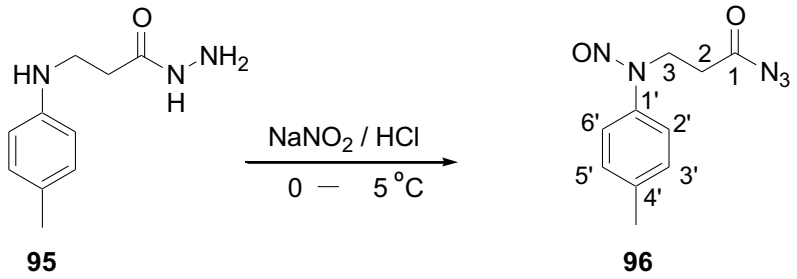
Methyl 3-(4-methylphenylamino)propanoyl hydrazide 95[91]



94 **95**

Ester **94** (2.0 g, 10.35 mmol) was dissolved in ethanol (15 ml), hydrazine monohydrate (3.11 g, 62.1 mmol) was added and the mixture was stirred at rt overnight. Formation of a white solid was observed, the reaction mixture was filtered, and the solid was then washed with copious amounts of cold ethanol and dried to give the hydrazide **95** (1.7 g, 85% yield) m.p. 138 – 140 ºC (lit. 138 – 139 ºC)[90]; ^{1}H NMR ($CDCl_3$) δ: 7.06 (br s, 1H, NHCO), 6.99 (½AB, 2H, *J* = 8.4 Hz, 3',5'-H), 6.55 (½AB, 2H, *J* = 8.4 Hz, 2',6'-H), 3.86 (br s, 3H, Ar-NH, CONHN<u>H</u>$_2$), 3.43 (t, 2H, *J* = 6.1 Hz, 3-H), 2.43 (t, 2H, *J* = 6.1 Hz, 2-H), 2.23 (s, 3H, CH_3-Ar); ^{13}C NMR ($CDCl_3$) δ: 172.5 (C-1), 145.2(C-1'), 130 (C-2',6'), 127.6 (C-4'), 113.7 (C-3',5'), 40.5 (C-3), 34.0 (C-2), 20.5 (CH_3-Ar); IR (KBr) 3312m (NH), 2901m (CH st), 1643s (CONH), 1525s (C=C), 803m (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES): m/z 194 (100%) $(M + H)^+$.

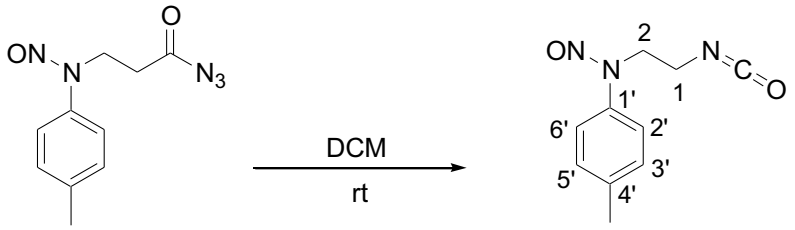
Methyl 3-(*N*-(4-methylphenyl)-*N*-Nitrosoamino)propanoyl azide 96



95 **96**

Hydrazide **95** (0.67 g, 3.47 mmol) was dissolved in a mixture of H_2O (10 ml) and DCM (10 ml) and was then stirred on an ice bath at 0 ºC, HCl (4 ml × 10 M) was added to the mixture slowly, then $NaNO_2$ (1.44 g, 20.8 mmol) solution in water (20 ml), was added to the reaction mixture, drop-wise keeping the exothermic reaction at 0 – 5 ºC. Further DCM (20 ml) was added and the DCM layer separated, washed with water, dried over $MgSO_4$ and then filtered. The IR and ^{1}H NMR spectra confirmed formation of the azide **96**. ^{1}H NMR ($CDCl_3$), δ: 7.32 (½AB, 2H, *J* = 8.3 Hz, 2',6'-H), 7.22 (½AB, 2H, *J* = 8.3 Hz, 3',5'-H), 4.29 (t, 2H, *J* = 7.1 Hz, 3-H), 2.55 (t, 2H, *J* = 7.1 Hz, 2-H), 2.34 (s, 3H, CH_3-Ar); ^{13}C NMR ($CDCl_3$), δ: 177.7 (C-1), 138.7 (C-1'), 138.0 (C-4'), 130.3 (C-2',6'), 120.2 (C-3',5'), 40.1 (C-3), 33.3 (C-2), 21.1 (CH_3); IR (liq film) 2925w (CH_2 st), 2142s (CON_3), 1713s (CO), 1512m (C=C aromatic), 1454s (NO)cm^{-1}.

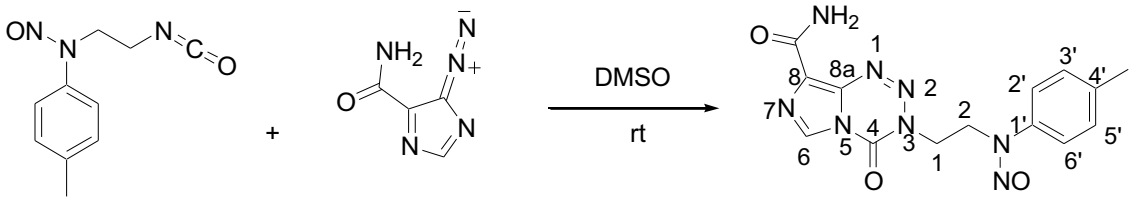
2-(*N*-(4-Methylphenyl)-*N*-Nitrosoamino)ethyl isocyanate 97



96 **97**

The azide **96** solution in anhydrous DCM was stirred under nitrogen at rt for 24 h. Isocyanate formation was confirmed by IR, which showed a strong band at 2273 cm^{-1}. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature). The isocyanate was collected as a pale brown oil (0.1 g, 59 %).^{1}H NMR ($CDCl_3$), δ: 7.49 (½AB, 2H, *J* = 8.3 Hz, 2',6'-H), 7.32 (½AB, 2H, *J* = 8.3 Hz, 3',5'-H), 4.12 (t, 2H, *J* = 7.1 Hz, 2-H), 3.48 (t, 2H, *J* = 7.1 Hz, 1-H), 2.34 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$), δ: 155 (NCO), 138.8 (C-1'), 138.1 (C-4'), 130.4 (C-2',6'), 120.5 (C-3',5'), 45.2 (C-2), 39.4 (C-1), 21.1 (CH_3); IR (liq film) 2953w (CH_2 st), 2273s (NCO), 1512m (C=C aromatic), 1454s (NO) cm^{-1}.

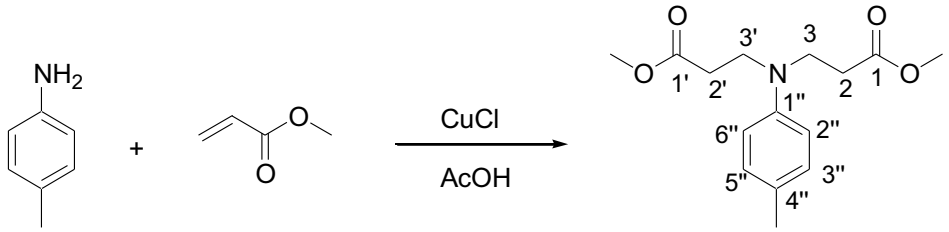
3-{2-[(4'-Methylphenyl)(nitroso)amino]ethyl}-4-oxo-3H,4H-imidazo[1,4-d][1,2,3,5]tetrazine-8-carboxamide 57



97 **20** **57**

Isocyanate **97** (1 g, 5.7 mmol) was dissolved in DMSO (1.5 ml) then added under nitrogen to a suspension of diazo-IC **20** (0.8 g, 5.7 mmol) in DMSO (1.5 ml), the mixture was stirred at r.t protected from light. The reaction was monitored by ^{1}H NMR it was found completed after 72 h. The reaction mixture was then suspended in water (15 ml), filtered, then the residue was washed with copious amounts of water until the washings came through colourless, then with cold $CHCl_3$ and dried to **57**, m.p. 148 – 150 ºC. ^{1}H NMR (DMSO-d_6) δ: 8.78 (s, 1H, 6-H), 7.77 & 7.65 (2 × br s, 2H, NH_2CO), 7.40 (½AB, 2H, *J* = 8.1 Hz 2',6'-H), 7.21 (½AB, 2H, *J* = 8.1 Hz 3',5'-H), 4.49 (t, 2H, *J* = 5.8 Hz, 1-H), 4.40 (t, 2H, *J* = 5.8 Hz, 2-H), 2.23 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 161.8 ($CONH_2$), 139.4 (C-4), 138.8 (C-1'), 137.9 (C-4'), 134.4 (C-8a), 131.7 (C-8), 130.4 (C-2',6'), 129.5 (C-6), 120.8 (C-3',5'), 46.3 (C-1), 42.4 (C-2), IR (KBr) 3458s (NH), 3120m (Ar-H st), 1742s (C(4)O), 1680s (CONH), 1595m (C=C), 1458s (NO), 818m cm^{-1}; MS(ES): m/z 313.3 (14%) ($C_{14}H_{15}N_7O_2$), 343.4 ($C_{14}H_{14}N_8O_3$) (100%) $(M+H)^+$; CHN Found C, 48.54; H, 3.90; N, 32.05. $C_{14}H_{14}N_8O_3 \cdot 0.25\ H_2O$, requires C, 48.48; H, 4.21; N, 32.31%.

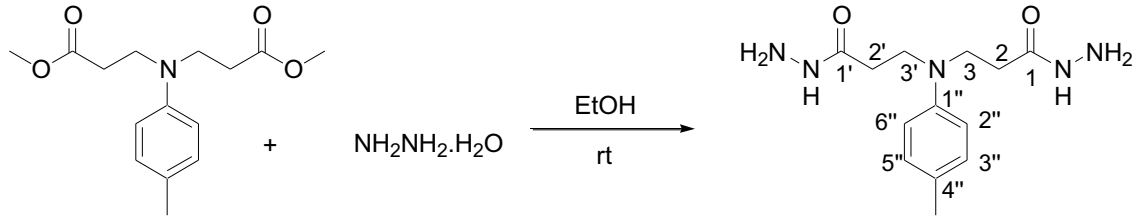
Dimethyl *N*-(4-methylphenyl)iminodipropanoate 101a[79]



81 **101a**

4-Methylaniline (1.2 g, 11.2 mmol), methyl acrylate (9.64 g, 112 mmol), CuCl (0.18g, 1.8 mmol) and acetic acid (1 ml) were mixed. The reaction mixture was stirred at 140 ºC for 72 h, monitored by ^{1}H NMR. ^{1}H NMR showed a mixture of the monoester **94** and the diester **101a**. The reaction mixture was partitioned between equal volumes of H_2O / Et_2O (25 ml); the Et_2O layer was separated, washed with three portions of H_2O (20ml), dried over anhydrous $MgSO_4$, and then filtered. The Et_2O was evaporated to give a yellow oil. The ester **94** was collected by fractional distillation at 100 – 108 ºC at 0.05 mmHg which solidified in the condenser, whereas ester **101a** was left behind pure (2 g, 64%), confirmed by ^{1}H NMR. ^{1}HNMR ($CDCl_3$) δ: 7.03 (½AB, 2H, J = 8.6 Hz, 3",5"-H), 6.62 (½AB, 2H, J = 8.6 Hz, 2",6"-H), 3.66 (s, 6H, 2 × OCH_3), 3.58 (t, 4H, J = 7.2 Hz 3,3'-H), 2.56 (t, 4H, J = 7.2 Hz 2,2'-H), 2.23 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) 172.7 (C-1,1'), 150.8 (C-1"), 130.1 (C-2",6"), 124.6 (C-4"), 113.4 (C-3",5"), 51.8 (OCH_3), 47.3 (C-3&3'), 32.4 (C-2&2'), 20.5 (CH_3); IR (liq film) 2951 (CH st), 1736s (CO), 1520m (C=C), 1173m (C-O), 804m (*p*-di-substituted aromatic ring) cm^{-1}; MS(ES) m/z: 280.1 (100%) $(M+H)^+$.

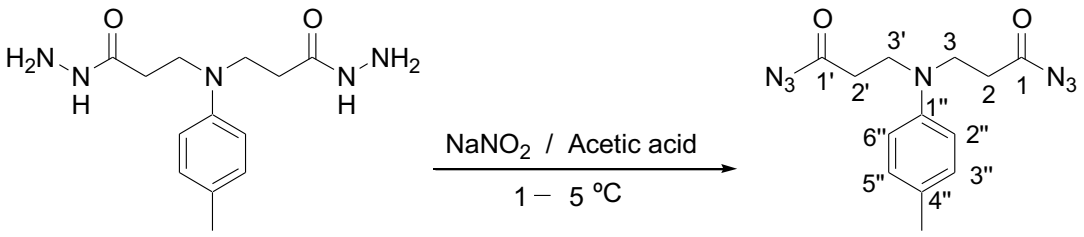
N-(4-methylphenyl)iminodipropanoyl dihydrazide 102a[79]



101a **102a**

Ester **101a** (1.2 g, 4.3 mmol) was dissolved in ethanol (10 ml), hydrazine monohydrate (2.1 g, 43.5 mmol) was added and the mixture stirred at rt for 2 h. Formation of a white solid was observed, the solid was collected by filtration, washed with copious amounts of cold ethanol and then dried. ^{1}H NMR confirmed the hydrazide **102a** (0.75 g, 63% yield) m.p. 162 – 163 ºC. ^{1}H NMR (DMSO-d_6) δ: 9.0 (br s, 2H, 2× CON<u>H</u>), 6.97 (½AB, 2H, *J* = 8.6 Hz 2",6"-H), 6.58 (½AB, 2H, *J* = 8.6 Hz 3",5"-H), 4.16 (br s, 4H, COHNN<u>H$_2$</u>), 3.43 (t, 4H, *J* = 7.1 Hz, 3,3'-H), 2.22 (t, 4H, *J* = 7.1 Hz, 2,2'-H), 2.16 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 170.6 (C-1,1'), 145.5(C-1"), 130.2 (C-2",6"), 124.8 (C-4"), 112.8 (C-3",5"), 47.6 (C-3,3'), 32.1 (C-2,2'), 20.4 (CH_3); IR (KBr) 3277s (NH), 2918m (CH st), 1641s (CONH), 1524s (C=C), 801m (p-di-substituted aromatic ring) cm^{-1}, MS (ES) m/z: 280.1 (100%) $(M+H)^+$.

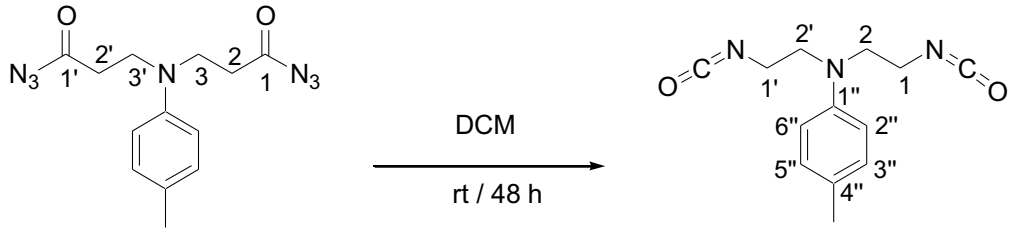
N-(4-Methylphenyl)iminodipropanoyl diazide 103a



102a **103a**

Hydrazide **102a** (0.1 g, 0.35 mmol) was dissolved in a mixture of aq. acetic acid solution (5 ml × 0.17 M) and DCM (5 ml). The mixture was stirred on an ice bath at 0 – 5 ºC; a dilute solution of $NaNO_2$ (1.44 g, 20.8 mmol) in H_2O (20 ml) was added gradually, keeping the exothermic reaction at 0 – 5 ºC. Further DCM (10 ml) was added, the DCM layer was separated, washed with H_2O (20 ml) dried over $MgSO_4$, and then filtered. Formation of the azide **103a** was confirmed by the IR and ^{1}H NMR spectra. ^{1}H NMR 400 MHz ($CDCl_3$), δ: 7.06 (½AB, 2H, *J* = 8.3 Hz, 3",5"-H), 6.65 (½AB, 2H, *J* = 8.3 Hz, 2",6"-H), 3.63 (t, 4H, *J* = 7.1 Hz, 3,3'-H), 2.61 (t, 4H, *J* = 7.1 Hz, 2,2'-H), 2.28 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$), δ: 179.2 (C-1,1'), 144.4 (C-1"), 130.2 (C-2",6"), 127.8 (C-4"), 114.2 (C-3",5"), 47.4 (C-3,3'), 35.1 (C-2,2'), 20.4 (CH_3); IR (liq film) 2919w (CH st), 2138s (CON_3), 1713s (CO), 1616m, 1521s (C=C aromatic), 804 (*p*-di-substituted aromatic ring) cm^{-1}.

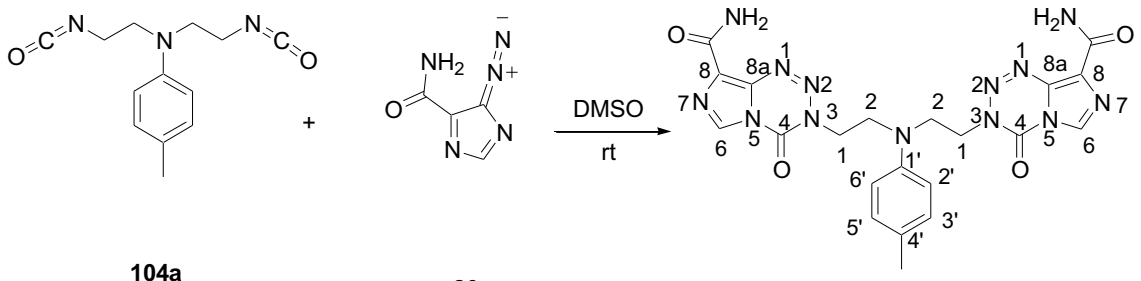
N,*N*-Di(2-isocyanatoethyl)-4-methylaniline 104a



103a **104a**

Azide **103a** solution in anhydrous DCM was stirred under nitrogen at rt for 48 h. IR confirmed the formation of the isocyanate **104a**, it showed the strong band at 2271 cm^{-1}. The DCM was evaporated to give a pale yellow oil (0.55 g, 68.6%). ^{1}H NMR ($CDCl_3$), δ: 7.03 (½AB, 2H, *J* = 8.6 Hz, 3",5"-H), 6.66 (½AB, 2H, *J* = 8.6 Hz, 2",6"-H), 3.47 (t, 4H, *J* = 6.2 Hz, 2,2'-H), 3.38 (t, 4H, *J* = 6.2 Hz, 1,1'-H), 2.20 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$), δ: 144.0 (C-1"), 130.3 (C-2",6"), 129.0 (C-4"), 124.2 (NCO), 115.4 (C-3",5"), 53.4 (C-2,2'), 41.0 (C-1,1'), 20.4 (CH_3); IR (liq film), 2925m (CH_2 st), 2271s (NCO), 1519m (C=C aromatic) cm^{-1}; MS (ES) m/z 245.7(100%) $(M+H)^{\cdot +}$, 146.4 (5%), 79 (100%) $(C_6H_6+H)^{+}$.

N,*N*-Bis(2-(8-Carbamoyl-4-oxo-3H,4H-imidazotetrazin-3-yl)ethyl)-4-methylaniline 58a

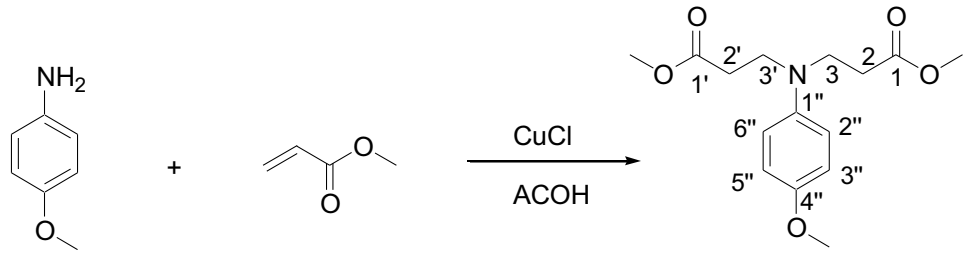


104a **20** **58a**

Isocyanate **104a** (0.06 g, 0.24 mmol) was dissolved in DMSO (0.75 ml) then added, under N_2, to a suspension of diazo-IC **20** (0.03 g, 0.24 mmol) in DMSO (0.75 ml). The mixture was stirred at 35 °C protected from light. The reaction was monitored by ^{1}H NMR and was shown complete after 72 h. The ^{1}H NMR spectrum showed the tetrazine **58a** with some impurities. The mixture was suspended in H_2O (10 ml), filtered, then the residue was washed with copious amounts of water until the washings came through colourless. The crude solid was collected and then subjected to purification by flash column chromatography with gradient elution, glacial acetic acid in chloroform was used, a yellow solid was collected from the fractions at 60% acetic acid. The solid was dissolved in DMF (2 ml) for further purification, filtered and then precipitated by addition of water, the yellow solid was collected by filtration washed with copious amounts of water and then dried (0.02 g, 16%), m.p. 195 – 196 ºC ^{1}H NMR (DMSO-d_6) δ: 8.65 (s, 2H, 6-H), 7.64 & 7.54 (2 × br s, 4H, N$\underline{H_2}$CO), 6.72 (½AB, 2H, *J* = 8.6 Hz, 3',5'-H), 6.58 (½AB, 2H, *J* = 8.6 Hz, 2',6'-H), 4.32 (t, 4H, *J* = 6.6 Hz, 1,1-H), 3.66 (t, 4H, *J* = 6.6 Hz, 2,2-H), 1.91 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 162.0 ($CONH_2$), 145.1 (C-1'), 139.7 (C-4), 134.8 (C-8a), 131.3 (C-8), 129.9 (C-2',6'), 129.3 (C-6), 126.0 (C-4'), 113.2 (C-3',5'), 49.1 (C-1,1), 46.8 (C-2,2), 20.3 (CH_3); IR (KBr), 3444m (NH), 2923w, (CH st), 1736s (C(4)O), 1682s (CONH), 1600&1521 (C=C),818m (*p*-di-

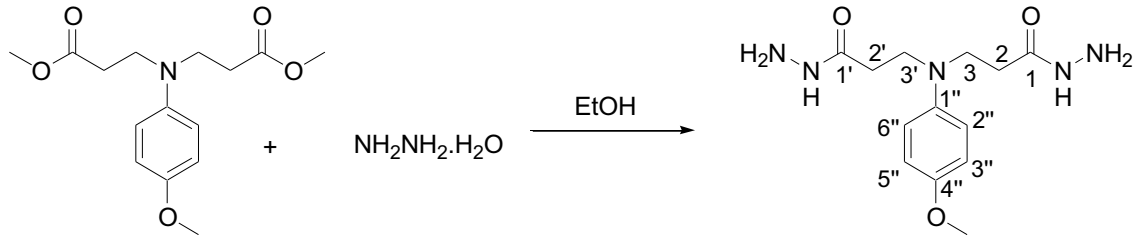
substituted aromatic ring) cm^{-1}; MS(ES): m/z 520(80%)$(M+H)^+$, 338(70%), 246 (70%)$(C_{13}H_{15}N_3O_2+H)^+$, 145(75%), 79(50%)$(C_6H_6+H)^+$; CHN Found: C, 48.13; H, 3.95; N, 34.35 $C_{21}H_{21}N_{13}O_4 \cdot 0.05CHCl_3 \cdot 0.1H_2O$, requires C, 47.95; H, 4.06; N, 34.54%.

Dimethyl *N*-(4-methoxyphenyl)iminodipropanoate 101b[79]



p-Methoxyaniline (2.88 g, 23.4 mmol), methyl acrylate (20 g, 234 mmol), CuCl (0.35g, 3.75 mmol) and acetic acid (20 ml) were mixed and stirred at 140 ºC for 48 h, monitored by ^1H NMR. The reaction mixture was partitioned between $CHCl_3$ (25 ml) and dilute aqueous Na_2CO_3 (pH 12) (25 ml); the $CHCl_3$ layer was separated, washed with three portions of H_2O (20ml), dried with anhydrous $MgSO_4$, decolourised with charcoal and then filtered through celite 521. The $CHCl_3$ was evaporated to give a yellow oil **101b** (5.52 g, 80%) ^1H NMR 400 MHz ($CDCl_3$) δ: 6.86 (½AB, 2H, J = 9.1 Hz, 2",6"-H), 6.76 (½AB, 2H, J = 9.1 Hz, 3",5"-H), 3.76 (s, 3H, OCH_3), 3.68 (s, 6H, 2× $COOCH_3$), 3.55 (t, 4H, J = 7.1 Hz, 3,3'-H), 2.55 (t, 4H, J = 7.1 Hz, 2,2'-H); ^{13}C NMR ($CDCl_3$) 172.7 (C-1,1'), 152.9 (C-4"), 141.5 (C-1"), 116.5 (C-3",5"), 115.0 (C-2",6"), 55.8 (CH_3), 51.7 (2× $COO\underline{C}H_3$), 48.2 (C-3,3'), 32.5 (C-2,2'); IR (liq film) 2951 (CH st), 1726s (CO), 1520m (C=C), 1173m (C-O), 804m (*p*-di-substituted aromatic ring) cm^{-1}; MS(ES): m/z 296.1 (100%) $(M+H)^+$.

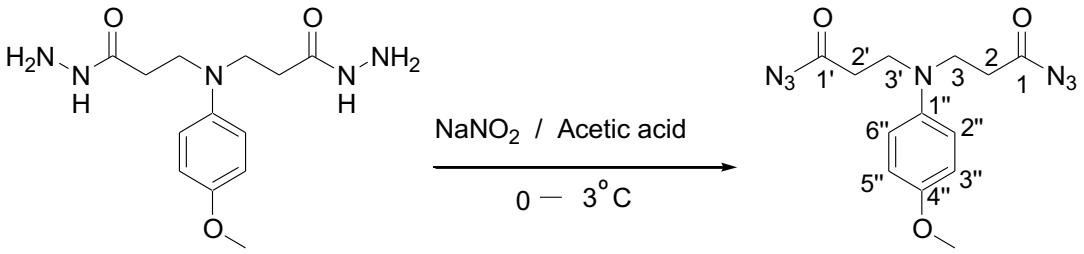
N-(4-Methoxyphenyl)iminodipropanoyl dihydrazide 102b[79]



101b **102b**

Ester **101b** (5.52 g, 18.7 mmol) was dissolved in ethanol (15 ml), then hydrazine monohydrate (9.34 g, 187 mmol) was added and the mixture stirred at rt for 24 h. White solid formation was observed; the solid was collected by filtration, washed with copious amount of cold ethanol and then dried. ^{1}H NMR confirmed hydrazide **102b** (4.01 g, 73%), m.p. 142 – 144 ºC, ^{1}H NMR ($CDCl_3$) δ: 7.40 (br s, 2H, 2× CONH), 6.88 (½AB, 2H, *J* = 9.1 Hz, 3",5"-H), 6.83 (½AB, 2H, *J* = 9.1 Hz, 2",6"-H), 4.10 (br s, 4H, HNN$\underline{H}_2$), 3.77 (s, 3H, OCH_3), 3.34 (t, 4H, *J* = 5.8 Hz, 3,3'-H), 2.29 (t, 4H, *J* = 5.8 Hz, 2,2'-H); ^{13}C NMR (DMSO-d_6) δ: 173.4 (C-1,1'), 155.0 (C-4"), 142.4 (C -1"), 121.5 (C-3",5"), 114.8 (C-2",6"), 55.7 (OCH_3), 50.8 (C-3,3'), 33.4 (C-2,2'); IR (KBr) 3277s (NH), 2918m (CH st), 1641s (CONH), 1524s (C=C), 801m (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES): m/z 296.2 (100%) $(M+H)^+$.

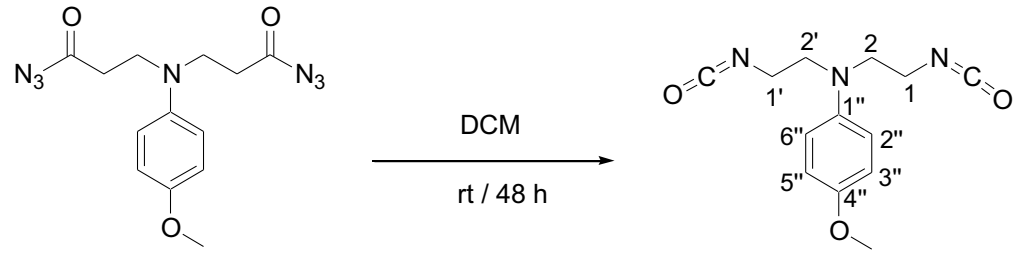
N-(4-Methoxyphenyl)iminodipropanoyl diazide 103b



102b **103b**

Hydrazide **102b** (0.37 g, 1.25 mmol) was dissolved in a mixture of aq. acetic acid solution (5 ml 0.64 M) and DCM (10 ml). The mixture was stirred on an ice bath at 0 – 5 º C; a dilute solution of $NaNO_2$ (0.86 g, 12.5 mmol) in H_2O (20 ml) was added gradually, keeping the exothermic reaction at 0 – 3 ºC. Further DCM (10ml) was added, the DCM layer separated, washed with H_2O (20ml) dried over $MgSO_4$ then filtered. Formation of azide **103b** was confirmed by IR and ^{1}H NMR. ^{1}H NMR ($CDCl_3$), δ: 6.84 (½AB, 2H, *J* = 9.1 Hz, 2",6"-H), 6.76 (½AB, 2H, *J* = 9.1 Hz, 3",5"-H), 3.75 (s, 3H, OCH_3), 3.50 (t, 4H, *J* = 7.1 Hz, 3,3'-H), 2.52 (t, 4H, *J* = 7.1 Hz, 2,2'-H); ^{13}C NMR ($CDCl_3$), δ: 179.3 (C-1,1'), 153.7 (C-4"), 141.1 (C-1"), 118.0 (C-3",5"), 115.1 (C-2",6"), 55.8 (OCH_3), 48.5 (C-3,3'), 35.3 (C-2,2'); IR (liq film) 2917w (CH_2 st), 2139s (N_3), 1712s (CO), 1513s (C=C), 1159m (C-O), 817m (*p*-di-substituted aromatic ring) cm^{-1}.

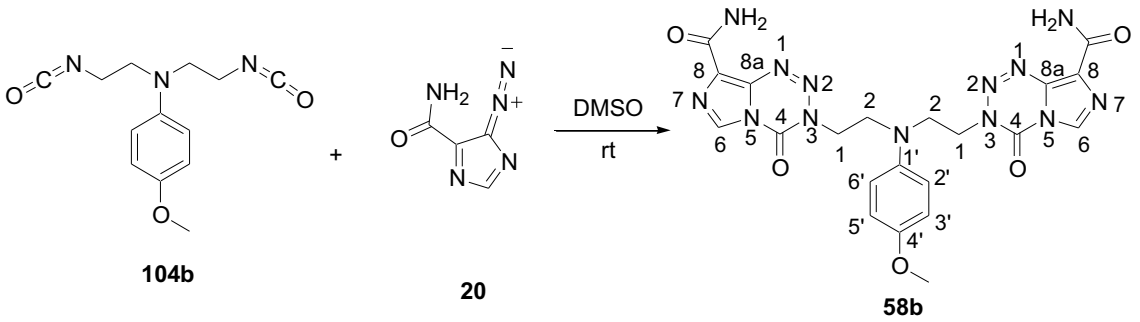
N,*N*-Di(2-isocyanatoethyl)-4-methoaniline 104b



103b **104b**

Azide **103b** solution in anhydrous DCM was stirred under nitrogen at r.t for 48 h. IR confirmed isocyanate **7** formation, it showed the strong band at 2271 cm^{-1}. The DCM was evaporated to a pale yellow oil **104b** (0.55 g, 69%). ^{1}H NMR ($CDCl_3$), δ: 6.87 (½AB, 2H, *J* = 9.1 Hz, 3",5"-H), 6.85 (½AB, 2H, *J* = 9.1 Hz, 2",6"-H), 3.77 (s, 3H, OCH_3), 3.43 (t, 4H, *J* = 6.2 Hz, 1,1'-H), 3.36 (t, 4H, *J* = 6.2 Hz, 2,2'-H); ^{13}C NMR ($CDCl_3$), δ: 154.6 (NCO), 140.5 (C-4"), 124.6 (C-1"), 119.6 (C-3",5"), 115.1 (C-2",6"), 55.7 (OCH_3), 44.6 (C-3,3'), 41.3 (C-2,2'); IR (liq film) 2919w (CH_2 st), 2268s (NCO), 1513s (C=C), 1181w (C-O), 818m (*p*-di-substituted aromatic ring) cm^{-1}; MS(ES) m/z: 262 (100%) $(M+H)^+$, 124 (97%) $(C_7H_9NO+H)^+$.

N,*N*-Bis(2-(8-Carbamoyl-4-oxo-3H,4H-imidazotetrazin-3-yl)ethyl)-4-methoxyaniline 58b

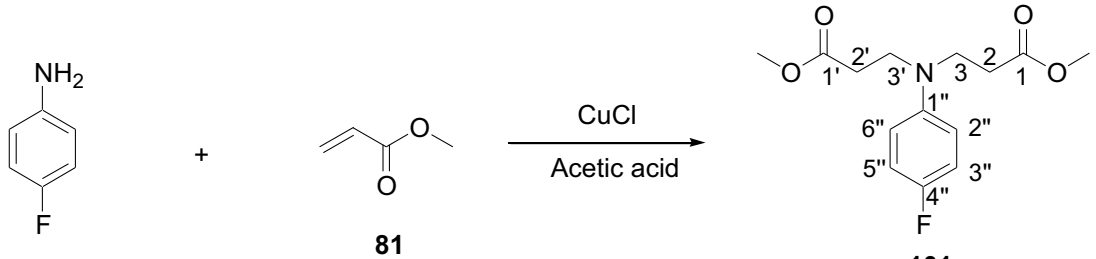


104b + 20 → 58b

Isocyanate **104b** (0.13 g, 0.49 mmol) was dissolved in DMSO (0.75 ml) then transferred under nitrogen to a suspension of diazo-IC **20** (0.07 g, 0.49 mmol) in DMSO (0.75 ml), the mixture was stirred at 35 °C protected from light. The reaction was monitored by ^{1}H NMR which was shown complete after 72 h. The ^{1}H NMR spectrum showed the imidazotetrazinone with some impurities. The mixture was suspended in H_2O (10 ml), filtered, then the residue was washed with copious amounts of water until the washings came through colourless. The crude solid (0.24 g) was collected and then subjected to purification by flash column chromatography eluted with 50% glacial acetic acid in $CHCl_3$. A red solid was collected from the fractions. The solid was re-dissolved in DMF (2 ml), filtered and then precipitated by addition of water. The red solid was collected by filtration, washed with copious amounts of water and then dried to **58b** (0.053 g, 22%), m.p. 178 – 179 ºC. ^{1}H NMR (DMSO-d_6) δ: 8.76 (s, 2H, 6-H), 7.74 & 7.65 (2 × br s, 4H, N$\underline{H_2}$CO), 6.76 (½AB, 2H, *J* = 9.1 Hz, 2',6'-H), 6.65 (½AB, 2H, *J* = 9.1 Hz, 3',5'-H), 4.42 (t, 4H, *J* = 6.4 Hz, 1,1-H), 3.75 (t, 4H, *J* = 6.4, 2,2-H), 3.58 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ: 162.0 ($CONH_2$), 152.1 (C-4'), 141.6 (C-1'), 139.6 (C-4), 134.8 (C-8a), 131.3 (C-8), 129.2 (C-6), 115.3 (C-2',6'), 115.0 (C-3',5'), 55.7 (OCH_3), 49.7 (C-1,1), 46.9 (C-2,2); IR (KBr), 3438m & 3341 (NH), 2914w, (CH st), 1745s (C(4)O), 1673s (CONH), 1612&1511 (C=C), 1093 (C-O), 826m (*p*-di-substituted aromatic ring)

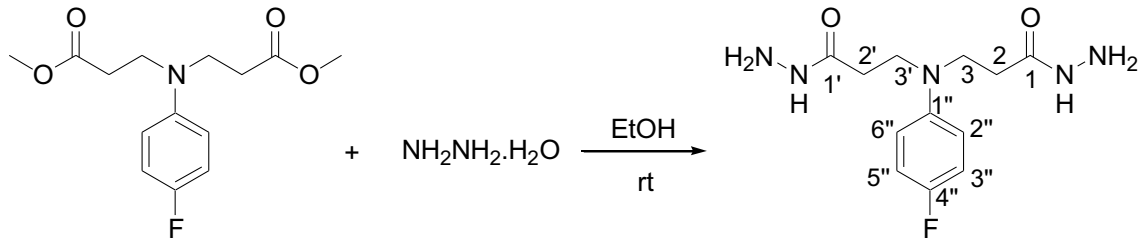
cm^{-1}; MS(ES): m/z 536.0 (73%)$(M+H)^+$, 558.0 (80%) $(M+Na)^+$, 261.9 (100%) $(C_{13}H_{15}N_3O_3+H)^+$; CHN Found: C, 47.16; H, 3.60; N, 33.44 $C_{21}H_{21}N_{13}O_5$, requires C, 47.1; H, 3.95; N, 34%.

Dimethyl *N*-(4-fluorophenyl)iminodipropanoate 101c[79]



p-Fluoroaniline (2.19 g, 19.7 mmol), CuCl (0.30 g, 3.15 mmol), acetic acid (20 ml) and methyl acrylate (17 g, 197 mmol) were mixed together. The reaction mixture was stirred at 140 ºC, overnight. TLC showed completion of the reaction, ^{1}H NMR confirmed product formation. The reaction mixture was then partitioned between equal volumes of $CHCl_3$/dil aqueous Na_2CO_3 (pH 12) (25 ml); the $CHCl_3$ layer was then separated, washed with three portions of H_2O (20ml), dried with anhydrous $MgSO_4$, decolourised with charcoal and then filtered through celite. The $CHCl_3$ was evaporated to give a yellow oil **101c** (4.4g, 78.6%). ^{1}H NMR 400 MHz ($CDCl_3$) δ: 6.95 (t, 2H, J_{HH} = J_{FH} = 9.1 Hz, 3",5"-H), 6.69 (dd, 2H, J_{HH} = 9.1 Hz, $^4J_{HF}$ = 4.1 Hz, 2",6"-H), 3.68 (s, 6H, 2 × CH_3), 3.60 (t, 4H, J = 7.1 Hz, 3,3'-H), 2.56 (t, 4H, J = 7.1 Hz, 2,2'-H); ^{13}C NMR 150.1 MHz ($CDCl_3$) 172.5 (C-1,1'), 156.0 (d, $^1J_{CF}$ = 235.5 Hz, C-4"), 143.6 (C-1"), 115.9 (d, $^2J_{CF}$ = 21.7 Hz, C-3",5"), 115.0 (d, $^3J_{CF}$ = 7.2 Hz, C-2",6"), 51.9 (OCH_3), 47.7 (C-3,3'), 32.3 (C-2,2'); IR (liq film) 3025m (Ar-H st), 2950m (CH st), 1725s (CO), 1525s (C=C). 1175m (C-O) cm^{-1}; MS(ES) m/z: 284.0 $(M+H)^+$.

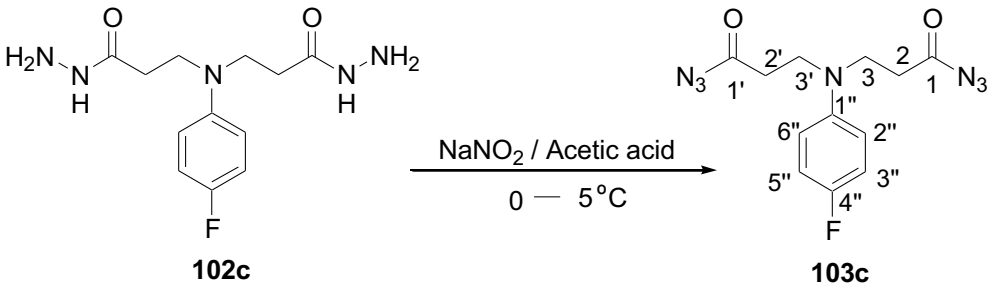
N-(4-Fluorophenyl)iminodipropanoyl diydrazide 102c[79]



101c **102c**

Ester **101c** (4.4 g, 15.53 mmol) was dissolved in ethanol (20 ml). Hydrazine monohydrate (7.8 g, 155.3 mmol) was then added and the mixture stirred at rt overnight. TLC confirmed the completion of the reaction. The volatile compounds were evaporated to give a viscous brown oil that crystallised on standing. The solid was re-crystallised from diethyl ether, collected and dried to give **102c** (3.8 g, 86%), m.p. 131 – 132 ºC; ^{1}H NMR ($CDCl_3$) δ: 7.26 (br s, 2H, 2 × CONH), 6.93 (t, 2H, $J_{HH} = J_{HF} = 9.1$ Hz, 3", 5"-H), 6.83 (dd, 2H, $J_{HH} = 9.1$Hz, $^{4}J_{HF} = 4.6$ Hz, 2",6"-H), 4.08 (br s, 4H, 2 × COHNNH_2), 3.39 (t, 4H, $J = 5.8$ Hz, 3,3'-H), 2.32 (t, 2H, $J = 5.8$ Hz, 2,2'-H); ^{13}C NMR ($CDCl_3$) δ: 173.2 (C-1,1'), 155.5 (d, $^{1}J_{CF} = 241.3$ Hz, C-4"), 144.5 (C-1") , 115.0 (d, $^{2}J_{CF} = 23.1$Hz, C-3",5"), 114.0 (d, $^{3}J_{CF} = 7.2$ Hz, C-2",6"), 50.1 (C-3,3'), 33.3 (C-2,2'); IR (KBr) 3245m (NH), 3032w (Ar-H st), 2947 w (CH st), 1620s (CONH), 1507m (C=C), 822m (*p*-di-substituted aromatic ring); MS (ES) m/z 284.2 (100%) $(M+H)^+$, 306 (60%) $(M+Na)^+$.

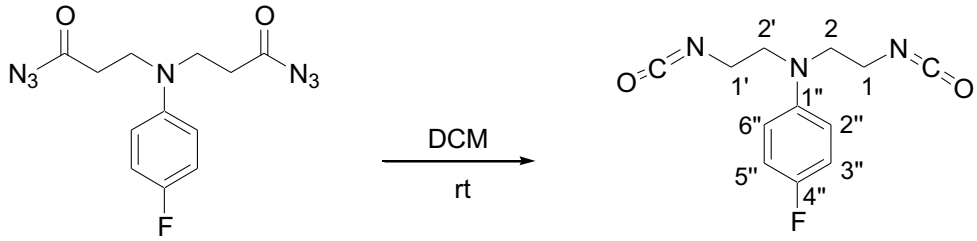
N-(4-Fluorophenyl)iminodipropanoyl diazide 103c



102c **103c**

Hydrazide **102c** (0.37 g, 1.31 mmol) was dissolved in a mixture of aq. acetic acid (0.66M, 15 ml) and DCM (15 ml), the mixture was then stirred on $CaCl_2$ ice bath at 0 ºC, a solution of $NaNO_2$ (0.9 g, 13.1 mmol) in H_2O (25 ml) was added dropwise keeping the exothermic reaction at 0 – 5 ºC. A Further amount of DCM (20 ml) was added, the DCM layer separated, washed with H_2O (50ml) dried over $MgSO_4$, then filtered. Formation of azide **103c** was confirmed by IR. ^{1}H NMR showed small amount of impurities. ^{1}H NMR ($CDCl_3$), δ: 6.94 (t, 2H, $J_{HH} = J_{HF} = 9.1$ Hz, 3", 5"-H), 6.67 (dd, 2H, $J_{HH} = 9.1$ Hz, $^4J_{HF} = 4.3$ Hz, 2",6"-H), 3.55 (t, 4H, $J = 7.1$ Hz, 3,3'-H), 2.55 (t, 4H, $J = 7.1$ Hz, 2,2'-H); ^{13}C NMR ($CDCl_3$), δ: 179.1 (C-1,1'), 155.6 (d, $^1J_{CF} = 235.5$ Hz, C-4"), 143.3 (C-1"), 117.0 (d, $^2J_{CF} = 23.1$Hz, C-3",5"), 116.1 (d, $^3J_{CF} = 7.2$ Hz, C-2",6"), 47.9 (C-3,3'), 35.1 (C-2,2'); IR (liq film) 2914w (CH st), 2137s (CON_3), 1711s (CO), 1511m (C=C), 815s (*p*-di-substituted aromatic ring) cm^{-1}.

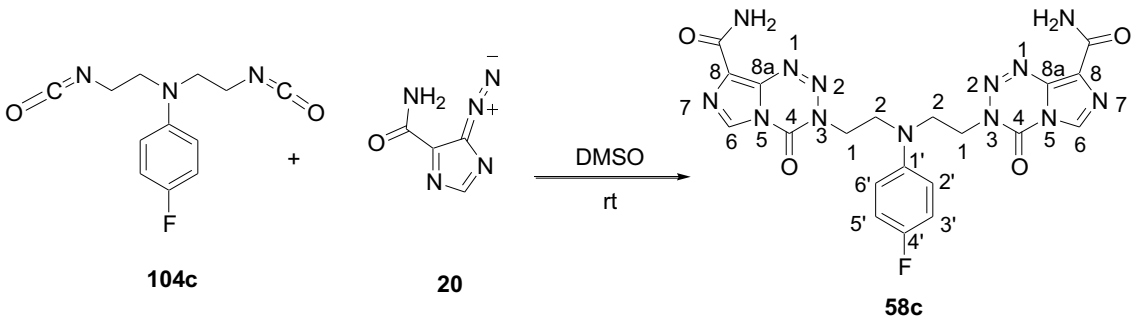
N,N-Di(2-isocyanatoethyl)4-fluoroaniline104c



103c **104c**

The anhydrous DCM solution of azide **103c** was stirred under nitrogen at rt overnight. Isocyanate **104c** formation was confirmed by IR and ^{1}H NMR. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature). Isocyanate **104c** was then collected as a yellow oil (0.192 g, 56.2 %). ^{1}H NMR ($CDCl_3$), δ: 6.97 (t, 2H, $J_{HH} = J_{HF} = 9.1$ Hz, 3",5"-H), 6.79 (dd, 2H, $J_{HH} = 9.1$. $^{4}J_{HF} = 4.3$ Hz, 2",6"-H), 3.49 (t, 4H, $J = 6.1$ Hz, 2,2'-H), 3.40 (t, 4H, $J = 6.1$ Hz, 1,1'-H); ^{13}C NMR ($CDCl_3$), δ: 157.3 (d, $^{1}J_{CF} = 239.9$ Hz, C-4"), 142.9 (C-1"), 124.3 (NCO), 117.6 (d, $^{2}J_{CF} = 7.2$ Hz, C-2", 6"), 116.3 (d, $^{3}J_{CF} = 23.1$ Hz, C-3", 5"), 53.8 (C-2,2'), 41.0 (C-1,1'); IR (liq film) 3057w (Ar-H st), 2917w (CH st), 2270s (NCO), 1512m (C=C), 1228m, 816s (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES) m/z 249.9 (100%) $(M+H)^{+}$, 206.8 (62%) $(C_{11}H_{12}FN_2O)^{+}$, 193.0 (59%) $(C_{10}H_{10}FN_2O)^{+}$.

N,N-Bis(2-(8-Carbamoyl-4-oxo-3H,4H-imidazotetrazin-3-yl)ethyl)-4-fluoroaniline 58c

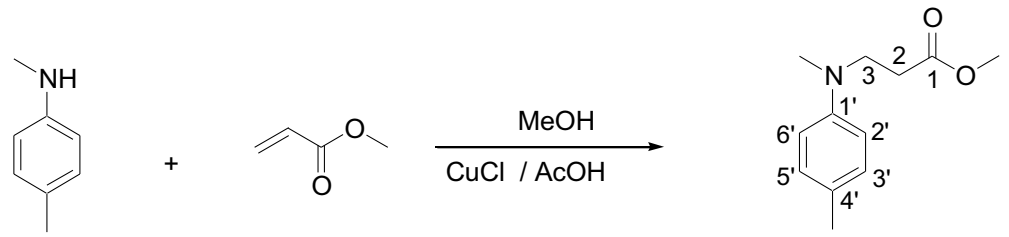


104c 20 58c

Isocyanate **104c** (0.192 g, 0.77 mmol) was diluted with DMSO (1.5 ml) under nitrogen then added to a suspension of diazo-IC **20** (0.11 g, 0.77 mmol) in DMSO (1.5 ml), the mixture was stirred at rt protected from light for 48 h and the reaction monitored by ^{1}H NMR. The reaction mixture was suspended in water (30 ml), filtered and the residue washed with copious amounts of water until the washings came through colourless, and then washed with ether. The dry yellow solid collected and purified by flash column chromatography eluting with 50% AcOH / $CHCl_3$. ^{1}H NMR showed some impurities from the silica; the solid was then dissolved in DMF, DMF solution filtered, and the imidazotetrazinone precipitated by addition of water. A yellow solid was collected by filtration and dried to give tetrazine **58c** (0.067 g, 17%) m.p. 290 – 291 ºC; ^{1}H NMR (DMSO-d_6) δ: 8.76 (s, 2H, 6-H), 7.70 & 7.61 (2 × br s, 4H, $CONH_2$), 6.92 (t, 2H, J_{HH} = J_{HF} = 9.1 Hz, 3',5'-H), 6.89 (dd, 2H, J_{HH} = 9.1 Hz, $J_{H\text{-}F}$ = 4.3 Hz, 2',6'-H), 3.44 (t, 4H, J = 6.7 Hz, 1,1-H), 3.78 (t, 4H, J = 6.7 Hz, 2,2-H); ^{13}C NMR ($CDCl_3$), δ: 162.0 ($CONH_2$), 155.3 (d, $^{1}J_{CF}$ = 235.5 Hz, C-4'), 144.1 (C-1'), 139.7 (C-4), 134.8 (C-8a), 131.4 (C-8), 129.3 (C-6), 116.0 (d, $^{2}J_{CF}$ = 20.2 Hz, C-3', 5'), 114.2 (d, $^{3}J_{CF}$ = 7.2 Hz, C-2', 6'), 49.5 (C-2, 2), 46.6 (C-1, 1); IR (KBr) 3464s (NH), 3133m (Ar-H st), 1731s (C(4)O), 1684s ($CONH_2$), 1512 s & 1458m, (C=C), 823m (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES): m/z 524.0 (10%) $(M+H)^+$, 250.1 (10%) $(C_{12}H_{12}FN_3O^2 + H)^+$;

CHN Found: C, 45.47; H, 3.04; N, 33.82 $C_{20}H_{18}FN_{13}O_4$, requires C, 45.09; H, 3.41; N, 34.01%.

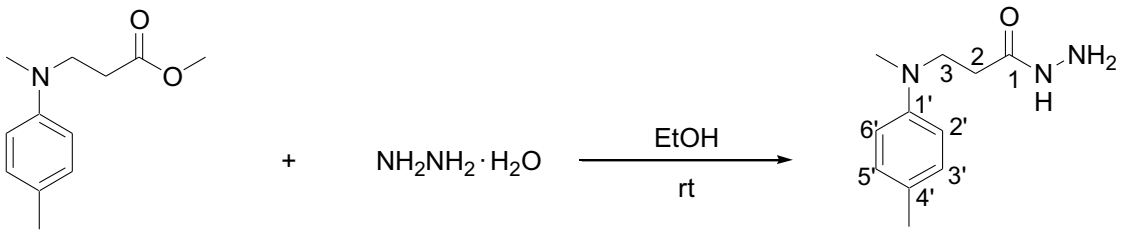
Methyl 3-(*N*-methyl-*N*-(4-methylphenyl)amino)propanoate 106a[79]



105a **81** **106a**

N-Methyl-*p*-tolylamine **105a** (2.0 g, 16.5 mmol) was dissolved in methanol (10 ml), then CuCl (0.16 g, 1.7 mmol), AcOH (2ml) and methylacrylate **81** (7.1 g, 82.5 mmol) were added. The reaction mixture was stirred at reflux, overnight. TLC showed the complete disappearance of the aniline, ^{1}H NMR confirmed the product formation. The reaction mixture was partitioned between equal volumes of $CHCl_3$ / dil aqueous Na_2CO_3 solution (pH 12) (25 ml); the $CHCl_3$ layer was then separated, washed with three portions of H_2O (20ml), dried over anhydrous $MgSO_4$, decolourised with charcoal and then filtered through celite 521. The $CHCl_3$ was evaporated to a yellow oil **106a** (2.3 g, 67.3%). ^{1}H NMR ($CDCl_3$) δ: 7.05 (½AB, 2H, J = 8.7 Hz, 3',5'-H), 6.67 (½AB, 2H, J = 8.7 Hz, 2',6'-H), 3.68 (s, 3H, OCH_3), 3.64 (t, 2H, J = 7.2 Hz, 3-H), 2.90 (s, 3H, NCH_3) 2.56 (t, 2H, J = 7.2 Hz, 2-H), 2.26 (s, 3H, Ar-CH_3); ^{13}C NMR ($CDCl_3$) δ: 172.9 (C-1), 146.7 (C-1'), 129.9 (C-2', 6'), 126.3 (C-4'), 113.1 (C-3', 5'), 51.8 (OCH_3), 49.1 (C-3), 38.5 (NCH_3), 31.5 (C-2), 20.3 (CH_3-Ar); IR (liq film) 2918w (CH st), 1736s (CO), 1616m, 1520s (C=C), 1169 (C-O), 803m (*p*-di-substituted aromatic ring) cm^{-1}; MS(ES) m/z: 208.2 (100%) $(M+H)^+$.

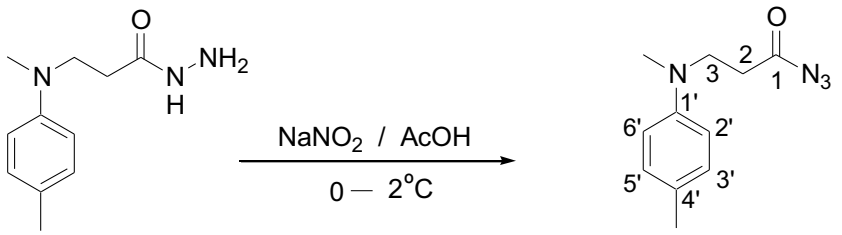
3-(*N*-Methyl-*N*-(4-methylphenyl)amino)propanoyl hydrazide 107a[79]



106a **107a**

Ester **106a** (1 g, 4.8 mmol) was dissolved in EtOH (10 ml). Hydrazine monohydrate (1.4 g, 28.8 mmol) was then added and the mixture stirred at rt overnight. TLC confirmed completion of the reaction. The volatile compounds were evaporated to give a viscous brown oil. The oil was dissolved in hot Et_2O and a white solid crystallised on cooling, the solid was then collected by filtration, washed with cold diethyl ether, collected and then dried as **107a** (0.71g, 71%), mp 73 – 75 ºC; ^{1}H NMR ($CDCl_3$) δ: 7.42 (br s, 1H, CONH), 7.05 (½AB, 2H, *J* = 8.6 Hz, 3',5'-H), 6.68 (½AB, 2H, *J* = 8.6 Hz, 2',6'-H), 3.89 (br s, 2H, COHNN$\underline{H_2}$), 3.59 (t, 2H, *J* = 6.7 Hz, 3-H), 2.86 (s, 3H, NCH_3), 2.37 (t, 2H, *J* = 6.7 Hz, 2-H), 2.23 (s, 3H, Ar-CH_3); ^{13}C NMMR ($CDCl_3$) δ: 172.7 (C-1), 146.7 (C -1'), 129.9 (C-2', 6'), 127.0 (C-4'), 113.8 (C-3', 5'), 49.7 (NCH_3), 39.1 (C-3), 31.9 (C-2), 20.3(CH_3-Ar); IR (KBr), 3314s (NH), 2915w (CH st), 1630s (CONH), 1601w, 1523 (C=C), 1366m, 800m (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES) m/z 208.2 (100%) $(M+H)^+$.

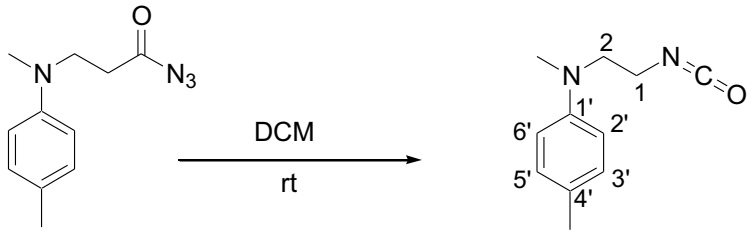
3-(*N*-Methyl-*N*-(4-methylphenyl)amino)propanoyl azide 108a



107a **108a**

Hydrazide **107a** (0.11 g, 0.53 mmol) was dissolved in a mixture of AcOH (0.16 M, 10 ml) and DCM (10 ml), the mixture was stirred on a $CaCl_2$ ice bath at 0 – 2 ºC, a solution of $NaNO_2$ (0.22 g, 3.2 mmol) in H_2O (15 ml) was added gradually keeping the exothermic reaction between 0 – 2 ºC. A further portion of DCM (15ml) was added, the DCM layer was separated, washed with H_2O (20ml) dried over $MgSO_4$, then filtered. The formation of the azide **108a** was confirmed by IR and ^{1}H NMR. ^{1}H NMR ($CDCl_3$), δ: 7.05 (½AB, 2H, *J* = 8.6 Hz, 3',5'-H), 6.66 (½AB, 2H, *J* = 8.6 Hz, 2',6'-H), 3.63 (t, 2H, *J* = 7.1 Hz, 3-H), 2.88 (s, 3H, NCH_3), 2.57 (t, 2H, *J* = 7.1 Hz, 2-H), 2.24 (s, 3H, CH_3-Ar); ^{13}C NMR ($CDCl_3$), δ: 179.4 (C-1), 146.5 (C-1'), 129.9 (C-2', 6'), 126.7 (C-4'), 113.3 (C-3', 5'), 48.9 (C-3), 38.6 (NCH_3), 34.2 (C-2), 20.3 (CH_3-Ar); IR (liq film) 2918w (CH st), 2137s (CON_3), 1714s (CO), 1522s (C=C), 1158m, 804m (*p*-di-substituted aromatic ring) cm^{-1}.

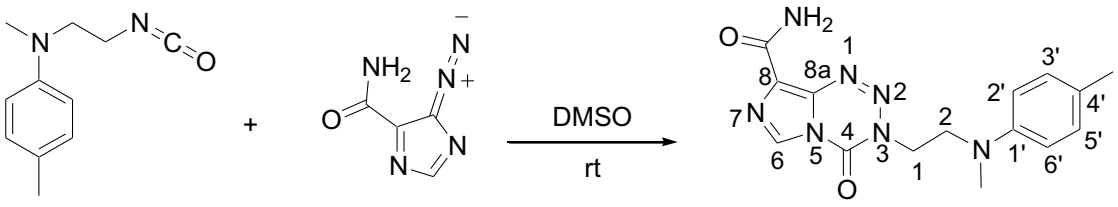
2-(*N*-Methyl-*N*-(4-methylphenyl)amino)ethyl isocyanate 109a



108a **109a**

The DCM solution of azide **108a (**0.1g, 0.5 mmol) was stirred under nitrogen at rt overnight. Isocyanate **109a** formation was confirmed by IR and ^{1}H NMR. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature) and isocyanate **109a** collected as a yellow oil (0.066 g, 69 %). ^{1}H NMR ($CDCl_3$), δ: 7.05 (½AB, 2H, *J* = 8.6 Hz, 3',5'-H), 6.65 (½AB, 2H, *J* = 8.6 Hz, 2',6'-H), 3.45 (t, 2H, *J* = 5.6 Hz, 2-H), 3.44 (t, 2H, *J* = 5.6 Hz, 1-H), 2.95 (s, 3H, NCH_3), 2.24 (s, 3H, CH_3-Ar); ^{13}C NMR ($CDCl_3$), δ: 154.8 (NCO), 146.9 (C-1'), 129.9 (C-2',6'), 127.0 (C-4'), 113.4 (C-3',5'), 54.1 (C-2), 41.0 (C-1), 39.4 (NCH_3), 20.3 (CH_3-Ar); IR (liq film) 2920m (CH st), 2268s (NCO), 1522s (C=C), 805m (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES) m/z 191 (100%) $(M+H)^+$, 213 (22%) $(M+Na)^+$.

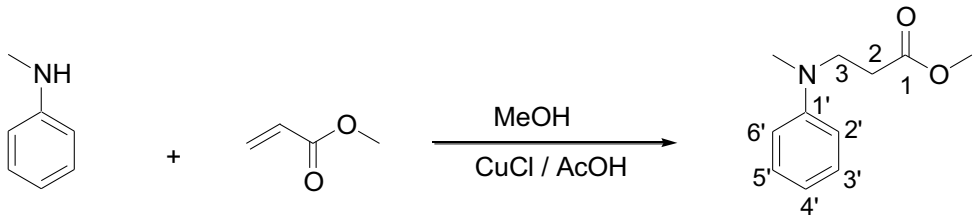
3-{2-[Methyl(4'-methylphenyl)amino]ethyl}-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 59a



109a **20** **59a**

Isocyanate **109a** (0.3 g, 0.15 mmol) was diluted with DMSO (0.75 ml), then added under nitrogen to a suspension of diazo-IC **20** (0.8 g, 5.7 mmol) in DMSO (0.75 ml), the mixture was stirred at rt protected from light for 48h. The ^{1}H NMR spectrum showed product formation. The reaction mixture was then suspended in water (10 ml) and filtered. The solid on the filter was washed with copious amounts of H_2O until the washings came through colourless, then with Et_2O, ^{1}H NMR showed some impurities which were washed out from the solid by chromatography through a 5 cm height silica column with 10% AcOH in $CHCl_3$. An orange solid was collected from the fractions **59a** (0.08 g, 14%), m.p. 172 – 173 ºC ^{1}H NMR (DMSO-d_6) δ: 8.72 (s, 1H, 6-H), 7.73 & 7.64 (2 × br s, 2H, NH_2CO), 6.83 (½AB, 2H, *J* = 8.3 Hz, 3',5'-H), 6.57 (½AB, 2H, *J* = 8.3 Hz, 2',6'-H), 4.45 (t, 2H, *J* = 6.3 Hz, 1-H), 3.77 (t, 2H, *J* = 6.3, 2-H), 2.87 (s, 3H, NCH_3), 2.04 (s, 3H, CH_3-Ar); ^{13}C NMR (DMSO-d_6) δ: 161.9 ($CONH_2$), 146.9 (C-1'), 139.5 (C-4), 134.7 (C-8a), 131.2 (C-8), 129.7 (C-2',6') , 129.1 (C-6), 125.3 (C-4'), 112.9 (C-3',5'), 51.5 (C-2), 43.4 (C-1), 38.1 (NCH_3), 20.3 (CH_3-Ar); IR (KBr) 3429s (NH), 2917w (CH st), 1736s (C(4)O), 1680 ($CONH_2$), 1523s (C=C), 1458s, 1361m, 975m, 804 (*p*-di-substituted aromatic ring) cm^{-1}; MS(ES): m/z 350.1 (100%) $(M+Na)^+$, 327.9 (50%) $(M+H)^+$, 191 (100%) $(C_{11}H_{14}N_2O+H)^+$; CHN Found: C, 53.69; H, 4.92; N, 28.03 $C_{15}H_{17}N_7O_2 \cdot 0.3\ C_2H_4O_2 \cdot 0.1CHCl_3$, requires C, 53.62; H, 5.22; N, 28.15%.

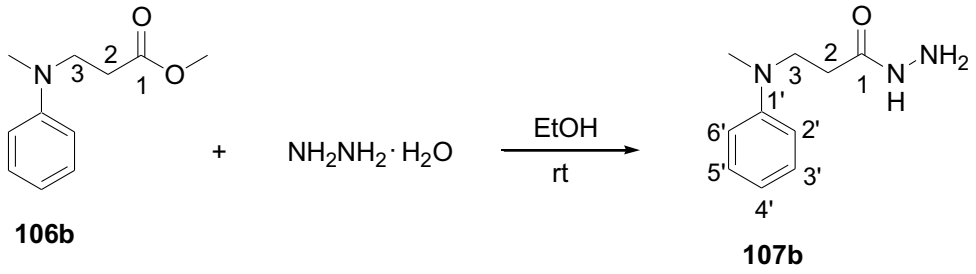
Methyl 3-(*N*-methyl-*N*-phenylamino)propanoate 106b[79]



105b **81** **106b**

N-Methylaniline **105b** (3.0 g, 28 mmol) was dissolved into methanol (10 ml) and then CuCl (0.28 g, 2.8 mmol), acetic acid (2ml) and methyl acrylate **81** (12.1 g, 138 mmol) were added. The reaction mixture was then stirred at reflux, overnight. The reaction mixture was partitioned between equal volumes of $CHCl_3$ / dil. aqueous Na_2CO_3 solution (pH 12) (30 ml); the $CHCl_3$ layer was then separated, washed with three portions of H_2O (25ml), dried on anhydrous $MgSO_4$, decolourised with charcoal and then filtered through celite. The $CHCl_3$ was evaporated to give a yellow oil **106b** (5.3 g, 97%). ^{1}H NMR ($CDCl_3$) δ: 7.24 (t, 2H, *J* = 8.5 Hz, 3',5'-H), 6.74 (d, 2H, *J* = 8.5 Hz, 2',6' -H), 6.74 (t, 1H, *J* = 8.5Hz, 4'-H), 3.68 (s, 3H, OCH_3), 3.67 (t, 2H, *J* = 7.2 Hz, 3-H), 2.93 (s, 3H, NCH_3) 2.58 (t, 2H, *J* = 7.2 Hz, 2-H); ^{13}C NMR ($CDCl_3$) δ: 172.7 (C-1), 148.7 (C-1'), 129.4 (C-3',5'), 116.9 (C-4'), 112.6 (C-2',6'), 51.7 (OCH_3), 48.7 (C-3), 38.3 ($N\underline{C}H_3$), 31.6 (C-2); IR (liq film) 2952 w (CH st), 1736s (CO), 1601m, 1507m (C=C), 1171 (C-O), 750&691 (mono-substituted aromatic ring) cm^{-1}; MS(ES): m/z 120.2 (60%) $(C_8H_{10}N)^+$, 194.2 (100%) $(M+H)^+$.

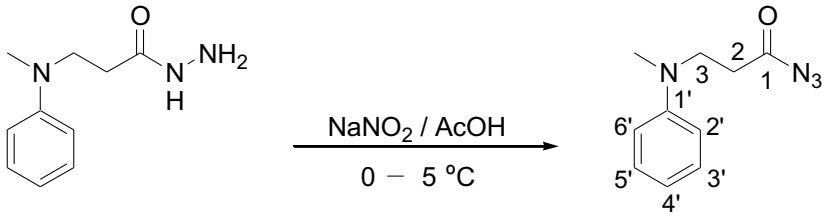
3-(*N*-Methyl-*N*-phenyl-amino)propanoyl hydrazide 107b[79]



106b **107b**

Ester **106b** (2.44 g, 12.6 mmol) was dissolved in ethanol (10 ml). Hydrazine monohydrate (3.8 g, 75.8 mmol) was then added and the mixture stirred at rt overnight. TLC confirmed the completion of the reaction. The volatile compounds were evaporated to give a viscous brown oil. The oil was suspended in diethyl ether with stirring, it was converted to a sticky solid. The suspension was heated and then the ether layer decanted, a white solid crystallised on cooling. The solid was then filtered, collected and dried to give white solid **107b** (2.3g, 94%), m.p. 66 – 68 ºC; ^{1}H NMR ($CDCl_3$) δ: 7.49 (br s, 1H, CONH), 7.21 (t, 2H, J = 8.1 Hz, 3',5'-H), 6.71 (t, 1H, J = 8.1 Hz, 4'-H), 6.70 (d, 2H, J = 8.1 Hz, 2',6'-H), 3.84 (br s, 2H, COHNN$\underline{H_2}$), 3.64 (t, 2H, J = 6.7 Hz, 3-H), 2.85 (s, 3H, NCH_3) 2.33 (t, 2H, J = 6.7 Hz, 2-H); ^{13}C NMR ($CDCl_3$) δ: 172.6 (C-1), 148.7 (C-1'), 129.4 (C-3',5'), 117.2 (C-4'), 113 (C-2',6'), 49.3 (C-3), 38.7 (NCH_3), 31.9 (C-2); IR (KBr), 3322m (NH), 3034 (Ar-H st), 2947w (CH st), 1635s (CONH), 1606w, 1496 (C=C), 750&691s (mono-substituted aromatic ring) cm^{-1}; MS (ES) m/z 194.2 (100%) $(M+H)^+$, 216.1 (18%) $(M+Na)^+$.

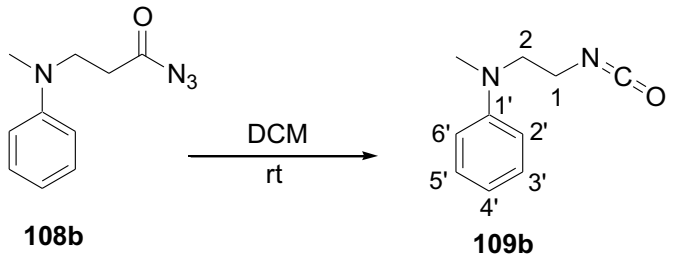
3-(*N*-Methyl-*N*-phenylamino)propanoyl azide 108b



107b **108b**

Hydrazide **107b** (0.54 g, 2.8 mmol) was dissolved in a mixture of aq. Acetic acid (0.32M, 10 ml) and DCM (10 ml), the mixture was stirred on a $CaCl_2$ ice bath at 0 ºC. A solution of $NaNO_2$ (1.2 g, 16.8 mmol) in H_2O (25 ml) was added gradually, keeping the exothermic reaction at 0 – 5 ºC. A further portion of DCM (10ml) was added, the DCM layer separated, washed with H_2O (20 ml) dried over $MgSO_4$, then filtered. Formation of the azide **108b** was confirmed by IR and ^{1}H NMR. ^{1}H NMR ($CDCl_3$), δ: 7.25 (t, 2H, *J* = 8.6 Hz, 3',5'-H), 6.73 (d, 2H, *J* = 8.6 Hz, 2',6'-H), 6.73 (t, 1H, *J* = 8.6 Hz, 4'-H), 3.68 (t, 2H, *J* = 7.1 Hz, 3-H), 2.91 (s, 3H, NCH_3), 2.60 (t, 2H, *J* = 7.1 Hz, 2-H); ^{13}C NMR ($CDCl_3$), δ: 179.3 (C-1), 148.5 (C-4'), 129.4 (C-3',5'), 117.2 (C-4'), 112.7 (C-2',6'), 48.5 (C-3), 38.4 (NCH_3), 34.3 (C-2); IR (liq film) 2923w (CH st), 2142s (CON_3), 1715s (CO), 1603s, 1523s (C=C) cm^{-1}.

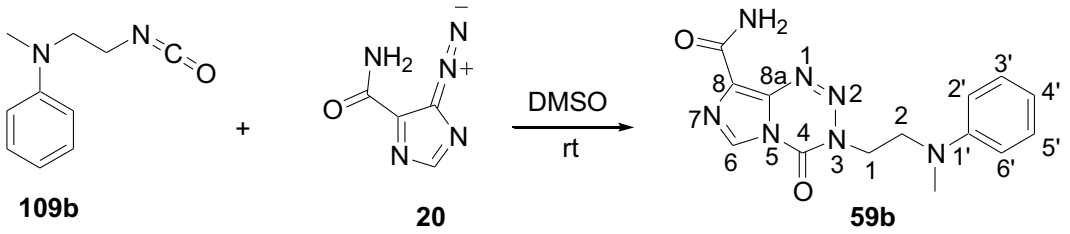
2-(*N*-Methyl-*N*-phenylamino)ethyl isocyanate 109b



108b **109b**

The anhydrous DCM solution of azide **108b** was stirred under nitrogen at rt overnight. The isocyanate formation was confirmed by IR and ^{1}H NMR. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature) and the isocyanate **109b** collected as a yellow oil (0.4 g, 70 %). ^{1}H NMR ($CDCl_3$), δ: 7.25 (t, 2H, *J* = 8.6 Hz, 3',5'-H), 6.76 (t, 1H, *J* = 8.6 Hz, 4'-H), 6.74 (d, 2H, *J* = 8.6 Hz, 2',6'-H), 3.53 (t, 2H, *J* = 6.1 Hz, 2-H), 3.48 (t, 2H, *J* = 6.1 Hz, 1-H), 3.16 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$), δ: 155.6 (NCO), 148.8 (C-1'), 129.4 (C-3',5'), 117.5 (C-4'), 112.8 (C-2',6'), 53.7 (C-2), 40.9 (C-1), 39.2 (NCH_3), IR (liq film) 2919w (CH st), 2270s (NCO), 1601s, 1506s (C=C), 750m, 694m (mono-substituted aromatic ring) cm^{-1}; MS (ES) m/z 177 (100%) $(M+H)^{+}$.

3-{2-[Methyl(phenyl)amino]ethyl}-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 59b

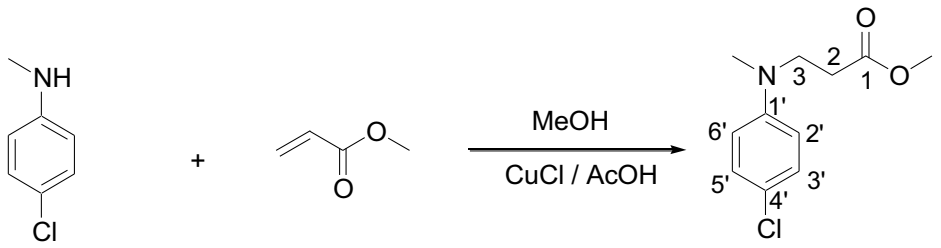


109b + **20** → **59b**

Isocyanate **109b** (0.4 g, 2.3 mmol) was diluted with DMSO (1.5 ml), then added under nitrogen to a suspension of diazo-IC **20** (0.3 g, 2.3 mmol) in DMSO (1.5 ml), the mixture was stirred at rt protected from light for 48 h. The ^{1}H NMR spectrum showed product formation. The reaction mixture was suspended in H_2O (10 ml) and filtered. The solid on the filter was washed with copious amounts of H_2O until the washings came through colourless, then with Et_2O, ^{1}H NMR showed some impurities which were washed out from the solid with $CHCl_3$. Further purification using short column chromatography eluted with 20% glacial AcOH in $CHCl_3$ gave a yellow solid collected from the fractions after evaporation of the solvent. The solid was then dissolved in DMF (3 ml), and the DMF solution filtered. The solid was precipitated from DMF by addition of water (10 ml). The solid was collected by filtration, washed with water and dried to give **59b** (0.16 g, 22%) m.p. 186 – 187 ºC. ^{1}H NMR (DMSO-d_6) δ: 8.78 (s, 1H, 6-H), 7.77 & 7.67 (2 × br s, 2H, $CONH_2$), 7.07 (t, 2H, *J* = 8.3 Hz, 3',5'-H), 6.70 (d, 2H, *J* = 8.3Hz , 2',6'-H), 6.51 (t, 1H, *J* = 8.3Hz, 4'-H), 4.46 (t, 2H, *J* = 6.6 Hz, H-1), 3.80 (t, 2H, *J* = 6.6, H-2), 2.93 (s, 3H, NCH_3); ^{13}C NMR (DMSO-d_6) δ: 161.9 ($CONH_2$), 149.0 (C-1'), 139.5 (C-4), 134.6 (C-8a), 131.3 (C-8), 129.4 (C-3',5') , 129.2 (C-6), 116.6 (C-4'), 112.4 (C-2',6'), 50.3 (C-2), 46.4 (C-1), 38.3 (NCH_3); IR (KBr) 3436m (NH), 3121w (Ar-H st), 2913 (CH st), 1748s (C(4)O), 1683 (CONH), 1599,1507, 1462 (C=C), 750m & 694m (mono-substituted aromatic ring) cm^{-1}; MS(ES): m/z 335.9 (100%)$(M+Na)^+$,

314 (20%) $(M+H)^+$, 176.9 (100%)$(C_{10}H_{12}N_2O+H)^+$, CHN Found: C, 53.61; H, 4.84; N, 31.63 $C_{14}H_{15}N_7O_2$, requires C, 53.67; H, 4.83; N, 31.29%.

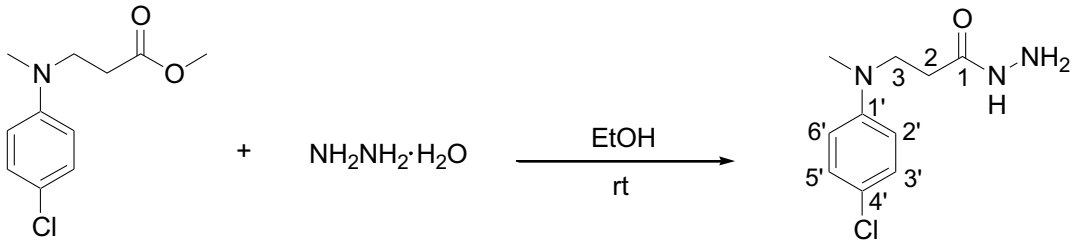
Methyl 3-(*N*-(4-chlorophenyl)-*N*-methylamino)propanoate 106c[92]



105c **81** **106c**

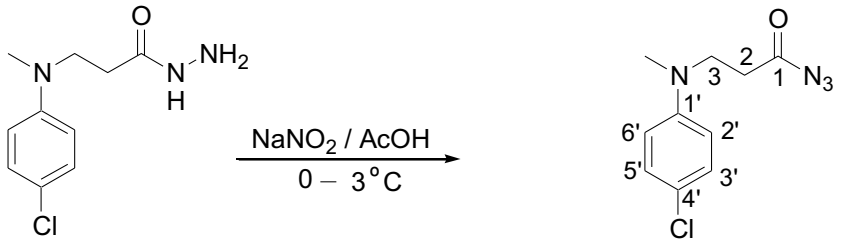
4-Chloro-*N*-methylaniline **105c** (0.55 g, 3.9 mmol) was dissolved into MeOH (5 ml), then CuCl (0.12 g, 1.24 mmol), AcOH (2 ml) and methylacrylate (1.7 g, 19.4 mmol) were added. The reaction mixture was then stirred at reflux for 48 h. TLC showed the completion of the reaction ^{1}H NMR confirmed product formation. The reaction mixture was then partitioned between equal volumes of $CHCl_3$ / dil aqueous Na_2CO_3 solution (pH 12) (25 ml); the $CHCl_3$ layer was separated, washed with three portions of H_2O (20ml), dried on anhydrous $MgSO_4$, decolourised with charcoal and then filtered through celite. The $CHCl_3$ was evaporated to give a yellow oil **106c** (0.70 g, 79%). ^{1}H NMR ($CDCl_3$) δ: 7.15 (½AB, 2H, *J* = 8.6 Hz, 3',5'-H), 6.62 (½AB, 2H, *J* = 8.6Hz, 2',6'-H), 3.65 (s, 3H, OCH_3), 3.62 (t, 2H, *J* = 7.1 Hz, 3-H), 2.89 (s, 3H, NCH_3) 2.54 (t, 2H, *J* = 7.1 Hz, 2-H); ^{13}C NMR ($CDCl_3$) 172.6 (C-1), 147.3 (C-1'), 129.1 (C-2',6'), 121.8 (C-4'), 113.7 (C-3',5'), 51.9 (OCH_3), 48.8 (C-3), 38.4 (NCH_3), 31.5 (C-2), IR (liq film) 3025 (Ar-CH st), 2950 w (CH st), 1725s (CO), 1601m, 1507m (C=C), 1171 (C-O) cm^{-1}; MS(ES): m/z 228.1 (100%) $(M+H)^+$.

3-(*N*-(4-Chlorophenyl)-*N*-methylamino)propanoyl hydrazide 107c[79]



Ester **106c** (0.7g, 3.1 mmol) was dissolved in EtOH (10 ml). Hydrazine monohydrate (0.9g, 18.4 mmol) was added and the mixture stirred at rt overnight. TLC confirmed the completion of the reaction. The volatile compounds were evaporated to a viscous brown oil that crystallised on standing. The solid was re-crystallised from Et_2O, collected by filtration and then dried, a second crop was collected from the filtrate: **107c** (0.6g, 85%), m.p. 103 – 105 ºC. ^{1}H NMR ($CDCl_3$) δ: 7.16 (½AB, 2H, *J* = 8.8 Hz, 3',5'-H), 6.82 (br s, 1H, CONH), 6.63 (½AB, 2H, *J* = 8.9 Hz, 2',6'-H), 3.85 (br s, 2H, COHNN$\underline{H_2}$), 3.64 (t, 2H, *J* = 6.7 Hz, 3-H), 2.88 (s, 3H, NCH_3), 2.35 (t, 2H, *J* = 6.7 Hz, 2-H); ^{13}C NMR ($CDCl_3$) δ: 172.2 (C-1), 147.3 (C-1'), 129.2 (C-2',6'), 122.1 (C-4'), 114.0 (C-3',5'), 49.3 (C-3), 38.9 (NCH_3), 31.8 (C-2); IR (KBr) 3312s (NH), 3036w (Ar-CH st), 2946 w (CH st), 1637s (CONH), 1593m, 1524m, 1500m (C=C), 810m (*p*-di-substituted aromatic ring) cm^{-1}; MS(ES): m/z 228.0 (100%) $(M+H)^+$, 250 (65%) $(M+Na)^+$.

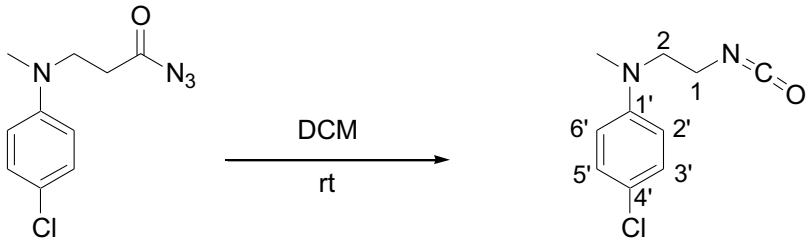
3-(*N*-(4-Chlorophenyl)-*N*-methylamino)propanoyl azide 108c



17c **18c**

Hydrazide **107c** (0.38 g, 1.6 mmol) was dissolved in a mixture of aq. Acetic acid (0.66 M 15 ml) and DCM (15 ml), the mixture was stirred on a $CaCl_2$-ice bath at 0 ºC. A solution of $NaNO_2$ (0.7 g, 10.0 mmol) in H_2O (25 ml) was added gradually, keeping the exothermic reaction between 0 – 3 ºC. A further portion of DCM (15ml) was added, the DCM layer was separated, washed with H_2O (20 ml) dried over $MgSO_4$, then filtered. Formation of the azide **108c** was confirmed by IR and ^{1}H NMR. ^{1}H NMR ($CDCl_3$), δ: 7.20 (½AB, 2H, *J* = 9.0 Hz, 3',5'-H), 6.66 (½AB, 2H, *J* = 9.0 Hz, 2',6'-H), 3.67 (t, 2H, *J* = 7.1 Hz, 3-H), 2.93 (s, 3H, NCH_3), 2.60 (t, 2H, *J* = 7.1 Hz, 2-H); ^{13}C NMR ($CDCl_3$), δ: 179.2 (C-1), 147.1 (C-1'), 129.2 (C-2',6'), 122.1 (C-4'), 113.9 (C-3',5'), 48.6 (C-3), 38.6 (NCH_3), 34.2 (C-2); IR 2913w (CH st), 2139s (CON_3), 1714s (CO), 1597m, 1501s (C=C), 812s (*p*-di-substituted aromatic ring) cm^{-1}.

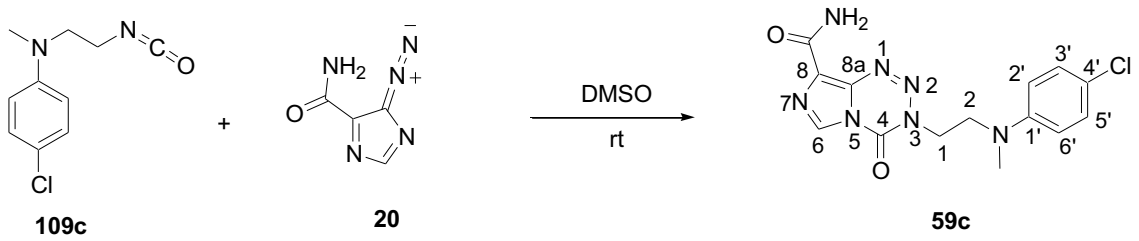
2-(*N*-(4-Chlorophenyl)-*N*-methylamino)ethyl isocyanate 109c



108c **109c**

The anhydrous DCM solution of azide **108c** was stirred under nitrogen at rt overnight. The isocyanate formation was confirmed by IR and ^{1}H NMR. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature) the isocyanate **109c** was collected as a yellow oil (0.37 g, 87.2 %). ^{1}H NMR ($CDCl_3$), δ: 7.17 (½AB, 2H, *J* = 9.0 Hz, 3',5'-H), 6.63 (½AB, 2H, *J* = 9.0 Hz, 2',6'-H), 3.49 (t, 2H, *J* = 5.5 Hz, 2-H), 3.45 (t, 2H, *J* = 5.5 Hz, 1-H), 2.97 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$), δ: 151.6 (NCO), 147.4 (C-1'), 129.2 (C-2',6'), 122.5 (C-4'), 113.9 (C-3',5'), 53.6 (C-2), 40.8 (C-1), 39.3 (NCH_3), IR (liq film) 2916w (CH st), 2268s (NCO), 1597m, 1500s (C=C), 811m (*p*-di substituted aromatic ring) cm^{-1}; MS (ES) m/z 211 (100%) $(M+H)^+$, 79 (50%) $(C_6H_6+H)^+$.

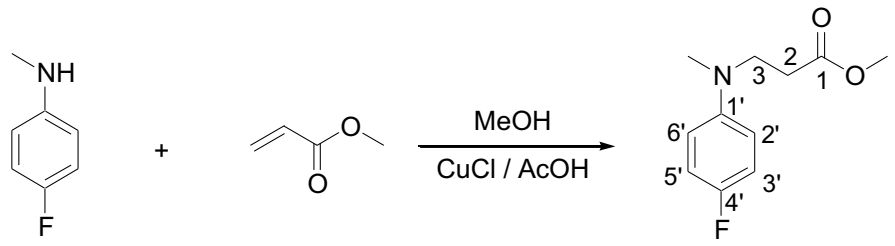
3-(2-(*N*-(4-Chlorophenyl)-*N*-methylamino)ethyl)-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamid 59c



109c + **20** → **59c**

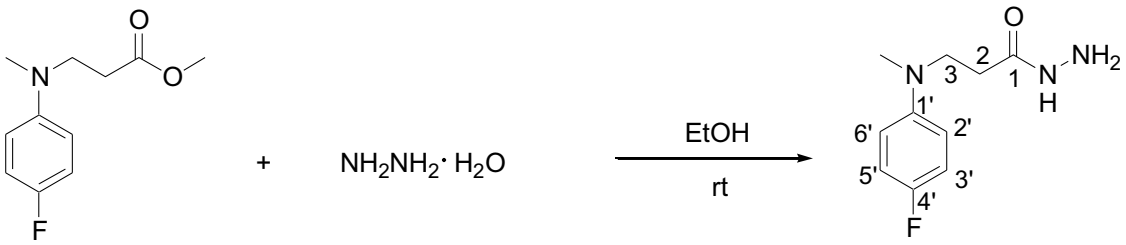
Isocyanate **109c** (0.37 g, 1.76 mmol) was diluted with DMSO (1.5 ml), then transferred under nitrogen to a suspension of diazo-IC **20** (0.24 g, 1.76 mmol) in DMSO (1.5 ml), the mixture was stirred at rt protected from light for 48 h. The ^{1}H NMR spectrum showed product **59c** formation. The reaction mixture was suspended in H_2O (10 ml) and filtered. The solid on the filter was washed with copious amounts of H_2O until the washings came through colourless and then with *i*-PrOH. The ^{1}H NMR showed a small amount of impurities of another tetrazine, the tetrazine **59c** was collected pure from the fractions of column chromatography eluted with 10% acetic acid in chloroform (0.08 g, 13.1%) m.p. 154 – 155 °C ^{1}H NMR (DMSO-d_6) δ: 8.78 (s, 1H, 6-H), 7.77 & 7.66 (2 × br s, 2H, $CONH_2$), 7.08 (½AB, 2H, *J* = 9 Hz, 3',5'-H), 6.70 (½AB, 2H, *J* = 9 Hz, 2',6'-H), 4.45 (t, 2H, *J* = 6.4 Hz, H-1), 3.79 (t, 2H, *J* = 6.4 Hz, H-2), 2.89 (s, 3H, NCH_3); ^{13}C NMR (DMSO-d_6) δ: 161.9 ($CONH_2$), 147.8 (C-1'), 139.6 (C-4), 134.7 (C-8a), 131.4 (C-8), 129.2 (C-6), 129.1 (C-2',6'), 120.3 (C-4'), 113.9 (C-3',5'), 50.4 (C-2), 46.3 (C-1), 38.5 (NCH_3); IR (KBr) 3441m (NH), 2911w (CH st), 1740s (C(4)O), 1672s ($CONH_2$), 1596 m, 1501m, 1454m (C=C), 1363m, 811(*p*-di substituted aromatic ring) cm^{-1}; MS(ES): m/z 347.9 (40%)$(M+H)^+$, 210 (100%) $(C_{10}H_{11}ClN_2O + H)^+$; CHN Found: C, 47.16; H, 3.98; N, 26.94 $C_{14}H_{14}ClN_7O_2 \cdot 0.1CHCl_3$, requires C, 47.08; H, 3.95; N, 27.26%.

Methyl 3-(*N*-(4-fluorophenyl)-*N*-methylamino)propanoate 106d[79]



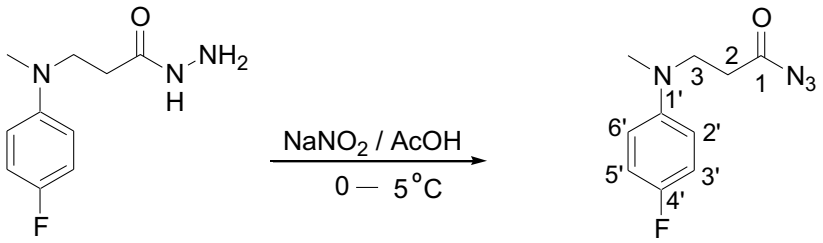
4-Fluoro-*N*-methylaniline **105d** (1.53 g, 12.2 mmol) was dissolved into methanol (7 ml), CuCl (0.12 g, 1.2 mmol), acetic acid (2 ml) and methyl acrylate **81** (5.3 g, 61.1 mmol) were added. The reaction mixture was stirred at reflux overnight. The reaction mixture was then partitioned between equal volumes of $CHCl_3$ / dil aqueous Na_2CO_3 solution (pH 12) (25 ml); the $CHCl_3$ layer was then separated, washed with three portions of H_2O (20ml), dried on anhydrous $MgSO_4$, decolourised with charcoal and filtered through celite. The chloroform was evaporated to give a yellow oil **106d** (2.1g, 81%). ^{1}H NMR ($CDCl_3$) δ: 6.92 (t, 2H, $J_{HH} = J_{HF} = 9.1$ Hz, 3',5'-H), 6.66 (dd, 2H, $J_{HH} = 9.1$ Hz, $J_{HF} = 4.3$Hz, 2',6'-H), 3.65 (s, 3H, OCH_3), 3.60 (t, 2H, $J = 7.1$ Hz, 3-H), 2.86 (s, 3H, NCH_3) 2.53 (t, 2H, $J = 7.1$ Hz, 2-H); ^{13}C NMR ($CDCl_3$) 172.8 (C-1), 156.0 (d, $^1J_{CF} = 237$ Hz, C-4'), 145.6 (C-1'), 115.8 (d, $^2J_{CF} = 23.1$ Hz, C-3',5'), 114.3 (d, $^3J_{CF} = 7.2$ Hz, C-2',6'), 51.8 (OCH_3), 49.5 (C-3), 38.7 (NCH_3), 31.5 (C-2); IR (liq film) 3025m (Ar-CH st), 2950m (CH st), 1725s (CO), 1525 (C=C). 1175m (C-O) cm^{-1}; MS(ES) m/z: 212.1 $(M+H)^+$.

3-(*N*-(4-fluorophenyl)-*N*-methylamino)propanoyl hydrazide 107d[79]



Ester **106d** (2.1g, 9.94 mmol) was dissolved in ethanol (10 ml). Hydrazine monohydrate (3g, 59.7 mmol) was then added and the mixture stirred at rt overnight. TLC confirmed the completion of the reaction. The volatile compounds were evaporated to give a viscous brown oil. Recrystallisation from Et_2O gave a white solid, **107d** (1.8g, 85%), m.p. 73 – 75 ºC; ^{1}H NMR ($CDCl_3$) δ: 7.03 (br s, 1H, CONH), 6.93 (t, 2H, $J_{HH} = {}^3J_{HF} =$ 9.1 Hz, 3',5'-H), 6.69 (dd, 2H, $J_{HH} =$ 9.1 Hz, $^4J_{HF}$ = 4.3 Hz, 2',6'-H), 3.85 (br s, 2H, COHNN$\underline{H_2}$), 3.57 (t, 2H, $J =$ 6.7 Hz, 3-H), 2.87 (s, 3H, NCH_3), 2.36 (t, 2H, $J =$ 6.7 Hz, 2-H); ^{13}C NMR ($CDCl_3$) δ: 172.4 (C-1), 155.8 (d, $^1J_{CF}$ = 237 Hz, C-4'), 145.7 (C-1'), 115.8 (d, $^2J_{CF}$ = 23.1 Hz, C-3',5'), 115.0 (d, $^3J_{CF}$ = 7.2 Hz, C-2',6'), 50.2 (C-3), 39.4 (NCH_3), 31.9 (C-2); IR (KBr) 3245m (NH), 3032w (Ar-CH st), 2947 w (CH st), 1620s (CONH), 1507m (C=C), 822m (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES) m/z 212.1(100%) $(M+H)^+$.

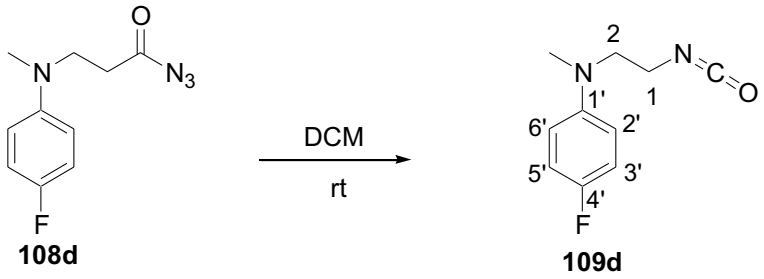
3-(*N*-(4-Fluorophenyl)-*N*-methylamino)propanoyl azide 108d



107d **108d**

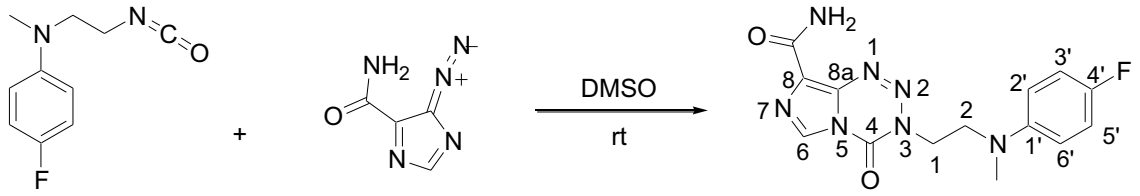
Hydrazide **107d** (0.25 g, 1.2 mmol) was dissolved in a mixture of aq. acetic acid (0.66M 15 ml) and DCM (15 ml), the mixture was then stirred on a $CaCl_2$-ice bath at 0 ºC, a solution of $NaNO_2$ (0.49 g, 7.1 mmol) in H_2O (25 ml) was added drop wise keeping the exothermic reaction at 0 – 5 ºC. A Further amount of DCM (20 ml) was added, the DCM layer separated, washed with H_2O (20ml) dried over $MgSO_4$, then filtered. Formation of azide **108d** was confirmed by IR. ^{1}H NMR showed a small amount of impurities. ^{1}H NMR ($CDCl_3$), δ: 6.93 (t, 2H, $J_{HH} = {}^3J_{HF}$ = 9.1 Hz, 3',5'-H), 6.66 (dd, 2H, J_{HH} = 9.1 Hz, $^4J_{HF}$ = 4.3 Hz, 2',6'-H), 3.60 (t, 2H, J = 7.1 Hz, 3-H), 2.86 (s, 3H, NCH_3), 2.55 (t, 2H, J = 7.1 Hz, 2-H); ^{13}C NMR ($CDCl_3$), δ: 179.2 (C-1), 155.3 (d, $^1J_{CF}$ = 237 Hz, C-4'), 145.2 (C-1'), 115.8 (d, $^2J_{CF}$ = 21.7 Hz, C-3',5'), 114.3 (d, $^3J_{CF}$ = 7.2 Hz, C-2',6'), 49.4 (C-3), 38.9 (NCH_3), 34.2 (C-2); IR (liq film) 2914w (CH st), 2137s (CON_3), 1711s (CO), 1511m (C=C), 815s (*p*-di-substituted aromatic ring) cm^{-1}.

2-(*N*-(4-Fluorophenyl)-*N*-methylamino)ethyl isocyanate 109d



The anhydrous DCM solution of azide **108d** was stirred under nitrogen at rt overnight. Isocyanate **109d** formation was confirmed by IR and ^{1}H NMR. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature). The isocyanate was collected as a yellow oil (0.2 g, 77%). ^{1}H NMR ($CDCl_3$), δ: 6.95 (t, 2H, $J_{HH} = {}^{3}J_{HF} = 9.1$ Hz, 3',5'-H), 6.69 (dd, 2H, $J_{HH} = 9.1$ Hz, $^{4}J_{HF} = 4.3$ Hz, 2',6'-H), 3.43 (m, 4H, 1,2-H), 2.94 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$), δ: 157.2 (NCO), 155.3 (d, $^{1}J_{CF} = 237$ Hz, C-4'), 145.2 (C-1'), 115.8 (d, $^{2}J_{CF} = 21.7$ Hz, C-3',5'), 114.4 (d, $^{3}J_{CF} = 7.2$ Hz, C-2',6'), 54.5 (C-3), 40.9 (C-2), 39.6 (NCH_3); IR (liq film) 3057w (Ar-CH st), 2917w (CH st), 2270s (NCO), 1512m (C=C), 1228m, 816s (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES) m/z 195 (100%) $(M+H)^{+}$.

3-(2-(*N*-(4-Fluorophenyl)-*N*-methylamino)ethyl)-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 59d

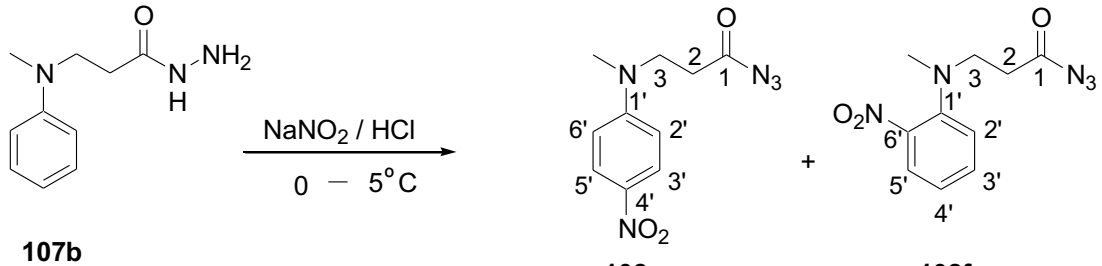


109d + **20** → **59d**

Isocyanate **109d** (0.2 g, 1.03 mmol) was diluted with DMSO (1.5 ml) under nitrogen then added to a suspension of diazo-IC **20** (0.14 g, 1.03 mmol) in DMSO (1.5 ml), the mixture was stirred at rt protected from light for 48 h. The ^{1}H NMR spectrum showed product **59d** formation. The reaction mixture was suspended in H_2O (30 ml) and filtered, the residue was washed with copious amounts of H_2O until the washings came through colourless, and then washed with Et_2O. The solid was purified by flash column chromatography, 10% AcOH in $CHCl_3$ was used for the elution; the fractions containing the product were evaporated to give an orange solid. The solid was then re-dissolved in $CHCl_3$ and filtered, the $CHCl_3$ evaporated and the residue re-suspended in Et_2O and then collected by filtration to give **59d** (0.057 g, 17%), m.p. 172 – 173 ºC . ^{1}H NMR (DMSO-d_6) δ: 8.77 (s, 1H, 6-H), 7.75 & 7.65 (2 × br s, 2H, $CONH_2$), 6.91 (t, 2H, $J_{HH} = J_{HF} = 9.1$ Hz, 3',5'-H), 6.69 (dd, 2H, $J_{HH} = 9.1$ Hz, $J_{HF} = 4.3$ Hz, 2',6'-H), 3.45 (t, 2H, $J = 6.4$ Hz, 1-H), 3.77 (t, 2H, $J = 6.4$ Hz, 2-H), 2.90 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$), δ: 161.89 ($CONH_2$), 155.0 (d, $^{1}J_{CF} = 235.5$ Hz, C-4'), 145.9 (C-1'), 139.5 (C-4), 134.7 (C-8a), 131.3 (C-8), 129.2 (C-6), 115.7 (d, $^{2}J_{CF} = 21.7$ Hz, C-3',5'), 113.6 (d, $^{3}J_{CF} = 7.2$ Hz, C-2',6'), 50.9 (C-2), 46.3 (C-1) 38.7 (NCH_3); IR (KBr) 3439s (NH), 3117m (Ar-CH st), 2914w (CH st), 1749s (C(4)O), 1682s ($CONH_2$), 1515 s, 1458m (C=C), 820m (*p*-di-substituted aromatic ring) cm^{-1}; MS (ES): m/z 331.9 (20%)

$(M+H)^+$, 353.9 (10%) $(M+Na)^+$, 195.0 (100%) $(C_{10}H_{11}FN_2O+H)^+$; CHN Found: C, 48.64; H, 4.22; N, 28.16 $C_{14}H_{14}FN_7O_2 \cdot 0.75H_2O \cdot 0.1CHCl_3$, requires C, 48.77; H, 4.53; N, 28.43%.

3-(*N*-Methyl-*N*-(4-nitrophenyl)amino)propanoyl azide 108e and 3(*N*-methyl-*N*-(2-nitrophenyl)amino)propanoyl azide 108f

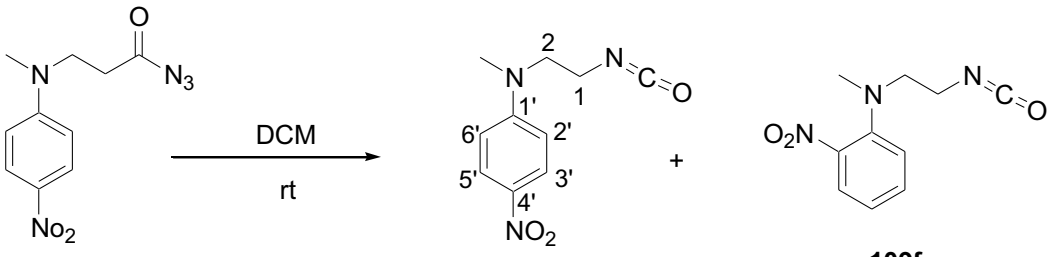


107b **108e** **108f**

Hydrazide **107b** (0.3g, 1.6 mmol) was dissolved in a mixture of DCM (10 ml) and HCl (10 ml, 14.8%), the solution was stirred at 0 ºC on a $CaCl_2$-ice bath, $NaNO_2$ (0.66 g, 9.6 mmol) solution in H_2O (10 ml) was added gradually, keeping the exothermic reaction between 0 – 5 ºC. A further portion of DCM (15 ml) was added, the DCM layer was separated, washed with two portions of H_2O (20 ml) dried over $MgSO_4$, then filtered. Formation of the azide **108e** was confirmed by IR and ^{1}H NMR which also showed the formation of small amount of the ortho nitrated azide **108f** in ratio 1:5 to azide **108e**. Azide **18 e** ^{1}H NMR ($CDCl_3$) δ: 8.12 (½AB, 2H, *J* = 9.5 Hz, 3',5'-H), 6.61 (½AB, 2H, *J* = 9.5 Hz, 2',6'-H), 3.77 (t, 2H, *J* = 7.0 Hz, 3-H), 3.10 (s, 3H, NCH_3) 2.62 (t, 2H, *J* = 7.0 Hz, 2-H); ^{13}C NMR ($CDCl_3$) δ: 178.5 (CON_3), 152.8 (C-4'), 133.2 (C-1'), 126.4 (C-3',5'), 110.6 (C-2',6'), 48.0 (C-3), 39.1 (NCH_3), 34.4 (C-2); IR (liq film) 2918w (CH st), 2140s (N_3), 1711s (CO), 1597s (NO_2 st as), 1518 s (C=C), 1311s (NO_2 st sy) cm^{-1}.

Azide **108f** ^{1}H NMR ($CDCl_3$) δ: 7.72 (d, 1H, *J* = 8.9 Hz, 5'-H), 7.42 (t, 1H, *J* = 8.9 Hz, 4'-H), 7.21 (d, 1H, , *J* = 8.9 Hz, 2'-H), 6.96 (t, 1H, *J* = 8.9 Hz, 3'-H), 3.46 (t, 2H, *J* = 7.1 Hz, 3-H), 2.80 (s, 3H, NCH_3) 2.62 (t, 2H, *J* = 7.1 Hz, 2-H).

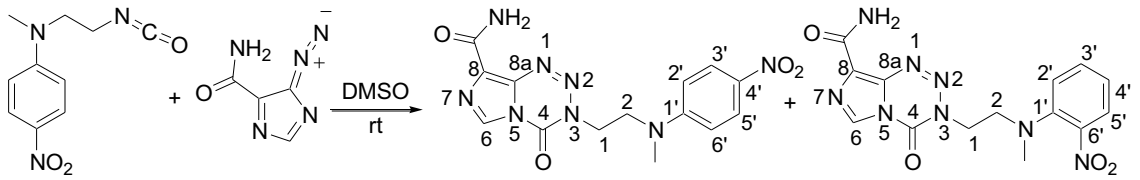
2-[(4-Nitrophenyl)methylamino]ethylisocyanate 109e



The anhydrous DCM solution of the crude azide **108e** was stirred under nitrogen at rt for overnight. Isocyanate formation was confirmed by IR. The ^{1}H NMR showed two isocyanates **109e** and **109f** in ration of 5:1. The DCM was evaporated under reduced pressure at low temperature (an ice bath was used to lower the temperature) and the crude isocyanate collected as a yellow oil (0.27 g, 90 %). ^{1}H NMR ($CDCl_3$) δ: 8.15 (½AB, 2H, *J* = 9.4 Hz, 3',5'-H), 6.67 (½AB, 2H, *J* = 9.4 Hz, 2',6'-H), 3.66 (t, 2H, *J* = 6.2 Hz, 2-H), 3.57 (t, 2H, *J* = 6.2 Hz, 1-H), 3.15 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$) δ: 153.1 (NCO), 145.2 (C-4'), 138.1 (C-1'), 126.4 (C-3',5'), 110.7 (C-2',6'), 52.6 (C-2), 40.7 (C-1) , 39.5 (NCH_3); IR (liq film) 2919w (CH st), 2270s (NCO), 1597s (NO_2 st as), 1517 s (C=C), 1311s (NO_2 st sy) cm^{-1}.

Isocyanate **109f** ^{1}H NMR ($CDCl_3$) δ: 7.74 (d, 1H, *J* = 8.4 Hz, 5'-H), 7.45 (t, 1H, , *J* = 8.4 Hz, 4'-H), 7.18 (d, 1H, , *J* = 8.4 Hz, 2'-H), 6.99 (t, 1H, *J* = 8.9 Hz, 3'-H), 3.48 (t, 2H, *J* = 6.0 Hz, 3-H), 2.90 (s, 3H, NCH_3), 3.31 (t, 2H, *J* = 7.1 Hz, 2-H).

3-(2-*N*-Methyl-*N*-(4-nitrophenyl)-*N*-methylamino)ethyl)-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 59e and 3-(2-(*N*-methyl-*N*-(2-nitrophenyl)-*N*-methylamino)ethyl)-4-oxo-3H,4H-imidazo[5,1d][1,2,3,5]tetrazine-8-carboxamide 59f



109e **20** **59e** **59f**

The crude mixture of the isocyanates **109e** & **f** (0.27 g, 1.22 mmol) was diluted with DMSO (1.5 ml) under N_2 then added to a suspension of diazo-IC **20** (0.17 g, 1.22 mmol) in DMSO (1.5 ml), the mixture was stirred at rt protected from light for 48 h. The reaction mixture was then suspended in water (30 ml) and filtered. The solid on the filter was washed with copious amounts of H_2O until the washings came through colourless, then with Et_2O. The dry solid was purified by flash column chromatography eluted with a gradient 5 – 20% AcOH in $CHCl_3$. ^{1}H NMR showed impurities from silica, the imidazotetrazinone solid was then dissolved in DMF and filtered, the imidazotetrazinone precipitated as a yellow solid by H_2O addition, the solid collected by filtration, washed with copious amounts of water and then dried to **59e** (0.025g, 6 %), m.p. 179 – 180 ºC. ^{1}H NMR (DMSO-d_6) δ: 8.81 (s, 1H, 6-H), 8.00 (½AB, 2H, *J* = 9.2 Hz, 3',5'-H), 7.77 & 7.67 (2 × br s, 2H, $CONH_2$), 6.82 (½AB, 2H, *J* = 9.2 Hz, 2',6'-H), 4.52 (t, 2H, *J* = 6.3 Hz, 1-H), 3.96 (t, 2H, *J* = 6.3 Hz, 2-H), 3.07 (s, 3H, NCH_3); ^{13}C NMR (DMSO-d_6) δ: 161.9 ($CONH_2$), 154.0 (C-4'), 139.7 (C-4), 136.6 (C-1'), 134.7 (C-8a), 131.6 (C-8), 129.5 (C-6), 126.3 (C-3',5'), 111.4 (C-2',6'), 50.3 (C-2), 46.4 (C-1) 39.2 (NCH_3); IR (KBr) 3443m (NH), 2919w (CH st), 1749s (C(4)O), 1684s (CONH), 1597s (NO_2 st as), 1457m (C=C), 1316s (NO_2 st sy) cm^{-1}; MS(ES): m/z 359.1(80%)

$(M+H)^+$, 381.1(100%) $(M+Na)^+$; CHN Found: C, 44.9; H, 3.70; N, 27.08 $C_{14}H_{14}N_8O_4$ ·0.6 AcOH·0.2 $CHCl_3$, requires C, 44.76; H, 4.03; N, 27.38%.

59f ^{1}H NMR (DMSO-d_6) δ: 8.83 (s, 1H, 6-H), 7.83 & 7.71 (2 × br s, 2H, $CONH_2$), 7.65 (d, 1H, *J* = 9.1 Hz, 5'-H), 7.46 (t, 1H, *J* = 9.1 Hz, 4'-H), 7.31 (t, 1H, , *J* = 9.1 Hz, 2'-H), 6.92 (d, 1H, *J* = 9.1 Hz, 3'-H), 4.52 (t, 2H, *J* = 6.1 Hz, 1-H), 3.59 (t, 2H, *J* = 6.1 Hz, 2-H), 2.83 (s, 3H, NCH_3).

E.4 Methods for Mechanistic Investigation of Aziridinium-ion-release-imidazotetrazinones in Chapter 3

E.4.1 NMR Study of the hydrolysis reaction mechanism of ^{13}C-labelled and unlabelled 3-{2-[Methyl(4'-methylphenyl)amino]ethyl}-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 59a

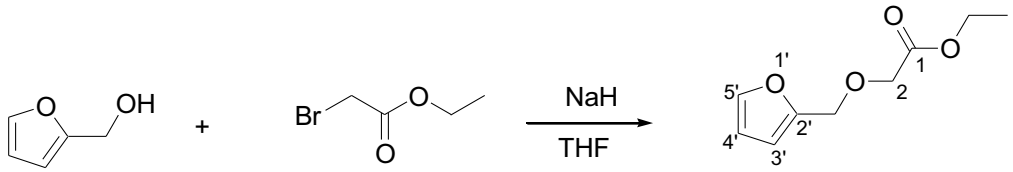
Imidazotetrazinone **59a**, (^{13}C-labelled and the unlabelled) stock solution was prepared by dissolving (1.5 mg, 4.6×10^{-3} mmol) in DMSO-d_6 (100 µl); 0.2 M deuteriated phosphate buffer pD 7.8 was prepared from disodium hydrogen phosphate (0.36g) in D_2O (10 ml), the pH was then adjusted to pD 7.8 (equivalent to 7.4) with 0.2 M sodium dihydrogen phosphate (0.28 g) in D_2O (10 ml); a digital pH-meter was used for the pH measurement. Buffer solution pD 7.8 (0.5 ml) was placed in a 5 mm diameter NMR tube. The tube was inserted into the magnet (Jeol ECA 600 instrument), incubated at 37 °C for 10 mins and then the magnet was shimmed. A 1D ^{1}H spectrum was acquired to determine the frequency of the residual water peak. Tetrazine stock solution (100 µl) was added and 1D ^{1}H spectra with water presaturation were recorded every 0.5 h for 15 h [16 scans, 0 – 12 ppm]. The reaction mixture of the final products was spiked with authentic samples of 2-(methyl-*p*-tolylamino)ethanol (9.08×10^{-3} mmol in buffer 10 µl) and AIC (6.9×10^{-6} mmol in 10 µl buffer), and further 1D ^{1}H spectra were acquired after each addition.

E.4.2 UV-Visible study of the pseudo 1st order hydrolysis kinetic of aziridinium-ion-release imidazotetrazinones.

Stock solutions 20 mM of the imidazotetrazinones synthesized for aziridinium ion release were prepared in DMSO. Buffer solutions were prepared from citric acid (0.1 M, 500 ml) and Na_2HPO_4 (0.2 M, 500 ml) a digital pH-meter was used for the pH adjusting; Aliquots of the stock solutions of the tetrazines (2 – 3.5 µl) were added to a quartz cuvette (1 cm pathlength) containing 1200 µl buffer solution maintained at 37 °C. [The buffer solutions used were citrate/phosphate pH 4, citrate/phosphate pH 7.4 and citrate/phosphate pH 8.] UV spectra (400 – 200 nm) were recorded every 4 min (pH 7.40, for 1 h), 1 min (pH 8 for 15 min) and 1h (pH 4 for 12 h). The decrease in the absorbance with time for the tetrazines and the appearance of AIC as a decomposition product at each pH were measured. Data were transferred to Excel for processing and the pseudo 1st order rate constant (k') and half life ($t_{1/2}$) calculated. All values were mean (± SD) of three separate determinations.

E.5 Synthesis of alcohol or phenol-release-imidazotetrazinone in Chapter 4

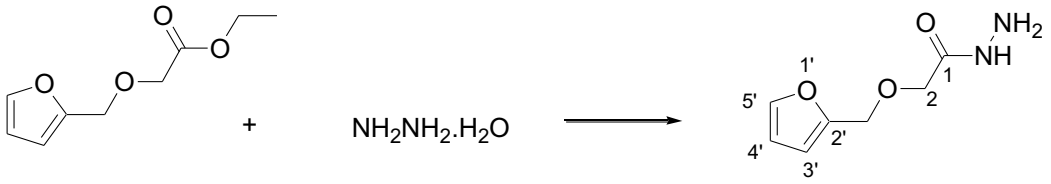
Ethyl 2-(furan-2'-ylmethoxy) acetate 118a[93]



118a

Furfuryl alcohol (1 ml, 11.6 mmol) was dissolved in anhydrous THF (20 ml), NaH (0.57g, 60% in oil, 14 mmol) was added portion-wise to the solution. After gas evolution ceased, ethyl bromoacetate (1.3ml, 11.7 mmol) was added and the mixture heated to reflux for 2.5 h, then cooled to ambient temperature overnight. The mixture was partitioned between equal volumes of EtOAc and H_2O (25 ml), the aqueous layer was separated and then extracted with another two portions of EtOAc (20 ml). The organic layers were combined and then washed with brine, dried over $MgSO_4$, filtered and then concentrated to a brown oil **118a** (2.1g), ^{1}H NMR confirmed the product. ^{1}H NMR ($CDCl_3$) δ: 7.41 (dd, 1H, J_{ab} = 1.8 Hz, J_{ac} = 0.9 Hz, 5'-H), 6.35 (dd, 1H, J_{bc}= 3.1 Hz, J_{ab} = 1.8 Hz, 4' H), 6.34 (dd, 1H, J_{bc}= 3.1 Hz, J_{ac} = 0.9 Hz, 3'-H), 4.59 (s, 2H, CH_2O), 4.21 (q, 2H, J = 7.1 Hz, $OC\underline{H}_2CH_3$), 4.08 (s, 2H, 2-H), 1.27 (t, 3H, J = 7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$) 170.3 (C-1), 150.8(C-2'), 143.3 (C-5'), 110.5 (C-4'), 110.3 (C-3'), 66.9 (OCH_2), 64.9 (C-2), 61.1 ($O\underline{C}H_2CH_3$), 14.3 ($OCH_2\underline{C}H_3$) ; MS(ES): m/z 207(100%)$(M+Na)^+$.

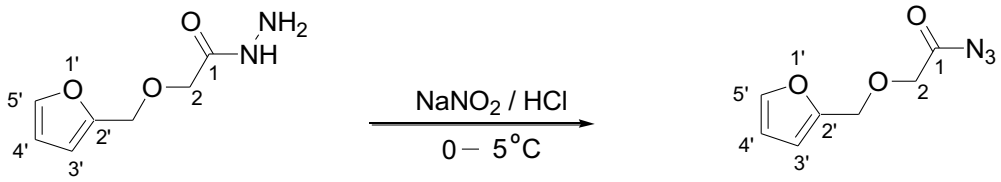
2-(Furan-2'-ylmethoxy)acetohydrazide 119a



118a **119a**

Ester **118a** (1.4g, 7.6mmol) was dissolved in EtOH (10 ml) then hydrazine monohydrate (2.2 ml, 45.6 mmol) was added and the mixture stirred at rt overnight. The volatile compounds were evaporated under vacuum at 70 ºC to give a thick brown oil (1.2g). ^{1}H NMR confirmed the product **119a**. ^{1}H NMR ($CDCl_3$) δ: 7.63 (bs, 1H, CON<u>H</u>NH_2), 7.42 (dd, 1H, J_{ab} = 1.8 Hz, J_{ac} = 0.9 Hz, 5'-H), 6.35 (dd, 1H, J_{bc}= 3.1 Hz, J_{ab} = 1.8 Hz, 4'-H), 6.34 (dd, 1H, J_{bc}= 3.1 Hz, J_{ac} = 0.9 Hz, 3'-H), 4.50 (s, 2H, CH_2O), 4.05 (s, 2H, 2-H), 3.81 (bs, 2H, CONHN<u>H_2</u>); ^{13}C NMR ($CDCl_3$) 169.8 (C-1), 150.3(C-2'), 143.6 (C-5'), 110.60 (C-4'), 110.55 (C-3'), 68.8 (O<u>C</u>H_2), 65.4 (C-2); MS(ES): m/z 193.1(100%)$(M+Na)^+$.

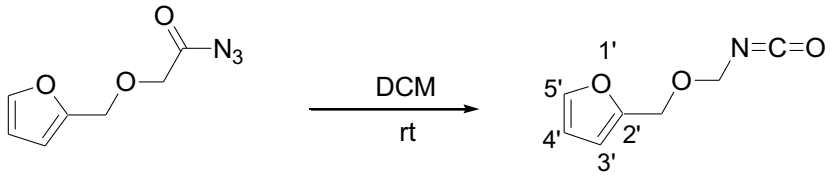
2-(Furan-2'-ylmethoxy)acetyl azide 120a



119a **120a**

Hydrazide **119a** (1.2 g, 7.1 mmol) was dissolved in mixture of H_2O and DCM (15 ml each) then aq. HCl (4 ml, 37%) was added, the mixture was cooled on a $CaCl_2$-ice bath to 0 ºC. $NaNO_2$ (2.9 g, 42.3 mmol) solution in (10 ml) H_2O was added gradually to the reaction mixture, keeping the exothermic reaction between 0 – 5 ºC. A further amount of DCM (20 ml) was added and then the DCM layer was then separated, washed with H_2O, dried over $MgSO_4$ and then filtered and kept to stir at rt under nitrogen for isocyanate preparation. ^{1}H NMR and IR confirmed the clean product azide **120a**. ^{1}H NMR 400 MHz ($CDCl_3$) δ: 7.46 (dd, 1H, J_{ab} = 1.8 Hz, J_{ac} = 0.9 Hz, 5'-H), 6.40 (dd, 1H, J_{bc}= 3.1Hz, J_{ab} = 1.8 Hz, 4'-H), 6.39 (dd, 1H, J_{bc}= 3.1 Hz, J_{ac} = 0.9 Hz, 3'-H), 4.61 (s, 2H, CH_2O), 4.15 (s, 2H, 2-H); ^{13}C NMR ($CDCl_3$) 177.6 (C-1), 150.3 (C-2'), 143.5 (C-5'), 110.7 (C-4'), 110.6 (C-3'), 68.8 (OCH_2), 65.1 (C-2); IR (liq film), 2919w (CH st), 2146s (N_3), 1731s (CO), 1187m (C-O), 1152s, 1057m, 750m cm^{-1}.

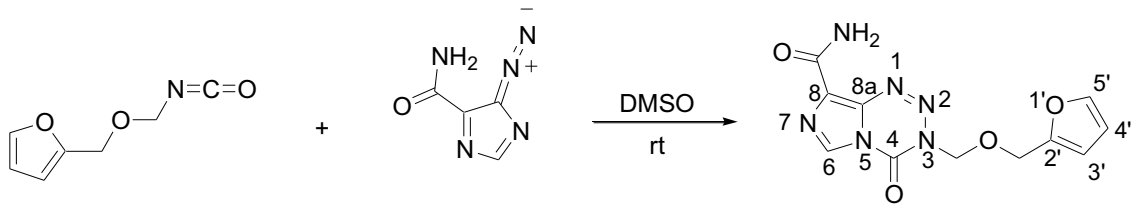
(Furan-2′-ylmethoxy)methylisocyanate 121a



120a **121a**

The anhydrous DCM solution of azide **120a** was stirred under nitrogen at rt overnight. DCM was evaporated at reduced temperature and pressure to a pale yellow oil, isocyanate **121a** (0.63 g, 58%). ^{1}H NMR ($CDCl_3$) δ: 7.42 (dd, 1H, J_{ab} = 1.8 Hz, J_{ac} = 0.9 Hz, 5'-H), 6.41 (dd, 1H, J_{bc}= 3.1 Hz, J_{ab} = 1.8 Hz, 4'-H), 6.38 (dd, 1H, J_{bc}= 3.1 Hz, J_{ac} = 0.9Hz, 3'-H), 4.79 (s, 2H, CH_2O), 4.59 (s, 2H, 2-H); ^{13}C NMR ($CDCl_3$) 150.0 (C-2'), 145.0 (NCO), 143.6 (C-5'), 110.7 (C-4'), 110.6 (C-3'), 74.0 (OCH_2), 62.5 ($\underline{C}H_2NCO$); IR (liq film), 2921w (CH st), 2254s (NCO), 1679 (C=C), 118m (C-O) cm^{-1}.

3-[(Furan-2'-ylmethoxy)methyl]-4-oxo-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 117a

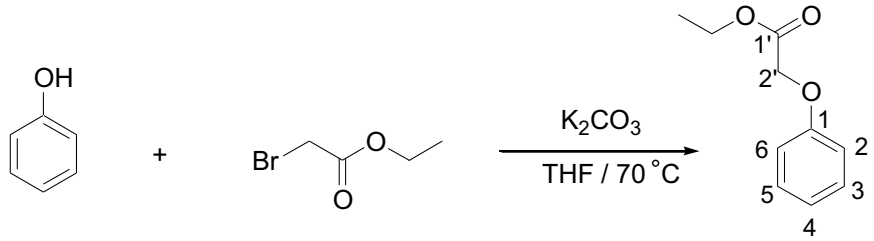


121a **20** **117a**

Isocyanate **121a** (0.63 g, 4.11 mmol) was diluted with DMSO (1.5 ml), under nitrogen then added to a suspension of diazo-IC **20** (0.56 g, 4.11 mmol) in DMSO (1.5 ml), the mixture was stirred at 35 °C protected from light for 48 h. The reaction mixture was then suspended in H_2O (30 ml) and extracted with three portions of $CHCl_3$ (20 ml), the $CHCl_3$ layers were combined, washed twice with H_2O (20 ml), dried over anhydrous $MgSO_4$ filtered and the $CHCl_3$ evaporated to give a brownish solid. The solid was then purified by column chromatography eluted with 10% AcOH in $CHCl_3$ to give a white solid. The solid was dissolved in DMF, filtered and precipitated by addition of water to the DMF solution of the imidazotetrazinone, the white solid was collected by filtration, washed with copious amounts of H_2O, Et_2O and then dried to **117a** (0.15 g, 13%), mp 138 – 140° C. ^{1}H NMR (DMSO-d_6) δ: 8.87 (s, 1H, 6-H), 7.83 & 7.70 (2 × br s, 2H, $CONH_2$), 7.62 (dd, 1H, J_{ab} = 1.8Hz, J_{ac} = 0.9Hz, 5'-H), 6.44 (dd, 1H, J_{bc}= 3.1Hz, J_{ab} = 1.8Hz, 4'-H), 6.42 (dd, 1H, J_{bc}= 3.1Hz, J_{ac} = 0.9Hz, 3'-H), 5.69 (s, 2H, 1'-H), 4.64 (s, 2H, 2'-H); ^{13}C NMR (DMSO-d_6) δ: 162.0 ($CONH_2$), 151.0 (C-3'), 144.1 (C-6'), 140.0 (C-4), 134.7 (C-8a), 131.9 (C-8), 130.1 (C-6), 111.1 (C-5'), 110.8 (C-4'), 77.3 (C-1'), 63.0 (C-2') ; IR (KBr) 3375m (NH), 3094m (Ar-CH st), 2923w (CH st), 1740s (C(4)O), 1693s ($CONH_2$), 1609 m, 1459m (C=C), 1092m (C-O) cm^{-1}; MS(ES): m/z 291.1

(100%)$(M+H)^+$, 137.9 (25%) (diazoIC + H)$^+$; Found: C, 45.67; H, 3.52; N, 28.84 $C_{11}H_{10}N_6O_4$, requires C, 45.52; H, 3.47; N, 28.96%.

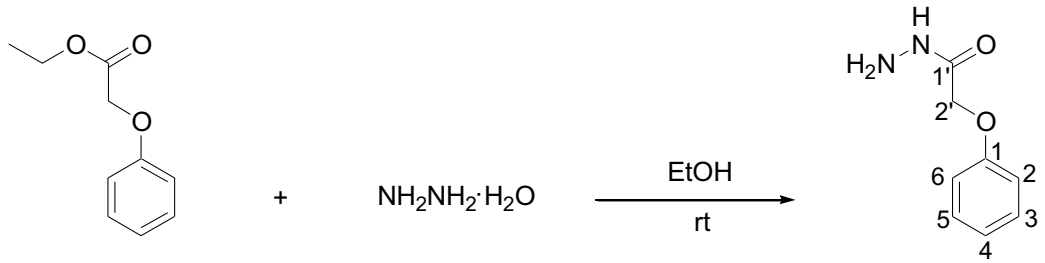
Phenoxyacetic acid ethyl ester 118b[94]



118b

Phenol (1.88 g, 20 mmol), ethyl bromoacetate (2.34 ml, 21 mmol) and K_2CO_3 (3.03 g, 22 mmol) were stirred in DMF (20 ml) at 70 °C for 18 h. The mixture was cooled to rt, then partitioned between Et_2O and H_2O (25ml each), the aqueous layer was separated and then extracted with another two portions of Et_2O (20 ml), the organic layers were combined and then washed with H_2O, then with brine, dried over $MgSO_4$, filtered and then concentrated to give a brown oil **118b** (1.8 g, 50%). ^{1}H NMR ($CDCl_3$) δ: 7.30 (t, 2H, *J* = 8.1 Hz, 3,5-H), 7.00 (t, 1H, *J* = 8.1 Hz, 4-H), 6.91 (d, 2H, *J* = 8.1 Hz, 2,6-H), 4.62 (s, 2H, 2'-H), 4.28 (q, 2H, *J* = 7.1 Hz, $OC\underline{H_2}CH_3$), 1.31 (t, 3H, *J* = 7.1 Hz, $OCH_2C\underline{H_3}$); ^{13}C NMR ($CDCl_3$) δ: 169.2 (C-1'), 157.9 (C-1), 129.7 (C-3,5), 121.9 (C-4), 114.8 (C-2,6), 65.5 (C-2'), 61.6 ($O\underline{C}H_2CH_3$), 14.3 ($OCH_2\underline{C}H_3$); IR (liq film) 2983w (CH st), 1757s (CO), 1597s (C=C), 1201s (C-O), 754&691 (mono-substituted aromatic ring) cm^{-1}; MS(ES) m/z: 181.1 (100%) $(M+H)^+$.

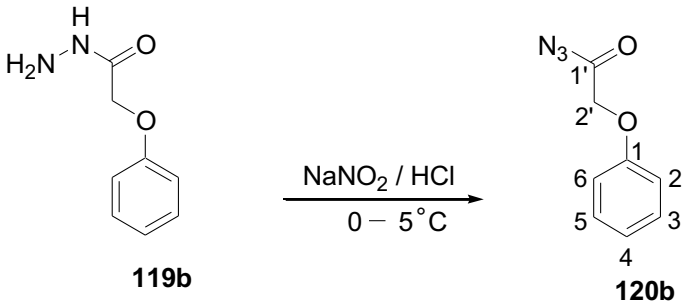
Phenoxyacetyl hydrazide119b[94]



118b **119b**

Ester **118b** (1.6 g, 8.88 mmol) was dissolved in EtOH (10 ml). Hydrazine monohydrate (2.7 g, 53.3 mmol) was then added and the mixture stirred at rt overnight. The volatile compounds were evaporated to leave a white solid. The solid was suspended in hot Et_2O, then collected by filtration and dried to give **119b** (0.8 g, 54%), m.p. 113 – 115 ºC (lit 112 ºC)[10]; ^{1}H NMR ($CDCl_3$) δ: 7.74 (br s, 1H, CONH), 7.30 (t, 2H, J = 8.1 Hz, 3,5-H), 7.02 (t, 1H, J = 8.1 Hz, 4-H), 6.89 (d, 2H, J = 8.1 Hz, 2,6-H), 4.56 (s, 2H, 2'-H), 3.91 (br s, 2H, NH_2); ^{13}C NMR ($CDCl_3$) δ: 168.7 (C-1'), 157.1 (C-1), 130.0 (C-3,5), 122.4 (C-4), 114.6 (C-2,6), 67.0 (C-2'); IR (KBr), 3313s (NH), 3042m (Ar-CH st), 1667 (CONH), 1543m&1497s (C=C), 1243 (C-O), 742&687s (mon-substituted aromatic ring) cm^{-1}; MS (ES) m/z 167.1 (100%) $(M+H)^+$.

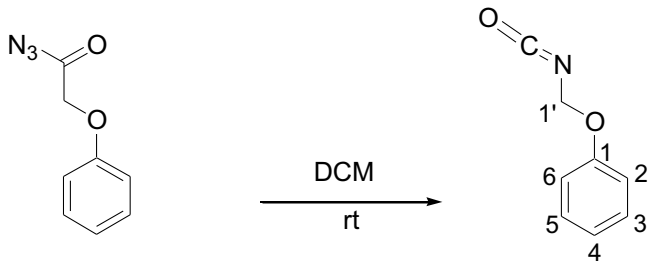
Phenoxyacetic acid azide 120b



119b **120b**

Hydrazide **119b** (0.75 g, 4.96 mmol) was dissolved in a mixture of H_2O (10 ml) and DCM (10 ml) then HCl (4 ml, 37%) was added, the mixture was stirred on a $CaCl_2$-ice bath at 0 °C. $NaNO_2$ (2.05 g, 29.8 mmol) solution in (10ml) H_2O was added gradually to the reaction mixture, keeping the exothermic reaction between 0 – 5 °C. A further portion of DCM (20 ml) was added and then the DCM layer was separated, washed with water dried over $MgSO_4$, filtered and kept stirred at rt under nitrogen for isocyanate preparation. ^{1}H NMR and IR confirmed the formation of azide **120b**. ^{1}H NMR ($CDCl_3$), δ: 7.29 (t, 2H, *J* = 8.1 Hz, 3,5-H), 7.00 (t, 1H, *J* = 8.1 Hz, 4-H), 6.88 (d, 2H, *J* = 8.1 Hz, 2,6-H), 4.65 (s, 2H, 2'-H); ^{13}C NMR ($CDCl_3$), δ: 176.5 (C-1'), 157.5 (C-1), 129.8 (C-3,5), 122.3 (C-4), 114.8 (C-2,6), 67.3 (C-2'); IR (liq film) 2920w (CH st), 2145s (CON_3), 1734s (CO), 1595s (C=C), 753&680m (mono-substituted aromatic ring) cm^{-1}.

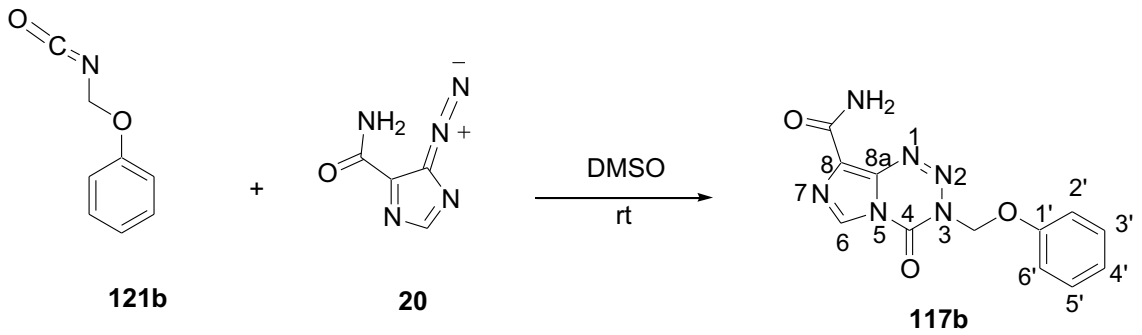
Phenoxymethylisocyanate 121b



120b **121b**

The anhydrous DCM solution of azide **120b** was stirred under nitrogen at rt overnight. IR and ^{1}H NMR confirmed the formation of the isocyanate **121b**. The DCM was evaporated at reduced temperature and pressure to give a pale yellow oil **121b** (0.54 g, 73%). ^{1}H NMR ($CDCl_3$), δ: 7.34 (t, 2H, *J* = 8.1 Hz, 3,5-H), 7.06 (t, 1H, *J* = 8.1 Hz, 4-H), 6.97 (d, 2H, *J* = 8.1 Hz, 2,6-H), 5.30 (s, 2H, 1'-H); ^{13}C NMR ($CDCl_3$), δ: 159.6 (NCO), 157.5 (C-1), 130.0 (C-3,5), 123.0 (C-4), 115.9 (C-2,6), 72.5 (C-1'); IR (liq film) 3066w (Ar-CH st), 2919w (CH st), 2257s (NCO), 1595s, 1495s (C=C), 755m, 680m (mono-substituted aromatic ring) cm^{-1}.

4-Oxo-3-(phenoxymethyl)-3H,4H-imidazo[1,5-d][1,2,3,5]tetrazine-8-carboxamide 117b



121b + 20 → 117b

Isocyanate **121b** (0.54 g, 3.6 mmol) was diluted with DMSO (0.75 ml) under nitrogen, then added to a suspension of diazo-IC **20** (0.5 g, 3.6 mmol) in DMSO (0.75 ml). The mixture was stirred at 35 °C protected from light for 48 h. The reaction mixture was then suspended in H_2O (30 ml) and then extracted with three portions of $CHCl_3$ (20 ml); the $CHCl_3$ layers were combined, washed twice with H_2O (20 ml), dried over anhydrous $MgSO_4$ filtered and the $CHCl_3$ evaporated to give an orange solid. The ^1H NMR showed impurities. The solid was then purified by flash column chromatography eluted with 10% AcOH in $CHCl_3$. The resultant solid was dissolved in DMF, the solution filtered and water added to precipitate the imidazotetrazinone as a white solid that was collected by filtration, washed with copious amounts of H_2O, Et_2O and then dried to give **117b** (0.064 g, 32%), mp 146 – 148 °C ^1H NMR (DMSO-d_6) δ: 8.87 (s, 1H, 6-H), 7.82 & 7.69 (2 × br s, 2H, $CONH_2$), 7.32 (t, 2H, J = 8.1, 3',5'-H), 7.15 (d, 2H, J = 8.1Hz, 2',6'-H), 7.03 (t, 1H, J = 8.1, 4'-H), 6.22 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ: 161.9 ($CONH_2$), 156.9 (C-1'), 139.7 (C-4), 134.4 (C-8a), 132.4 (C-8), 130.4 (C-6), 130.3 (C-3',5'), 123.1 (C-4'), 117.1 (C-2',6'), 76.3 (CH_2); IR (KBr) 3426m (NH), 3092m (Ar-H st), 2920w (CH st), 1741s (CO-4), 1669s ($CONH_2$), 1609 m, 1493m (C=C), 1217m (C-O), 742m, 689 (mono substituted aromatic ring) cm^{-1}; MS(ES): m/z 138.2 (80%)

(diazoIC + H)$^+$, 287.1 (100%)(M+H)$^+$; CHN Found: C, 50.27; H, 3.21; N, 29.63. $C_{12}H_{10}N_6O_3$, requires C, 50.35; H, 3.52; N, 29.36%.

E.5.2 ^{1}H NMR Study of hydrolysis reaction mechanism of alcohol- and phenol-release imidazotetrazinones (117a and b)

The imidazotetrazinone **117a** stock solution was prepared by dissolving (0.002 g, 6.89×10^{-3} mmol) in DMSO-d_6 (50 µl), the rest of the experiment performed same as in E 4.1, 50 µl tetrazine stock solution was used for the study. ^{1}H NMR confirmed the hydrolysis of the tetrazine and showed decomposition products. The reaction mixture of the finished products was spiked with authentic samples of furfurlal cohol and AIC (6.9×10^{-6} mmol each in 10 µl deuteriated phosphate buffer), subsequent ^{1}H NMR spectra confirmed their identities.

The experiment repeated for imidazotetrazinone **117b** (0.002 g, 6.99×10^{-3} mmol) in DMSO-d_6 (100 µl), and the instrument programmed to record a ^{1}H NMR spectrum at intervals of 0.5 h for 12 h. ^{1}H NMR confirmed the decomposition of the tetrazine and showed hydrolysis products. The reaction products mixture was spiked with authentic samples of phenol (3.2×10^{-8} mmol in deuteriated phosphate buffer 10 µl) and AIC (13.8×10^{-6} mmol in 20 µl buffer). ^{1}H NMR spectra confirmed their formation.

E.5.3 UV-Visible study of the kinetic of decomposition of alcohol / phenol-release-imidazotetrazinones

This experiments was performed exactly same as in E 4.2, the calculated rate constants (k′) and $t_{1/2}$ showed that hydrolysis was faster than the aziridinium ion release tetrazines.

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