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THE DIELS-ALDER-REACTION WITH INVERSE-ELECTRON-DEMAND - A REVIEW OF AN EFFICIENT & ATTRACTIVE CLICK-REACTION CONCEPT

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ABSTRACT: Here, we review the development of prospective image processing systems in molecular diagnostics and of pharmacologically active ingredients for patient-specific therapeutic approaches. These projects require not only high demands on quality, safety, and specificity but also, rapid, efficient and irreversible ligation routes during the synthesis of future pharmaceuticals. The Diels-Alder ligation reaction with inverse electron demand (DAR_{inv}) is an eligible technology not restricted to medical applications, but valuable in selective modification by functionalization of polymers, organic and inorganic surfaces and micro arrays. Additionally, the DAR_{inv} technology is considered as an attractive strategy-platform for efficient syntheses of promising pharmacologically active components and derivatives of natural molecules with an optimized therapeutic index. We like to encourage scientists working with the brilliant concept of Sharpless's "Click chemistry", to intensify their research with this valuable DAR_{inv} technology able to open the door for regioselective, stereospecific, and bioorthogonal exigent syntheses of substances of highest quality inconceivable so far.

INTRODUCTION: The pioneering work of Otto P.H. Diels and Kurt Alder started in 1926 in the field of chemical reactions between reaction partners with one ene- and diene- containing components to cyclohexene products. This cyclo-addition methodology was documented as "Diels-Alder-Reaction" (DAR) and started a meteoric rise in ligation chemistry. The Nobel Prize in Chemistry has been awarded in 1950 [1].

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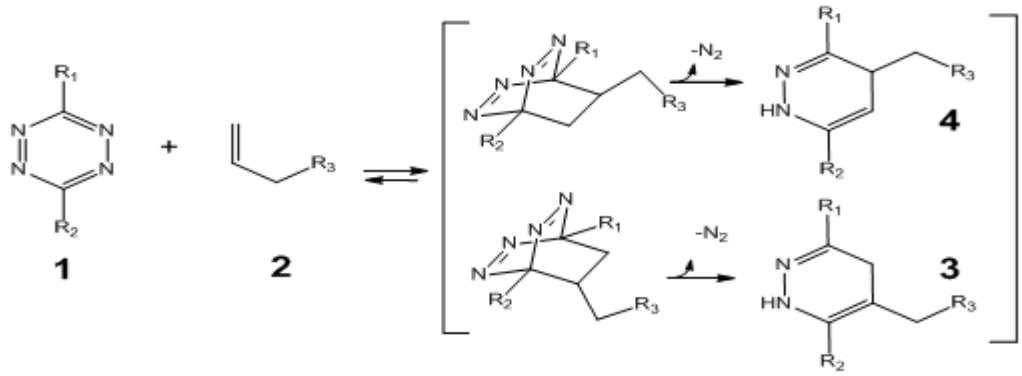
The DAR principle and its chemical potential for the pharmaceutical research were well investigated [2-8]. Dependent on the chemical properties of the dienes and the dienophiles, the reaction led to different but preferred variants. One variant was documented as "Hetero-Diels-Alder-Reaction" and described the chemical reaction with a heteroatom substitution of the diene- and the dienophile-components [9, 10].

The DAR also fulfils the criteria of the "Click-Chemistry" compiled by Sharpless (Scheme 1) [11], comprising a cornucopia of qualified ligation reactions as shown in Huisgen's work [12]. The Staudinger ligation [13, 14] and Bertozzi's variant [15, 16] which were reviewed by Wiessler [17].

Under normal conditions the reaction rate was either low at room temperature or a catalyst was required. In the classical DAR, the dominating orbital interaction (corresponding to the lowest HOMO-LUMO energy separation) was between HOMO diene and LUMO dienophile [18-20]. In contrast, the Diels-Alder Reaction with inverse-electron demand (DAR_{inv}) was controlled mainly by the interaction of HOMO dienophile and LUMO diene and required an electron-rich dienophile and an electron-poor diene [21-25].

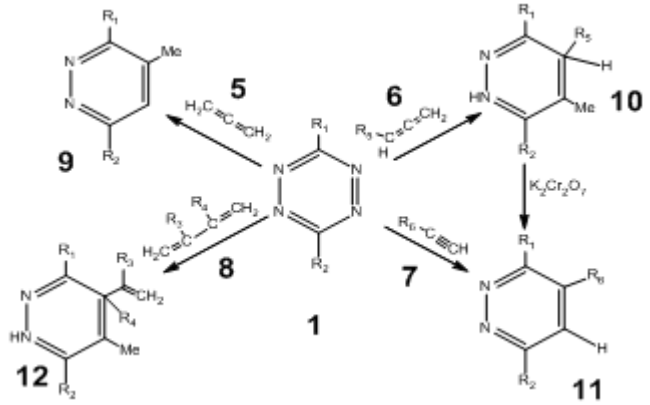
Substituents with pushing electrons increased and, with pulling electrons reduced the electron density of the dienes as used in the DAR_{inv}. All ligation products were generated after a rapid and complete reaction at room temperature in organic solutions. The reaction of this ligation chemistry led to high purity, but with hurdles and difficulties on the way to the multifunctional drug-imaging-conjugates appropriate to multimodal "theranostic" approaches [26]. The synthesis of complex nature identical pharmacologically active molecules was mentioned here [17].

The wide spectrum of this universal and powerful DAR_{inv} methodology in the field of the complex macromolecular architecture is expounded here.



Scheme 1: The scheme points out that the DAR_{inv} fulfils the criteria of the "Click"-Reaction principle [3, 27, 28]. R_1 and R_2 represent different functional moieties harbouring –I and/or –M effects on the diene **1**, which induces a decrease of the electron density of the tetrazine ring. In contrast, the R_3 features a +I effect resulting in a relatively high electron density in the dienophile compound **2**. The stepwise reaction from **1** and **2** results in the stereoisomers **3** and **4**, which can be attributed to the two different variants of intermediates (bracketed) after elimination of molecular nitrogen. As shown here, the reverse reaction is impossible (modified from Wiessler [17]).

The DAR_{inv} can help to overcome obstacles, like long reaction times, stringent conditions and the need of catalysts for the ligation reactions [12, 29, 30]. The first DAR_{inv} documentation by Carboni and Lindsey in 1959 [27] describes the chemical reactions between tetrazines **1** and unsaturated compounds **5-8**. These are dienes, or acetylenes **9-12** and are referred as the Carboni-Lindsey Reaction [31-33] (Scheme 2). The scientific interest on this technology increased exponentially.



Scheme 2: illustrates the variability of synthetic routes of substituted tetrazines **1** with unsaturated compounds (dienes, acetylenes) **5-8** to the formation of 3,6-disubstituted pyridazines **9-12**. R_1 and R_2 represent different functional moieties harbouring –I and/or –M effects on the diene **1**. (R_3 – R_6 = H; Me; alkyl) (modified from Carboni and Lindsay [27]).

It is obvious that in this rapidly increasing field of the syntheses of pharmacologically active substances, the DAR_{inv} technology offers a tremendous potential for the development of pharmaceutically interesting active nature identical molecules.

The almost infinite variability of the DAR_{inv}-products is supported by the huge reservoir of different diene and dienophile compounds as reaction partners which are referred in detail as follows.

Diene components: Heterocyclic molecules have a prominent role in the DAR_{inv} and were exemplarily reported elsewhere [34-36].

1. **Synthesis of tetrazine compounds:** The most frequently used diene in the DAR_{inv} is the easily available 1,2,4,5-tetrazine-3,6-di-carbonic acid **17**, which can be produced in three-steps starting with diazo etylacetate **13** as shown in Scheme 3. The carboxylic acids **14** and **15** were synthesized first by Curtius [37]. The diazo ester and the hydrazine molecule **16** were isolated and described by Sauer [36]. Almost all these molecules underwent a quantitative reaction at room temperature within minutes as proposed by Nenitzescu. The research groups of Boger and Largeron comprehensively described regioselective properties of substituted 1,2,4,5-tetrazines in the DAR_{inv} [38]. In 2012, derivatized tetrazines were lodged as an US Patent application for bio-orthogonal coupling agents [39].

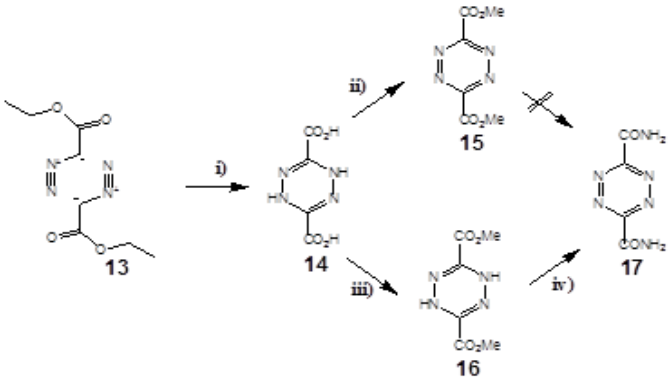
   The electro negativity was crucial for maintaining the high diene-activity. The stability of functionalized tetrazine esters, however, proved to be insufficient, whereas functionalized tetrazine amides provided the ability for the synthesis of many functionalized derivatives. These could be obtained via the dihydrotetrazine-amides followed by an oxidation step. Nevertheless the use of these tetrazine derivatives as described in Scheme 3 turned out to be problematic for two reasons:

a) Their insufficient stability and

b) Their poor solubility in aqueous solutions obviates applications in living systems.

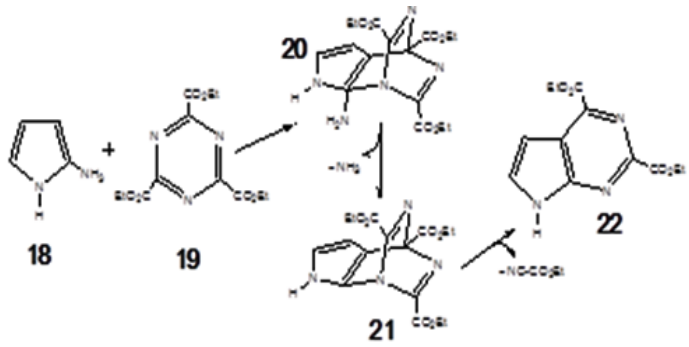
   The sensitivity against nucleophiles also seemed to be the cause of the low stability of these tetrazine derivatives in aqueous solutions. Because of these chemical decomposition processes, tetrazines modified with the dicarboxylic acid **15** were not qualified for ligation under conditions for the solid phase peptide synthesis (SPPS) and use under physiological conditions as proven in our experiments.

   Considerations to circumvent these limitations the development gave rise to the synthesis of aryl substituted tetrazines featuring –I attributes. Following the reaction, the colour change during DAR_{inv} from a tetrazine (magenta) to a diazine (yellow) under degassing nitrogen occurred rapidly [40].



Scheme 3: Illustrates the chemical route of the synthesis of the diamide of the tetrazine dicarbonic acid **17**. The reaction steps were initiated and carried out in i) **13**, a) 50% NaOH, b) H_2SO_4; ii) $NaNO_2$ in glacial acetic acid; iii) $SOCl_2$, MeOH; iv) $NaNO_2$ (modified from Wiessler [17]).

2. **Synthesis of triazine components:** Wu and Gomez-Galeno documented studies to investigations of the DAR_{inv}-based synthesis of purine and pyrimidine analogues **22** with 1,3,5-triazine derivatives **19** as diene compounds as reaction partners [23, 41, 42] (Scheme 4). De Rosa and Arnold investigated the electronic and steric effects of the DAR_{inv}'s reaction mechanism with 1, 3, 5-triazines **19** [43].



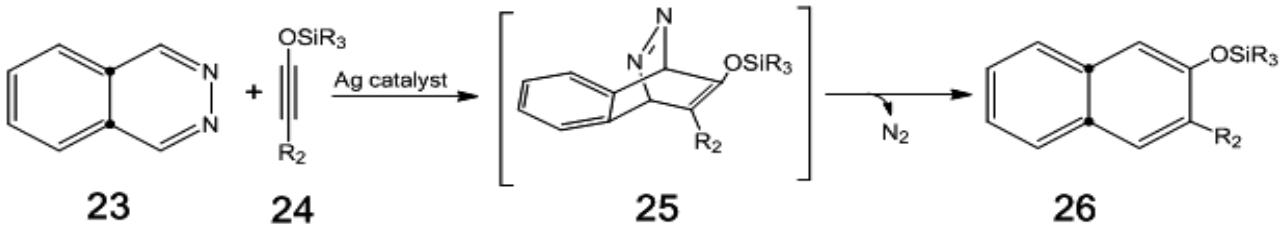
Scheme 4: Illustrates, strongly simplified, the chemical route of the reaction of 1,3,5-triazine derivative **19** and 2-aminopyrrole **18** to the final aromatic cycloadduct 22 (modified from De Rosa [43]).

Yu and co-workers published in 2001 a detailed theoretical study of a DAR_{inv}-based reaction mechanism with 1,3,5-triazine and 2-amino pyrrole as diene and dienophile reaction partners for the synthesis of purine analogues [23].

The huge potential of triazines was substantiated by the work of Branowska who documented a direct chemical synthesis route with 5,5'-bi-1,2,4-triazines with bicyclo[2.2.1]hepta-2,5-dienes to disubstituted-2, 2'-bipyridine derivatives [44-46].

Whereas the Synder group showed the importance of the triazine as a diene reaction partner in the DAR_{inv} in 1,2,4-triazine studies [47-49], the Boger group systematically investigated the 1,2,3-triazines [50, 51]; earlier, the group exhibited expertise in the research of the DAR_{inv} azadiene chemistry in the field of the total synthesis of nature identical molecules [4, 5, 52].

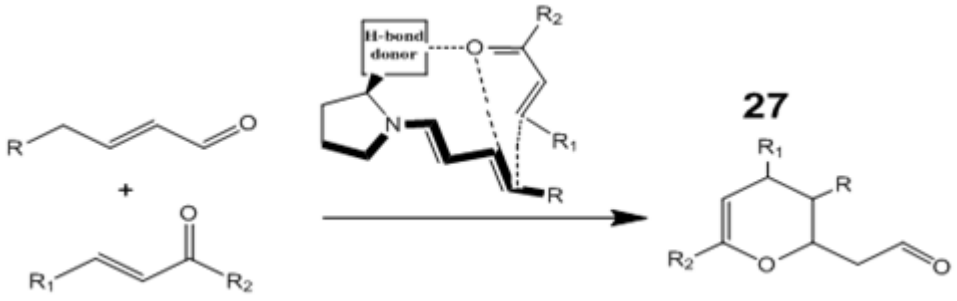
Diazines as diene reaction partners: Recently, in 2012, the DAR_{inv} of 1,2-diazines as azadienes and siloxy alkynes, which were silver-catalyzed, was published by the Rawal group. An example for a silver-catalyzed reaction phthalazines **23** and siloxy alkynes **24**, reacting under loss of nitrogen to silyl-protected 2-naphthols **26** [53] is shown in Scheme 5.



Scheme 5: illustrates the chemical route of the DAR_{inv} of 1,2-diazine **23** and siloxy alkyn **24** to the silyl-protected 2-naphthol (modified from Turkmen [53]).

The van der Plas group investigated intramolecular DAR_{inv}–based cycloadditions and documented the synthesis of 7,7-dicyano-6,7dihydro-5h-1-pyridines from the intermediacy of cycloadducts 2-(1,1-dixyanopent-4-yn-1-yl)pyrimidine and 2-(1,1-dicyanohex-5-yn-1-yn)pyrimidine after reaction of 2-chloro- or 2-methylsulfonyl-pyrimidines and the sodium salt of 5,5-dicyanopent-1-yne and 6,6-dicyanohex-1-yne [54].

Dienamine intermediates as diene reaction partners: In 2012, Albrecht and co-workers described an asymmetric variant of the DAR_{inv} using metal catalysis and organocatalysis facilitating the synthesis of optical active dihydropyran derivatives **27**. This was achieved by DAR_{inv} using dienamines intermediates as reaction partners (Scheme 6). The high stereo- and regiocontrol was realized by use of a bifunctional H-bond aminocatalyst [55].

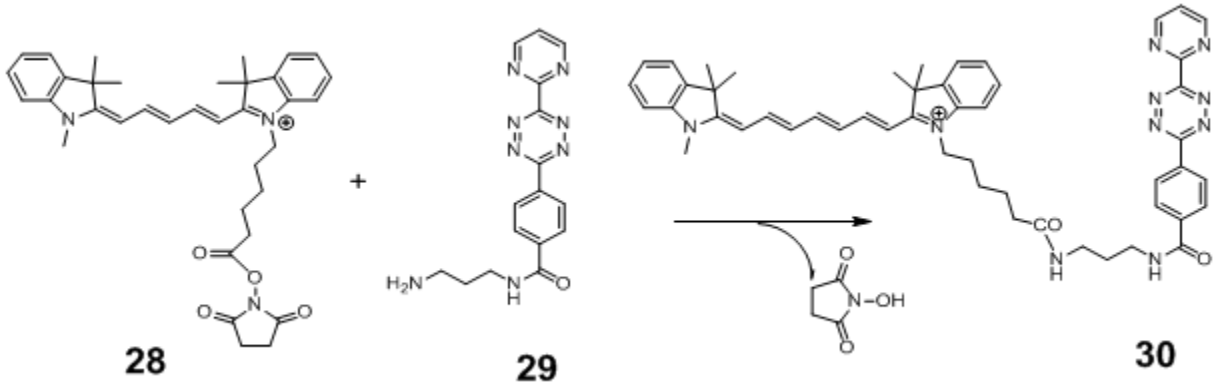


Scheme 6: Illustrates the catalyst facilitated hetero DAR_{inv} (modified from Albrecht [55]). Further, aminocatalytic DAR and DAR_{inv} via HOMO activation were documented using dienamine species from α, β-unsaturated aldehydes which act either as electron-rich dienes in normal-electron-demand DAR or as dienophiles in DAR_{inv}. All these reactions occur with high chemo-, regio-, and stereoselectivity as mentioned above [56].

Functionalization of dienes with imaging components:

1. **Dienes can be considered as coupling molecules for diagnostic as well as for therapeutic use:** The Devaraj group intensively investigated benzylamino-tetrazines functionalized with different fluorescent dyes in imaging studies [57-60]. Tetrazines derivatized with functional groups like 3,6-diaryl-*s*-tetrazines, suitable for labeling with biomolecules were demonstrated by the Fox group. They developed an $\alpha_v\beta_5$ integrin targeted PET tracer by DAR_{inv}. reaction [61].

For optical imaging studies the Wiessler group developed $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin addressed to cyclic RGD-BioShuttle carrier molecules. The chemical reaction to the tetrazine diene, exemplarily functionalized with the polymethine-based Cyanine dye Cy7 **30** as an imaging component suitable for NIR imaging, is illustrated in Scheme 7.



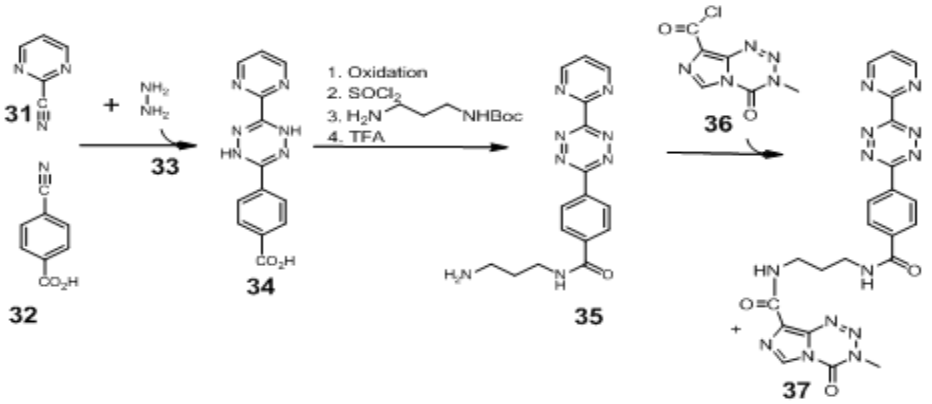
Scheme 7: Illustrates the chemical reaction of **28** and **29**. The Cy7 reaction product **30** acts as a diene reaction partner for the DAR_{inv}.

The conjugate **30** is composed of the diene *N*-(2-aminopropyl)-4-(6-(pyrimidine-2-yl)-1, 2, 4, 5-tetra zine-3-yl)benzamide **29** and the indotricarbo cyanine fluorescent dye Cy7 **28** [26]. The synthesis of further diene building blocks improved the variability of the "Click Chemistry" for fluorescence imaging as exemplarily documented here by the coupling of 5-(dimethylamino)-naphthalene-1-sulfonyl (dansyl) **28** and bis2,6-[5-carboxylic acid-pyrid-2-yl]-tetrazine **29** [62, 63].

Hilderbrand *et al*, designed an asymmetric aryl-tetrazine derivative, functionalized with the chelators 1,4,7,10-tetraazacyclo-dodecane 1,4,7,10-tetraacetic acid (DOTA) or desferrioxamine (DFO) bearing the positron emitting radioisotopes ^{64}Cu or ^{89}Zr. These functionalized components react in turn under the DAR_{inv} route with trastuzumab (Herceptin®), an antibody against the human epidermal growth factor receptor HER2/neu norbonene-modified [64].

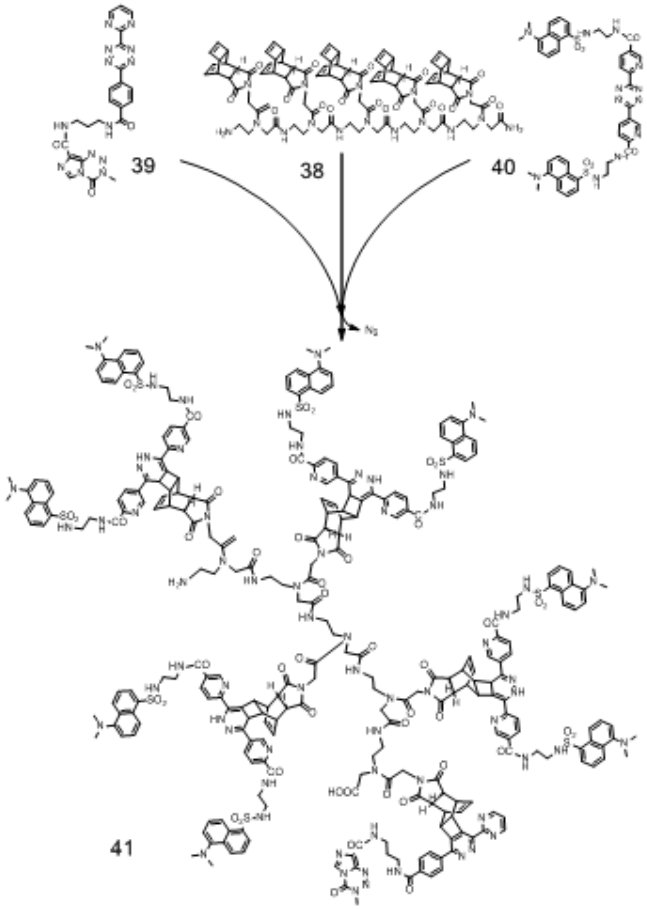
Functionalization of tetrazines with biologically active molecules: In future, dienes, functionalized with pharmacologically active molecules are definitely conceivable. The functionalization of a diaryl-tetrazine with "old fashioned" drugs like temozolomide (TMZ) and the effects of the DAR_{inv}. reaction product were first documented by the Wiessler group as an important example of reformulation of established drugs [65].

The synthesis of dienes used in our TMZ-BioShuttle studies was highly efficient but the synthesis of functionalized tetrazines suitable for the DAR_{inv} posed an experimental challenge [66]. As illustrated in Scheme 8, the nitriles 2-cyanopyrimidine **31** and 4-cyanobenzoic acid **32** reacted with hydrazine **33** to the dihydro-intermediate **34**. The oxidation and the conversion to the acid chloride which in turn reacted with the Boc-mono-protected 1, 3-propylenediamine are the next steps to product **35.** After deprotection, the amino group reacted with the acid chloride derivative of the TMZ **36** to the final product TMZ-diaryl-tetrazine **37** acting as diene reaction partner for the DAR_{inv}–mediated ligation [17].



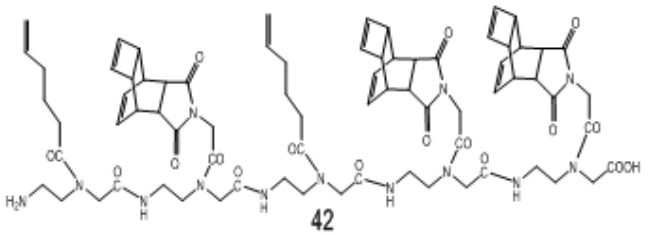
Scheme 8: Illustrates the synthesis route of the TMZ tetrazine derivative **37** which reacts as a diene partner for DAR_{inv}. The 1,3-diaminopropyl modified 4-diaryl-3, 8-dihydro-1,2,4,5-tetrazine **35** is reacted with the acyl chloride derivative of the TMZ **36** (modified from Wiessler [17]).

Functionalization of the peptide nucleic acid-backbone (PNA) with dienophile compounds: Our group also developed the following prototype of a theranostic agent (Scheme 9).



Scheme 9: The simplified illustration shows the reaction route to the ligation product **41** after the DAR_{inv} of the Reppe anhydride double functionalized peptide nucleic acid (PNA) pentamer **38**. This is completely loaded with four diaryl-1,2,4,5-tetrazine-3,6 functionalized with two dansyl chloride 40 and the *N*-(2-aminopropyl)-4-(6-(pyrimidine-2-yl)-1,2,4,5-tetrazine-3-yl)benzamide. It is functionalized with 4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo[4.3.0]nona-2,7,9-triene-9-carboxamide (temozolomide) **39** (modified from Wiessler [67]).

Here, the synthesis of **41** was performed by a combined DAR_{inv}-mediated ligation of diene-functionalized pharmacologically active substances, like TMZ **39**, with fluorescent dyes like 5-(dimethylamino)naphthalene-1-sulfonyl chloride (Dansyl chloride) **40**. The Cyanine dyes C5 and C7 as further imaging components were also documented. A further possible reaction partner was a PNA-based polymer whose building blocks were functionalized with different dienophiles (instead of nucleobases) **42** (Scheme 10).



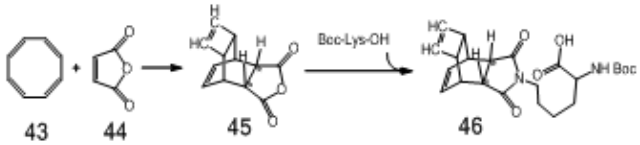
Scheme 10: Illustrates a PNA pentamer backbone which consists of momoners functionalized with dienophile structures offering different reactivity, like pentenoic acid and the Reppe anhydride **42** (modified from Wiessler [67]).

The variability of the charge of a polymer consisting of different molecules in desired ratios confers the key properties to the DAR_{inv} products. They reach optimal effects and minimal adverse reactions and are simultaneously able to monitor metabolic processes at the cellular level. This was feasible with the theranostic molecule in expressing cells and tissues after coupling to functional peptides like the cRGD for targeting to the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [26].

1. **Synthesis of dienophile components:** It should be mentioned that a lot of educts harbouring reactive terminal double bonds or double bonds in ring systems are commercially available or are easy to prepare. By this means a wide range of dienophilic compounds is available for certain DAR_{inv} reactions. In order to obtain reaction times in the range of minutes for the DAR_{inv}, the reactivity of the dienophile is decisive for the rapid reaction process besides the reactivity of the tetrazines as reactants. The allyl-group, a component of numerous chemical compounds which are easily accessible indeed may offer dienophile-activity, but nevertheless it is not qualified for a rapid chemical reaction. Therefore symmetric dienophiles with higher reactivity should be favoured.

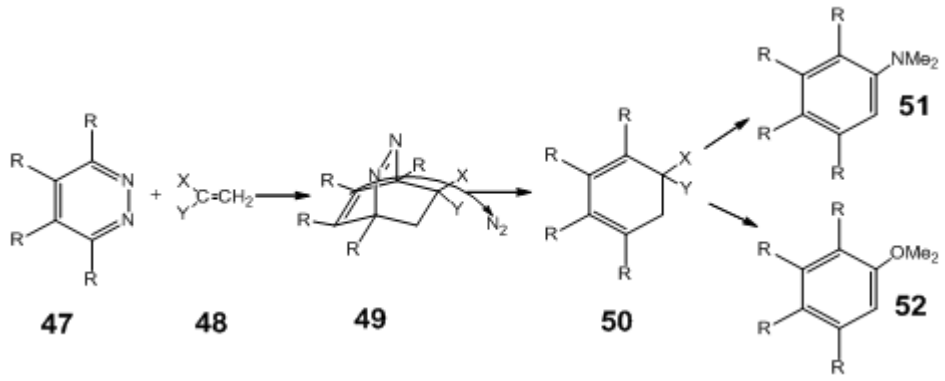
2. **Synthesis of the "Reppe-Anhydride":** Already in 1966, Sauer documented a very high dienophile activity of double bonds in cyclic ring systems adjoining to the terminal double bonds [68]. Incipient with strained rings like cyclopropene possessing very high dienophile reactivity, the reaction rate was reciprocally proportional to the ring's size; the minimum was reached with the six-atom ring and in larger rings the reaction rate increased slightly. In case of cyclobutene the derivatives disposed a sufficient stability as well as excellent dienophile reactivity.

A tetracyclic anhydride, synthesized and well described by Reppe (best-known under the name of Reppe-Anhydride), turned out to be the ideal compound for our purpose **45**. It was easily available by the classic DAR of cyclooctatetraene **43** and maleic anhydride **44** [69-71]. (Scheme 11 exemplifies the chemical route). Other ring systems, dedicated for chemical reactions, as described above, are the cyclobutene-3,4-dicarboxylic acid anhydride [72, 73].



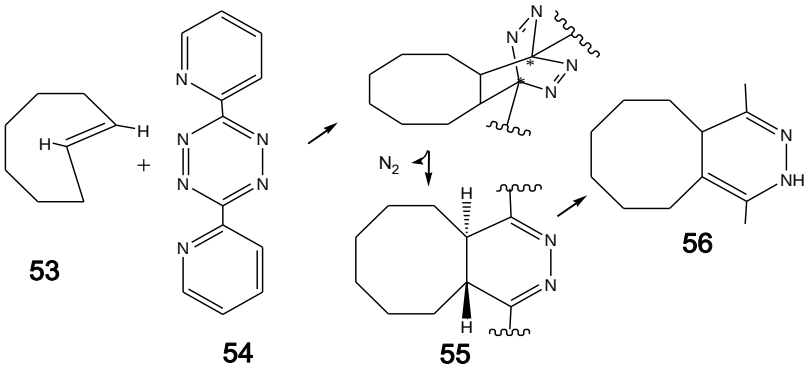
Scheme 11: Illustrates the synthesis of a versatile building block for modification of peptides. The syntheses of the Reppe-Anhydride **44** and the corresponding (±) Boc-Lys derivative **46** were described [65].

A valuable input to the fundamental research for the DAR_{inv} was given by Sauer's group from 1966 to 2004 [28; 74-79], An intensive research in the field of cycloaddition reactions of azabenzols **47** with different reaction partners [80-85] was conducted by Neunhoeffer's group (exemplified in Scheme 12).



Scheme 12: Describes the chemical reaction of diazobenzol derivatives **47** with ethylene-based vinylamines using as an example 1-methoxy-N,N-vinylamine [R = H; Me]; [X = OMe; $Y = NMe_2$] 48 to the structurally isomeric products **51** and **52** (modified from Neunhoeffer and Werner [86]).

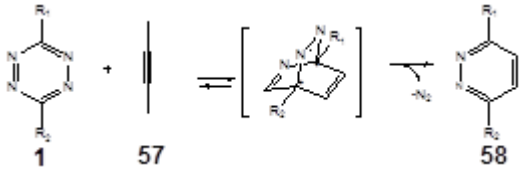
Molecules with reactivity as dienophiles: The bioorthogonal reaction is characterized by rapid reaction rates and without need for catalysis. In 1962, the Sauer group first documented the cyclooctene as a dienophile [28]. The dienophile cyclooct-4-enol also was lodged in the US patent application US2011/0268654 A1 [87]. The Fox and the Robillard groups investigated the *trans*-cyclooctene **53** as dienophile partner for DAR_{inv} with substituted *s*-tetrazines **54** as diene reaction partners with an excellent bioorthogonal reactivity [88, 89] (Scheme 13).



Scheme 13: Illustrates the DAR_{inv}.of 3,6-di-(2-pyridyl)-*s*-tetrazine **54** and *trans*-cyclooctene 53 to the (±)-4,5-dihydropyridizine **56** (modified from Blackman).

Schoch and co-workers first documented a bioorthogonal reaction established with DNA building blocks functionalized with cyclooctene-based dienophiles to rapidly synthesize oligonucleotides as reporter molecules for FRET studies [90].

Alkenes, alkynes: The use of isolated olefinic double or triple bonds in hydrocarbon rings or linear systems as dienophile components **57** was comprehensively published by Wiessler [91].



Scheme 14: Shows the DAR_{inv} "Click"-Reaction of the diene **1** with the alkyne **57**. R_1 and R_2 represent functional moieties harbouring –I and/or –M effects on the diene **1**.

In 2012, Fox and co-workers published coupling reactions with 3-substituted cycopropenes in organic and aqueous solvents [39] and documented, already in 2006, that chiral cyclopropenes are considered as eligible dienophile reaction partners for diastereoselective synthesis of methylene cyclopropanes [92].

Alkenes and alkynes also could act as typical dienophile counterparts for chemical reactions with dienes like dicyanocyclohexa-1,3-dienes and substituted phthalonitriles resulting in the synthesis of a broad class of heterocyclic derivatives like dicyano-indoles, and -carbazoles [93].

The use of 2(*1H*)quinolones acting as dienophile components was not restricted to the synthesis of 5(*6H*)-Phenanthridone derivatives, pharmacologically active as inhibitors of poly (ADP-ribose)-polymerase (PARP) [94].

Mendez and co-workers investigated ethylene as a dienophile partner with oxazole under a DAR_{inv} whose reaction was facilitated by addition of Brønsted or Lewis acids [95].

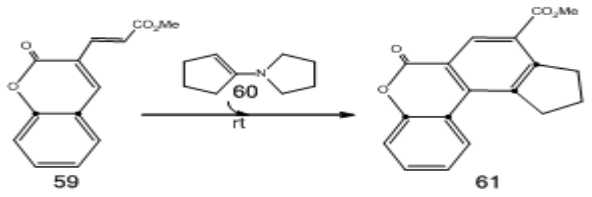
The Largeron group first documented a multistep electrochemical synthesis of polyfunctional 1,4-benzoxazine derivatized by alkylamino substituents via a DAR_{inv}-based reaction route [96, 97]. Due to their pharmacological activity 1,4-benzoxazine derivatives could play a role as neuroprotective agents [98].

During the cycloaddition reaction via the regiospecific and distereospecific DAR_{inv}, the reactions of *o*-iminoquinone with secondary alkylenamines were generated *in situ* and produced

aryl-*2H*-3,4-dihydro-1,4-benzoxazine intermediates which were unstable and chemically not accessible [99].

Enamines: Bodwell *et al*, comprehensively described the synthesis of (±)-6*H*-dibenzo[*b*,*d*]pyran-6-one derivatives using the DAR_{inv}. Coumarin-fused electron-deficient 1,3-dienes [100] were synthesized and reacted with a series of electron-rich enamines derived from acyclic carbonyl compounds, cyclic ketones and pyrrolidines. They led to the functionalized products dibenzopyranones **61** after aromatization by oxidation of the nondehydrogenated precursor intermediates which were due to be produced (Scheme 15). It is important to note, that the tested enamines **60** were chemically accessible by their generation before or *in situ* [101].

The key importance of dibenzopyranones **59** as educts and intermediates in the synthesis of molecules with multi-faceted pharmacologically activities is clearly documented. We mention here a few examples of steroid hormone analogues, receptor agonists, growth factor inhibitors and flavonoid derivatives with protective properties against cardiovascular diseases and cancer [101-105].

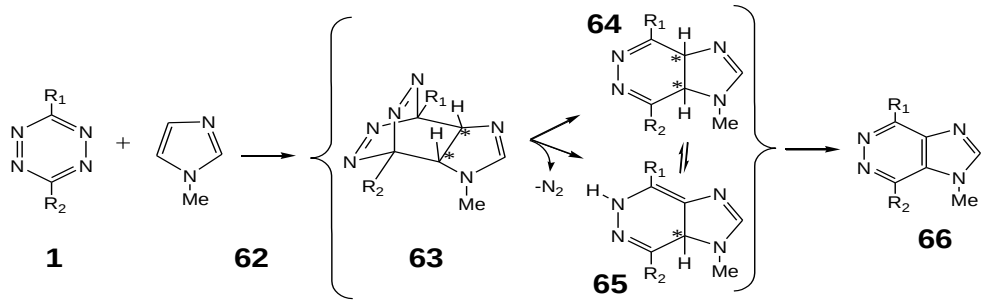


Scheme 15: Illustrates in a simple way the DAR_{inv}-based step of the chemical route for the synthesis of (±)-6*H*-dibenzo[*b*,*d*]pyran-6-one **61** with the enamine dienophile **60** (produced by reacting cyclopentenone and pyrrolidine) with the coumarin diene compound **59** as reaction partner (modified from Bodwell [100]).

An additional attractive DAR_{inv}-strategy, documented by the Dujardin group [106], is based on the reaction groups of electron-rich chiral enamines *N*-vinyl-2-oxazolidonones with the β,γ-unsaturated α-ketoesters to the heterocycloadducts *N*-2-deoxyglykosyl-oxazolidinones. The distinctive feature was the oxazolidine compound, which acted not only as a chirally inducing agent but also as a monomer which allowed a stereocontrolled de novo synthesis of *N*-2-deoxyglykosides or sugar-α-amino acid hybrid derivatives.

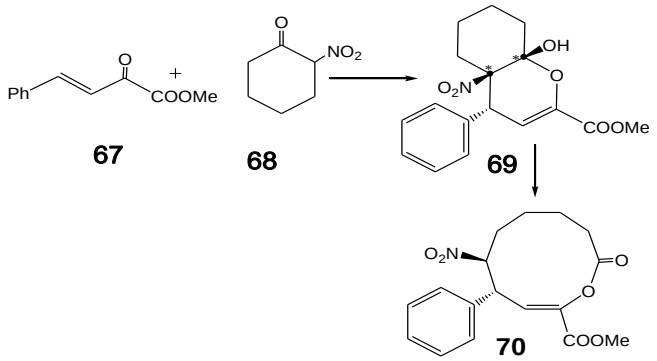
Total synthesis of pharmacologically active nature identical products: The old fashioned total synthesis of naturally occurring molecules was representative for undesired adverse reactions, which hampered the therapeutic success. The DAR_{inv}-methodology could help to use specific modifications of the molecular structure during synthesis of the active molecules realizing an optimization of their therapeutic index.

Synthesis of purine analogues with antibiotic properties: DAR_{inv}-based cycloaddition reactions with different π-electron rich heteroaromatic "inverse" dienophiles like furanes, pyrroles, thiophenes, and N-methylimidazoles **62** and a negatively substituted tetrazine derivative **1** were described by the Seitz group [107-110] **Scheme 16**). They provided a promising reaction for purine analogues which were pharmacologically highly active as repellents against microorganisms.



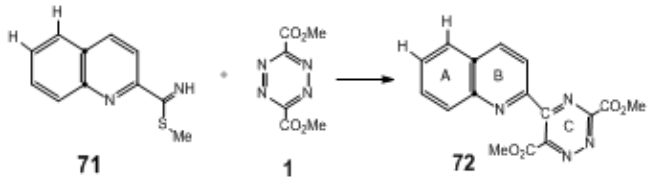
Scheme 16: Describes the reaction steps of the [4+2] cycloaddition of the tetrazine derivative **1** with *N*-methylimidazole **62** to the corresponding not isolatable cycloaddition intermediate (bracketed) **63** [**64** and **65** represent tautomeric structures after nitrogen elimination] which are oxidized under recovery of the aromatic imidazole ring to the resulting reaction product **66** (modified from Seitz and Kämpchen [111]).

Synthesis of macrolides: The macrolide structural element is found in several pharmacologically active molecules like alkaloids and antibiotics. Despite the efforts in the development of synthetic lactones a challenge remained for the chemical synthesis. The Wang group presented the first approach of the macrolide synthesis. They used the asymmetric hetero DAR_{inv} to construct **69** after chemical reaction of cyclic ketones **68** with enones **67**, thereby affording densely functionalized bicyclic skeletons in (±)-macrolides **69**, **70** [112] as shown in Scheme 17.

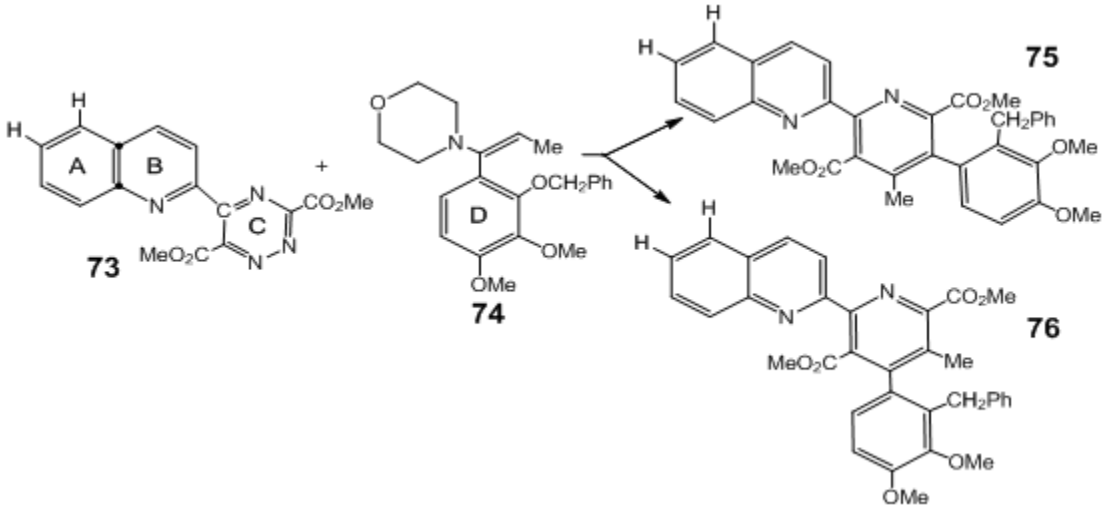


Scheme 17: Illustrates the catalytic asymmetric hetero DAR_{inv} during the synthesis of chiral (±)-macrolides **69** (modified from Jiang [112]).

Synthesis of antibiotics with antitumor properties: The Boger and Snyder groups succeeded in the total synthesis of multifaceted substances identical to nature: as an example, the complex synthesis of streptonigrin **76** [113]. It is an antibiotic with antitumoral properties. A sequential implementation of the steps a and b of the DAR_{inv} methodology is illustrated in the highly simplified Scheme 18 [114; 115].

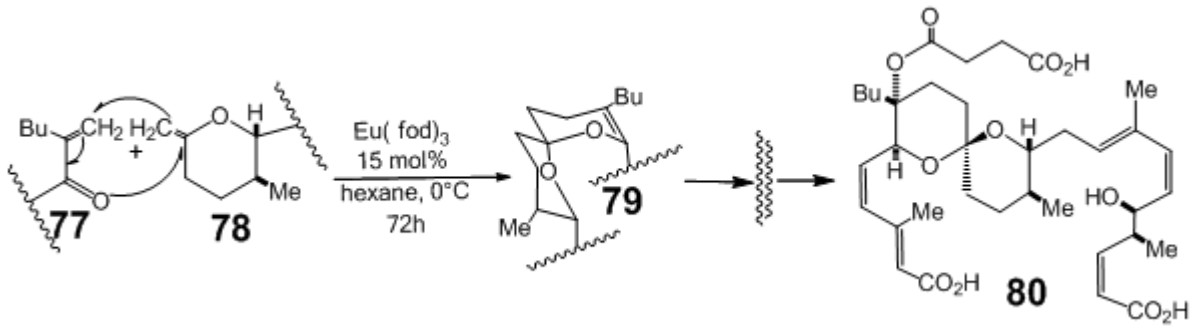


Scheme 18 exemplifies the key chemical reactions of: The cycloaddition of tetrazine derivative dimethyl 1,2,4,5-tetrazine-3,6-dicarboxylate **1** with the heterodienophile S-methyl 6-methoxy-5-nitro-2-quinolinethio-imidate **71** for the formation of the 1,2,4-triazine streptonigrin ABC ring system **72**.



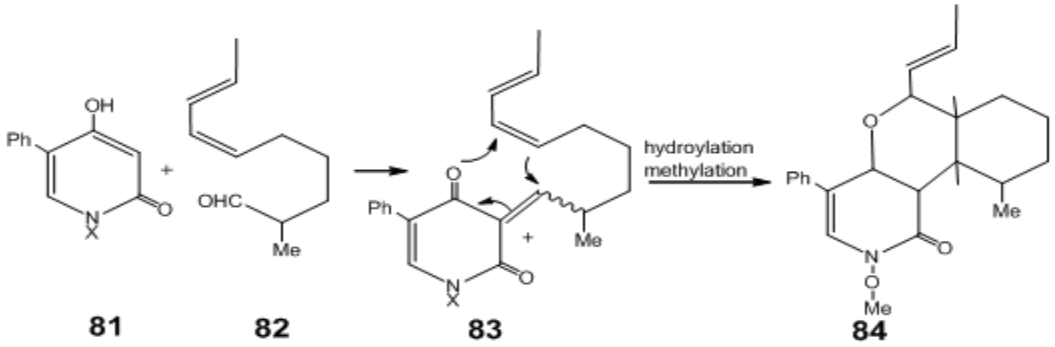
a) The [4+2] cycloaddition of the dimethyl 5-(6-methoxy-5-nitro-2-quinolyl)-l,2,4-tri azine-3,6-dicarboxylate **73** with the morpholino enamine derivative 2-(benzyl oxy)-3,4-dimethoxypropiophenone **74** to the formation of the CD biaryl ring system in a mixture which contains the Diels-Alder adducts **75** and **76**. The adduct 76 is composed of streptonigrin's framework (modified from Boger [114]).

Total synthesis of the antimitogenic (-)-Reveromycin A: El Sous in the Rizzacasa group reported the total synthesis of (-)-Reveromycin A **80** using the DAR_{inv} in a "hetero strategy" to construct the critical spiroketal moiety of the molecule [116, 117]. The central step, the Lewis acid catalyzing the hetero-DAR_{inv}, is visualized in Scheme 19.



Scheme 19: illustrates the key step of the Lewis acid [Eu(fod)$_3$]. Hexane as solvent at O°C promoted the cycloaddition of the diene **77** with methylene pyran **78** to the spiroketal fragment **79**. The intermediate to the final product (-)-Reveromycin A is **80**. The hetero DAR$_{inv}$ was catalyzed by Eu(fod)$_3$. without solvent (modified from El Sous [117]). The natural product (-)-Reveromycin A **80** (**Scheme 19**) derived from a member of the soil actinomycete family *Streptomyces sp*. acts as an inhibitor of the mitogenic activity of the epidermal growth factor (EGF). It inhibited the human tumor cell lines KB [118] and K-562 [119] by blocking of the isoleucyl-tRNA-synthetase [120-122].

Synthesis of antiinsectans: In the year 1996, Snider and Lu described the first total synthesis of the antiinsectans (±)-Leporin A 123, isolated from the sclerotia of Aspergillus-Leporis by the Gloer group in 1991 [124]. The speciality of this synthesis was the "tandem Knoevenagel condensation" and the intramolecular DARinv. It was first documented by the Tietze group in 1995 [125] and qualified to construct the unstable tricyclic intermediate o-quinone methide **83** from 4-hydroxy-5-phenyl-2-pyridone **81** and the acyclic 2-methyl-6E,8E-decadienal **82**. This molecule underwent the intramolecular DARinv followed by hydroxylation and methylation steps resulting in the hydroxypyridone product (±)-Leporin A **84** as shown in **Scheme 20**.



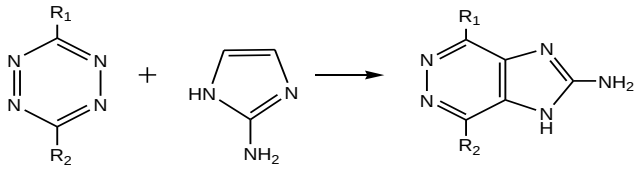
Scheme 20: Shows the chemical tandem Knoevenagel-intramolecular DAR$_{inv}$ reaction steps of the (±)-Leporin A synthesis **84** (modified from Snider [123]).

Total synthesis of alkaloids: The multi-facetted pharmacological potential of alkaloids and the increasing demand for therapeutic approaches are in contrast to their availableness because of their extensive isolation and purification procedures. The complex chemical synthesis hampered their clinical breakthrough, unfortunately.

Therefore the DAR$_{inv}$ increasingly comes into the pharmaceutical research's field of vision, as demonstrated exemplarily by the following syntheses.

Synthesis of Zarzissine: Here the synthesis of the cytotoxic guanidine alkaloid, an imidazole pyridazine derivative is documented. It was originally extracted from the Mediterranean sponge *Anchinoe paupertas*.

Zarsissine was first isolated and characterized by the Puel group [126]. The synthesis route by DAR_{inv} was described by Snyder [115] (Scheme 21).

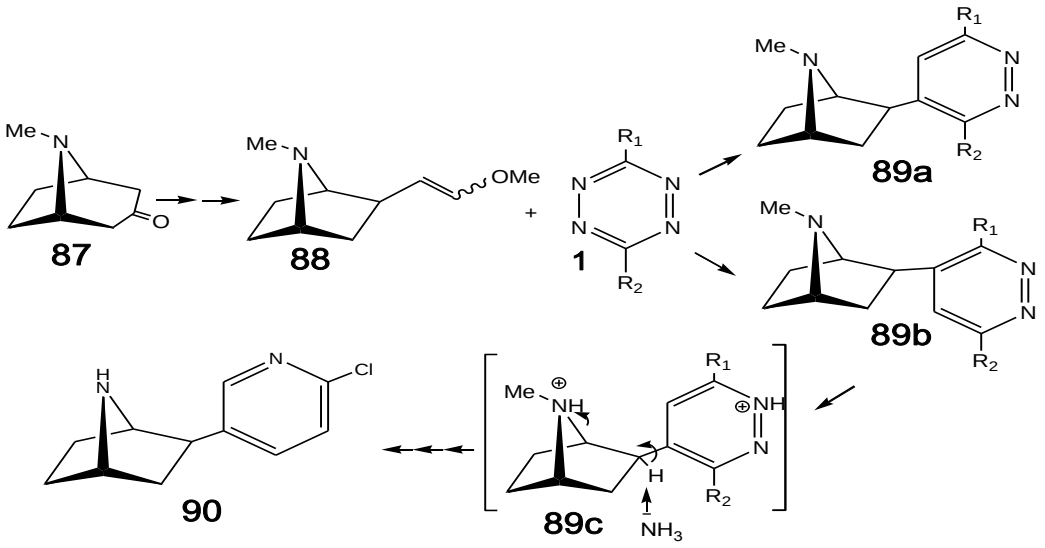


Scheme 21: Shows the simplified chemical reaction of the cycloaddition of 2-aminoimidazole 85 with 1,2,4,5-tetrazine derivative 1 to the final product 2-amino-1*H*-imidazo[4,5-*d*]pyridazine derivative (zarzissine: $R_1 = R_2 = H$) **86** (modified from Synder [115]).

The DAR_{inv} bases on the reaction of dienophilic heteroaromatic compounds **1** and imidazoles **85** as documented by Seitz [109]. Due to the pharmacological activity the imidazo[4,5-*d*]pyridazines [127] as purine analogues were in the focus of the pharmaceutical research of heart diseases [128; 129].

Synthesis of (±)-Epibatidine: The synthesis of the pyridazine analogue (±)-Epibatidine **90** is representative for the fact that the DAR_{inv} technology is not limited to the synthesis of derivatives offering structural similarity to naturally occurring molecules. The DAR_{inv} was also critical in the synthesis of nicotinic acetylcholine receptor agonists like the pyridazine analogue (±)-Epibatidine **90** which was exemplified by the Methfessel group [130]. Their synthetic route to **90** started with the commercially available 3-tropanone **87**.

After the conversion to the corresponding racemic ester and transformation to the electron-rich dienophile enol ether **88** reacted with the electron-deficient diazadiene 1,2,4,5-tetrazine **1**. It was carried out in a $LUMO_{diene}/HOMO_{dienophile}$-regulated DAR_{inv} after inverse [4+2]-cycloaddition and elimination of nitrogen via the racemic intermediates **89a**/**89b**. This leads to the pyridazine analogon product (±)-Epibatidine **90** (**Scheme 22**).



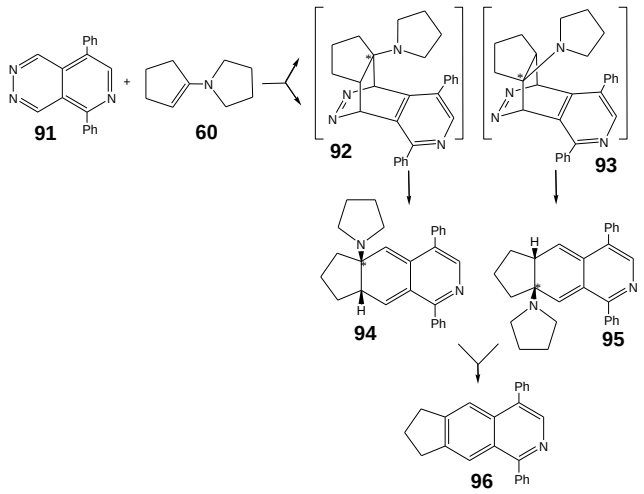
Scheme 22: Shows the chemical DAR_{inv} reaction steps of the synthesis of (±)-Epibatidine products **90** (modified from Methfessel in a simple form [130]).

Epibatidine derivatives were used as highly effective non-opioid analgesic molecules, but due to their toxicity the therapeutical application had to to be restrained [131; 132].

The synthesis of Epibatidine homologues may produce relief for approaches to potential nicotinic acetylcholine receptor ligands [133].

Synthesis of isoquinoline derivatives: The Haider lab scrutinized the [4+2] cycloaddition potential of the DAR_{inv} on condensed pyridazines for the construction of higher order annealed ring systems [134-138].

Here, the key synthesis of the isoquinoline derivative **96** by DAR_{inv} of pyrido[3,4-d]pyridazine **91** with 1-pyrrolidino-1-cyclopentene **60** [139] was illustrated (Scheme 23).



Scheme 23: Shows the reaction of the biphenyl-substituted pyrido[3,4-*d*]pyridazine **91** with 1-pyrrolidino-1-cyclopentene **60** to the two not-isolatable isomeric dihydro intermediate products **92** and **93** (in brackets) reacting to the two corresponding cycloadducts **94, 95**. The elimination of pyrrolidine and the following aromatization resulted in the formation of the single final isoquinoline derivative product **96** (modified from Haider [139]). It is worth to note, that the isomeric cyclopentane-fused dihydroiso quinolines **94** and **95** react to the single final product **96**.

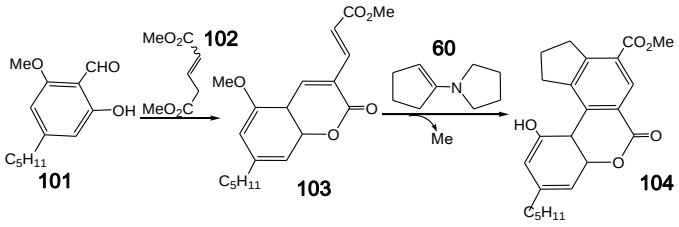
Tetrahydroquinoline synthesis: Quinolines were of great interest for the pharmaceutical and chemical research as basic drug molecules and for the synthesis of herbicides and fungicides. The synthetic access by isolation from the gas tar exclusively was documented by Masson [140]. The synthesis route of 2,3,4-trisubstituted tetrahydro quinolines **100** using the DAR_{inv} modified as a three-component aza-DAR_{inv} of aldehydes (R2) **99**, anilines **98** and isoeugenol **97** [140] is shown in Scheme 24.

Scheme 24: Illustrates the synthesis step of the three-component aza-DAR_{inv} of the synthesis of 2,3,4-trisubstituted tetrahydroquinolines **100**. [R2= -CHO] (modified from Masson [140]) .

The synthesis route is complex but the reaction mechanism was well investigated and described as a nucleophilic attack of isoeugenol to the N-arylimine under cyclization and formation of intermediates [140].

Total synthesis of cannabinol and derivatives: The molecular class of cannabinoids consists of **70** natural products isolated from *Cannabis sativa*.

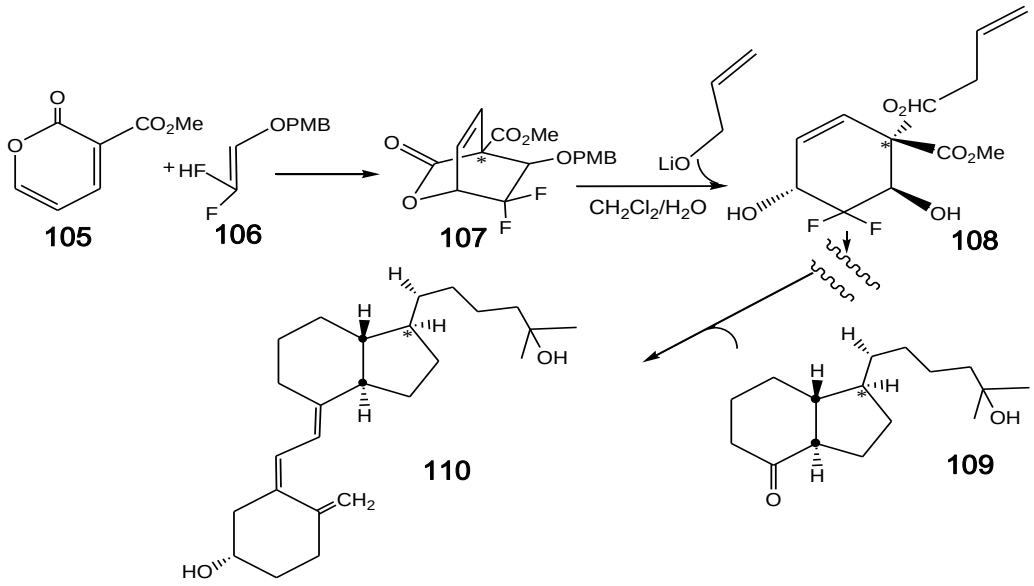
This alkaloid's synthesis is extensive and requires several steps. The Bodwell group clearly documented an efficient methodology (yields up to 79%) employed in a concise total synthesis of cannabinol **104** whose intermediate 6*H*-dibenzo[*b,d*]pyranone (DBP) **103** was formed by an asymmetric DAR_{inv} of the compounds 6-methoxy-3-pentyl-salicylaldehyde **101** and dimethyl glutaconate **102** [141] (Scheme 25). The generation of the enamine **60** acting as an electron-rich dienophile was documented by Bodwell and co-workers as herein mentioned before [100]. The process was already shown in Scheme 15.



Scheme 25: Describes the key steps and the DAR_{inv}-based reaction of 6-methoxy-3-pentyl-salicylaldehyde **101** and dimethyl glutaconate **102** and the enamine 1-pyrrolidino-1-cyclopentene **60** to 6*H*-dibenzo[*b,d*]pyranone (DBP) **103**. After elimination of pyrrolidine and the following aromatization step (not shown), it shows the condensed route to the final cannabinol derivative product **104** (modified from Nandaluru [141]).

Total synthesis of analogues of natural hormones: The Posner group depicted the first DAR_{inv}-based synthesis step of dihydroxyvitamin D3 analogues by [4+2]-cycloaddition between the commercial 3-methoxycarbonyl-2-pyrone **105** as a diene and the difluorinated electron-rich vinyl ether 106 as a dienophile. The reaction product was the racemic (±)-lactone cycloadduct intermediate **107** [142; 143] which reacted under a nucleophilic opening of the lactone ring with the Li-allyloxide under formation of the polyfunctionalized racemic (±)-cyclohexene **108**.

This happened without inter- or intramolecular displacement of fluoride ions and the coupling of the enantiomerically pure ketone **109**. Additional reaction steps resulted in the final reaction product 1α, 25-dihydroxyvitamin D3 **110** (shown in the condensed Scheme 26).

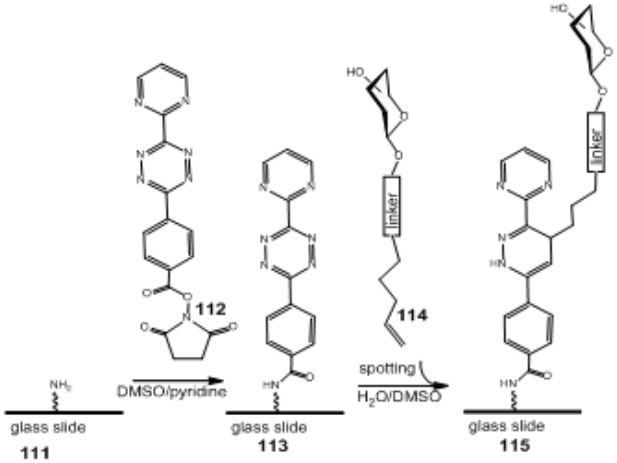


Scheme 26: Simply shows the DAR_{inv} reaction steps of the synthesis of the 1α, 25-dihydroxyvitamin D3 product **110** (modified from Posner [143]).

The pharmacologic properties of dihydroxyvitamin D3 analogues were well investigated [144]. Currently, the analogues 1α, 25-dihydoxy-19-nor-vitamin D2 and calcipotriol are in clinical use for therapeutic intervention for hyperparathyroidism and psoriasis [145].

Functionalization of Polymer Surfaces:

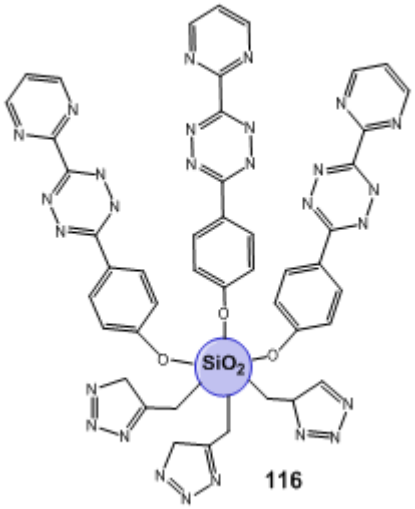
Glass carbohydrate-microarrays: The Wittmann group developed a promising carbohydrate microarray platform [146] with inorganic SiO_2-surfaces functionalized with tetrazine derivatives as diene molecules **113**. Molecules, like norbonenes or terminal alkenes which were ligated with carbohydrates **114** act as dienophile reaction partners for DAR_{inv} chemistry whose reaction kinetics realized an immobilization of the carbohydrates in a high homogeneity. This fulfilled the requirement criteria of glycomix high-throughput screening studies of carbohydrate-protein interactions [147-150].



Scheme 27: Describes the preparation of carbohydrate microarrays **115**. The amine-coated glass slides **111** were diene-functionalized with **112** in DMSO/pyridine solution. Then follows the DAR_{inv} based ligation of **113** and the dienophile **114**. This is connected via a linker to a carbohydrate (modified from Wittmann [146]).

Biocompatible silicon surfaces: The Beck-Sickinger group investigated the potential of inorganic SiO_2-surfaces as possible linkers and coupling sites for functional peptides in biomaterials. The Scheme 28 highlights the SiO_2-surface **116** functionalized with different linkers realizing independently ligation reactions.

The 1,4-diaryl-1,2,4,5-tetrazine reacts with dienophiles like the Reppe anhydride in a DAR_{inv} click reaction**.** The well-established copper(I) catalyzed Huisgen azide**-**alkyne [3+2] cyclo-addition (CuAAC) is possible using the cyclic azides.



Scheme 28: Describes schematically the structure of the inorganic SiO_2 material whose surface is functionalized with silica binding peptides which in turn are connected to different linkers like the diaryl tetrazine as diene component for DAR_{inv} (upper part) and the cyclic azide for the copper (I) catalyzed Huisgen azide-alkyne cycloaddition (CuAAC) (lower part). (Modified from Beck-Sickinger).

The functionalization was exemplarily carried out with the well investigated cRGD [151-153], a small molecule which acts as a ligand for specific binding to the $\alpha_v\beta_5$ integrin receptor, involved in neoangiogenic and metastasis processes [154-156]. The characteristics of the used DAR_{inv}-allowed a bioorthogonal click reaction strategy [157-159] for a bridge formation between inorganic surfaces and biological active molecules [160].

CONCLUSION: The DAR_{inv} chemistry is an increasing and expanding technology for rapid, efficient, irreversible ligations of user-defined functional molecules or genetic materials. These properties fulfil not only the requirements for the "Click Chemistry", but also for chemical reactions under conditions for bioorthogonal chemistry. These topics were introduced and developed further by Bertozzi in 2000 [15; 161; 162], and are currently in the focus of the scientific interest.

Additionally, these reaction products are dedicated as a combination of cargo to carrier and as biological address-molecules to realize high local concentrations of active substances in living cells. These chemical reaction products are able to overcome cellular membrane barriers during a safe and efficient transfer of therapeutic and / or diagnostics cargos into target cells and tissues. It is important to point out that this DAR_{inv} technology is not restricted to application and easier handling of our developed BioShuttle delivery platform [67, 163] and other related carrier molecules.

An enhancement towards functionalization of surfaces and polymers by a proper ligation at surfaces like biotechnical arrays for genome or transcriptome analysis can be realized by this technique. Depending on the scientific subject and formulation of the scientific project, the coupling of dienes and dienophiles at a defined surface could be demonstrated.

The following points support this technology:

I) The presented kinetic data with high reaction rates demonstrate the potential of the Diels-Alder-Reaction with inverse electron demand (DAR_{inv}) as a method of choice for ligation of molecules.

II) The reaction process could be easily monitored by use of photometrical methods with a decreasing absorption maximum at 520 nm, which is typical for tetrazines.

III) We could demonstrate that the DAR_{inv} features all the conditions for the successful "Click"-Chemistry and as a consequence, it turns out to be a dedicated tool for ligation reactions important but not restricted to the medical and pharmaceutical science.

The great advantage of DAR_{inv} is based on

A) Compounds' accessibility [164],

B) The high and quantitative reaction rate,

C) Bioorthogonal reactions,

D) The potential for selective multiple reactions at the identical molecule,

E) High regioselectivity,

F) The ease of monitoring of the chemical reaction and

G) The feasibility of the reaction on surfaces.

With this report we like to emphasize the great potential of the DAR_{inv} technology in many attractive fields. It was exemplarily documented in the field of drug-re-formulation, which dramatically increased the therapeutic potential of classic drugs as pointed out here with the alkylating agent temozolomide (TMZ). It was also able to enhance the therapeutic spectrum in malignant gliomas or in hormone-refractory prostate cancer [65, 66, 164]. Additionally this DAR_{inv} technology attracts increasing notice to further medical applications, especially in oncological diagnostics and therapy at the cellular and molecular level.

As illustrated above impressively, the DAR_{inv} technology is considered as an attractive strategy-platform for efficient syntheses of promising pharmacologically active components and derivatives of natural molecules (Scheme 16 – Scheme 26) with an optimized therapeutic index. The DAR_{inv} methodology contributed to the enhancement of the quality of already established syntheses of natural materials which are difficult to isolate and enrich [165]. The technical superiority like chemoselectivity, regio- and stereo-selectivity allow the synthetic access to increasingly more and more complex target structures [166].

ACKNOWLEDGEMENTS: This work was supported in part by grant from the Deutsche Krebshilfe Foundation (Project No. 106335).

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with p-substituted styrenes. 1999, 82(7): 1122-1134.
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