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Separation, characterisation and biological evaluation of the individual isomers of the rat selective toxicant norbormide – isolated using a chemical derivatization strategy

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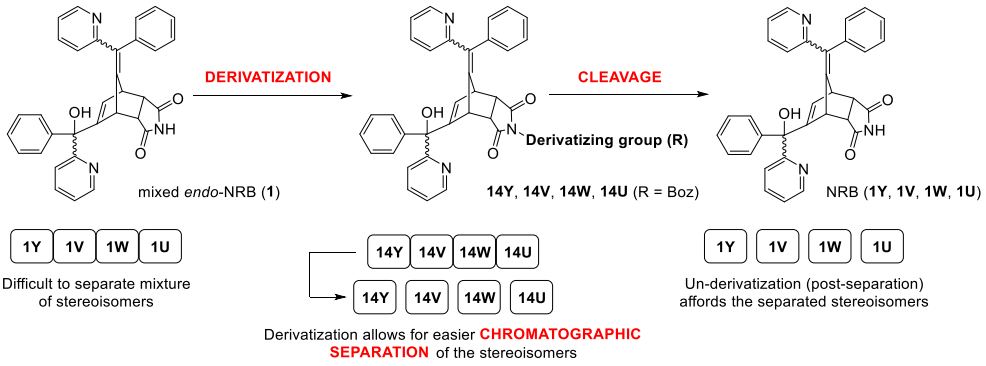
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Abstract

Norbormide [5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide] (NRB, **1**), an existing but infrequently used rodenticide, is known to be uniquely toxic to rats but relatively harmless to other rodents/mammals. However, as an acute vasoactive, NRB has a rapid onset of action, often leading to sub-lethal uptake/bait shyness. Recently, it was brought to our attention that baits containing two independently sourced batches of NRB (which differed noticeably in their stereochemical composition) displayed markedly different palatability/efficacy profiles in rats. Accordingly, with a view to independently evaluating the individual isomers of NRB in rats by means of a palatability and efficacy bait trial, this research describes the isolation of the individual isomers of *endo*-NRB (Y, V, W and U) from the parent mixture, by means of a chemical derivatization strategy.



Keywords: Norbormide, stereochemistry, rodenticide, rat toxicant, particle size

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Introduction

Discovered in the 1960s, norbormide (NRB, **1**) (Figure 1), a vasoactive also marketed under the trade names Shoxin® and Raticate,® was found to be uniquely toxic to rats but relatively harmless to other rodents and mammals.[1,2] NRB is also known to display unique stereo- and species-specific activity both *in vitro*[3,4] and *in vivo*;[5] the mechanisms behind these effects have yet to be fully clarified.[6,7] Disappointingly, to date, efforts to establish NRB as a viable rodenticide have been largely unsuccessful. Over time, rats have developed an evolutionary trait relating to how they sample food, particularly novel food; this survival strategy is most likely linked to their lack of an emetic centre, and thus their incapacity, as a species, to vomit. As an acute poison, NRB has a rapid onset of action in rats with toxic symptoms being registered within minutes of ingestion, and available evidence suggests that rats can develop a learnt aversion to this poison following the consumption of a sub-lethal dose during sampling, a phenomenon referred to as bait-shyness.[8,9] NRB is also known to be relatively unpalatable to rats.[10-12] Although efforts to address this palatability problem using microencapsulation technologies have had varying degrees of success, the onset of symptoms has yet to be sufficiently delayed to significantly improve palatability,[13,14] and the potential for sub-lethal dosing remains a major hurdle. More recently, a prodrug approach[15-17] revealed a series of NRB derivatives which were demonstrated to be more palatable/efficacious to rats.

Recently, it was brought to our attention[18] that baits (of the same formulation) containing two independently sourced batches of NRB (herein termed NRB#A and NRB#B) displayed markedly different palatability/efficacy profiles in rats; as demonstrated using a two-choice bait trail, where each rat was offered both poisoned and non-poisoned food (Table 1). Upon closer inspection, NRB batches #A and #B were both demonstrated to be of high purity, but differed noticeably in their stereochemical composition (Figure 1 and Table 2); NRB exists as a mixture of up to eight stereoisomers.[4] NRB#A was revealed to contain elevated levels of isomers V (NRB V, **1V**) and, to a lesser extent, U (NRB U, **1U**); relative to NRB#B. Given that consumption levels were demonstrated to be lower for bait containing NRB#A, it was tentatively postulated that the increased presence of NRB V may, at least in part, be accountable for such an observation; NRB V is known to be the most toxic NRB isomer to rats (Table 2).[5] With a view to independently evaluating the individual isomers of NRB in rats by means of a palatability and efficacy bait trial, this research describes the isolation of the individual isomers of NRB (Y, V, W and U) from the parent mixture by means of a chemical derivatization strategy.

Table 1. Two-choice bait-palatability and efficacy trial observations for NRB batches #A and #B, in *Sprague-Dawley* rats (see Figure 1 and Table 2).

Norbormide batch	Mean bait consumption (g) (as a % of body mass ±SE)[a]	Mean NRB dose consumed ±SE (and range) (mg/kg)	Mean NRB dose consumed that led to death ±SE (and range) (mg/kg)	Mortality[b]
#A	1.35 (0.39±0.05)	39±5 (17-70)	44±7 (29-61)	5/12
#B	1.89 (0.55±0.06)	55±6 (35-110)	56±6 (35-110)	11/12

[a] Rats were presented with a choice of standard lab pellets (20 g) and a bait formulation containing 1% w/w toxicant (20 g) for 2 days, following 3 days 'pre-feeding' with a choice of standard lab pellets and a bait formulation free of toxicant (*n* = 12, 6 male rats and 6 female rats). [b] Total number of rat deaths recorded/total number of rats participating in the experiment

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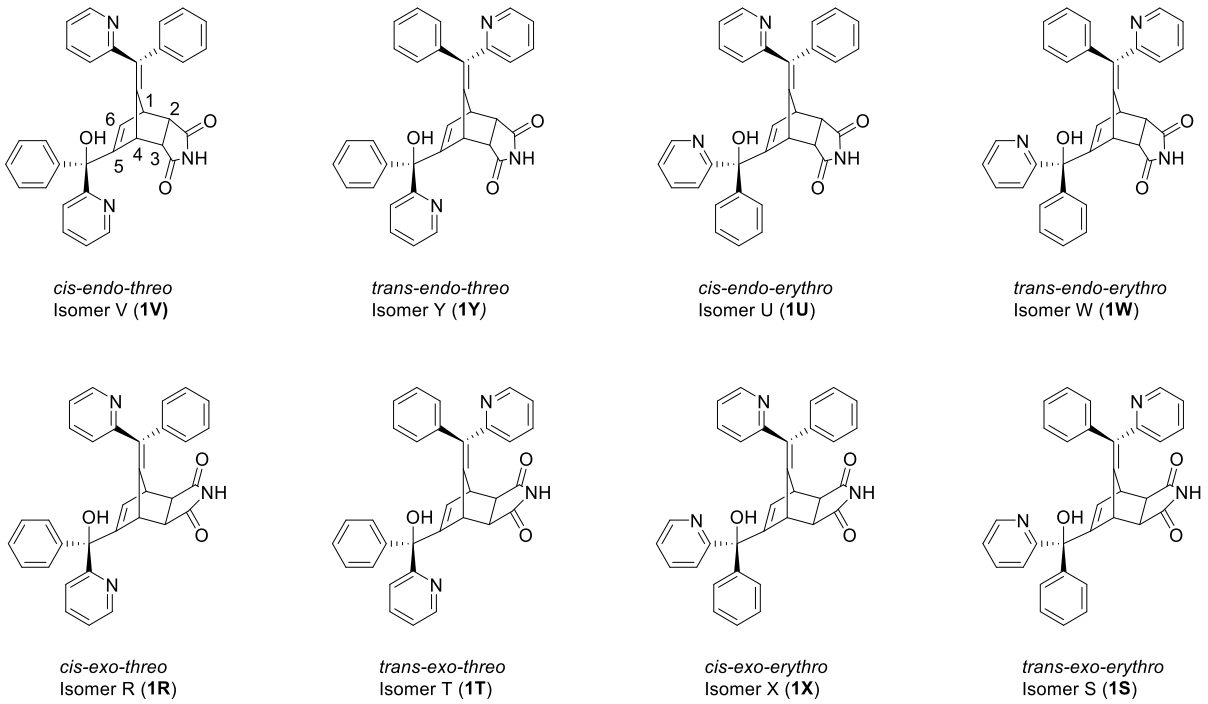


Figure 1. Naming and numbering of the individual stereoisomers of NRB (**1**).[4]

Table 2. Stereoisomeric composition of NRB batches #A and #B, and individual NRB isomer toxicity in rats (see Figure 1).[5]

	Stereoisomeric composition (%)[a]							
Norbormide batch	Y	V	W	U	R	T	X	S
#A	36	32	16	13	0	0	3	0
#B	45	22	23	10	0	0	0	0
LD_{50} (rat, *i.v.*) mg/kg	0.50	0.15	5.0	1.5	>10	>8.5	>10	>8.0
LD_{50} (rat, *p.o.*) mg/kg	<5	2.1	n/a	n/a	n/a	n/a	>100	n/a

[a] As determined by achiral RP-HPLC analysis (AUC, λ_{254} nm), in correlation with ^{1}H NMR.[4] n/a = Not available.

Results and Discussion

Chemical synthesis. Original research conducted by Mohrbacher and Poos over fifty years ago successfully accomplished NRB isomer separation using an exhaustive mixture of fractional recrystallization and preparative TLC methods.[19] Recoveries were reported to be extremely low (<0.01% yield), generating mere milligram quantities of the isolated NRB isomers from kilograms of mixed NRB; yet sufficient material was obtained to allow for the individual NRB isomers to be evaluated for toxicity in rats by *i.v.*, and in some cases, *p.o.* (Table 2).[5] Accordingly, in-house efforts to achieve a chromatographic separation of NRB using modern-day techniques were undertaken, but with only limited success. Using normal phase flash chromatography methods it was not possible to attain a clean separation of all the isomers, regardless of stationary phase or eluent.

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In parallel, work continued on developing a strategy which would ultimately enable the isolation of the individual isomers of NRB, on a scale sufficient for extended *in vivo* biological evaluation. Previous studies working with NRB derivatives[16,17] had revealed that chromatographic differentiation between the isomers could be achieved through the installation of a group on the *N*-dicarboximide nitrogen. Employing a chemical derivatization strategy largely built on established literature protecting group chemistry, a selection of novel *N*-dicarboximide derivatives of NRB were synthesized in an effort to induce greater differentiation between the isomers during chromatographic separation. Requirements for such a strategy fell into three main categories: 1) derivatization must be clean, and the resulting derivative be of sufficient stability to allow for its isolation; 2) a clear chromatographic separation must be attained to reveal all four major isomers present in mixed NRB (NRB Y, V, W, U); and 3) the derivatizing group must be removable under conditions to which NRB is stable. Given that NRB's tolerance to various cleavage environments was unknown, a broad range of derivatizing groups were considered.

Using Kocienski's classification of protecting groups,[20] *N*-dicarboximide derivatives **2**-**28** were split into 10 distinct groups based on the primary method of cleavage used for their removal (Table 3). Acid-labile (both Brønsted and Lewis acid) derivatives **2** (R = Tr), **11** (R = EOM), **15** (R = Boc) and **24** (R = Ts) were considered as exemplars within this particular category. Base sensitive examples included derivatives **3** (R = Ac), **4** (R = TFA), **5** (R = Bz) and **14** (R = Boz). Derivatives **6** (R = Bn), **7** (R = NAP), **12** (R = BOM) and **16** (R = Cbz) where primarily prepared with a view to removing the derivatizing group using hydrogen in the presence of a transition metal catalyst such as palladium(0); under mild hydrogenolysis conditions it was presumed that the tri- and tetra-substituted olefin bonds of NRBs would be stable to such reducing environments. Conversely, following an oxidation approach, PMB and DMB groups are known to undergo single electron transfer in the presence of certain oxidants, for example ammonium cerium(IV) nitrate (CAN) or 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ); which was to be probed using derivatives **8** (R = PMB) and **9** (R = DMB). Transition metal catalysis was to be employed in the cleavage of derivatives **10** (R = All) and **17** (R = Aloc), using palladium(0) catalysts to afford π-allyl palladium complexes which could then be trapped by mild nucleophiles such as dimedone or morpholine. Two distinct families of derivatizing groups cleaved by fluoride ions (fluoride-induced fragmentation) were also explored; those where the silyl group was directly attached to the *N*-dicarboximide, for example derivatives **21** (R = TBDMS), **22** (R = TBDPS) and **23** (R = TIPS), and those which proceeded via a *β*-elimination mechanism, such as derivatives **13** (R = SEM) and **18** (R = Teoc). Reductive elimination (also following a *β*-elimination pathway) was to be explored using zinc-acetic acid (Zn/AcOH) in the cleavage of 2,2,2-trichloroethyl derivatives such as derivative **19** (R = Troc). Furthermore, alternative cleavage techniques may be employed other than the primary methods outlined above; for example derivatives **6** (R = Bn), **7** (R = NAP), **16** (R = Cbz) and **24** (R = Ts) are all known to cleave under dissolving metal reduction conditions, such as sodium in liquid ammonia (Na/NH_3) or lithium di-*tert*-butylbiphenyl (LiDBB). Mild-base catalyzed removal of the 9-fluorenylmethoxycarbonyl group, such as that found in derivative **20** (R = Fmoc), provided a further example of a *β*-elimination-type approach. Finally, nosyl, and more recently tosylvinyl, groups are known to cleave under mild conditions employing thiols (*e.g.* PhSH) in combination with bases such as DIPEA, providing examples **25** (R = 2-Ns), **26** (R = 4-Ns), **27** (R = *E*-Tsv) and **28** (R = *Z*-Tsv).

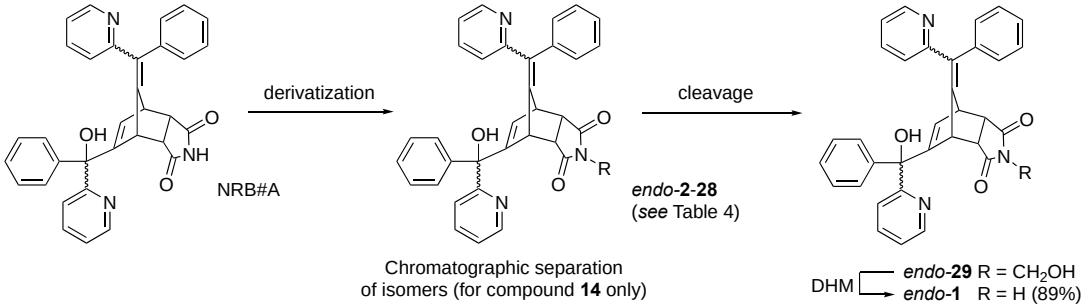
Table 3. Common methods for the removal of protecting groups used in this study.

Method[a]	Reagent examples	Protecting groups
Acid	HCl, TFA, $AlCl_3$	Tr, EOM, Boc, Ts
Basic solvolysis	K_2CO_3, NaOH	Ac, TFA, Bz, Boz
Hydrogenolysis	Pd/C, $Pd(OH)_2/C$	Bn, NAP, BOM, Cbz

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Oxidation	CAN, DDQ	PMB, DMB
Transition metal catalysis	Pd^0/dimedone	All, Aloc
Fluoride ions	TBAF	TBDMS, TBDPS, TIPS, SEM, Teoc
Reductive elimination	Zn/AcOH	Troc
Dissolving metal reduction	Na/NH_3, LiDBB	Bn, NAP, Cbz, Ts
β-Elimination	piperidine	Fmoc
Other	PhSH/DIPEA	Ns, Tsv

[a] Classification and abbreviations according to Kocienski.[20]



Scheme 1. General conditions. Derivatization (in conjunction with Table 4): TrCl, Et_3N, DCM, rt, 24 h (to give **2**); Ac_2O, Et_3N, DMAP, DCM, rt, 24 h (**3**); TFAA, Et_3N, DCM, rt, 24 h (**4**); BzCl, Et_3N, DCM, rt, 24 h (**5**); BnCl, K_2CO_3, DMF, rt, 16 h (**6**); NAPBr, K_2CO_3, DMF, rt, 16 h (**7**); PMBCl, K_2CO_3, DMF, rt, 16 h (**8**); DMBCl, K_2CO_3, DMF, rt, 16 h (**9**); AllBr, K_2CO_3, TBAI, DMF, rt, 16 h (**10**); EOMCl, NaH, DMF, rt, 3 h (**11**); BOMCl, Et_3N, DCM, rt, 24 h (**12**); SEMCl, NaH, DMF, rt, 3 h (**13**); BozCl, K_2CO_3, DMF, rt, 16 h (**14**); Boc_2O, DMAP, DCM, rt, 16 h (**15**); CbzCl, K_2CO_3, THF, rt, 16 h (**16**); AlocOSu, NaH, DMF, rt, 3 h (**17**); TeocONp, Et_3N, DCM, rt, 24 h (**18**); TrocCl, Et_3N, DMF, rt, 16 h (**19**); FmocOSu, NaH, THF, rt, 3 h (**20**); TBDMSCl, NaH, DMF, rt, 16 h (**21**); TBDPSCl, NaH, DMF, rt, 3 h (**22**); TIPSCl, NaH, DMF, rt, 3 h (**23**); TsCl, Et_3N, DCM, rt, 24 h (**24**); 2-NsCl, Et_3N, DCM, rt, 24 h (**25**); 4-NsCl, Et_3N, DCM, rt, 24 h (**26**); tosylacetylene, DMAP, MeCN, 50 °C, 20 h (**27**); tosylacetylene, Et_3N, MeCN, 0°C to rt, 0.5 h (**28**).

Cleavage (in conjunction with Table 5): H_2, Pd/C, aq. HCl (conc.), MeOH, 3 atm., 40 °C, 18 h; H_2, $Pd(OH)_2$/C, aq. HCl (conc.), MeOH, 3 atm., 40 °C, 18 h (Method A); H_2, $Pd(OH)_2$/C, AcOH, MeOH, 1 atm., rt, 18 h (Method B); Na/NH_3, THF, -60 °C, 2 h; LiDBB, THF, -78 °C, 2 h; CAN, MeCN, H_2O, rt, 18 h; DDQ, DCM, H_2O, 70 °C, 18 h; 4 M HCl/1,4-dioxane, MeOH, 70 °C, 18 h; TFA, 60 °C, 18 h; $AlCl_3$, PhMe, 80 °C, 18 h; TBAF, THF, 60 °C, 4 h; K_2CO_3, MeOH, rt, 18 h. Dehydroxymethylation (DHM): i) Et_2NH, DCM, rt, 1 h, ii) $NaBH(OAc)_3$, AcOH, rt, 1 h (**1**).

The synthesis of derivatives **2**, **5**-**17**, **20**, **23**, **24**, **27** and **28** (starting from NRB#A), proceeded smoothly using standard literature methods; conversely, no product formation was observed in the preparation of compounds **18** and **25**, whereas derivatives **3**, **4**, **19, 21**, **22** and **26** were all found to be of insufficient stability and/or contained inseparable side-products (Scheme 1, Table 4). Disappointingly, on attempting chromatographic separation, derivatives **2**, **5**, **9**-**11**, **15**-**17**, **20**, **23**, **24**, **27** and **28** failed to clearly reveal all 4 major isomers present within the parent NRB#A mixture, and as a consequence these particular derivatives were abandoned. Of the derivatives which were found to separate (**6**-**8** and **12**-**14**) (Scheme 1, Tables 4 and 5), initial efforts to cleave benzyl derivative **6** using hydrogenolysis (both Pd/C and $Pd(OH)_2$/C) proved

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unsuccessful, even at elevated temperature and pressure (all cleavage experiments were conducted on the purified stereoisomeric mixture, unless stated otherwise). Similarly, 2-naphthylmethyl derivative **7**, prepared as a further example of a benzyl-type protecting group with known enhanced lability towards palladium(0)-catalyzed hydrogenolysis,[21] was also revealed to be stable. As an alternative method of cleavage, attempts were next made to un-derivatize compounds **6** and **7** via a dissolving metal reduction approach. Disappointingly, reactions using Na/NH_3 led to a complex mixture of products, as was also found to be the case using LiDBB.

Table 4. Norbormide *N*-dicarboximide derivatization and chromatographic separation (in conjunction with Scheme 1).

Compound	R	Yield (%)[a]	Separation	Entry	Compound	R	Yield (%)[a]	Separation
2	Tr	18	No	15	16	Cbz	95	No
3	Ac	-[b]	-	16	17	Aloc	36	No
4	TFA	-[b]	-	17	18	Teoc	-[c]	-
5	Bz	14	No	18	19	Troc	-[b]	-
6	Bn	87	Yes	19	20	Fmoc	30	No
7	NAP	96	Yes	20	21	TBDMS	-[b]	-
8	PMB	57	Yes	21	22	TBDPS	-[b]	-
9	DMB	72	No	22	23	TIPS	92	No
10	All	77	No	23	24	Ts	49	No
11	EOM	59	No	24	25	2-Ns	-[c]	-
12	BOM	78	Yes	25	26	4-Ns	-[d]	-
13	SEM	75	Yes	26	27	*E*-Tsv	80	No
14	Boz	98	Yes	27	28	*Z*-Tsv	83	No
15	Boc	99	No					

Derivatization group (R) abbreviations, according to Kocienski[20]: Ac = acetyl; All = allyl; Aloc = allyloxycarbonyl; Bn = benzyl; Boc = *tert*-butyloxycarbonyl; BOM = benzyloxymethyl; Boz = benzoyloxymethyl; Bz = benzoyl; Cbz = benzyloxycarbonyl; DMB = 3,4-dimethoxybenzyl; EOM = ethoxymethyl; Fmoc = 9-fluorenylmethoxycarbonyl; NAP = 2-naphthylmethyl; 2-Ns = 2-nosyl; 4-Ns = 4-nosyl; PMB = 4-methoxybenzyl; SEM = 2-(trimethylsilyl)ethoxymethyl; TBDMS = *tert*-butyldimethylsilyl; TBDPS = *tert*-butyldiphenylsilyl; Teoc = 2-(trimethylsilyl)ethoxycarbonyl; TFA = trifluoroacetyl; TIPS = triisopropylsilyl; Troc = 2,2,2-trichloroethoxycarbonyl; Tr = trityl; Ts = tosyl; Tsv = 2-(4-tosyl)vinyl. [a] Sum of all isomers. [b] Product was found to be unstable. [c] Reaction failed. [d] Inseparable side-products formed.

Moving our focus onto derivatizing groups designed to cleave under oxidative conditions, PMB derivative **8** was subsequently revealed to be stable in the presence of both CAN and DDQ; on the strength that both benzyl and 2-naphthylmethyl ethers are known to be more resistant to oxidative cleavage than their PMB counterparts, efforts to deprotect derivatives **6** and **7** under oxidative conditions were not pursued.[20,22] On a related note, while PMB ethers have been reported to undergo hydrogenolysis, given that benzyl ethers are known to cleave more readily in the presence of H_2-Pd/C,[20] and with the knowledge that derivative **6** had proven stable to such conditions, this experiment was similarly discounted. Likewise, since PMB ethers are recognized as being more stable to dissolving metal reductions than benzyl ethers,[23] the deprotection of derivative **8** using such methods was not attempted. Instead, efforts to remove the PMB group of derivative **8** using Brønsted acids were investigated. Again, disappointingly, derivative **8** failed to cleave in the presence of

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neat trifluoroacetic acid (TFA), even at elevated temperature, while treatment with aqueous hydrochloric acid (HCl) led only to decomposition products. Lewis acid (aluminium chloride ($AlCl_3$)) methods also failed to deprotect derivative **8**. Knowing that PMB ethers are generally less stable to acid than their unsubstituted benzyl counterparts, attempts to cleave derivatives **6** and **7** using either Brønsted or Lewis acids were not attempted.[20]

Table 5. Norbormide *N*-dicarboximide derivative cleavage method/reagent (in conjunction with Scheme 1).

Cleavage method	Reagent	R (Compounds)	Observations	Yield (%)[a]
Hydrogenolysis	H_2, Pd/C; $Pd(OH)_2$/C (A)	Bn (**6**), NAP (**7**)	Starting material recovered	-
	H_2, $Pd(OH)_2$/C (B)	BOM (**12**)	Gave compound **29**	75
Dissolving metal reduction	Na/NH_3; LiDBB	Bn (**6**), NAP (**7**)	Inseparable side-products formed	-
Oxidation	CAN; DDQ	PMB (**8**)	Inseparable side-products formed	-
Acid	HCl	PMB (**8**)	Inseparable side-products formed	-
	TFA; $AlCl_3$	PMB (**8**)	Starting material recovered	-
	TFA	BOM (**12**)	Gave compound **29**	76
	HCl	SEM (**13**)	Gave compound **29**	82
	HCl	Boz (**14**)	Gave compound **29**	91
Fluoride	TBAF	SEM (**13**)	Starting material recovered	-
Basic solvolysis	K_2CO_3	Boz (**14**)	Gave compound **29**	73

[a] Sum of all isomers; cleavage method and conditions initially trialled on unseparated mixture.

As a further example of a derivatizing group which permitted the required chromatographic separation of isomers, BOM derivative **12** was now subjected to an array of cleavage conditions. Initial approaches focused on catalytic hydrogenolysis, using H_2-$Pd(OH)_2$/C in the presence of dilute aqueous acid. Contrary to expectation, NRB was not revealed as the product of the hydrogenolysis, instead the reaction was found to terminate at *N*-hydroxymethyl NRB derivative **29** (in 75% yield); it was originally anticipated that intermediate **29** would be unstable and spontaneously collapse *in situ* to furnish NRB. The benzyl group of BOM derivative **12** could similarly be removed under heating in TFA, again leading to *N*-hydroxymethyl NRB **29** (76% yield). Analogous observations were noted in the cleavage of SEM derivative **13** using HCl (only starting materials were recovered using tetra-*n*-butylammonium fluoride (TBAF)), and in the deprotection of Boz derivative **14** using either aqueous acid (HCl) or base (potassium carbonate (K_2CO_3)), in all instances affording *N*-hydroxymethyl NRB **29** (82%, 91% and 73% yield, respectively).

It has been reported that *N*-hydroxymethyl dicarboximides can be hydrolyzed under basic conditions to furnish the corresponding unsubstituted dicarboximides.[24-29] Ensuing attempts to hydrolyze *N*-hydroxymethyl NRB **29** using ammonium hydroxide (NH_4OH)[25-27] led only to dicarboximide ring-opened side-products. In an effort to limit such unwanted hydrolysis, a selection of tertiary amine bases were screened (to be used under non-aqueous conditions).[28,29] Reactions employing either *N*-methylmorpholine (NMM), 1,4-diazabicyclo[2.2.2]octane (DABCO), triethylamine (TEA) or *N*,*N*-diisopropylethylamine (DIPEA) (in order of increasing base strength) resulted in only the partial conversion of *N*-hydroxymethyl NRB **29** to NRB (12%, 9%, 16%, 81%, respectively, rt, 24 h; according to achiral RP-HPLC of the reaction mixture). Conversely, the reaction proceeded to completion within 15 minutes in the presence of strongly basic, non-nucleophilic 1,8-

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diazabicyclo[5.4.0]undec-7-ene (DBU) (pK_a 12).[30] Unexpectedly, upon isolation (standard aqueous work-up, pH 6.5) of the product, despite the reaction having apparently proceeded to >99% completion (according to achiral RP-HPLC), an inseparable mixture of NRB and *N*-hydroxymethyl NRB **29** was observed (*ca.* 3:1, respectively). It is tentatively postulated that an equilibrium is in existence between *N*-hydroxymethyl NRB **29** and the corresponding reaction breakdown products, NRB and formaldehyde (thus allowing the reaction to proceed in the reverse direction during work-up) (Figure 2). Similar observations have been reported for unrelated *N*-hydroxymethyl dicarboximides.[29]

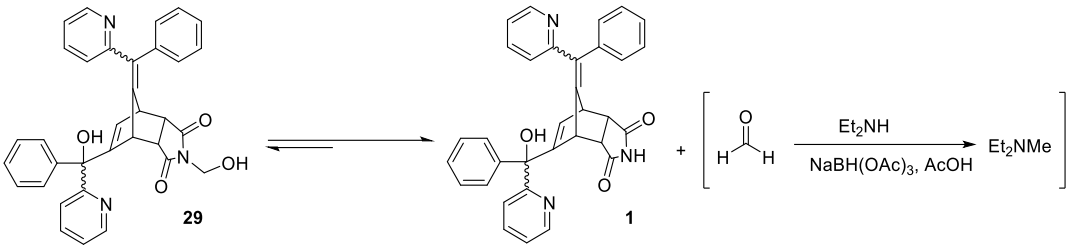


Figure 2. Postulated equilibrium between *N*-hydroxymethyl NRB derivative **29** and NRB (**1**) in the presence of formaldehyde (scavenged by means of *in situ* reductive amination).

On the premise that the formaldehyde generated in the reaction was indeed responsible for the *N*-hydroxymethylation of NRB during product isolation, attempts were made to sequester it from the reaction mixture using various 'scavenging' techniques. A number of reagents have been shown to selectively react with formaldehyde, including sodium bisulfite,[31] tris(hydroxymethyl)aminomethane (Tris),[32] semicarbazide[33] and urea.[34] Alternatively, the selective reduction of aldehydes in the presence of other carbonyl-containing functional groups has been achieved using sodium triacetoxyborohydride ($NaBH(OAc)_3$).[35] Subsequent attempts to induce the breakdown of *N*-hydroxymethyl NRB **29** in the presence of Tris failed, likely owing to its lower basicity (pK_a 8.5).[36] On repeating the reaction in the presence of both Tris (as a formaldehyde scavenger) and DBU (to promote *N*-dehydroxymethylation), again, a mixture of NRB and *N*-hydroxymethyl NRB **29** was observed post work-up. Instead, in an effort to scavenge formaldehyde by means of hydride reduction, a sequential one-pot two-step process commencing with DBU-induced *N*-dehydroxymethylation, followed by the addition of $NaBH(OAc)_3$/AcOH in an attempt to consume the formaldehyde by-product, again led to a mixture of NRB and *N*-hydroxymethyl NRB **29**. Replacing $NaBH(OAc)_3$ with the more powerful hydride source, sodium borohydride ($NaBH_4$), led to partial reduction of the dicarboximide ring. On a related note, $NaBH(OAc)_3$ has been demonstrated to act as a mild hydride reductant in the reductive amination of both aldehydes and ketones.[37,38] Correspondingly, it was proposed that substituting DBU (a tertiary amine) with a strongly basic secondary amine, for example diethylamine (pK_a 10.5),[39] with a view to generating an iminium ion which in turn could then undergo mild selective reduction *in situ* (using $NaBH(OAc)_3$), could offer an alternative way of preventing the reformation of *N*-hydroxymethyl NRB **29** post cleavage (Figure 2). To our immense gratification, using this procedure, NRB was afforded quantitatively upon work-up (and in 89% yield after purification) (Scheme 1); note, using diethylamine in the absence of $NaBH(OAc)_3$ again resulted in a mixture of NRB and *N*-hydroxymethyl NRB **29** post work-up.

Table 6. Isolated isomer recovery from NRB#A using a chemical derivatization strategy (via Boz-protected derivative **14**; following method B) (see Figure 1).

% Isomer Recovery[a]			
NRB Y[b]	NRB V	NRB W	NRB U[c]

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64[d]	49[d]	66[d]	65[d]

[a] % recovery based on the maximum theoretical yield (100%) of each isomer available within the parent NRB#A mixture (1.3 g scale). [b] Contains ≤5% isomer U. [c] Contains ≤3% isomer W. [d] Combined yield over 3 stages: derivatization, chromatographic separation, cleavage (using aqueous HCl method).

With a successful chemical derivatization/cleavage strategy now unveiled, we next set out to obtain sufficient quantities of each NRB stereoisomer to supply the intended palatability and efficacy bait trial. Basing selection on a combination of the fundamental requirements involved in the derivatization strategy (ease of derivatization, chromatographic separation and cleavage), an approach proceeding via Boz derivative **14** was nominated as the best available route (Scheme 1, Tables 4 and 5). Starting from mixed NRB, sequential derivatization and chromatographic separation afforded the isolated individual isomers of Boz derivative **14** in high recovery: **14Y** (92%, based on the maximum theoretical yield of each isomer available within the parent NRB#A mixture), **14V** (85%), **14W** (94%) and **14U** (92%). Subsequent deprotection (HCl), via *N*-hydroxymethyl NRB **29** (not isolated), which in turn was *N*-dehydroxymethylated using diethylamine in combination with $NaBH(OAc)_3$/AcOH, successfully furnished NRB Y, V, W and U. Dependably, overall isomer recovery ranged between 49-66%, emphatically surpassing previous literature isolation methods employing fractional recrystallization and preparative TLC techniques (<0.01% yield) (Table 6).[19]

Scanning electron microscopy (SEM). SEM analysis of the isolated individual NRB isomers (NRB Y, V, W and U) revealed a high degree of similarity among the samples (being largely comprised of widely polydisperse particle sizes exhibiting similar crystalline morphology; minor surface-scale deviations were observable at 4000× magnification) (Figure 3).

Biological evaluation: bait palatability and efficacy trial in rats. Having now successfully isolated the individual *endo*-isomers of NRB using the aforementioned chemical derivatization strategy in sufficient quantity to undergo palatability and efficacy evaluation in rats, NRB Y, V, W and U were now assessed in a bait trial; for completeness, an exclusively *exo*-NRB mixture (NRB R/S/T/X)[40] was appraised in parallel to determine what effect, if any, the non-lethal (to rats) isomers of NRB have on palatability.[5] Following 1 day 'pre-baiting' with a highly palatable bait formulation free of toxicant, rats (*Sprague-Dawley*) were fed bait containing either NRB Y, V, W, U or R/S/T/X, at a concentration of either 0.3% or 0.1 w/w (Table 7); NRB#B was employed as a positive control. Upon exchanging the non-toxic 'pre-feed' bait for bait containing either NRB Y (0.63±0.16, as a % of body mass), NRB V (0.77±0.18) or NRB U (0.86±0.09), consumption was found to be largely comparable across the different isomers; and similar to that of the NRB control bait (NRB#B 0.90±0.20). Conversely, baits containing either NRB W (1.50±0.24) or NRB R/S/T/X (1.59±0.01) were revealed to be consumed in significantly greater quantities. With respect to mortality, baits containing either NRB Y (3 death recorded from a total of 4 rats) or NRB V (2/3) displayed a level of efficacy similar to that of the NRB control (NRB#B 3/3), whereas baits containing NRB W (0/3), NRB U (0/3) or NRB R/S/T/X (0/3) were all demonstrated to be non-lethal (in the quantities consumed). Given that NRB isomers W, U, R, S, T and X have all previously been demonstrated to be of relatively lower toxicity to rats (when compared directly to isomers V and Y),[5] such an observation was not entirely unexpected. Note, earlier studies working with baits containing 0.1% w/w toxicant (chosen on the basis of best minimizing the impact of mortality on the ensuing palatability data by working with lower % loadings of the toxicant) failed to allow for differentiation between the toxic baits, since all baits were consumed in their entirety; no lethal endpoints were recorded (data not presented).

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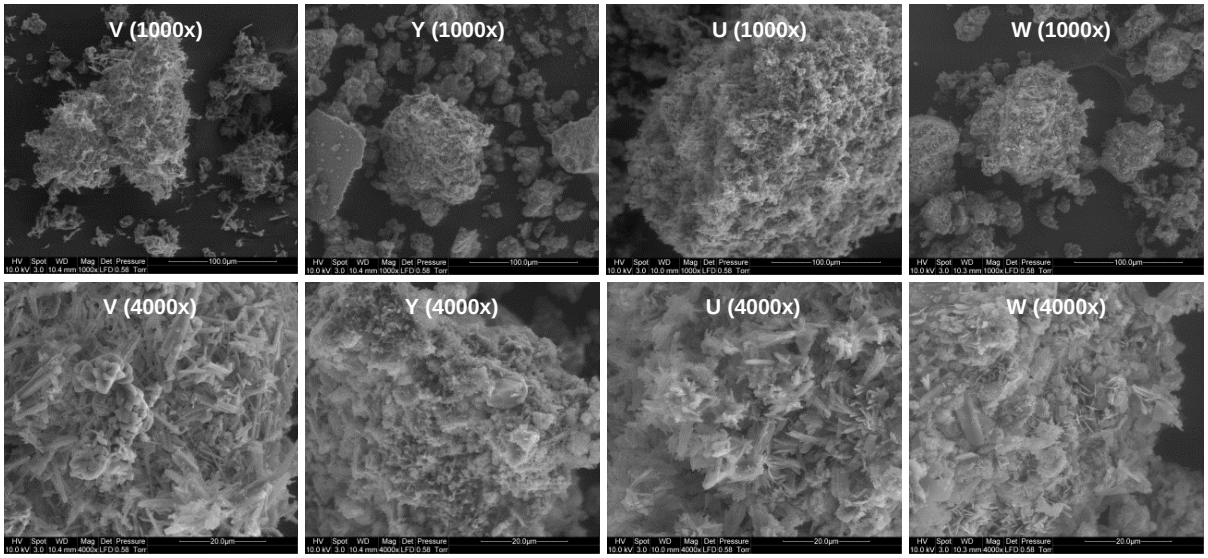


Figure 3. Scanning electron micrographs of NRB V, Y, U and W; at 1000× and 4000× magnification.

Table 7. No-choice bait-palatability and efficacy trial observations for isolated NRB isomers Y, V, W, U and R/S/T/X, alongside NRB#B (as a control), in *Sprague-Dawley* rats (see Figure 1).

	Consumption/rat[a]			
Norbormide Batch	Mean bait consumption (g)[b]	Mean bait consumption as a % of body mass ±SE	Mean NRB dose consumed ±SE (mg/kg)	Mortality[c]
Y	1.66	0.63±0.16	19± 6	3/4
V	2.12	0.77±0.18	23±5	2/3
W	4.42	1.50±0.24	45±7	0/3
U	2.73	0.86±0.09	26±3	0/3
R/S/T/X[d]	5.00	1.59±0.01	48±1	0/2
NRB#B	2.72	0.90±0.20	27±6	3/3

[a] Rats were presented with the toxicant in a highly palatable bait formulation (5 g, 0.3% w/w toxicant) following 1 day 'pre-feeding' with a highly palatable bait formulation free of toxicant (starting with n = 5 for each test compound; rats which unequivocally refused to consume the 'pre-feed' bait were subsequently removed from the trial). [b] Over 2 h. [c] Total number of rat deaths recorded/total number of rats participating in the experiment. [d] R/S/T/X, 26:14:14:46 ratio.

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Conclusions

Each of the *endo* stereoisomers of NRB (Y, V, W and U) was successfully isolated using a novel chemical derivatization strategy (using methods accessible to those skilled-in-the-art, and without the need for expensive apparatus). Succinctly, through the reversible introduction of a Boz-substituent onto the *N*-dicarboximide nitrogen of NRB, chromatographic differentiation between the stereoisomers could be achieved. Subsequent cleavage of the separated NRB Boz-isomers (compound **14**), using a two-step one-pot reaction procedure (via *N*-hydroxymethyl NRB **29**), revealed NRB Y, V, W and U in good yield. Disappointingly, given the privileged pharmacological tools available as a product of this research, no clear relationship between stereochemistry and palatability/efficacy in rats was observed.

Experimental Section

General. All reagents were used as supplied, unless stated otherwise. Solvents were purified using standard methods. Analytical thin layer chromatography (TLC) was carried out on pre-coated silica gel plates (*Merck*/UV254) and products were visualized by UV fluorescence. Flash chromatography was performed using silica gel (*Riedel-de Haën*, particle size 0.032–0.063 mm). Distillation of cyclopentadiene was carried out using a *Vigreux* apparatus.

5-(*α*-Hydroxy-*α*-2-pyridylbenzyl)-7-(*α*-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide (**1Y**).[4] To a solution of **14Y** (159 mg, 0.25 mmol) in MeOH (3 mL) was added a solution of HCl in 1,4-dioxane (3 mL, 4 M), and the mixture stirred at 70 °C for 18 h. The solvent was then removed *in vacuo* and the resulting residue taken up in DCM, washed with aq. NaH_2PO_4/Na_2HPO_4 (0.5 M, pH 6.6), dried over anhydrous $MgSO_4$, filtered and the filtrate concentrated *in vacuo*. The resulting residue was then taken up in a solution of Et_2NH (25 µl, 0.25 mmol) in DCM (3 mL), and stirred at r.t. for 1 h. $NaBH(OAc)_3$ (52 mg, 0.25 mmol) and AcOH (15 µl, 0.25 mmol) were then added and the mixture stirred at r.t. for a further 1 h. The mixture was then diluted with aq. NaH_2PO_4/Na_2HPO_4 (0.5 M, pH 6.6; to achieve a soln. pH of 6.6) and extracted with DCM. The combined organic extracts were then dried over anhydrous $MgSO_4$, filtered and the filtrate concentrated *in vacuo*. Purification by column chromatography (petroleum ether/AcOEt, 1:1) afforded **1Y** (88 mg, 70%) as a white solid (*for spectral data, see supporting information*).

5-(*α*-Hydroxy-*α*-2-pyridylbenzyl)-7-(*α*-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide (**1V**).[4] A similar procedure to that previously described for the preparation of **1Y** was followed using **14V** (345 mg, 0.53 mmol) and HCl (5 mL, 4 M in 1,4-dioxane) in MeOH (5 mL), followed by Et_2NH (53 µl, 0.53 mmol) in DCM (2 mL), then $NaBH(OAc)_3$ (113 mg, 0.53 mmol) and AcOH (30 µl, 0.53 mmol). Purification by column chromatography (petroleum ether/AcOEt, 1:2) afforded **1V** (160 mg, 58%) as a white solid (*for spectral data, see supporting information*).

5-(*α*-Hydroxy-*α*-2-pyridylbenzyl)-7-(*α*-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide (**1W**).[4] A similar procedure to that previously described for the preparation of **1Y** was followed using **14W** (84 mg, 0.13 mmol) and HCl (2 mL, 4 M in 1,4-dioxane) in MeOH (2 mL), followed by Et_2NH (13 µl, 0.13 mmol) in DCM (1 mL), then $NaBH(OAc)_3$ (28 mg, 0.13 mmol) and AcOH (7 µl, 0.13 mmol). Purification by column chromatography (petroleum ether/AcOEt, 1:1) afforded **1W** (47 mg, 70%) as a white solid (*for spectral data, see supporting information*).

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5-(α-Hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide (**1U**).[4] A similar procedure to that previously described for the preparation of **1Y** was followed using **14U** (95 mg, 0.15 mmol) and HCl (2 mL, 4 M in 1,4-dioxane) in MeOH (2 mL), followed by Et_2NH (15 µl, 0.15 mmol) in DCM (1 mL), then $NaBH(OAc)_3$ (31 mg, 0.15 mmol) and AcOH (9 µl, 0.15 mmol). Purification by column chromatography (petroleum ether/AcOEt, 1:1) afforded **1U** (53 mg, 71%) as a white solid (*for spectral data, see supporting information*).

***N*-(Benzoyloxymethyl)-5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene)-5-norbornene-2,3-dicarboximide** (**14Y**, **14V**, **14W**, **14U**).[15] To a solution of NRB#A (1.30 g, 2.54 mmol) and K_2CO_3 (0.53 g, 3.81 mmol) in DMF (5 mL) was added chloromethyl benzoate[15] (0.51 g, 3.05 mmol), and the mixture stirred at r.t. for 16 h. The mixture was then diluted with AcOEt, washed with brine, dried over anhydrous $MgSO_4$, filtered and the filtrate concentrated *in vacuo*. Purification by column chromatography (petroleum ether/AcOEt, 2:1) afforded (post separation) **14Y** (540 mg, 92%), **14V** (445 mg, 85%), **14W** (246 mg, 94%) and **14U** (197 mg, 92%) (% recovery based on maximum theoretical yield of each isomer available within the parent NRB#A mixture), as white solids (*for spectral data, see supporting information*).

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Supplementary Material

Experimental protocol describing the synthesis of compounds **1**, **2**, **5**-**17**, **20**, **23**, **24**, **27**-**29**, along with the ^{1}H-NMR spectra and RP-HPLC chromatograms for compounds **1Y**/**1V**/**1W**/**1U** and **14Y**/**14V**/**14W**/**14U**, are available as Supplementary data in the online version of the text.

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