Synthesis and Biological Investigation into the Metabolism of Retinoids

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October 2008 - September 2009

Abstract

Retinoids have a wide variety of applications. They can act as potent inducers of stem cell differentiation, as chemopreventative and chemotherapeutic agents, and have been utilised for the treatment of dermatological conditions. However, natural retinoids readily undergo photo-isomerisation in the presence of natural light and / or in physiological conditions. This propensity for isomerisation is believed to be the cause of many unwanted problems / side effects. Synthetic retinoids have been made by replacing key functional groups with more robust pharmacophores which retain the key functionalities of natural retinoids but are resistant to isomerisation. Previous studies have shown that synthetic retinoids, such as EC23 and EC19 have very high stability and are more potent than their natural analogues ATRA and 13*cis*RA.

The high potency of synthetic retinoids such as EC23 implies that they do not undergo metabolism. An *in vivo* investigation into the metabolism of retinoids was initiated. AG1-X2 polymer beads were used to deliver various concentrations of AT-retinol solutions in DMSO to the anterior margin of a stage 20 chick limb bud which was then observed at stage 36 for signs of abnormal development such as digit duplication. Occurrence of digit duplication would suggest that AT-retinol had been metabolised to ATRA, a known inducer of teratogenic effects, within the limb bud; however, only normal development was observed. This suggests that an alternative route for delivering uncharged molecules to limb buds is required.

Sonogashira cross coupling reactions were employed to synthesise ether acetate analogues of EC23 and EC19, (EC23-OAc and EC19-OAc) from TMNA with acetic acid 4-bromo-benzyl ester and acetic acid 3-iodo-benzyl ester respectively, with the intention of hydrolysing them into EC23-OH and EC19-OH, i.e. synthetic analogues of AT-retinol, to be investigated for teratogenic effects in the chick limb-bud as above. If successful delivery of AT-retinol produced limb defects, while EC23-OH did not, this would be consistent with the idea that EC23 and its analogues are refractory to normal retinoid metabolism.

A variety of useful retinoid precursors were also synthesised from cross coupling reactions and borylation techniques. Synthetic retinoids methyl 5',6',7',8'-tetrahydro-2,2'-binaphthyl-6-carboxylate and (E)-methyl-3-(4-(5,6,7,8-tetrahydronaphthalen-2-yl)phenyl)acrylate were made by Suzuki-Miyaura Cross-coupling reactions, also to be tested *in vivo*.

Acknowledgements

I'd like to give a huge thank you to my supervisor Todd for his endless patience, encouragement enthusiasm. I would also like to give a big thanks to Paul, who has also spent a great deal of time, explaining and teaching me biological techniques. Andy too has shown much support and given many great suggestions, for which I'm very grateful.

I would not have made it this far if it had not been for the warmth and friendliness of the rest of the group. I'm grateful for Jon and Jonathan who were always on hand to teach me techniques and help me out and for Meng Guan and Manolo for their reassurance. Thank you to Pete and Andrew for their continued help and patience – especially with my seemingly never ending columns – and for making me laugh. Thanks also to Andreas for the hours spent helping me with written work. I am incredibly thankful for the support of Emma and Marie Hélène for making me tea when it was all too much and not letting me drop that potato! Bianca – It's difficult to feel anything but positive when you're around! Thanks also to Christian, Nim, Liu Chao and Hazmi for great ideas, fantastic cakes and lots of laughs. I've never known such a lovely group. I've really enjoyed getting to know you and will miss you all. *Thank You!*

Contents

Abbreviations

9*cis*RA	9-*cis* retinoic acid
13*cis*RA	13-*cis* retinoic acid; Isotretinoin
A	Absorbance
A.C.S.	A.C.S. grade reagent meets the specifications of the American Chemical Society
AER	Apical ectodermal ridge
APL	Acute promyelocytic leukaemia
ASAP	Atmospheric pressure solids analysis probe
AT	All-*trans*
ATRA	All-*trans* retinoic acid; Tretinoin
CNS	Central nervous system
CRABP	Cellular retinoic acid binding protein
CRBPs	Cellular retinol binding protein
DCM	Dichloromethane
dppf	1,1'-bis(diphenylphosphino)ferrocene
dtbpy	4,4´-di-*tert*-butyl-2,2´-bipyridine
ESI	Electro spray ionisation
EC	Embryonal carcinoma
GC-MS	Gas chromatography – Mass spectrometry
HRMS	High Resolution Mass Spectrometry
IPS cells	Inducible pluripotent stem cells
LBP	Ligand binding pocket
LRAT	Lecithin retinol-acyl-transferase
neop	Neopentylglycolato
NMR	Nuclear magnetic resonance
pin	Pinacolato ($OCMe_2CMe_2O$)
ppm	Parts per million
QTOF	Quadrupole time of flight

RA	Retinoic acid
RAR-α,β,γ	Retinoic acid receptors; α, β and γ isotypes
RXR-α,β,γ	Retinoid X receptors; α, β and γ isotypes
Shh	Sonic hedgehog gene
S-phos	2-Dicyclohexylphosphino-2',6'-dimethoxybiphenyl
TMNA	6-Ethynyl-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene
tlc	Thin layer chromatography
ZPA	Zone of polarising activity

Chapter 1: Introduction

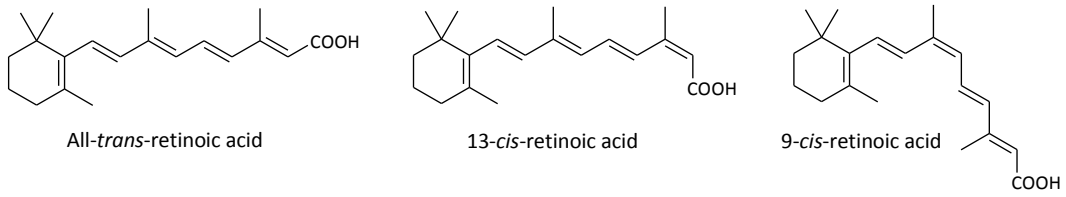
1.1 Natural Retinoids

The retinoids are a group of natural and synthetic molecules which have a structure and / or function related to vitamin A. Natural retinoids are important for embryonic development and play key roles in the development of organs and systems such as the heart[1] and central nervous system.[2] They also play crucial roles in the adult organism, regulating important biological processes including vision,[3] immune defence,[3] sleep regulation[4] and cellular regulation and differentiation.[2] Disregulation of the retinoid pathway is associated with neurological disorders such as Schizophrenia, Alzheimer's and motor neurone disease.[2]

Close regulation of vitamin A levels is crucial in maintaining bodily processes. Vitamin A cannot be synthesised by mammals and must be ingested either in the form of provitamin A from plants or preformed vitamin A from animal products. In humans, the major storage site is the liver, where it is stored in the form of retinyl palmitate. It is then transported in the form of retinol bound to retinol binding protein to tissues in the body as required. Diets either too high or too low in vitamin A can result in severe disorders and extreme deviations from optimum levels may be fatal.[5]

Vitamin A deficiency is characterised by xerophthalmia (dry eyes), night blindness and impaired immune responses which can be fatal.[2] At the other extreme, excessive intake of vitamin A, known as hypervitaminosis A, can result in problems with the skin, nervous system, circulation, bone formation and immune system.[2] Hypervitaminosis A is also associated with osteoporosis.[6] Abnormal vitamin A levels can cause teratogenic effects, as evidenced by the occurrence of birth defects in the offspring of mothers with either excessive or

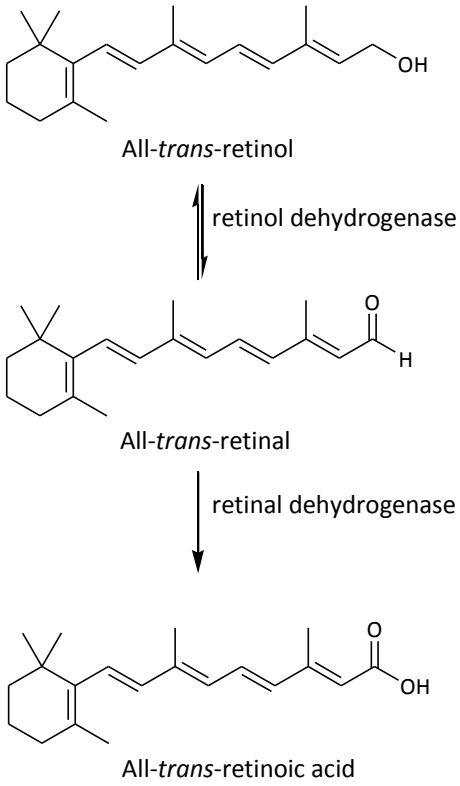
restricted intakes during early pregnancy.[7] Regulation of vitamin A levels is therefore crucial both for normal development and regulation of biological processes.



All-*trans*-retinoic acid 13-*cis*-retinoic acid 9-*cis*-retinoic acid

Figure 1: Common isomeric forms of retinoic acid.

Retinoic acid (RA), a well studied metabolite of vitamin A, exists in several isomeric forms including all-*trans*-retinoic acid (ATRA), 13-*cis*-retinoic acid (13*cis*RA) and 9-*cis*-retinoic acid (9*cis*RA), as shown in Figure 1. Interconversion between the different isomers is facile, especially in the presence of light. ATRA is the most thermodynamically stable and is the major isomer at equilibrium. It is converted in the body from ingested retinol, via a two step reaction,[8] shown in Scheme 1. Retinol is reversibly converted to retinal by retinol dehydrogenase, which is then irreversibly converted to ATRA by retinal dehydrogenase.[9] Retinoid activity arises mainly from the activation of three retinoic acid receptors (RAR α, β and γ) and three retinoic X receptors (RXR α, β and γ).[10] The receptors have varying affinity for the different isomers.



Scheme 1: Enzymatic conversion of ATRA to AT-retinol.

1.2 Applications

The high biological activity of retinoids lends them to a wide variety of medical applications. For example, retinoid-based therapies show promise in the treatment of eye diseases such retinal dystrophies[11] and macular degeneration.[12] An early use of ATRA was in the treatment of skin conditions such as acne[13, 23] and psoriasis.[26] Use of retinoids such as ATRA and 13*cis*RA have considerably improved the management and prognosis of certain cancers including prostate[14,15,16] and breast cancers,[17] leukaemia,[19, 21,20] and neuroblastoma.[18] The ability of retinoids to induce differentiation of stem cells has been utilized to grow tissues *in vitro.*[34]

Applications: Cancer Therapy (Leukaemia)

Retinoids are able to control differentiation and apoptosis of tumour cells, giving them therapeutic potential for cancer treatment and prevention. The introduction of ATRA into the therapy of acute promyelocytic leukaemia (APL) has completely revolutionised its management and outcome. A characteristic of this disease is that the cells are blocked at an early stage of their development and are unable to differentiate into functional mature cells.[19] Whilst traditional cytotoxic therapies simply kill cancer cells, ATRA can reverse malignant cell growth.[20] The new treatment theoretically avoids the undesirable side effects of cytotoxic therapies and has drastically improved remission and cure rates.[19]

The major difficulty with ATRA therapy is the rapid resistance patients develop to the treatment.[9,19,20] Both *in vivo* and *in vitro* studies have shown RA metabolism increases with increasing ATRA therapy.[20] It is therefore suggested that metabolism plays a key role in ATRA resistance.[9] Improvements in drug delivery methods such as encapsulation in liposomes have been shown to slow ATRA metabolism;[20] however, currently the most effective way to combat ATRA resistance is combined therapy with arsenic trioxide (ATO).[19,21]

The side effects of ATRA therapy, whilst much milder than those caused by traditional cytotoxic therapies, are still unpleasant and undesirable. They include hepatoxicity (chemical-driven liver damage), headaches, nausea, vomiting, abdominal pain and mucocutaneous dryness (dryness of skin found at the orifices)[20] and are attributed to isomers of ATRA, such as 13*cis*RA, which are formed readily under physiological conditions. The development of synthetic retinoids which have the same key functionalities as ATRA and 13*cis*RA, yet are resistant to metabolism and isomerisation, may be a new approach to overcoming the resistance and side effects of current retinoid treatment.

Applications: Skin Treatment

Retinoids are used widely and successfully in the treatment of skin conditions such as acne and psoriasis. The effects of topical ATRA (also known as tretinoin) was first reported by Stuttgen and Beer in 1962.[22] Since then, three generations of topical retinoids have been developed. Today, the major commercially available retinoids are tretinoin, tazarotene and adapalene (Figure 2).

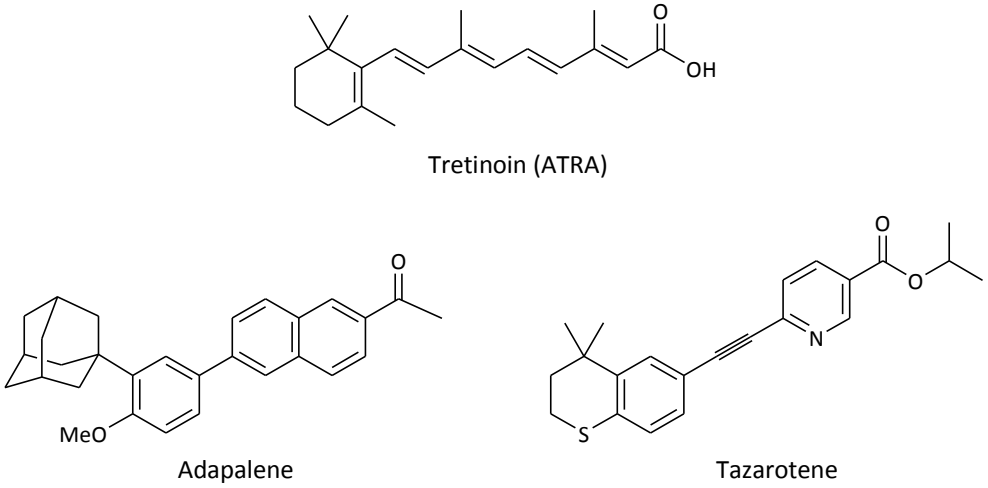


Figure 2: Major commercially available retinoids: First generation retinoid tretinoin, along with third generation retinoids adapalene and tazarotene.

In 1972, ATRA became the first topical retinoid used for the treatment of acne.[23] Derivatives of ATRA, namely retinol and 13*cis*RA (also known as isotretinoin) were also amongst the early topical retinoids. It is believed that 13*cis*RA is isomerised to ATRA[24] which then interacts with RAR receptors to regulate pathogenic factors associated with acne, specifically, normalising keratinisation

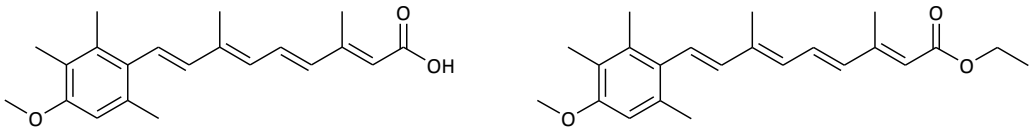
of the follicular infundibulum[i], normalising propionibacterium acnes[ii] mediated responses and reducing inflammation.[23] Early preparations caused severe skin irritation such as redness, scaling, itching, burning, blistering and thinning of the skin. ATRA is currently the only topical retinoid available in generic formulations and is therefore significantly cheaper than others.[23] For this reason, despite its unpleasant side effects and the fact that it is a reported human teratogen,[25] ATRA is still used widely as a skin treatment.

Acitretin (shown in Figure 3) is a synthetic systemic retinoid taken orally for the treatment of severe psoriasis.[26] It is a non selective activator of retinoic acid receptors RAR α, β and γ[26] and acts to reduce the severity of scaling, erythema (redness of the skin) and induration[27] (hardening of the skin). The side effects associated with oral retinoids are more severe than those of their topical counterparts and include elevation of liver enzymes, hyperostosis[28] (excessive bone growth) and elevation of cholesterol;[27] therefore, acitretin is only prescribed to patients who have not responded to topical retinoid treatment. Acitretin can cause teratogenic effects up to three years after discontinuation and has accordingly been assigned as a pregnancy category X.[29]

Etretinate (shown in Figure 3), the ethyl ester form of acitretin, was also administered to treat psoriasis until it was discovered it had an elimination half-life of 80-175 days.[30] Its long elimination half life is attributed to it being highly lipophilic and therefore it readily accumulates in skin tissue. As a build up of teratogenic drugs is highly undesirable, etretinate was withdrawn in favour of the acid derivative, acitretin, which has an elimination half life of only 2-3 days. However, it was later discovered that in the presence of ethanol, acitretin can be esterified to etretinate.[31] It is therefore important that patients taking acitretin do not consume alcohol. This should also serve as a warning as to the extreme

[i] I.e. regulation of the formation of skin around the hair follicles. In acne patients, improper regulation of this process leads to a build up of dead skin cells which block the pores.
[ii] A bacteria with the potential to break down the walls of pores in the skin.

care and attention required when taking, administering and working with retinoids. Whilst the exact metabolic pathways are unknown it should be presumed that all forms of retinoids have the potential to be converted into active and / or long lived forms. All retinoids, especially synthetic ones, should be treated as highly teratogenic.



Acitretin Etretinate

Figure 3: Second generation retinoids, Acitretin and Etretinate.

Third generation synthetic retinoids have been developed to show greater receptor specificity and lesser side effects than their first and second generation counterparts. Adapalene (shown in Figure 2) was the first synthetic retinoid used in the topical treatment of acne. Like ATRA, it regulates keratinisation and has anti-inflammatory properties; however, unlike ATRA it is selective for RAR *β/γ* and does not bind to CRABPs (cellular retinoic acid binding proteins). Tazarotene, (shown in Figure 2) also used in the treatment of acne and psoriasis, is rapidly converted into tazarotenic acid, its active metabolite. The primary effects of Tazarotene are regulation of cell differentiation and down-regulation of proinflammatory mediators.[23] Tazarotene is able to bind all three RAR*α/β/γ* but shows increased affinity to RAR*β* and *γ.* Both Adapalene and Tazarotene have less unwanted side reactions than the RAR pan agonist ATRA and this is attributed to their greater selectivity.

Difficulties with the use of Retinoids in Skin Treatment

Retinoid therapies have shown excellent results in the treatment of skin

conditions; however, t hey should be administered with caution due to their potential teratogenic effects. The teratogenic risks posed by oral retinoids is much greater than those for topical retinoids. All retinoid treatment is contraindicated for use in the early stages of pregnancy, and it is recommended that oral treatment must be ceased at least three years prior to conception.[26]

The facility of natural retinoids to isomerise can cause further problems with their use as skin treatment agents. Under physiological conditions, their conversion into other isomers[iii] may induce additional biological pathways, resulting in unwanted side effects. Synthetic third generation retinoids generally have milder side effects than natural first generation retinoids and this may be attributed to their enhanced receptor specificity along with their increased resistance to isomerisation. The ability of natural retinoids to isomerise readily in natural light results in degradation of the active ingredient in the formulation, and therefore leads to reduced potency. An interesting approach to this problem is a recommendation to "use at night" on patient leaflet![32] A more practical solution is the microencapsulation of ATRA, which has been shown to dramatically reduce photodegredation and also reduce skin irritation.[32] A photostable synthetic derivative of ATRA, discussed later, has been developed. This could be a future, more stable, alternative to the use of natural retinoids.

Applications: Stem Cell Differentiation

Retinoids are also utilised to grow tissues *in vitro.* Generally embryonal carcinoma (EC) cells, the stem cells of tetracarcinomers, differentiate to form a wide variety of cell types; however, certain EC cell lines do not have the capacity to differentiate and are propagated primarily as stem cells. It is possible to

[iii] Although it is important to note that isomerisation could be important in their mode of action[20]

induce differentiation in these cell lines with small molecules, such as ATRA.[49] For example TERA.cl.SP12 cells are known for their ability to form neurons in response to ATRA,[33] [34] thus facilitating the production of neural tissues which can potentially be used for drug screening, toxicological testing, research into neuron disorders and the development of therapies.

Once again, the propensity for ATRA to isomerise is undesirable. Murayama *et al.,* issued a warning in 1997, stating that when exposed to fluorescent lamps, a solution of ATRA undergoes significant isomerisation within 30 minutes. [35] The outcome of cell differentiation is often dependent on both the concentration and identity of isomer(s) present, thus uncontrolled isomerisation leads to variation in cell population and irreproducible results. Previous attempts to control isomerisation of ATRA include the use of additives[iv] which inhibit either *cis-trans* interconversion or oxidation.[34] However, none has been found to completely prevent isomerisation and the additives may themselves affect cell behaviour. A more successful solution has been found in the use of stable, non-isomerisable, synthetic retinoids.

[iv] Such as bovine serum albumin, fibrogen, lysozyme, phosphatidylcholine N-ethylmaleimide and vitamin C

1.3 Developing Synthetic Derivatives of Natural Retinoids

It is a recurrent theme throughout the applications described previously that the problems incurred with the use of natural retinoids are frequently attributed to either lack of receptor specificity, propensity for metabolism and / or susceptibility to isomerisation in both natural light and physiological conditions.

The key functionalities of ATRA and other natural retinoids are susceptible to isomerisation. ATRA and its isomers have a polyene chain containing five conjugated double bonds, which is important in maintaining π-electron delocalisation across the molecule. However, this structure is an excellent chromophore, absorbing light in the region of 300-400 nm. Therefore, natural retinoids readily undergo photo-isomerisation in the presence of fluorescent light. This leads to a mixture of geometric isomers, each inducing very different biological effects.

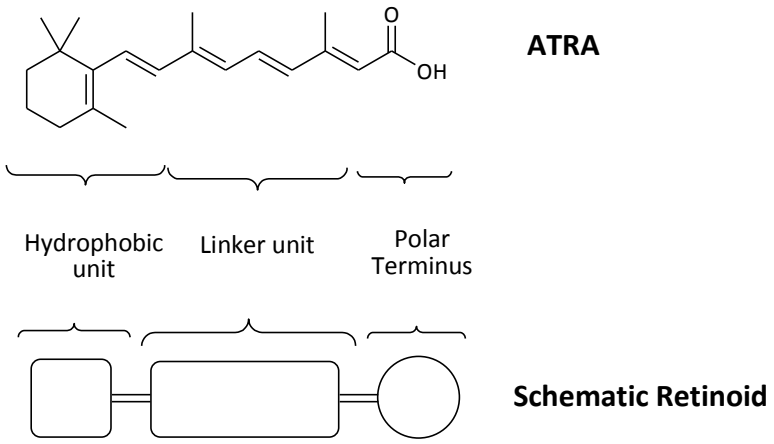


Figure 4: Illustration of the different units which comprise a retinoid.

A retinoid can be thought of as comprising of three units, a hydrophobic end group, a linker region and a polar terminus (See Figure 4). Careful modification of these units can result in the formation of new, synthetic retinoids. Many synthetic retinoids which retain the key functionalities of natural retinoids, yet are resistant to isomerisation and metabolism have been designed[36].

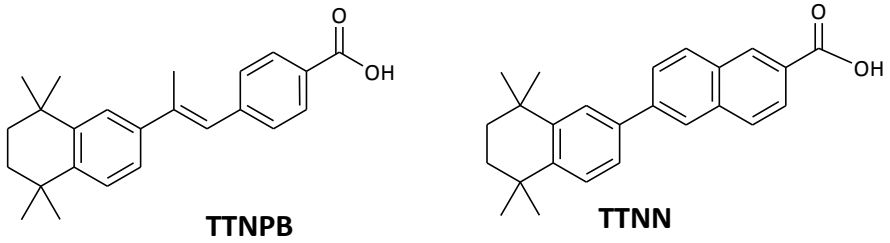


Figure 5: The structure of arotinoids TTNPB and TTNN

Synthetic retinoids TTNPB[37] and TTNN[38] shown in Figure 5 are functionally similar to ATRA, however, contain an a 1,1,4,4,-tetramethyl-1,2,3,4-tetrahydro-naphthalene moiety in place of the trimethylcyclohexenylvinyl unit, and the conjugated double bond linker is replace by stilbene or biaryl respectively. TTNPB and TTNN exhibit both high stability and high biological activity.

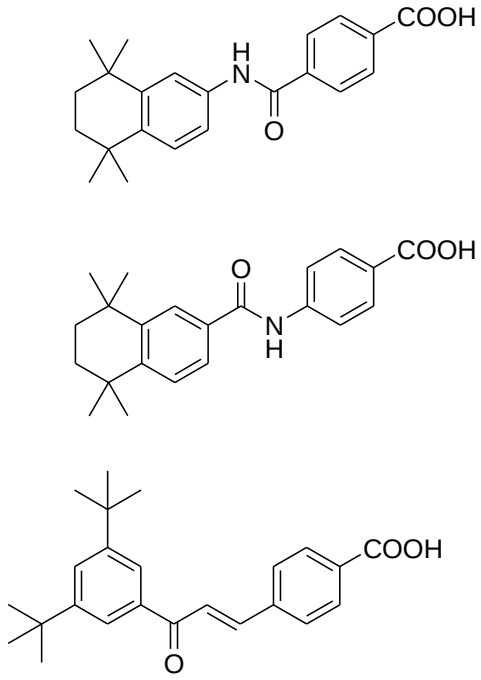


Figure 6: Synthetic retinoids Am80 (top), Am580 (middle), and Ch55 (bottom).

Synthetic retinoids Am80, Am580 and Ch55 have also been developed. Again, structural modifications have been carried out to introduce stability to the molecule whilst retaining key functional components of ATRA. Am80 and Am580 contain the same hydrophobic end group and polar terminus as TTNPB and TTNN, but differ in that they contain an amide linker region. Chick limb bud experiments have shown them to have similar biological activity to ATRA.[45](This is elaborated on in the following section).

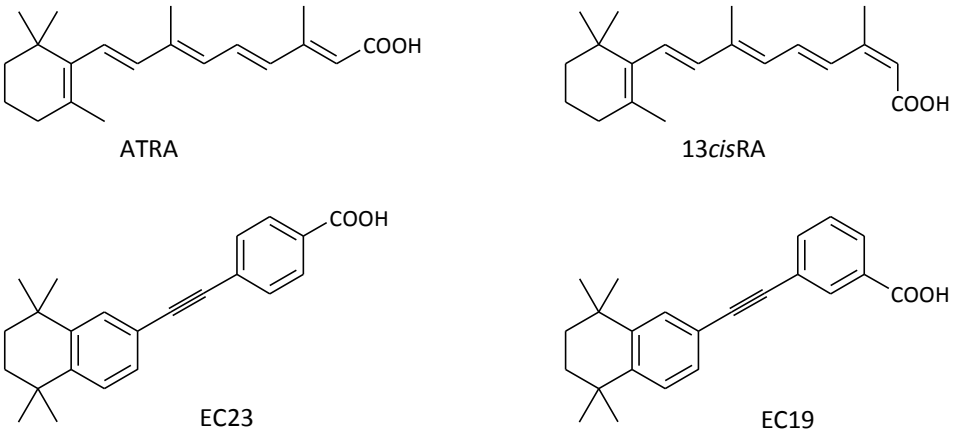


Figure 7: Natural retinoids ATRA and 13*cis*RA and their synthetic derivatives, arotinoids EC23 and EC19. The *para* / *meta* positioning of the carboxylic acid group in EC23 and EC19 reflects the spacial positioning of the carboxylic acid group in ATRA and 13*cis*RA respectively.

EC23 and EC19 are recent additions to the synthetic retinoid collection. Again, they contain the same hydrophobic end group and polar terminus as TTNPB and TTNN, but in these cases, the linker unit is a non-isomerisable acetylene unit. The general structures of ATRA and 13*cis*RA were replicated by placing a carboxylate function in the *para*- (EC23) and *meta*- (EC19) positions of the aromatic ring respectively.34 (See Figure 7.) As with the other synthetic retinoids discussed in this section, EC23 and EC19 exhibit both high stability and high biological activity.

NMR studies have shown that EC23 and EC19 are stable in natural light. They remain completely unchanged even after three weeks exposure to fluorescent light.[34] In comparison, their natural counterparts ATRA and 13*cis*RA undergo substantial isomerisation such that within 3 days exposure less than 40% of the

original compound remains.[34]

Biological studies carried out demonstrate that EC23 and EC19 possess the ability to induce differentiation of human pluripotent stem cells to the same degree or better than that observed for ATRA and 13*cis*RA.[34] EC23, like ATRA, induces neural tissues; whereas, EC19 behaves like 13*cis*RA in that it induces epithelial cells. Subsequent proteomic studies support these findings that the biological behaviour of EC23 and EC19 mimics that of ATRA and 13*cis*RA respectively.[39] Preliminary evidence is suggesting that the difficulties encountered with the use of natural retinoids may be mitigated through replacement with more stable, synthetic analogues.

1.4 Chick Limb Development

Studies have shown that EC23 and EC19 are more potent than their natural analogues. This may be attributed to the inherent stability of the synthetic retinoids. It is well documented that natural retinoids are metabolised by the body[8] and it is believed that this metabolism plays a large role in problems with their use, such as the rapid build up of resistance to retinoid therapies, and to the reduced potency of natural retinoids when used as stem cell derivatising agents. It is hypothesised that the body is unable to metabolise EC23 or EC19 and study was devised to test this hypothesis *in vivo*. The following introduction to proposed models of limb bud development is to help contextualise the study carried out.

The chick limb bud has previously been used to demonstrate the teratogenicity and potency of EC23 and EC19.[63] It is a good system in which to investigate retinoid effects as it is easy to access, its natural development has long been studied therefore it is fairly well understood, and it facilitates quantitative analysis of the teratogenic and toxic effect of the compounds studied.

There are three orthogonal axes to consider in the chick limb bud. The proximodistal axis runs from the shoulder to the tip of the digits; the anteroposterior axis runs from digit 2 to digit 4 (the equivalent of thumb to little finger in humans); and the dorsoventral axis runs from the back of the wing to the front (equivalent to back of the hand to the palm in humans). Chick limbs, like all vertebrate limbs, develop from small buds of mesenchyme cells. The cells are specified to form limb digits long before they actually do. As they grow the cells differentiate into the various tissues of the limb.[40]

There are two main stages of pattern formation. In the first stage, *specification,* the cells are informed of their position and acquire what is known as a positional value. During the second stage, *differentiation,* the cells then interpret this value to form the appropriate structures.[41]

Anteroposterior, proximodistal and dorsoventral patterning are regulated by distinct but interdependent pathways. For a discussion of how these models may be interlinked, see the recent review by Towers and Tickle.[41] Growth also plays an essential role in the specification of positional values in the early limb bud and a recent growth/morphogen model of chick wing pattering has been discussed by Towers *et al.*[42] For simplicity, the rest of this introduction will focus on anteroposterior patterning.

The Morphogen Gradient Model

This classical model proposes that a group of mesenchyme cells at the posterior limb bud margin, known as the zone of polarizing activity (ZPA), produce a morphogen which diffuses across the limb bud, creating a diffusion gradient. In regions where the morphogen concentration is high, i.e. close to the polarizing region, the cells will form posterior digits. As the distance the cells are from the

ZPA decreases, the morphogen concentration decreases and the cells progressively form more anterior digits.[43,41] In the normal chick limb, the digits are formed in a 234 pattern.

A concentration gradient of ATRA plays an important role in the pattern formation of digits

Both the *Shh* gene and ATRA are thought to play important roles in the pattern formation of digits. ATRA may be synthesised enzymatically in the posterior tissue from retinol and is thought to form a concentration gradient along the AP axis.[44] Several experiments which demonstrate the roles of the ZPA and ATRA in digit patterning have been carried out.

The ZPA and dHAND are important in digit pattern formation

When the ZPA is removed from the limb bud no digits form.[45] Grafting tissue taken from the ZPA of one limb bud to the anterior margin of another limb bud gives rise to a symmetrical 4 3 2 2 3 4 digit pattern.[41] This is evidence that the ZPA is important in instructing digit pattern in developing limbs.

dHAND, a basic helix-loop-helix transcription factor also plays an important role in regulation of digit development. It is expressed during the onset of limb bud outgrowth, and is localised in the posterior region of the limb bud. Studies have shown it to be an upstream activator of *Shh* expression and a regulator of limb development.[46] Embryos devoid of *dHAND* show underdeveloped limbs and those with ectopic expression in the anterior region show digit duplication.

Evidence that a morphogen is important in controlling digit pattern formation

Application of ATRA to the anterior margin of the limb bud also gives rise to a symmetrical 4 3 2 2 3 4 digit pattern.[41] A diagram of the procedure can be seen in Figure 8. The extent of digit duplication was found to be dependent on the concentration of ATRA applied[47] showing that the limb bud can be used to

assess quantitatively the potency of retinoids.[v] The position at which the exogenous ATRA is applied is important. When ATRA was applied to the posterior margin of the limb bud at concentrations that would induce digit duplication when applied to the anterior, normal digit patterning was seen. This supports theories that a concentration gradient is necessary in determining normal digit patterning and that disrupting this leads to abnormal digit growth.

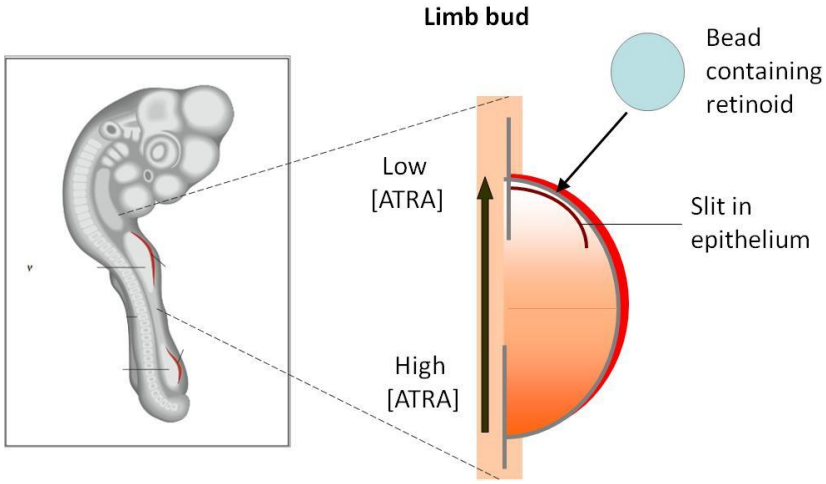


Figure 8: Application of a polymer bead soaked in retinoid solution applied to the anterior margin of the limb bud may affect the concentration gradients occurring naturally in the developing limb.

Application of certain synthetic retinoids to the anterior margin of the limb bud can also give rise to a symmetrical 4 3 2 2 3 4 digit pattern.

Synthetic retinoids Am80, Am580 and Ch55, shown in Figure 6, also mimic the action of ATRA when applied to the anterior margin of the limb bud.[45] The extent of digit duplication was shown to increase progressively (i.e. 3234, 43234, 432234, 4334) as the retinoid concentration was increased from 0.001 to 1.000 mg/ml. The effective dose necessary to produce each effect was similar to that required of ATRA. Interestingly, when these synthetic retinoids were applied to the anterior margin of a ZPA-free limb bud (i.e. ZPA had been removed), a

[v] It should also be noted that the speed at which retinoids are released from the bead also affect the extent of digit duplication seen.[62]

reverse digit pattern was observed (432).[45]

EC23, a synthetic analogue of ATRA, has been evaluated in the chick limb bud system and was also found to produce the 432234 phenotype at concentrations of 0.1 mg/ml (shown in Figure 9). Concentrations above 1.0 mg/ml were found to be toxic. EC19 was also investigated and found to induce collapse of facial features (shown in Figure 9). As found in the stem cell differentiation studies, EC23 shows very similar biological effects to ATRA, but is much more potent.[48]

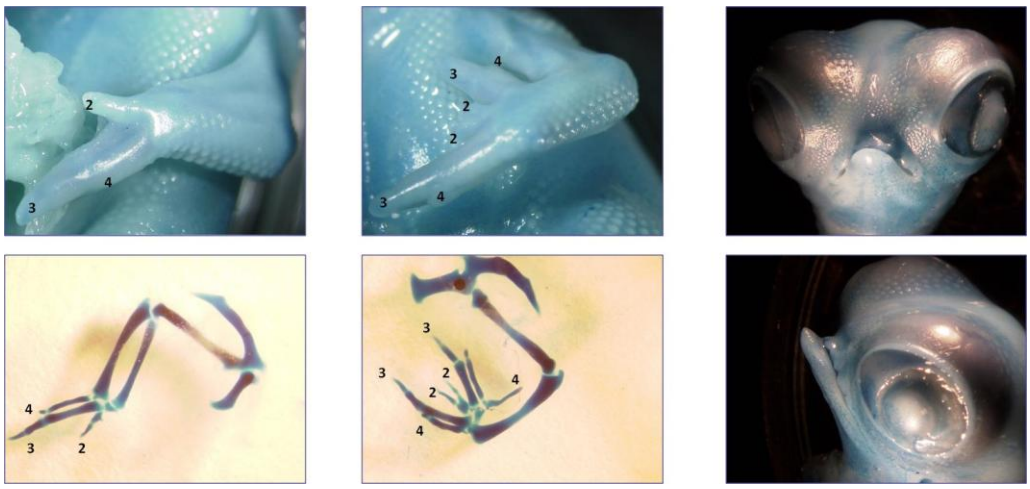
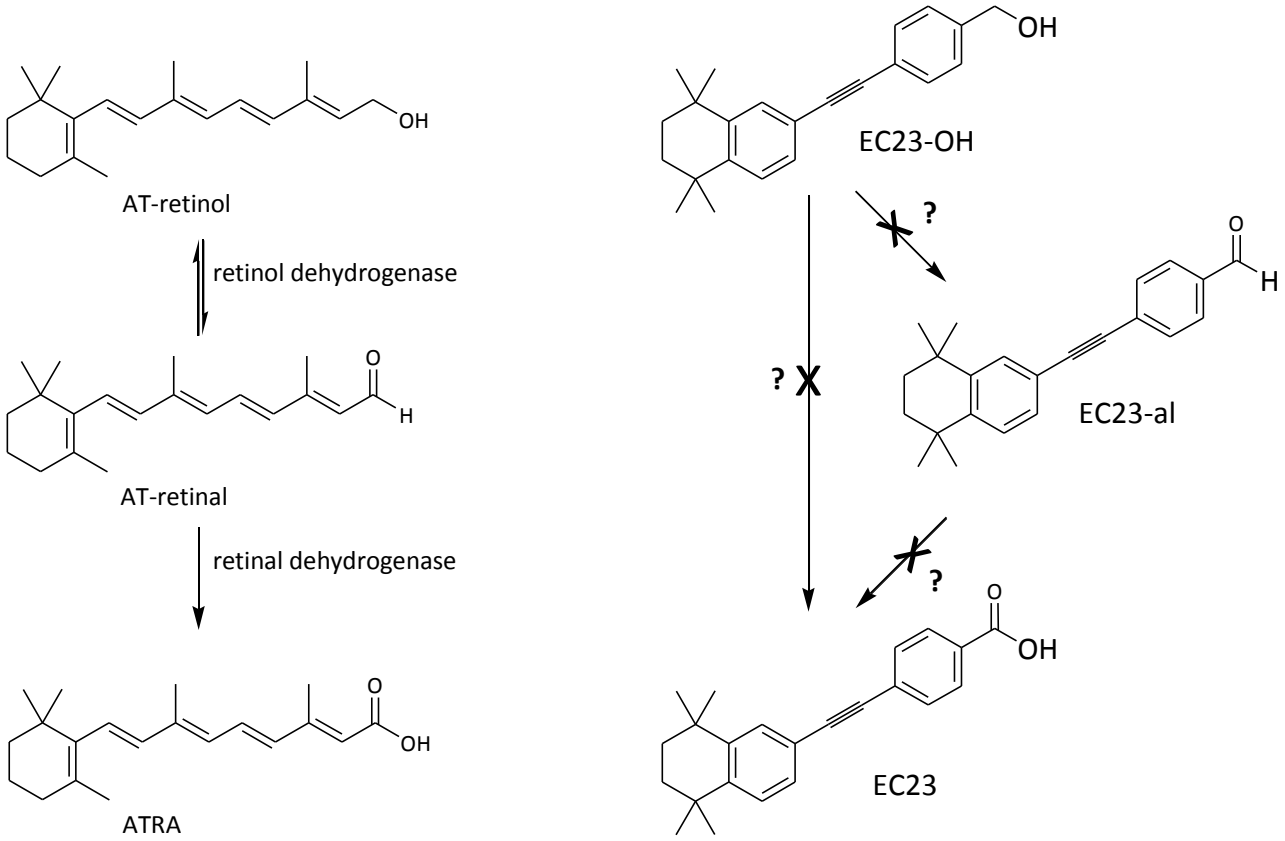


Figure 9: Previous results from chick limb bud investigations carried out by insertion of a loaded AG1-X2 polymer bead into to the anterior margin of the limb bud of a stage 20 chick embryo. The above images show the development observed at stage 36. **Left:** Control. Limb showing normal, 234 development after being treated with a saline soaked bead. **Middle:** Embryo limb showing 432234 digit pattern after being treated with a bead soaked in 0.1 mg/ml EC23; **Right:** Facial disfiguration caused by the insertion of a bead soaked in 0.1 mg/ml EC19 solution (DMSO) [48]



Scheme 2: Left hand side*:* A schematic showing the metabolism of AT-retinol to ATRA, via AT-retinal. There is much evidence that this occurs. **Right hand side:** It is currently believed that the body is unable to metabolise synthetic retinoids such as EC23 along the same route as its natural analogue.

It is hypothesised that the body is unable to metabolise EC23, due to its robust structure and resistance to isomerisation. If correct, this could explain its increased potency compared to that of ATRA. The following study was devised to test this hypothesis. It is known that the body is able to enzymatically convert AT-retinol to ATRA,[49] and that when suitable concentrations of ATRA are applied to the anterior margin of the limb bud the result is digit duplication. It was thus

hypothesised that if retinol was applied exogenously to the anterior margin, it would be converted to ATRA and thus cause digit duplication. An extension of this reasoning would be to apply the synthetic derivative of retinol, EC23-OH, (see Scheme 2) to the anterior margin of the limb bud and look for abnormal phenotypes. If the body *is* able to metabolise EC23-OH, it would be expected it would be converted to EC23 and hence digit duplication would occur. If, however, as is predicted, *normal* develpment is observed, this would provide further evidence that EC23 is refractory to normal retinoid metabolism. In order to test this hypothesis, the effects of natural retinol on the limb bud had to be evaluated and the synthetic equivalent, EC23-OH, had to be synthesised.

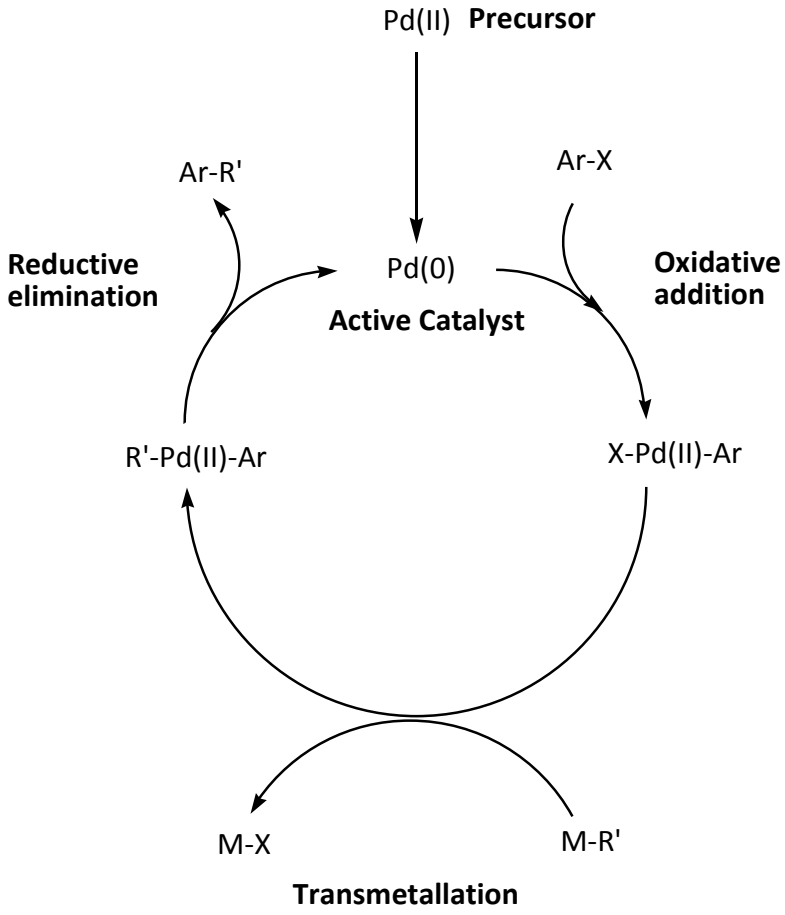
1.5 Synthetic Strategy: Pd-Catalysed Cross-Coupling Reactions

Pd is one of the most versatile, powerful and widely used transition metal catalysts. Pd is in group 10 of the periodic table and is therefore an electron rich transition metal. Pd complexes can exist in three different oxidations states 0, II and IV, and interconversion between them is often facile. This, along with the fact it can support different coordination numbers, enables Pd to be a catalyst. It is widely used in the synthesis of many functionalised organics used for wide ranging applications from liquid crystals to biologically active molecules which will be discussed in this report.

Despite recent debate, it is generally believed that Pd(0) is the active catalytic species. This can either be obtained directly from a source of Pd(0), e.g. $Pd(PPh_3)_4$, or can be formed in situ by reduction of a Pd(II) source such as $Pd(PPh_3)_2Cl_2$. The latter approach is normally used as generally Pd(II) sources are soluble in most organic solvents and fairly stable towards air and moisture, making them easier to handle and store than Pd(0).

Over the years, many variations of Pd-catalysed cross-coupling systems have

been developed such as Suzuki-Miyaura, Heck, Stille, Negishi, Sonogashira, etc. However, they are all mechanistically similar and follow a catalytic cycle comprising oxidative addition, transmetallation and reductive elimination (see Scheme 3).



Scheme 3: Generic catalytic cycle for palladium-catalysed cross-coupling reactions.

If a Pd(II) catalyst precursor is used, it must first be reduced to form a nucleophilic Pd(0) species. It is not fully understood how this mechanism occurs for each different cross coupling reaction, but it has been thoroughly studied for the Sonogashira reaction and will be discussed briefly later.

Oxidative Addition

The first step in the catalytic cycle is oxidative addition of Ar-X (where Ar is generally an aryl, vinyl or allyl and X is a halide or triflate) to the Pd(0) catalyst to form an electrophilic Pd(II) intermediate. In the past, this was usually the rate determining step of the cycle so much work has been done to increase the rate of oxidative addition. For example, optimisation of the donor ligands leads to increased electron density on the Pd centre, increased nucleophilicity and therefore an increased rate of oxidative addition. For example, $^{t}Bu_3P$ has proved to be an excellent ligand, and facilitates the reaction of otherwise unreactive, or very slow reacting reagents such as aryl chlorides.[50] $^{t}Bu_3P$ is strongly donating, which promotes oxidative addition, yet bulky which helps promote reductive elimination.[vi] Choice of Ar-X substrate also influences the rate; this is briefly elaborated on later.

Transmetallation

The Pd(II) intermediate formed during the oxidative addition step then reacts with a nucleophilic organometallic reagent, M-R', to form a diorganyl-palladium(II) species via transmetallation. The M-X salt formed simultaneously is the thermodynamic driving force for this reaction. It is generally the transmetallation step that differs between the different cross coupling-reactions, and the choice of metal used determines the name of the reaction. For example, in the Suzuki-Miyaura reaction, M = $B(OR)_2$ whereas in the Sonogashira reaction, a copper acetylide is used.

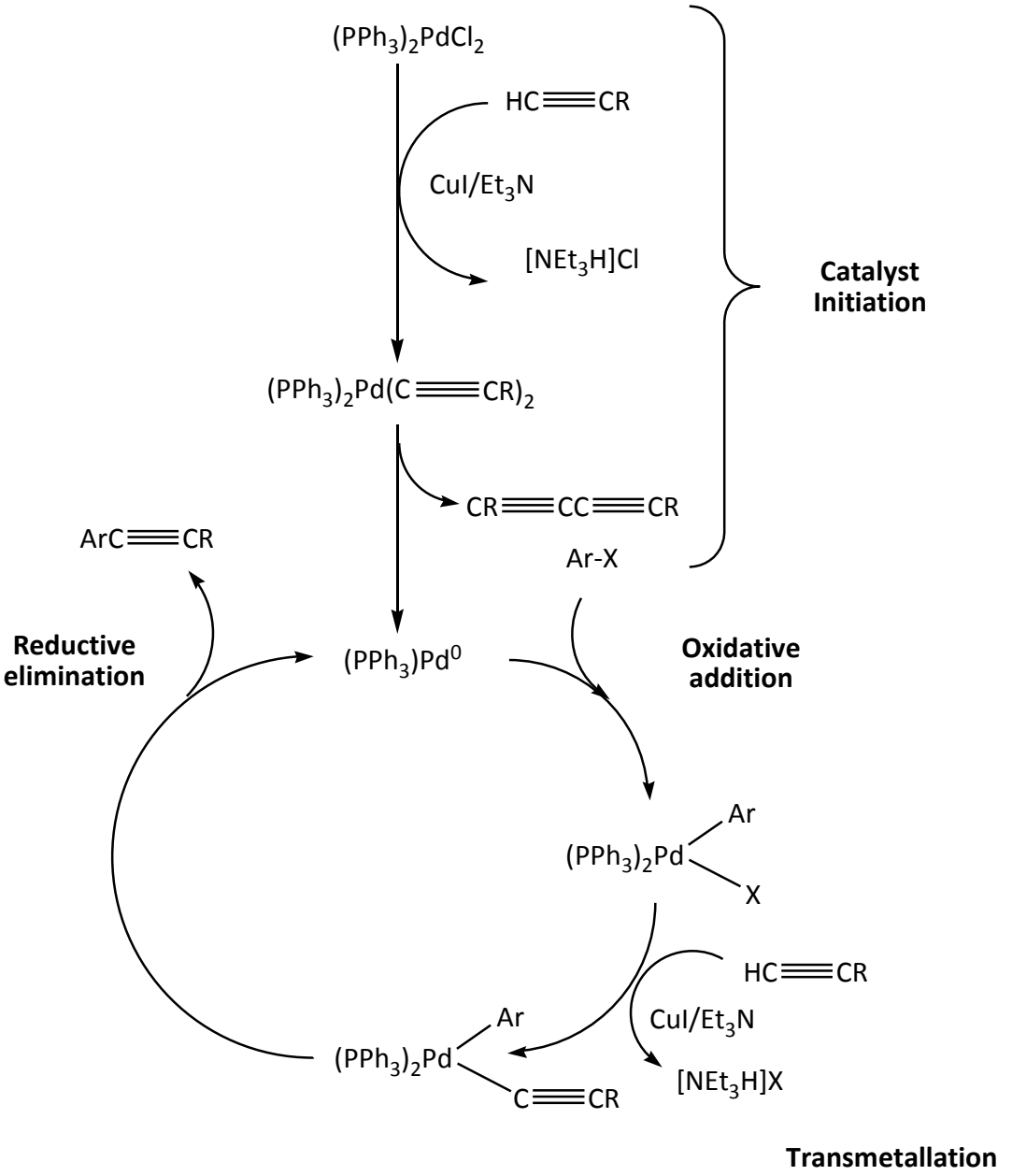
[vi] It is important to maintain a balance – whilst electron donating ligands promote oxidative addition; bulky, electron withdrawing ligands facilitate reductive elimination. A balance must therefore be struck as both processes are essential to the catalytic cycle.

Reductive Elimination

The final step is reductive elimination. During this step the two organic fragments are joined by the formation of a new bond and the Pd(0) catalyst is regenerated. This is believed to be a concerted process and in order for it to happen a *trans-cis* isomerisation must first occur to align the two fragments.

The Sonogashira Reaction

The palladium-catalysed cross-coupling of terminal alkynes with aryl halides was first reported in 1975 independently by the groups of Cassar[51] and Heck.[52] Shortly after, Sonogashira et al. reported an improvement to the reaction by the addition of Cu(I) salts, which allowed the reaction to proceed under mild conditions.[53] Scheme 4 shows the proposed catalytic cycle. Further improvements and modifications have been made over the years and it is now an important route to many organic compounds. It is a key route to alkyne containing synthetic retinoids.



Scheme 4: Proposed Catalytic Cycle for the Sonogashira Cross- Coupling Reaction [adapted from Sonogashira *et al.*[53]]

Unless a practicality requires the direct use of a Pd(0) catalyst, Pd(II) precursors[vii] are generally used for the Sonogashira reaction. They are reduced

[vii] Pd(II) complexes are generally much more stable to air and moisture that Pd(0) complexes and are therefore easier to handle.

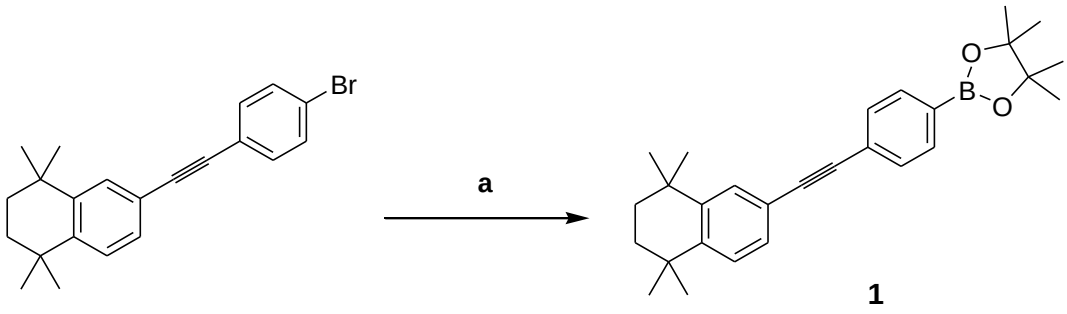
in situ to Pd(0) before the catalytic cycle can begin. This mechanism has been studied extensively[54] and is well understood for the Sonogashira reaction. Generation of the active catalyst Pd(0) is achieved by transmetallation of two equivalents of terminal Cu-acetylide to Pd(II) followed by reductive elimination. CuI facilitates the deprotonation of a terminal alkyne by an amine to give Cu-acetylide *in situ*. Two equivalents of Cu-acetylide are then exchanged for the two halide ligands on the Pd centre. *Trans-cis* isomerisation followed by reductive elimination of one equivalent of homocoupled diyne generates the active catalyst Pd(0). Generally, it is a facile process to separate the homocoupled from the desired product during purification. If however, this would be difficult, e.g. if the two compounds had very similar polarities, then the direct use of a Pd(0) catalyst would be advised.

Much work has also gone into the study of the oxidative addition step. It is well documented that the rate of oxidative addition of an aryl halide to a Pd catalyst is correlated to the strength of the C-X bond, with weakly bound C-I having a high rate of reaction in comparison to more strongly bound C-Cl. Also, the presence of electron withdrawing groups *para* to the halide, further increases the rate of oxidative addition. The mechanism of oxidative addition was examined theoretically with the aid of DFT calculations in a recent study by Lam, Marder and Lin.[55]

Chapter 2: Chemical Synthesis, Results and Discussion

2.1 Synthesis of Starting Materials

Aryl boronic acids and esters are versatile reagents in organic synthesis for the formation of carbon-carbon, carbon-nitrogen and carbon-oxygen bonds. They are especially useful for cross coupling reactions as they generally have high stability and low toxicity. The compound 4,4,5,5-tetramethyl-2-(5,6,7,8-tetrahydronaphthalene-2-yl)-1,3,2-dioxaborolane (**1**) was made for use as a precursor to synthetic retinoids.



Scheme 5: Synthesis of retinoid precursor 4,4,5,5-tetramethyl-2-(5,6,7,8-tetrahydronaphthalene-2-yl)-1,3,2-dioxaborolane (**1**). a) 1 eq. B_2pin_2, 2% $Pd(OAc)_2$ / 5% S-phos, 2 e.q. $K_3PO_4 \cdot 2H_2O$, dioxane, 80 °C

Buchwald conditions were utilised to prepare **1** from 6-(4-Bromo-phenylethynyl)-1,1,4,4-tetramethyl-1,2,3,4-tetrahydro-naphthalene (bromo-TTNPB) (See Scheme 5). Buchwald reported that the ligand s-phos, (shown in Figure 10) facilitates the palladium catalysed borylation of aryl chlorides at room temperature.[56] As aryl bromides are generally more reactive than aryl chlorides, it was could be expected that the reaction of bromo-TTNPB would occur readily at room temperature. However, it had been found previously to take two weeks of room temperature stirring followed by a further week of heating at 60 °C before

complete borylation of bromo-TTNPB was observed by GC-MS. It was therefore decided to carry out the reaction at 80 °C in order to speed up the reaction time. GC-MS analysis showed 60% consumption of starting materials after 18 hours, and this increased to 80% after the reaction had been left to continue for 48 hours.

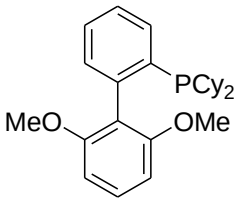
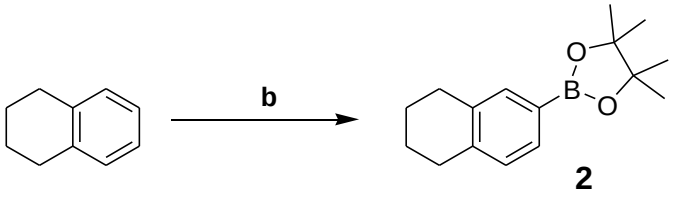


Figure 10: Structure of Buchwald's S-phos ligand.

The aryl boronic ester, 4,4,5,5-tetramethyl-2-(5,6,7,8-tetrahydronaphthalene-2-yl)-1,3,2-dioxaborolane **2**, was also synthesised for future use as a retinoid precursor.



Scheme 6: Synthesis of retinoid precursor 4,4,5,5-tetramethyl-2-(5,6,7,8-tetrahydronaphthalene-2-yl)-1,3,2-dioxaborolane **2.** b) 1 e.q. B_2pin_2, 1 % $[Ir(OMe)(COD)]_2$ / 2 % dtbpy, hexane, 80 °C / N_2

As $[Ir(OMe)(COD)]_2$ / dtbpy is known to be effective catalyst for borylation of heteroaromatic substrates,[57] it was used to borylate tetrahydronaphthalene (see Scheme 6). The reaction proceeded cleanly and was shown to have gone to completion by GC-MS analysis after being stirred for 18 hours at 80 °C. The product was separated from the reaction mixture by passing through a short

silica plug with a mixture of dichloromethane and hexane. Once the solvent was removed, a transparent oil remained. This was crystallised from methanol at -20 °C as colourless needles. A yield of 93% was obtained. Boron, proton and carbon NMR confirm the identity of **2** and the crystal structure is shown in Figure 11, below.

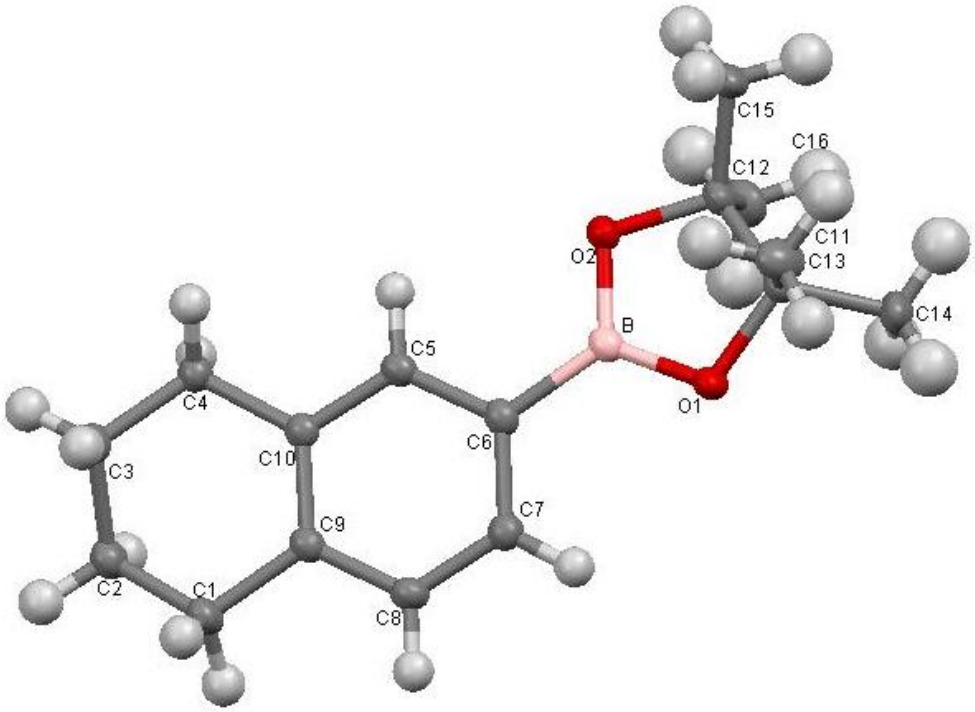
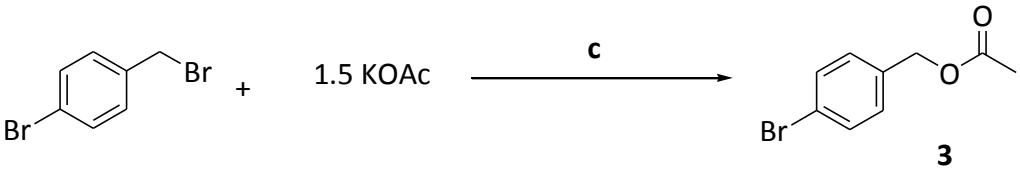


Figure 11: Molecular structure of 4,4,5,5-tetramethyl-2-(5,6,7,8-tetrahydronaphthalene-2-yl)-1,3,2-dioxaborolane **2.**

Acetic acid 4-bromo-benzyl ester **(3)** is a starting material for the benzyl-alcohol equivalent of EC23. It was synthesised from 4-bromobenzyl bromide and potassium acetate using a method adapted from a paper by Wolfe *et al,*[58] as shown in Scheme 7. 4-bromobenzyl bromide was reacted with potassium acetate in acetonitrile. After 5 days GC-MS analysis showed the reaction had gone to completion. No complications were encountered and a yield of 87% was

obtained. Full characterisation was carried out and the crystal structure is shown in Figure 12 below.



Scheme 7: Synthesis of acetic acid 4-bromo-benzyl ester **(3)**;
c) MeCN, reflux, N_2, 24 h.

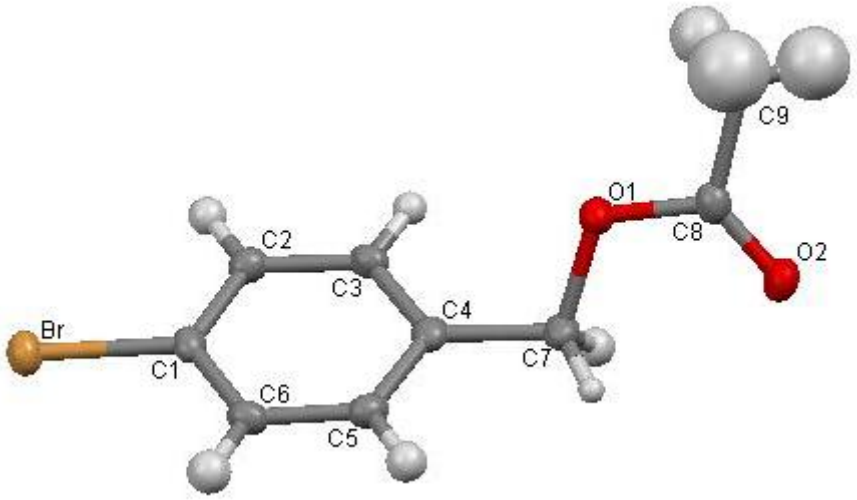
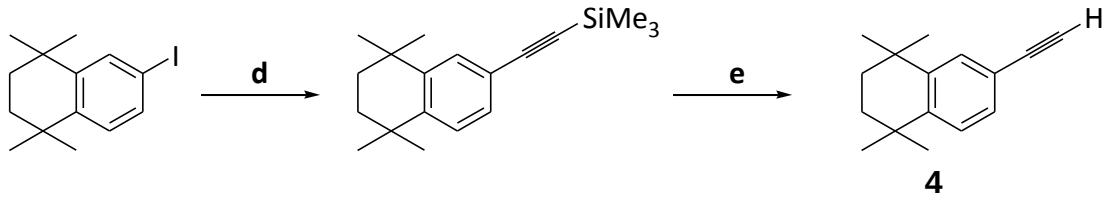


Figure 12: Molecular structure of acetic acid 4-bromo-benzyl ester **(3)**.

6-Ethynyl-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene (TMNA) is a key starting material for synthetic acetylenic retinoids. It is not yet available commercially, but a synthesis from an aryl methyl ketone has previously been reported by Wuest *et al.*[59] A more straightforward synthesis from commercially available 1,1,4,4-tetramethyl-2,2,3,3-tetrahydronaphthalene has been developed

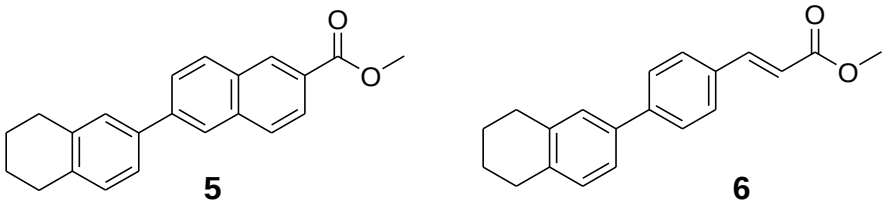
in the Marder lab, as shown in Scheme 8. It was synthesised in bulk prior to further retinoid synthesis.



Scheme 8: Synthesis of TMNA (4). d) 1.2 eq. TMSA, 1% $Pd(PPh_3)_2Cl_2$, 1% CuI, Et_3N; e) 4 eq. Na_2CO_3, MeOH/10% H_2O

The reaction of 6-iodo-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene and trimethylsilyl was catalysed by $Pd(PPh_3)_2Cl_2$ and copper iodide in dry triethylamine. The reaction went to completion at room temperature within 18 hours. The reaction mixture turned dark brown / black, but most of the colour was removed on extraction with dichloromethane to give a 72% yield of a pale orange crystalline solid. GC-MS and NMR spectra are consistent with those expected. In the ^1H NMR spectra, the doublet seen at δ 1.29 integrating to 12 protons is typical of the CMe_2 groups of the TMNA moiety. The three peaks seen in the aromatic region are consistent with being the three aromatic protons and the singlet at δ 3.02 corresponds to the **H**-C≡C proton.

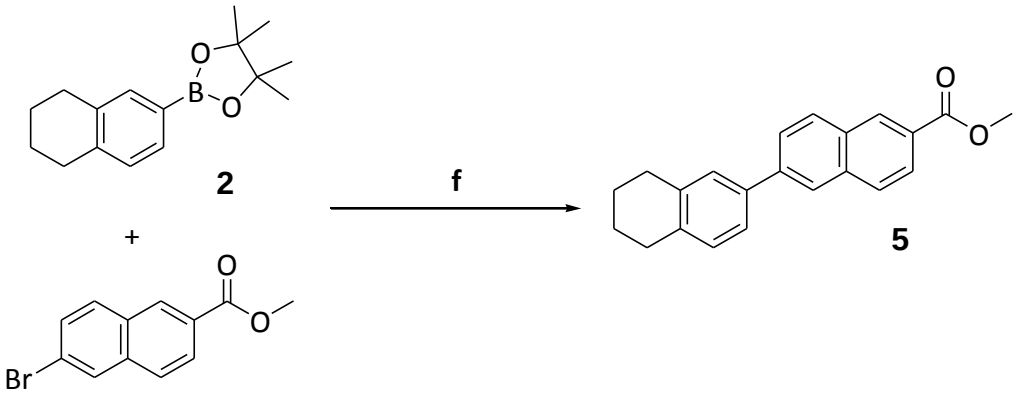
2.2 Retinoids from Suzuki-Miyaura Cross-coupling



5 **6**

Figure 13: Methyl 5',6',7',8'-tetrahydro-2,2'-binaphthyl-6- carboxylate **(5)** and *(E)*-methyl 3-(4-(5,6,7,8-tetrahydronaphthalen- 2-yl)phenyl)acrylate **(6)**.

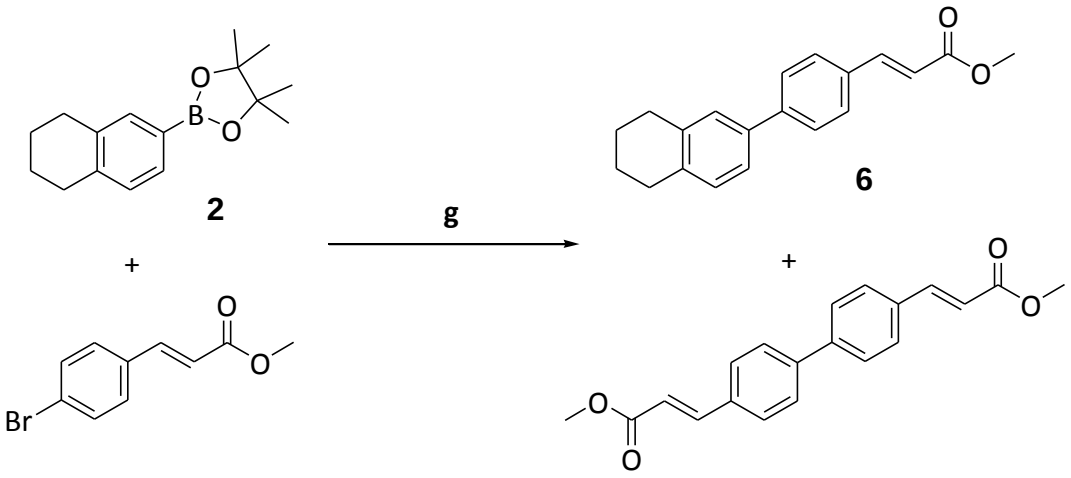
The synthesis of compounds methyl 5',6',7',8'-tetrahydro-2,2'-binaphthyl-6-carboxylate **(5)** and *(E)*-methyl 3-(4-(5,6,7,8-tetrahydronaphthalen-2-yl)phenyl)acrylate **(6),** shown in Figure 14, was attempted in order to add these compounds to our growing library of retinoids and to be tested for biological activity. They possess several of the key moieties necessary for retinoid activity, such as a polar terminus and a system of conjugated double bonds. However, a key difference between these molecules and other known retinoids such as ATRA, EC23 and TTNPB[37] (discussed in section 1.3), is that they do not possess CMe_2 groups on the naphthalene ring. It is thought that the CMe_2 groups are involved in receptor binding[36] and it would therefore be of interest to investigate the biological activity of structurally similar molecules with the absence of these groups.



Scheme 9: Synthesis of methyl 5',6',7',8'-tetrahydro-2,2'-binaphthyl-6-carboxylate **(5)** f) 2% Pd(dppf)Cl_2, 2 eq. K_3PO_4, 5:1^{i}PrOH/H_2O, 80°C

Suzuki-Miyaura conditions were utilised to couple **2** with methyl-6-bromo-2-naphthoate[viii] to form **5** (see Scheme 9). GC-MS analysis showed some product had formed after four hours. After five days an orange / brown solution had formed and GC-MS analysis showed that the reaction had gone to completion. The volatiles were removed and the organic product was extracted into dichloromethane. Further removal of volatiles yielded the product as a yellow solid. ^{1}H NMR and GC-MS analysis showed that **5** had been made but that it was impure. Flash chromatography with dichloromethane and hexane significantly improved the purity. The number and size of impurity peaks was reduced considerably and elemental analysis results agreed to within 0.7% of those calculated.

[viii] Methyl-6-bromo-2-naphthoate is available commercially, CAS# 3362-98-1



Scheme 10: Synthesis of (*E*)-methyl-3-(4-(5,6,7,8-tetrahydronaphthalen-2-yl)phenyl)acrylate **(6)** showing homocoupled by-product. g) 2% $Pd(dppf)Cl_2$, 2 eq. K_3PO_4, 5:1^{i}PrOH/H_2O, 80°C.

2 was also coupled with (*E*)-methyl 3-(4-bromophenyl)acrylate, again using Suzuki-Miyaura conditions, to form **6**. (see Scheme 10). GC-MS, ^{1}H and ^{13}C NMR analysis confirmed that **6** had been made. However small additional peaks in the spectra indicate traces of an impurity. Flash chromatography using dichloromethane/hexane increased the purity; however, the yield was reduced to 5% and small impurity peaks remained. Inspection of the GC-MS spectra showed the impurity had a mass of 320, suggesting it was likely to be the homocoupled product of the boronate ester (shown in Scheme 10). Time constraints prevented further attempts at purification; however, a less polar solvent system, such as hexane/ether or hexane/ethyl acetate may lead to greater success in isolating **6** from the homocoupled product in the future.

2.3 Route to Benzyl Alcohol

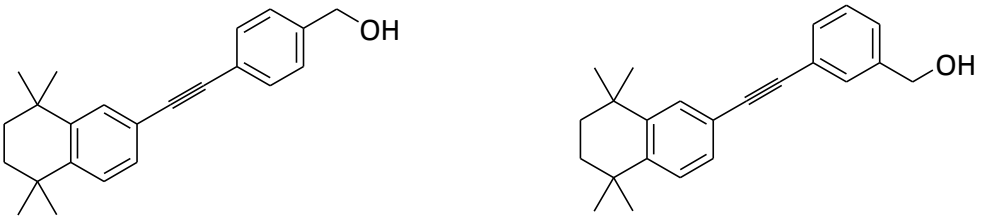
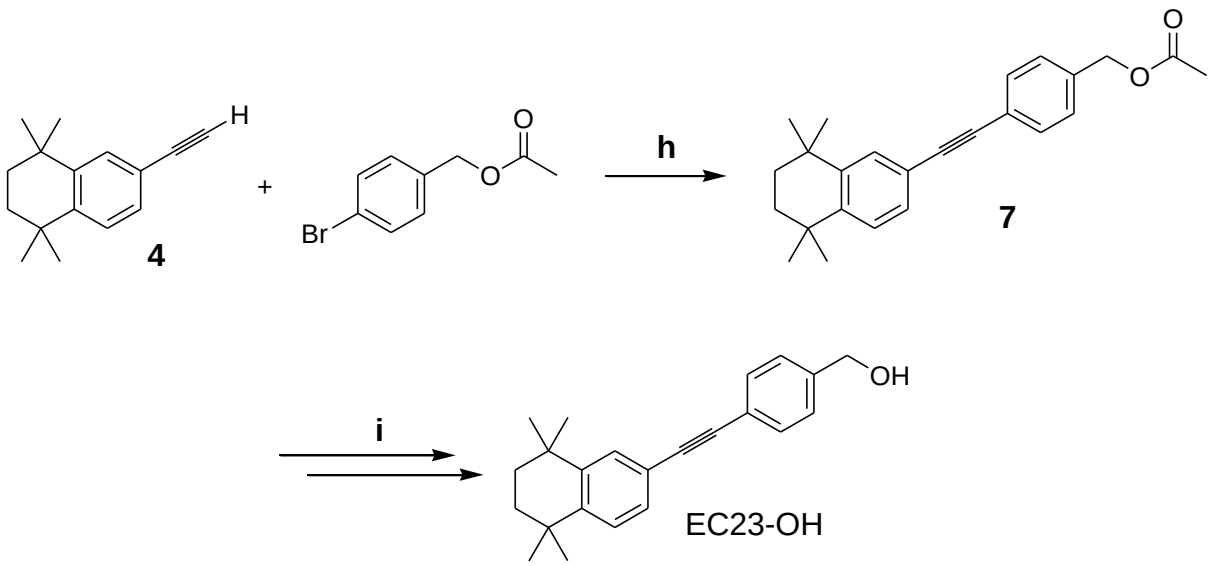


Figure 14: EC23-OH (left) and EC19-OH (right); Benzyl alcohol analogues of EC23 and EC19 respectively.

In order to determine whether or not synthetic retinoids EC23 and EC19 can be metabolised in the same manner as the naturally occurring retinoid ATRA, as discussed in the introduction, it was necessary to first synthesise the benzyl alcohol analogues, EC23-OH and EC19-OH (Figure 14) which could then be tested in the chick-limb system.

It had previously been thought that EC23-OH and EC19-OH could be made simply by a reduction of the methyl esters of EC23 and EC19 respectively. High conversions (100% by GC-MS) were obtained using lithium borohydride in diglyme at 80 °C. However, work up proved difficult and only low yields were obtained, so a new approach was taken.

Readily available starting materials were instead used to synthesise EC23-OAc and EC19-OAc with the intent that they could then be easily hydrolysed to EC23-OH and EC19-OH respectively when needed. The direct synthesis of EC23-OH and EC19-OH was considered, however previous experience has shown that ether acetate analogues have the advantage of being easier to work up and purify.



Scheme 11: Proposed synthesis of EC23-OH, via EC23-OAc **(7)**.
h) 1 % $Pd(PPh_3)_2Cl_2$, 1 % CuI, Et_3N, 80°C, 2 d; i) Hydrolysis.

A Sonogashira reaction was employed to couple **4** with acetic acid 4-bromobenzyl ester (see Scheme 11) to form EC23-OAc **(7)**. GC-MS analysis showed that complete consumption of starting materials was achieved within 24 hours at 80 °C using a 1% $Pd(PPh_3)Cl_2$/CuI in Et_3N catalyst system. The volatiles were removed from the resulting black solution to give a tan coloured solid. This was purified using flash chromatography, eluting with DCM/Hexane. The final yield of pure product was only 1 % - Much lower than expected for this type of

coupling reaction. It is believe that much product was lost in purification. If repeated, a different solvent system should be used to elute. Crystals obtained at room temperature in DCM were analysed by x-ray diffraction, which confirmed them to be the desired compound, crystal structure shown in Figure 15) ^{13}C and ^{1}H NMR spectra also conform to that expected for **7** and elemental analysis agrees to within 0.5% of that calculated.

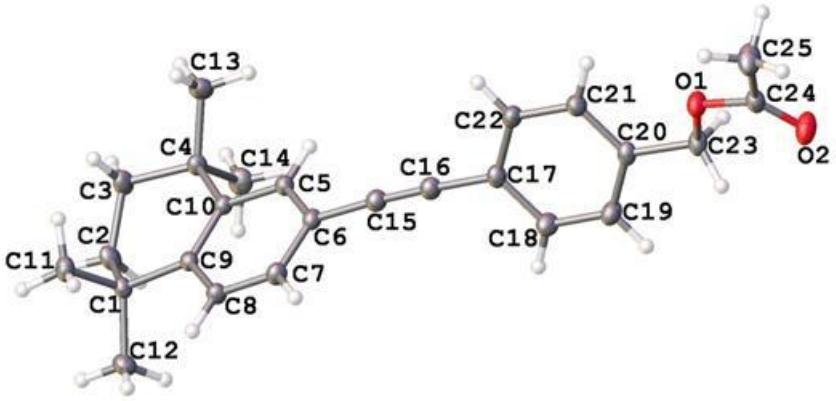
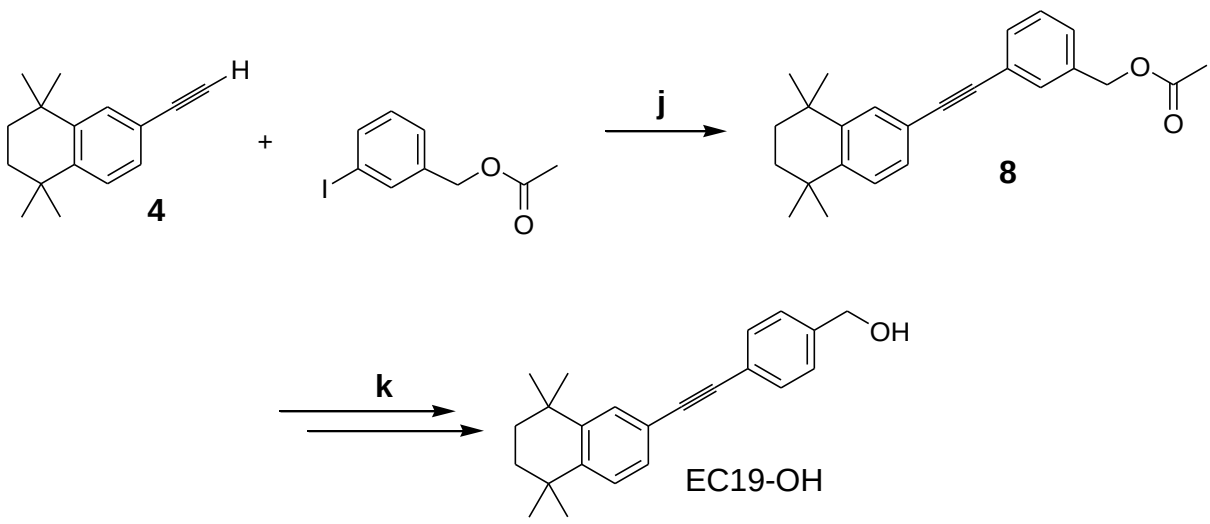


Figure 15: Molecular structure of EC23-OAc [4-((5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)ethynyl)benzyl acetate].



Scheme 12: Proposed synthesis of EC19-OH, via EC19-OAc **(8)**.
j) 1 % $Pd(PPh_3)_2Cl_2$, 1 % CuI, Et_3N, 21°C, 2 d; k) Hydrolysis.

Another Sonogashira reaction was employed to couple the iodide precursor, acetic acid 3-iodo-benzyl ester with **4** to synthesise EC19-OAc, as shown in Scheme 12. The same catalyst system was used as for the previous reaction; however, in this case the aryl iodide was sufficiently electrophilic to react at room temperature. GC-MS analysis revealed that complete consumption of starting materials was achieved within 21 hours. After washing the crude product through a silica plug with Et_2O, tlc analysis showed that two compounds were still present. These were separated using a silica plug, eluting first with hexane, and then with hexane/Et_2O (1:5). Removal of the solvent yielded 30 % yield of a pale orange / brown solid. It is likely that some product was lost during purification, and expected that this yield could be improved upon. Full characterisation has been carried out and HR-MS measured an excellent mass accuracy of 0.06 ppm!

Chapter 3: Biological Results and Discussion

3.1 Serial Dilution of Retinoid Solutions

The volumes and concentrations of retinoid solutions required for the investigations were small and low. Precision in measuring was therefore crucial and a serial dilution was carried out to determine pipetting accuracy. This was performed using 0.1 mg/ml solutions of EC23 and AT-retinol in DMSO. [See section 6.2 for procedure details.] A NanoDrop spectrophotometer was used to measure the absorbance of the diluted solutions at λ = 305 nm and λ = 335 nm for EC23 and AT-retinol respectively. Both series gave straight lined graphs (see Figures 16 and 17 below), showing that the pipette can be used to accurately make up solutions with concentrations as low as 0.0016 mg/ml (0.467 x 10^{-5} mol·dm^{-3}).

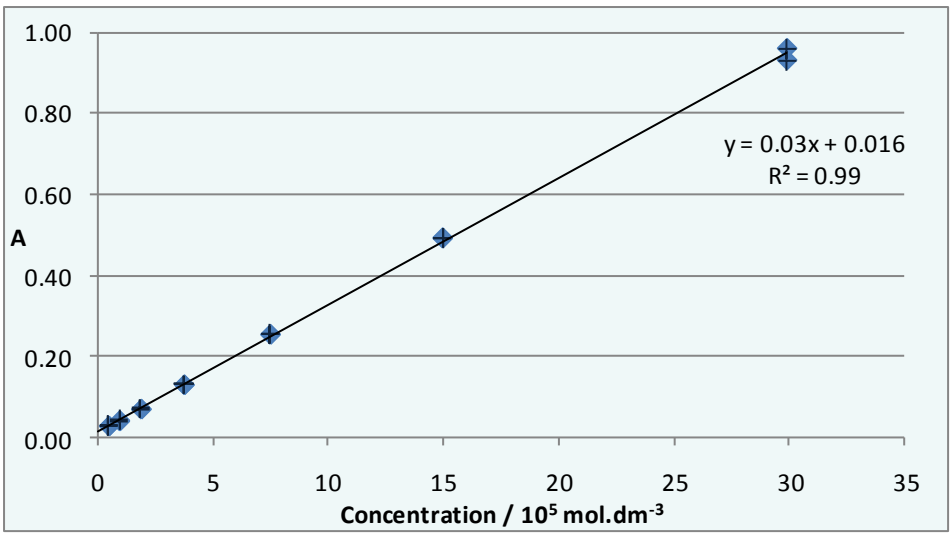


Figure 16: Serial dilution of EC23. Solutions were made up at a range of concentrations from 0.10000 mg/ml to 0.00156 mg/ml. The absorbance (A) of each solution at λ = 306 nm was measured and plotted against concentration. R^2 (the coefficient of determination)[ix] was calculated to be 0.99, showing excellent agreement between the concentration and the absorbance measured, confirming pipetting accuracy.

[ix] R^2 (the coefficient of determination) returns a value between 1 and 0, where 1 corresponds to complete correlation of data and 0 implies no correlation at all.

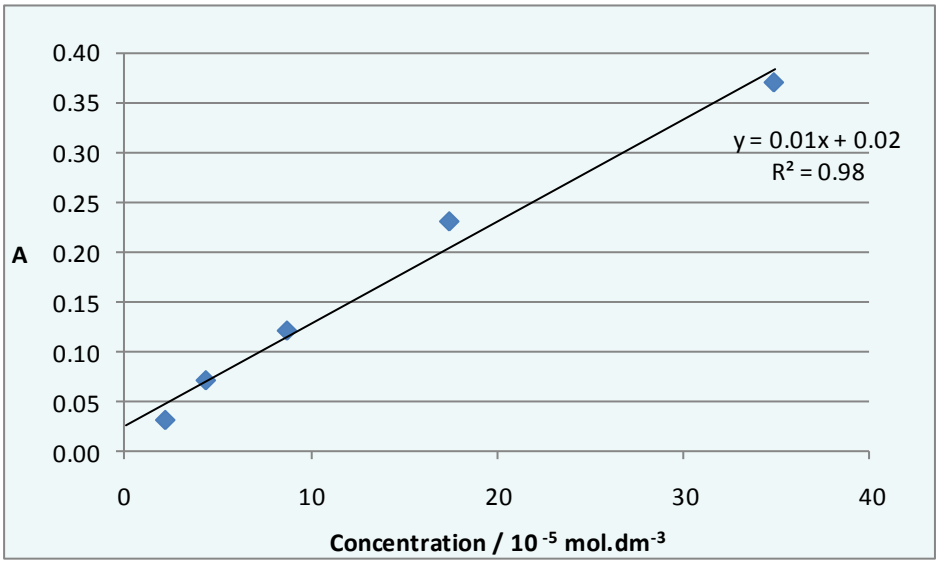


Figure 17: Serial Dilution of AT-retinol. Solutions were made up at a range of concentrations from 0.10000 mg/ml to 0.00156 mg/ml. The absorbance (A) of each solution at λ = 335 nm was measured and plotted against concentration. R^2 (the coefficient of determination)**Error! Bookmark not defined.** was calculated to be 0.98, showing excellent agreement between concentration and the absorbance measured, confirming pipetting accuracy.

3.2 Development of Procedures for *In Vivo* Analysis of Retinoid Action

The procedure used to apply retinoids to the chick limb bud is technically challenging, due to the small scale and delicacy of the embryo. Therefore a significant amount of practice is required to become competent in this technique. It is important the operation is performed when the embryo is at stage 20 of the Hamburger and Hamilton[60] staging system as this is the critical period at which the chick limb bud is susceptible to malformation by teratogens[5] such as ATRA. First, forceps are used to remove the outer shell to create a window through which the embryo can be viewed and accessed. Great care must be taken to

apply just enough force to break through the tough outer shell, whilst leaving the membrane intact to protect the embryo from falling shell. The outer membrane is then peeled back to reveal the embryo. Biological saline containing nutrients, antibiotics and antimitotics is applied and the egg is placed under a microscope. A further two membranes are cleared using a tungsten tipped needle, before access to the limb bud is gained. The needle is then used to cut a slit, approximately 220 µm in length (fractionally longer than the diameter of the bead) along the anterior margin of the limb bud. Fine forceps are used to insert an AG1-X2 bead into the opening and gentle pressure is applied to ensure it is securely embedded. The embryo is observed for a few seconds to ensure the bead and limb-bud move up and down together when the heart beats. This is an indication of successful bead attachment. The egg is quickly sealed with "Sellotape Original",™[x] then returned to the incubator for 7 days. It is essential to ensure a complete seal around the opening – this prevents the embryo from drying out and also provides protection from infection. The whole procedure should be carried out quickly and carefully. The faster the operation, the greater the chance of survival.

Beads soaked in biological saline were used initially whilst practicing the technique. The embryos developed normally, showing that neither the act of carrying out the operation nor insertion of a bead causes digit duplication. The abnormal effects seen in other experiments can therefore be attributed to the retinoid applied. However, it is important to use spherical beads, as previous studies have shown the implantation of irregular shaped beads can cause malformation.

21 operations were carried out using 0.1mg / ml retinol in DMSO. 2 embryos survived to examination and were found to have successfully attached beads. They both showed normal development. A further 3 embryos survived to stage

[x] Other types of tape were found to be much less effective. Sellotape Original was found to give the best seal of the tapes tried and therefore the highest survival rate.

24 or beyond and also all showed signs of normal development. 9 embryos became infected and / or died before reaching stage 24, and were not yet advanced enough in development to analyse. The remaining embryos were found to have either the bead attached in the wrong position, or that it had migrated elsewhere in the embryo and were therefore discounted in the study.

In summary, 10 embryos containing correctly positioned beads soaked in 0.1 mg/ml AT-retinol survived to a suitable age to assess accurately their development. They all exhibited normal development, suggesting that under these conditions, 0.1 mg/ml AT-retinol does not cause digit duplication when applied anteriorally to the limb bud. This result was surprising as AT-retinol is a potent teratogen[61], documented to induce severe birth defects when ingested by pregnant mice. These results appear to suggest that AT-retinol does not induce teratogenic effects when applied to this region of the limb bud; however, alternative explanations include the possibility that the beads may not be delivering AT-retinol to the limb-bud or that the concentration of AT-retinol used may be too low to cause an effect. These later possibilities must be investigated before conclusions can be drawn.

In order to see if a higher concentration of AT-retinol may cause an effect, a further 14 operations were carried out using 1.0 mg/ml AT-retinol. All of the embryos surviving to stage 24 or beyond, with correctly positioned beads showed normal development. Again, this is a surprising result, as it was expected this high concentration of retinol would be toxic. Further investigation into whether or not the beads were delivering retinol was required.

3.3 Loading and Unloading Assays

AG1-X2 beads consist of a styrene-divinylbenzene cross-linked lattice with attached quaternary ammonium groups. They act as ion exchange beads and in solution the electrostatically bound formate ions are exchanged for polar molecules such as ATRA,[62] EC23 and EC19. Thaller and Eichele used chromatography to demonstrate that AG1-X2 beads deliver radioactive AT-retinol to the limb-bud and that it is then converted to AT-retinal and ATRA.[49] AG1-X2 beads have also been used successfully in our group to deliver ATRA, EC23 and EC19 to chick limb-buds and digit duplications have been recorded.[63] It should therefore be reasonable to expect the beads to deliver AT-retinol.

Loading and unloading assays were carried out in triplicate to assess whether or not retinol was being taken up by the beads and delivered to the embryos. A fresh solution of 0.1 mg/ml AT-retinol was made up using spectroscopic grade DMSO, to ensure impurities from the solvent would not interfere with the results. (*In vivo* experiments were carried out using retinoid solutions made up in biological grade DMSO). The beads were soaked in the retinol solution and 5 µm aliquots were removed at regular time intervals. The samples were quenched by freezing to preserve them until they could be analysed spectroscopically to assess the concentration of retinol remaining in solution. [See section 6.2 for full details of method.]

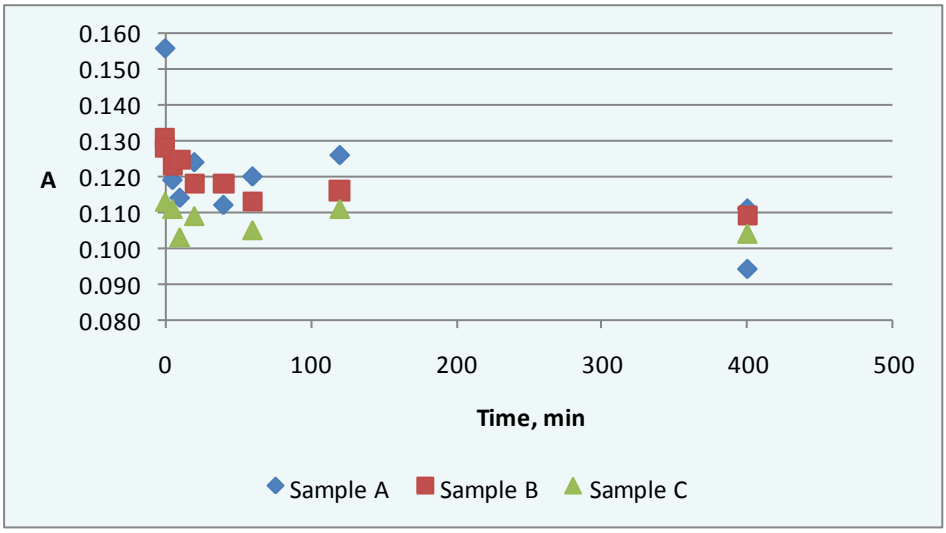


Figure 18: AT-Retinol loading assay. 40 AG1-X2 beads were soaked in a solution of AT-retinol in A.C.S. DMSO. The absorption measured at λ = 284 nm appears to decrease over time.

Before analysing the samples, the absorbance of pure A.C.S. DMSO was measured. No peak was observed at λ = 284 nm, implying any peak observed at this wavelength is representative of AT-retinol. The results are shown in Figure 18. Overall the absorbance at λ = 284 nm appears to decrease over time, suggesting the concentration of retinol in solution is decreasing, indicating that the beads *are* taking up retinol. However, the increase in absorbance seen around 2 hours is inconsistent with the expected results and gives reason to question these findings. Whether or not the beads are taking up retinol is inconclusive from these data.

An unloading assay was also performed to assess the release of AT-retinol from the beads. Beads were soaked in 1.0 mg/ml and 10.0 mg/ml retinol respectively for 11 days. The excess retinol solution was removed and replaced with saline. 5 µl samples were taken at time intervals for spectroscopic analysis. The results are displayed in Figure 19 below and are not as expected for an unloading

assay[xi] (the expected curve for an unloading assay is shown in Figure 20). The results are difficult to interpret, and it is not possible to deduce accurately whether or not retinol is being released from the beads. It is likely that isomerisation of retinol is taking place, and that this is interfering with the results.

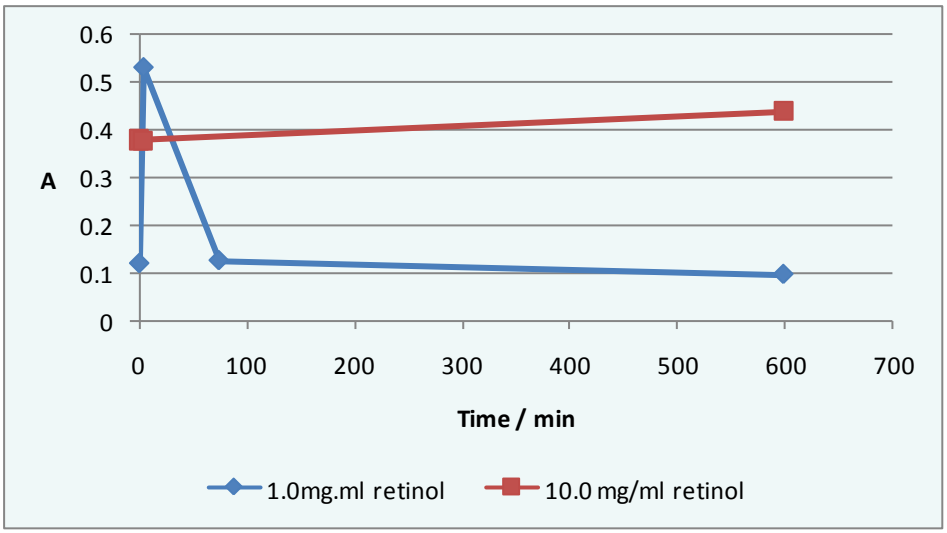


Figure 19: AT-Retinol unloading assay. AG1-X2 beads were soaked in AT-retinol in DMSO solution. The excess solution was removed and replaced by fresh DMSO. The absorbance at λ = 284 nm of samples was plotted against time to assess the rate at which retinol is released from the beads.

[xi] In a similar investigation, Thaller and Eichele report the amount of compound released per unit time increases in direct proportion to the concentration of compound adsorbed by the beads.[62]

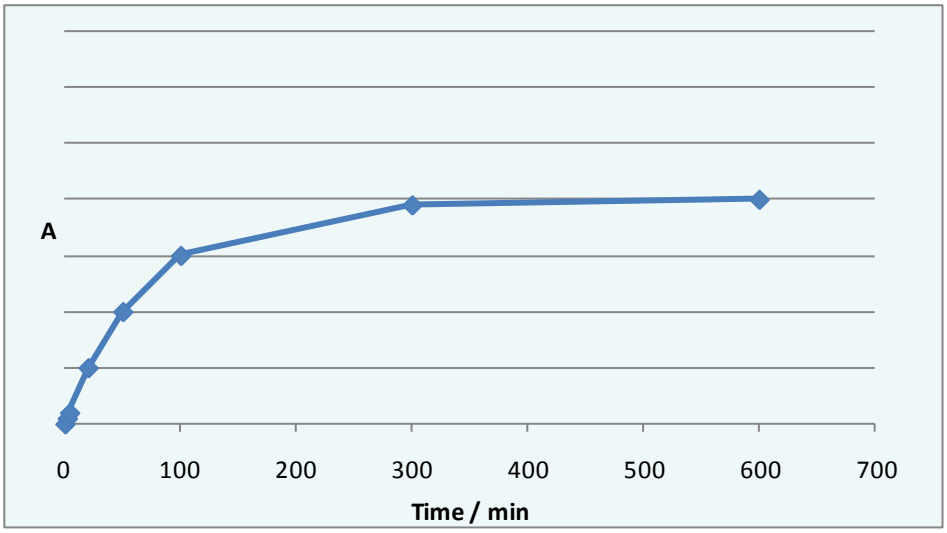


Figure 20: Expected shape of an unloading curve.

A control assay was carried out to show that the peak at λ = 284 nm was from the AT-retinol, and was not due to either the beads or the DMSO. AG1-X2 beads were soaked in A.C.S. grade DMSO and the absorbance of samples taken immediately and at time intervals afterwards were measured. The absorbance at λ = 284 nm was consistently zero.

3.4 Effect of EC23 on Chick Limb Development

The results from the loading and unloading assays were inconclusive and an alternative way in which to test the beads was sought. It is known that EC23 induces significant digit duplication in chick wings,[63] so 21 operations were carried out using 0.01 mg/ml EC23 (the optimum concentration). Surprisingly, no evidence of abnormal development was observed. This unexpected result provided further evidence that the beads were *not* delivering the compounds to the embryos.

The previous beads were discarded, and a fresh set of AG1-X2 beads were derivatised using formic acid (See section 6.2 for details of procedure). There was no apparent difference in appearance between the old and the new beads.

Operations using 0.01 mg/ml EC23 solution were repeated and carried out within 2 weeks of the beads being derivatised. 17% of embryos were still alive at the point of observation and all clearly showed that a 2,2,3,4 digit pattern had developed, i.e. a second digit 2 had developed. A further 33% of embryos survived until stage 31 and also showed clear duplication of digit 2. Another 17% of embryos also showed signs of digit duplication, but only survived to stage 27. It was therefore too early to be able to accurately determine whether or not abnormal development has occurred. Unfortunately, in one case the bead had become attached to the membrane, so had to be discounted from the study. The key results are summarised in Figure 21. These positive findings show that the technique is working and the beads are positioned correctly. It strongly suggests that the previous beads were not delivering retinoids to the limb bud and that the beads need to be freshly derivatised frequently.

Embryos showing abnormal development (%)	83
Embryos observed with 2,2,3,4 digit pattern (%)	50

Figure 21: Summary of abnormalities observed after the application of 0.01 mg/ml EC23 solution to the anterior margin of a stage 20 chick limb bud. Observations made at stage 36.

3.5 Effect of Retinol on chick limb development

A further twenty-seven operations were carried out using freshly derivatised beads to deliver 0.1 mg/ml AT-retinol solution to the limb bud. Seven embryos survived to examination, had correctly positioned beads and yet still all showed normal development. A further six embryos had correctly positioned beads and survived beyond stage 24 and also all showed normal development. As Thaller and Eichele assert that AG1-X2 beads are suitable to deliver retinol[62], and we now have evidence they are successfully delivering EC23, it is highly likely the beads are now delivering retinol, yet digit duplication is still not seen.

These results appear to suggest that a concentration of 0.1 mg/ml does not induce digit duplication in chick embryos. However, care must be taken when interpreting these findings. As discussed in the introduction, AT-retinol is very unstable in both natural light and physiological conditions, and undergoes isomerisation readily[xii]. It is feasible that the retinol underwent isomerisation, in solution even before it was applied to the limb buds. It is therefore not known what concentration, if any was delivered to the embryos. In fact, a major driving force behind this project is to develop synthetic retinoids which mimic the biological activity of natural retinoids, yet be resistant to isomerisation, in order to avoid just these types of problems. Further investigation is required before firm conclusions can be made about the effect of retinol on the limb bud. Suggestions are made in “future work” section.

[xii] An NMR study on the stability of ATRA revealed that after only three days exposure to ordinary laboratory light, substantial isomerisation / degradation had occurred, such that approximately only 37% ATRA remained[34]. The structure of AT-retinol is closely related to ATRA, differing only by one end functional group. As the chromophoric unit, is identical in each compound it is assumed that AT-retinol is equally susceptible to photo-isomerisation as ATRA.

3.6 Proposed Explanation of Findings

The lack of abnormal development following implantation of an AT-retinol containing bead was unexpected, as Thaller and Eichele have demonstrated that retinol is converted to ATRA under physiological conditions.[47,49] AT-retinol is also a known potent teratogen[61]. However, our results suggest that retinol is a precursor, and is not biologically active itself. They also suggest that a key regulatory step is the control of the rate of conversion of retinol to ATRA. Thaller and Eichele state that the concentrations of endogenous retinol, retinal and ATRA in the limb bud were found to be about 600 nM, 15 nM and 25 nM respectively.[49] The natural concentration of retinol in the limb is considerably higher, approximately 25 times, than that of ATRA. This is consistent with a theory that retinol serves as a precursor for ATRA and is converted enzymatically as required.

3.7 Conclusions

The technique to test retinoids on chick limb buds has been optimised. It was found that the shorter the time taken to perform the operation, the greater the chance of survival. With practice, operation times were reduced to below 10 minutes. One of the most common causes of embryos not surviving to examination is a poor seal. It is essential to ensure the Sellotape™ is firmly stuck down to make a complete seal around the opening.

Digit duplications from EC23 have successfully been reproduced but normal development was seen when AT-retinol was applied to the anterior margin of the limb bud. An explanation for the lack of phenotype seen has been postulated; however, further investigation is necessary before firm conclusions can be drawn about the effect of AT-retinol on the limb bud.

3.8 Future Work

Further Investigation into Delivery of AT-Retinol to the Limb Bud

It was initially thought that exogenous retinol applied to the limb bud may be converted to ATRA and that digit duplications similar to those observed when ATRA is directly applied to the limb bud would occur. However, despite numerous attempts, no digit duplication was seen on application of AT-retinol to the chick limb bud. It was discussed previously in this report, that some kind of biological control may be effective, preventing exogenous AT-retinol from being converted into teratogenic amounts of ATRA. However, before concluding that exogenous AT-retinol does not result in a teratogenic affect, it must first be ascertained that AT-retinol *is* actually being delivered to the limb bud.

Eichele, Tickle and Alberts investigated the suitability of several different beads for the delivery of compounds such as ATRA, retinyl acetate and prostaglandin E_1 to the chick limb bud.[62] Both *in vitro* loading / unloading assays and *in vivo* studies were carried out. It was concluded that positively charged ion exchange resins, such as AG1-X2 and AG1-X8 were the most suitable for the controlled release of ATRA, and that uncharged acrylic ester polymer beads such as Amberlite XAD-7 and XAD-8 were better for controlled release of uncharged retinoids such as retinyl acetate. Loading and unloading assays should be repeated using freshly derivatised AG1-X2 beads and also carried out using Amberlite XAD-7 and XAD-8 beads[xiii] to determine the best bead for delivery of AT-retinol. It should also be taken into account that AT-retinol isomerises readily in natural light and physiological conditions[xiv], which is a major reason for the

[xiii] XAD-7 and XAD-8 beads are the same size (150 – 200 µm diam) but have differing pore sizes. AG1-X8 are slightly smaller (75 – 150 µm diam)

[xiv] An NMR study on the stability of ATRA demonstrated that after only three days exposure to ordinary laboratory light, substantial isomerisation / degradation had occurred, such that approximately only 37% ATRA remained[34]. The structure of

development of more stable analogues such as EC23! Carrying out these assays in a dark room would reduce the likelihood of isomerisation occurring. Once suitable beads have been found, the *in vivo* experiments should be repeated using 0.1, 1.0 and 10.0 mg/ml concentrations of AT-retinol. Again, carrying out these operations in a dark room would reduce the likelihood of isomerisation occurring.

Alternative Technique for Assessing Teratogenicity and Toxicity

Currently operations on chick limb buds, as described in this report, are carried out to test the affects of retinoids on the developing limb. This method is advantageous in that it provides quantitative data about the teratogenicity and toxicity of the compounds tested. However, this delicate procedure is technically challenging and very time consuming. Démarchez *et al* have reported an alternative method in which retinoid solutions are simply injected directly into the amniotic cavity of 10-day old chick embryos[64]. At 16 days of incubation the eggs are then opened and observed as normal, however in this case rather than looking for limb abnormalities, the number of embryos presenting club-shapped feathers was recorded. Log-probit analysis was then used to calculate the lethal embryotoxic dose ($LED50_{d16}$) which provokes the death of 50% of the embryos at 16 days of incubation and the effective dose (ED_{50}) which induces production of club-shaped feathers in 50% of surviving embryos. Démarchez *et al* found that the more active retinoids were also more toxic, which is consistent with our previous findings. In this alternative method, significantly less time is required for each operation, which is doubly advantages. Firstly, more experiments can be carried out in a given time and secondly, the length of time the embryo is exposed to the atmosphere is reduced, thereby reducing the likelihood of infection. Infection is a major cause of void results. This alternative method could be used to quickly and quantitatively assess the teratogenicity and the

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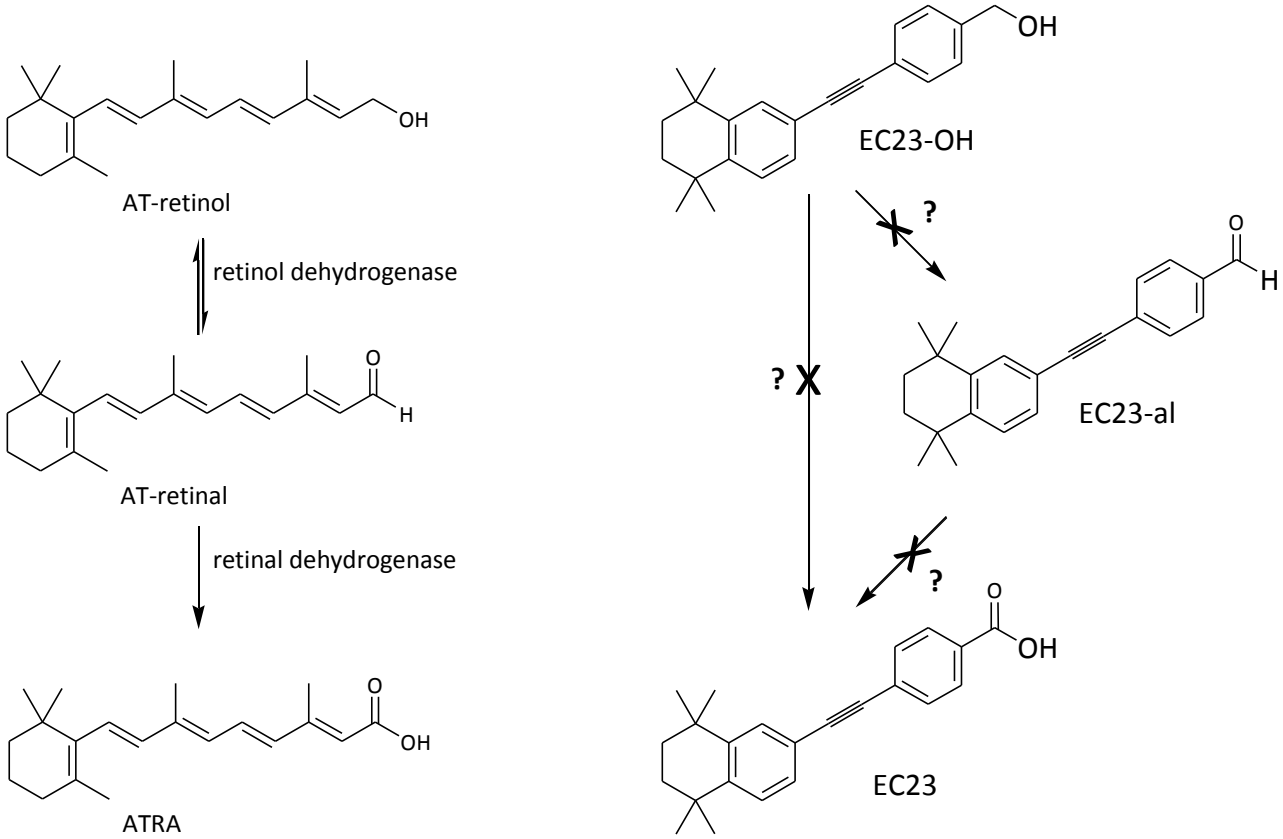
AT-retinol is closely related to ATRA, differing only by one end functional group. As the chromophoric unit, is identical in each compound it is assumed that AT-retinol is equally susceptible to photo-isomerisation as ATRA.

toxicity of new retinoid compounds synthesised.

Further Investigation into the Metabolism of Synthetic Retinoids



Scheme 2: *Left hand side:* A schematic showing the metabolism of AT-retinol to ATRA, via AT-retinol. There is much evidence that this occurs. *Right hand side:* It is currently believed that the body is unable to metabolise synthetic retinoids such as EC23 along the same route as its natural analogue.

As discussed previously, it is well documented that AT-retinol is metabolised to ATRA, via AT-retinal in the body.[9] It was hypothesised that the metabolism of EC23 could be investigated by applying EC23-OH, the EC23 analogue of AT-retinol, to the limb bud by the methods described earlier and observing its effect on limb development. EC23 is known to cause digit duplication, so

occurrence of digit duplication on application of EC23-OH may suggest that the chick limb bud is capable of metabolising EC23-OH to EC23, see Scheme 2. In order to carry out this study, EC23-OH should be made by hydrolysis of **7** (EC23-OAc). It would also be of interest to synthesis EC23-al (i.e. a synthetic analogue of AT-retinal) and also investigate this in the chick limb bud. Again the occurrence of digit duplication would provide evidence of metabolism. For completeness, the above investigations could be repeated using EC19 analogues in the place of EC23 analogues.

An alternative idea for further investigation into the metabolism of ATRA and EC23 has been inspired by a paper by on the role of ATRA in the morphogenesis of the neural tube.[65] Maden *et al* have reported findings that quail embryos that have developed in the absence of ATRA undergo abnormal neural tube formation. For example, neural tubes developed in these conditions had a wider floor plate, a thicker roof plate and a different dorsoventral shape and as development continued, the proliferation rate was found to decline drastically and neuronal differentiation was found to be highly deficient.

We hypothesise that quail embryos developed in the absence of ATRA could be rescued by the timely application of exogenous ATRA. Initial experiments would have to be conducted to determine the amount of ATRA required and the optimum stage at which to apply it order to prevent / reverse the abnormal development of neural tubes. If successful, then this could provide the bases for further investigation into the metabolism of synthetic retinoids. The following hypothesises are made on the presumption that ATRA *would* successfully rescue quail embryos developed in the absence of ATRA.

It is predicted that either AT-retinol or AT-retinal exogenously applied to embryos developed in the absence of ATRA would also induce rescue, and that development would continue in the manner seen for those rescued directly by ATRA. The reasoning for this is that AT-retinol and AT-retinal would be

metabolised to ATRA (see Scheme 2). It is predicted that exogenous application of EC23, a synthetic analogue of ATRA, would also rescue the quail embryos and again development would be expected to continue in a similar manner to those rescued directly by ATRA. Although, it is likely that much lower concentrations would be required than needed for ATRA, in accordance to previous studies showing that EC23 is much more potent than ATRA. Finally, it is predicted that exogenous application of either EC23-OH or EC23-al to quail embryos developed in the absence of ATRA *would not* rescue the quail embryos (see Scheme 2). If the above predictions turn out to be correct, this would provide further evidence that EC23 behaves in a similar manner to ATRA, but that the body is unable to metabolise EC23 or its derivatives. Alternatively, if EC23-OH and/or EC23-al *did* induce rescue, this would be evidence that the body *is* able to metabolise synthetic retinoids.

Synthesis of Luminescent Retinoids

Currently much evidence of the role of ATRA within a developing organism has been obtained from gene reporter assays. For example, studies carried out on *Raldh2* null mutant mice by Mic, Duester *et al.*[66] have demonstrated the synthesis of / requirement for ATRA in several different areas of the organism during development. However, the limitation of these genetic studies is that they provide direct information only as to the whereabouts of particular genes / enzymes, from which assumptions are then made as to the location of ATRA. In general gene reporter assays will only reveal the places where a subset of retinoid targets can be activated and it is possible that gene reporter assays may under represent the retinoid activity in tissues.

An alternative approach to learn more about the pathways of synthetic retinoids *in vivo* would be to synthesis retinoids that can be 'tracked'. For example, the synthesis of tritiated or ^{14}C labelled retinoids, or perhaps more simply, the synthesis of retinoids with extended polyene chains which are highly

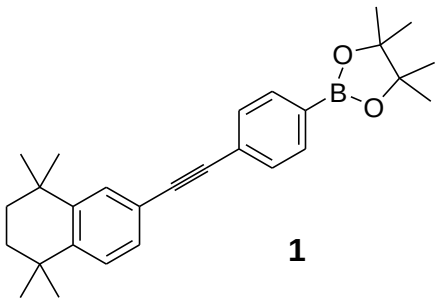
luminescent. Once in the system, luminescent retinoids could then be observed, thus facilitating direct evidence for their distribution.

Chapter 4: Experimental

All air or moisture sensitive reactions were carried out under a dry nitrogen atmosphere using standard Schlenk techniques or in a glove box. Glassware was oven dried before transfer into the glove box. Hexane and THF were dried over sodium / benzophenone and acetonitrile was dried over CaH_2 and all were distilled under nitrogen. The solvents 1,4-dioxane, DMF, THF and DMSO were degassed by 3 freeze-pump-thaw-cycles. Toluene was dried and deoxygenated by passage through columns of activated alumina and BASF-R311 catalyst under Ar pressure using a locally modified version of the Innovative Technology, Inc. SPS-400 solvent purification system. $[Ir(\mu\text{-}OMe)COD]_2$, was synthesised by literature procedures. Hydrochloric acid was obtained from Fisher Scientific and all other compounds were obtained from Aldrich Chemical Company and tested for purity by GC-MS. NMR spectra were recorded at ambient temperature on Bruker 400 Ultrashield (^1H, $^{13}C\{^1H\}$,^{11}B and $^{11}B\{^1H\}$) Varian Unity 300 (^{11}B and $^{11}B\{^1H\}$) and Bruker AC200 ($^{13}C\{^1H\}$) instruments. GC-MS analyses were performed on an Agilent 6890N gas chromatograph, equipped with a 5973 inert mass selective detector and a 10 m fused silica capillary column (DB-5ms). HRMS (ESI^+) was performed on ThermoFinnagan 7.0T LTQ-FT. HRMS ($ASAP^+$) was performed on Waters Xevo QTOF.

6.1 Chemical Synthesis

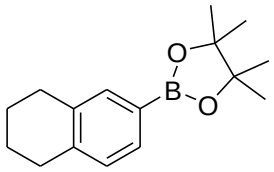
4,4,5,5,-Tetramethyl-2-[4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-ylethynyl)-phenyl]-[1,3,2]dioxaborolane



1

6-(4-Bromo-phenylethynyl)-1,1,4,4-tetramethyl-1,2,3,4-tetrahydro-naphthalene (0.44 g, 1.17 mmol), $B_2(pin)_2$ (0.30 g, 1.17 mmol) and $K_3PO_4 \cdot 2H_2O$ (0.58 g, 2.34 mmol) were suspended in dioxane (3 ml). A solution of $Pd(OAc)_2$ (0.005 g, 0.023 mmol) and S-phos (0.024 g, 0.059 mmol) in dioxane (1 ml) was added and the reaction mixture was heated at 80 °C for 2 days whilst stirring. The reaction was followed by GC-MS analysis. After addition of water (100 ml) the product was extracted with diethyl ether (100 ml) and the organic fractions were washed with water (2 x 100 ml) then dried over $MgSO_4$. The ether solution was concentrated *in vacuo*. The mixture was filtered through a silica plug with 20% dichloromethane/hexane and the solvent was removed *in vacuo* to yield an orange/yellow oil (0.08 g, 0.23 mmol, 20%). ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 7.77 (d, J_{H-H} = 7 Hz, 2H, Ar,), 7.51 (d, J_{H-H} = 7 Hz, 2H, Ar), 7.47 (s, 1H, Ar), 7.27 (s, 2H, Ar) [No ortho splitting observed], 1.69 (s, 4H, CH_2), 1.35-1.24 (m, 24H, CH_3). $^{13}C\{^1H\}$ NMR (125MHz, $CDCl_3$) δ: 145.79, 145.2 (Ar), 134.70 (Ar), 130.88 (Ar), 130.14 (Ar), 128.86, 126.81 (Ar), 120.17 (Ar), 111.46 (Ar), 91.47 (Ar-C≡C), 88.58 (C≡C-Ar), 84.07 (O$\underline{C}(CH_3)_2$, pin), 35.10, 32.03 (CH_2), 34.49, 34.36 (Ar), 31.91, 31.84 (CH_3), 25.03, 25.00 (CH_3). The carbon alpha to boron was not observed due to fast relaxation induced by the boron nucleus. MS (EI) *m/z*: 414 (M^+), 399 (M-CH_3).

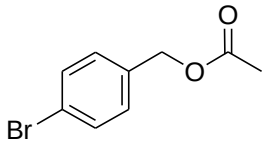
4,4,5,5-tetramethyl-2-(5,6,7,8-tetrahydronaphthalene-2-yl)-1,3,2-dioxaborolane



2

$[Ir(OMe)(COD)]_2$ (26 mg, 0.04 mmol), dtbpy (21 mg, 0.08 mmol) and B_2pin_2 (50 mg, 0.20 mmol) were mixed in hexane (3 ml). The pre-catalyst solution was added to a stirred solution of tetrahydronaphthalene (528 mg, 4 mmol) and B_2pin_2 (950 mg, 3.74 mmol) in hexane (5 ml). The reaction mixture was stirred at 80 °C for 18 hours. The progress of the reaction was followed by GC-MS. The reaction mixture was diluted with dichloromethane/hexane (20/80 v/v) and passed through a short silica plug (5 cm) eluting with dichloromethane/hexane (20/80 v/v). Removal of solvent *in vacuo* gave a colourless, transparent oil. The product was crystallised from methanol at -20 °C as colourless needles (0.96 g, 93% yield). Mp 62.5 °C. ^{11}B NMR (128 MHz, $CDCl_3$) δ 31.05 (Bpin), ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.52 (d, 2H, Ar, J_{H-H} = 7 Hz), 7.08 (d, 1H, Ar, J_{H-H} 7 Hz), 2.78 (m, 4H, CH_2), 1.79 (m, 4H, CH_2), 1.34 (s, 12H, CH_3). $^{13}C\{^{1}H\}$ NMR (100 Hz, $CDCl_3$): δ 140.78 (Ar), 136.52 (Ar), 135.71 (Ar), 131.74 (Ar), 128.64 (Ar) 83.59 ($\underline{C}Me_2$), 29.70 (Cy), 29.20 (Cy), 24.86 (Me), 23.27 (Cy), 23.13 (Cy), [C-B not observed due to quadrapolar broadening]. Analysis Calcd for $C_{16}H_{23}BO_2$: C, 73.79, H, 8.67 Measured – C-74.40, H-9.08; HRMS ($ASAP^+$) Calcd for $C_{16}H_{23}BO_2$: 257.1827, found 257.1793

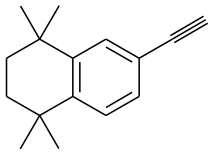
Acetic acid 4-bromo-benzyl ester



3

4-bromobenzyl bromide (2.5 g, 10.00 mmol) and potassium acetate (1.47 g, 15.00 mmol) were added to a 500 ml round bottomed flask equipped with stirring bar. Acetonitrile (150 ml) was added. A condenser was fitted and the reaction mixture was heated at 70 °C for 5 days. GC-MS analysis showed the reaction had gone to completion. The reaction mixture was filtered through a Celite pad then partitioned between diethylether and H_2O. The organic layer was washed with H_2O (150 ml), dried ($MgSO_4$) and the solvent removed *in vacuo*, to give a yellow oil (2 g, 8.73 mmol, 87% yield). This was purified via column chromatography and crystallisation. mp 33.3 – 34.1°C IR (nujol mull, KBr disc, cm^{-1}) 1750 (C=O), 1598 (C-O) ^1H NMR (400 MHz, $CDCl_3$) δ_H: 7.49 (d, 2H, Ar, J_{H-H} = 7 Hz) 7.24 (d, 2H, Ar, J_{H-H} = 7 Hz), 5.06 (s, 2H, CH_2), 2.11 (s, 3H, CH_3); $^{13}C\{^1H\}$ NMR (100 Hz, $CDCl_3$): δ 170.86 (C=O) 135.21 (Ar) 131.90 (Ar, CH) 130.11 (Ar, CH), 122.46 (Ar), 65.65 (CH_3), 21.13 (CH_2); Analysis Calcd for $C_9H_9O_2Br$: C, 47.19, H, 3.96 Measured – C-47.21, H-3.97; MS (EI) *m/z* 228 [M^+]

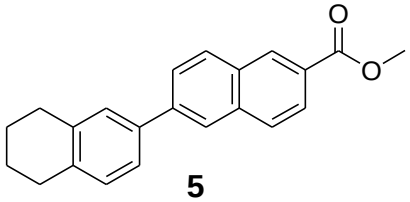
6-Ethynyl-1,1,4,4-tetramethyl-1,2,3,4-Tetrahydronaphthalene



4

6-iodo-1,1,4,4-tetramethyl-1,2,3,4-tetrahydronaphthalene (10 g, 31.8 mmol), copper iodide (60 mg, 0.32 mmol) and $Pd(PPh_3)_2Cl_2$ (0.224 g, 0.32 mmol) were added to a 500 ml round bottomed flask under N_2. Dry toluene (150 ml) and dry triethylamine (75 ml) were added via cannula and trimethylsilyl acetylene (3.28 g, 33.4 mmol, 4.7 ml) was added via a syringe. The reaction was stirred for 18 hours at room temperature and changed from yellow to brown / black. The volatiles were removed *in vacuo* and the residue was dissolved in a mixture of sodium carbonate, methanol and water. Excess solvent was removed *in vacuo* and the product was extracted into dichloromethane and dried over magnesium sulphate and filtered. The volume of solvent was reduced on a rotary evaporator, then evaporation at ambient temperature for 48 hours yielded the product as an orange / brown crystalline solid (4.84 g, 22.8 mmol, 72%); ^1H NMR (400 MHz, $CDCl_3$) δ_H: 7.47 (1H, s, Ar), 7.26 (2H, m, Ar, + $CHCl_3$), 3.02 (1H, s, H-C≡C), 1.71 (4H, s, CH_2), 1.29 (12H, d, CH_3, J_{HH}=3 Hz)

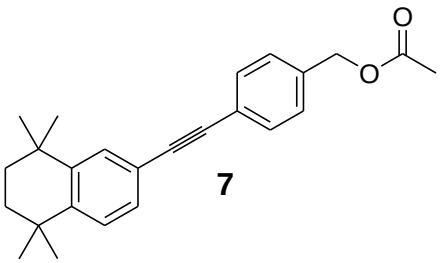
Methyl 5',6',7',8'-tetrahydro-2,2'-binaphthyl-6-carboxylate



5

6-pinacolboryl-1,2,3,4-tetrahydronaphthalene (0.24 g, 0.93 mmol), methyl 6-bromo-2-naphthoate (0.234 g, 0.89 mmol), [$PdCl_2$(dppf)] (15 mg, 0.0186 mmol) and $K_3PO_4 \cdot 2H_2O$ (0.46 g, 1.86 mmol) were suspended in ^{i}propanol / H_2O (5:1; 6 ml) and stirred at 80 °C for 5 days to give an orange/brown solution containing a grey precipitate. The volatiles were removed under reduced pressure, the brown residue was redissolved in DCM/NaCl (aq.) and the organic layer was separated. After removal of the volatiles *in vacuo* the crude product was obtained as a yellow solid (0.14 g, 0.44 mmol, 50 %). Flash chromatography [dichloromethane/hexane (25/75 v/v)] increased the purity and yielded a white solid; however, traces of an homocoupled product can still be seen in NMR and GC-MS spectra. Melting range 131.0 – 131.9 °C. IR (nujol mull, KBr disc, cm^{-1}) 1717 (C=O), ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 8.59 (s, 1H, Ar), 8.05 (dd, J = 9 Hz, 2 Hz 1H, Ar), 8.02 (br s, 1H, Ar), 7.98 (d, J = 9 Hz, 1H, Ar), 7.90 (d, J = 9 Hz, 1H, Ar), 7.78 (dd, J = 9 Hz, 2 Hz, 2H, Ar), 7.44 (m, 2H, Ar), 7.18 (d, J = 8 Hz, 1H, Ar) 3.79 (s, 3H, CH_3) 2.84 (m, 4H, CH_2), 1.84 (m, 4H, CH_2). Other small peaks also present. $^{13}C\{^{1}H\}$ NMR (100 Hz, $CDCl_3$): δ 167.52 (C=O), 141.54, 138.17, 138.16, 137.53, 136.30, 131.91, 131.22, 130.19, 130.13, 128.52, 127.56, 126.85, 126.01, 125.56, 124.97 (Ar), 51.67 (Me), 29.56, 29.17, 23.19, 21.97 (alkyl), Other small peaks also present. Analysis Calcd for $C_{22}H_{20}O_2$: C, 83.51, H, 6.37 Measured – C-82.80, H-6.23; MS MS (EI) *m/z* 316 [M^+]

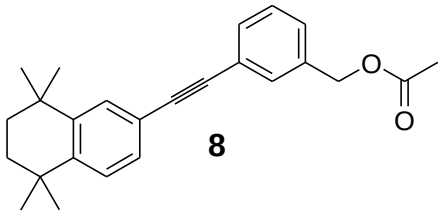
4-((5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)ethynyl)benzyl acetate



7

6-ethynyl-1,1,4,4,-tetramethyl-2,2,3,3,-tetrahydro-naphthalene (0.97 g, 4.59 mmol), $Pd(PPh_3)_2Cl_2$ (30 mg, 0.043 mmol) and CuI (8 mg, 0.041 mmol) were placed in a Schlenk tube. Acetic acid 4-bromo-benzyl ester (1.00 g, 4.37 mmol) was added, followed by triethylamine (100 ml) via cannula. The reaction was stirred at 80 °C for 6 days. GC-MS analysis showed complete consumption of starting materials. The solvent was removed *in vacuo* to give a tan coloured solid. This was purified using flash chromatography, eluting with DCM/hexane (1:10). The product was crystallised from DCM at 21 °C (0.1 g, 0.03 mmol, 1 % yield). Mp 140 °C. X-ray analysis confirms the product to be 4-((5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)ethynyl) benzyl acetate. IR (nujol mull, KBr disc, cm^{-1}) 1733 (C=O), [No signal observed for C≡C, attributed to lack of dipole moment in the molecule] ^1H NMR (400 MHz, $CDCl_3$) δ_H: 7.50 (dm, J = 8 Hz, 2H, Ar) 7.45 (t, J = 2 Hz, 1H, Ar), 7.37 (dm, J = 8 Hz, 2H, Ar), 7.26 (d, J = 2 Hz, 2H, Ar) 5.09 (s, 2H, CH_2), 2.10 (s, 3H, CH_3), 1.67 (s, 4H, CH_2), 1.27 (d, J = 8 Hz 12H, CH_3), $^{13}C\{^1H\}$ NMR (100 Hz, $CDCl_3$): δ170.79 (C=O), 145.66, 145.09, 135.72, 131.72, 129.93, 128.72, 128.09, 126.67, 123.59, 120.04 (Ar), 90.44, 87.87 (C≡C), 65.89 (Me), 34.99, 34.92, 34.35, 34.23 (Cy) 31.78, 31.70 (Me) 20.98 (CH_2) Anal. Calcd. for $C_{16}H_{28}O$: C, 83.29, H, 7.83 Measured – C, 83.05, H, 7.81, MS (EI) *m/z* 360 [M^+], 345 [M-CH_3].

3-((5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)ethynyl)benzyl acetate



8

6-Ethynyl-1,1,4,4,-tetramethyl-2,2,3,3,-tetrahydronaphthalene (0.806 g, 3.80 mmol), $Pd(PPh_3)_2Cl_2$ (36 mg, 0.05 mmol) and CuI (36 mg, 0.19 mmol) were transferred to a 150 ml round bottom flask with tap, equipped with stirrer bar. Triethylamine (60 ml) was added via cannula followed by acetic acid-3-iodo-benzyl ester (1.00 g, 3.62 mmol), giving a dark yellow / orange solution. The reaction was stirred at room temperature for 3 d. The volatiles were removed *in vacuo*. The crude product was filtered through a silica plug with Et_2O as the elutant (orange / yellow solution). The solvent was removed *in vacuo* to give an orange / brown solid; this was dissolved in hexane/Et_2O (1:5; 15 ml) and filtered through a silica plug with hexane (250 ml) then hexane/Et_2O (1:5; 300 ml). The solvent was removed in vacuo to give a pale orange / brown solid (3.84 g, 1.07 mmol, 30 % yield). Mp 72.4 – 72.8°C, IR (nujol mull, KBr disc, cm^{-1}) 2332 (C≡C), 1742 (C=O), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.52 (s, 1H, Ar), 7.47 (m, 2H, Ar), 7.32 (m, 1H, Ar), 7.27 (m, 3H, Ar), 5.08 (s, 2H, CH_2), 2.15 (s, 4H, Cy), 2.10 (s, 3H, CH_3), 1.27 (d, J = 7 Hz, 12H, CH_3). $^{13}C\{^1H\}$ NMR (100 Hz, $CDCl_3$): δ 170.89 (C=O), 145.77, 145.19, 136.28, 131.44, 131.35, 130.05, 128.80, 128.64, 127.87, 126.78, 124.09, 120.10 (Ar), 90.47, 87.92 (C≡C), 65.90 (Me), 35.09, 35.02, 34.45, 34.33 (Cy), 31.88, 31.80 (Me), 21.08 (CH_2). Analysis Calcd for $C_{25}H_{28}O_2$: C, 83.29, H, 7.83, Measured – C, 80.79, H, 8.12; MS MS (EI) *m/z* 360 [M^+], 345 [$M-CH_3$] HS-MS submitted 24/6/10 HRMS ($ASAP^+$) Calcd for $C_{25}H_{28}O_2$: 361.21621, found 361.21623

6.2 Biological Procedures

Storage and Pre-treatment of Eggs

Eggs were refrigerated until needed. Before incubation, the egg shell was cleaned with 70% ethanol. A small hole (~2 mm diameter) was made through the shell and a small amount of albumen removed with a syringe. The eggs were them incubated at 37 °C for 4 days. Increasing or decreasing the incubation temperature respectively speeds up or slows down development. The optimum stage for the operations discussed here is Hamburger and Hamilton stage 20,[60] reached after approximately 4 days of incubation at 37 °C.

Formate Derivatisation of AG1-X2 Beads

(Method adapted from ”AG1, AG MP-1 and AG 2 Strong Anion Exchange Resin Instruction Manual” BIO-RAD p. 10-11)

1 M Formic Acid

1.15 g of formic acid (powder) was added to a 50 ml centrifuge tube and the solution volume was made up to 25 ml with deionised water.

1 M NaOH

1 g of NaOH (solid) was added to a 50 ml centrifuge tube and the solution volume was made up to 25 ml with deionised water.

1 g AG1-X2 beads were added to a 50 ml centrifuge tube. NaOH (aq) (10 ml x 1 M) was added and left agitating for 1 h. The suspension was left to settle then the excess NaOH (aq) was removed with a plastic pipette. The remaining NaOH solution (15 ml) was added and returned to the agitator for 1 h. The excess NaOH (aq) was removed and the beads were rinsed with phosphate buffered saline (2 x 30 ml) and then with deionised water (4 x 30 ml). Deionised water (25 ml) was added to the beads which were agitated (30 min). The excess water was removed and the beads were given a final rinse with deionised water (25 ml). Formic acid solution (12 ml x 1 M) was added and the beads were left

agitating for 1 h. The suspension was left to settle and then the excess formic acid (aq) was removed with a plastic pipette. The remaining formic acid (13 ml) was added and the tube was returned to the agitator for 1 h. The excess formic acid (aq) was removed. The beads were dried on filter paper for 48 h and then were transferred to a screw topped centrifuge tube until required.

Biological Saline (Made up Daily)

5 ml Tyrode's solution (contains: NaCl, KCl, $CaCl_2 \cdot 6H_2O$, $NaHCO_3$, NaH_2PO_4, glucose and distilled water)
0.050 g glucose
0.050 g $NaHCO_3$
0.5 ml antibiotic / antimitotic
50 ml reverse osmosis purified water

Implantation of Bead

The surface of the egg was wiped with 70 % ethanol to clean it. Forceps were used to remove a small area of shell, whilst keeping the inner membrane intact to protect the embryo from falling shell. The membrane was then peeled back to access the embryo. 2-4 drops of biological saline were added to protect the embryo and improve clarity. Whilst viewing under a microscope, a tungsten needle is used to gently peel back the outer vitelline membrane to the reveal area surrounding the limb bud (approx $1.5cm^3$). The membranes close to the limb bud also had to be cleared using a tungsten needle. Once the limb-bud was exposed, the needle was used to cut a 'flap', slightly longer than the diameter of the bead, along the anterior margin. Fine forceps were used to remove a bead (AG1-X2, formate derivatised) from the retinoid solution (e.g. 0.1 mg/ml AT-retinol in biological grade DMSO); this was dipped briefly (3 sec) in biological saline and then inserted under the 'flap'. The bead was released from the

forceps by squeezing, then releasing. Sellotape Original™ was used to create air tight seal over the window and the embryo was incubated at 37 °C for 7 days. After this time, scissors were used to reopen the window and observe the embryo.

Dilution Series (Carried out in duplicate)

A set of small Eppendorf tubes were labelled from A to G. 10 µl of DMSO was pipetted into tubes B-G. 20 µl x 0.1 mg/ml of EC23 in DMSO was added to tube A, which was then centrifuged (12 sec) and vortexed (5 sec). 10 µl of the EC23 solution from tube A was transferred to tube B using a Gilson pipette. Tube B was then centrifuged (12 sec), and vortexed (5 sec), and 10 µl of the solution was transferred to tube C. This process was continued until each tube contained a solution of EC23. The absorbance of each sample at λ = 306 nm was measured using a NanoDrop spectrophotometer ND-1000.

The above process was repeated using a stating solution of 0.1 mg/ml EC23 in DMSO.

Loading Assay (Carried out in triplicate)

40 x AG1-X2 beads were soaked in A.C.S. spectrophotometric grade DMSO (200 µl) in a small Eppendorf tube for 2 h. The Eppendorf tube was vortexed, then centrifuged, then DMSO (180 µl) was replaced with AT-retinol in DMSO solution (180 µl x 0.1 mg/ml). Samples were immediately vortexed and centrifuged and a 5 µl aliquot was immediately taken. Further 5 µl aliquots were taken at given times, vortexing and centrifuging before each. The samples were frozen for 7 days before being analysed on a NanoDrop spectrophotometer.

Unloading Assay (Carried out in triplicate)

The beads from the loading assay (above) were left soaking in the AT-retinol in DMSO solution for 24 h. The excess retinol solution was then removed and replaced with saline (200 µl). The samples were immediately vortexed and centrifuged and a 5 µl aliquot was immediately taken. Further 5 µl aliquots were taken at given times, vortexing and centrifuging before each one. The samples were analysed on a NanoDrop spectrophotometer.

Control Assay (Carried out in duplicate)

40 x AG1-X2 beads were put in a small Eppendorf tube and centrifuged (30 sec). A.C.S. DMSO (200 µl) was added and the tube was vortexed (10 sec) and centrifuged (30 sec). A 5 µl sample was taken immediately. The beads were left to soak for 24 h, then 180 µl of DMSO was removed and replaced by fresh DMSO. The sample was vortexed (10 sec), centrifuged (30 sec) and a 5 µl sample was taken immediately. A final 5 µl sample was taken after 5 min. The absorbance of all samples at $\lambda = 284$ nm was measured and found to be zero.

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