Photoactivation

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A Straightforward Approach towards Cyclic Photoactivatable Tubulysin Derivatives**

*Judith Hoffmann and Uli Kazmaier**

Abstract: *The development of a new photolabile protecting group containing an additional allyl functionality allows the synthesis of cyclic photoactivatable natural products. Cyclization occurs between the allyl moiety in the protecting group and a second double bond in the target molecule by means of ring-closing metathesis. Cyclization should increase the metabolic stability towards proteases. On the other hand, the conformational change should cause diminished biological activity. As illustrated for tubulysin derivatives, cyclic and photoactivatable drug candidates can easily be obtained in only two steps from simple building blocks through Ugi reaction and ring-closing metathesis. The photolabile protecting group is introduced by means of the isocyanide component during the Ugi reaction.*

Since ancient times light has been used for the treatment of skin diseases. In ancient Egypt, psoralen[1] already found application in UV therapy, a protocol known today as PUVA (psoralen + UVA) therapy .[2] Psoralen and its more frequently used 8-methoxy derivative boost the sensitivity of the skin towards UV irradiation, increasing the efficiency of the light therapy.[3] Another approach, photodynamic therapy, combines UV irradiation with the administration of porphyrin derivatives as photosensitizers, and is used in tumor therapy.[4]

A rather modern concept uses photoswitches, molecules which change their conformation upon irradiation.[5] Although azobenzenes are by far the most commonly used photoswitches,[6] other substance classes such as 1,2-dithienylalkenes are also widespread.[7] The light-induced conformational change can be used to open or close ion channels[8] and to modulate the affinity of drugs towards their receptor.[9] Besides these medical applications, photoactivatable compounds find also application in cell biology.[10] Here, biologically active molecules can be deactivated by modification with a photolabile linker,[11] and upon irradiation, the active compound is released into the cell.[12] This approach can be applied for the release of neurotransmitters[13] and hormones.[14] One of the first described examples was the photochemical release of ATP.[15]

Several different photocleavable groups are suitable candidates for these types of applications.[10a,11] The *o*-nitrobenzyl group (NB) plays a dominant role (Figure 1), although the *o*-nitrobenzaldehyde formed in the cleavage step might

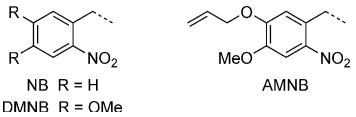

Figure 1. Photocleavable protecting groups.

cause damage in cellular systems.[16] The introduction of two additional methoxy groups to NB (DMNB) lowers the quantum yield, but also shifts the absorption maximum to longer wavelengths (365 nm), making DMNB an ideal candidate for application in biological systems.[17]

Our research group has been involved in the syntheses of peptidic natural products the past few years.[18] An especially interesting class of compounds are the tubulysins and their derivatives. We are particularly interested in pretubulysin, a biosynthetic precursor of the tubulysins (Figure 2), which

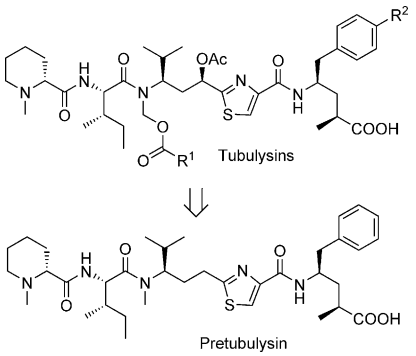

Figure 2. Tubulysins and pretubulysin.

inhibits the growth of a wide range of tumor cells in the low nanomolar range.[19] Like the tubulysins, pretubulysin interacts with the microtubuli skeleton,[20] suppressing angiogenesis,[21] and is therefore an excellent candidate for the development of antitumor drugs.[22] Structure–activity relationship (SAR) studies indicate that pretubulysin-derived esters are 3–6 times less active than pretubulysin, while amides are even less active (10–20fold) depending on the derivative. Therefore one might assume that these derivatives might act as prodrugs.

[*] J. Hoffmann, Prof. Dr. U. Kazmaier
Institute for Organic Chemistry, Saarland University
P.O. Box 151150, 66041 Saarbrücken (Germany)
E-mail: u.kazmaier@mx.uni-saarland.de
Homepage: http://www.uni-saarland.de/fak8/kazmaier

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We recently became interested in applying the concept of drug photoactivation for the directed treatment of easily accessible tumors, such as melanoma and colon cancer. Our aim was to convert pretubulysin into an "inactive" prodrug, which could be activated by light (Figure 3). The major

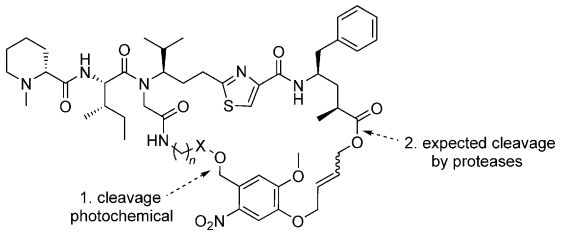
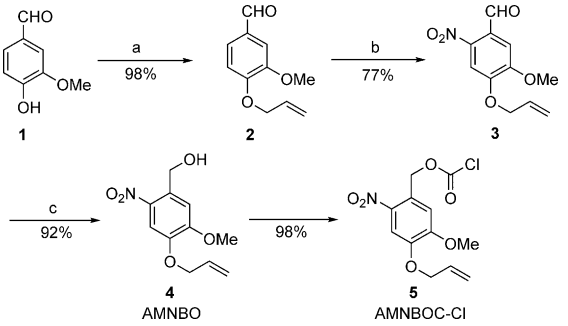


Figure 3. Photocleavable pretubulysin derivatives.

difference between pretubulysin and the tubulysins is the missing *N*-acylal side chain. Therefore, this position should be suitable for the introduction of a photolabile side chain as well, allowing cyclization with the C-terminus of the peptide. One might expect that the conformation of the cyclic pretubulysin derivative should differ significantly from the open-chain form, resulting in a (significantly) diminished binding affinity towards tubulin. Upon irradiation, the ring is cleaved and the molecule should take up its natural conformation for binding. In this case, the photolabile protecting group is still bound to prebutulysin through the ester linkage. Proteolytic cleavage of this bond might liberate the active drug.

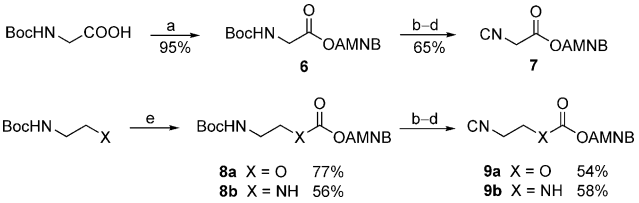
Based on our good experience with peptide cyclizations through ring-closing metathesis (RCM),[23] we explored a short synthetic sequence for the 4-allyloxy-5-methoxy-3-nitrobenzyl (AMNB) protecting group, starting from vanillin (**1**, Scheme 1). While the O-allylation of **1** proceeded almost quantitatively, subsequent nitration was not a trivial issue owing to several side reactions. However, when exactly 1 equiv KNO_3 was used in trifluoroacetic acid, the desired product **3** could be obtained in good yield and purity. Reduction of the aldehyde provided the photolabile benzylalcohol AMNBO (**4**), which can be used directly for the protection of carboxylic acids. For the synthesis of the corresponding carbonates/carbamates, **4** was stirred with an



Scheme 1. Synthesis of photolabile protecting groups suitable for subsequent metatheses: a) allyl bromide, K_2CO_3, EtOH, 78 °C, 16 h; b) KNO_3 (1.0 equiv), CF_3COOH, RT, 16 h; c) $NaBH_4$, EtOH, RT, 4 h; d) $COCl_2$ (20% in toluene, 10 equiv), THF, RT, 2 h.

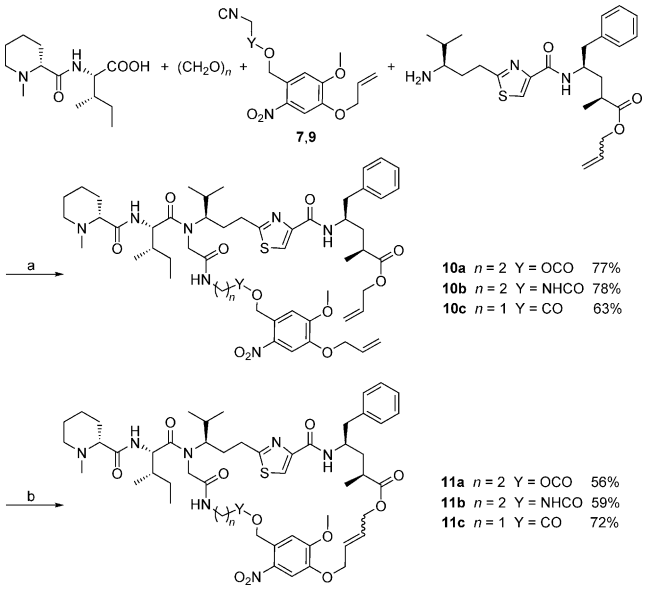
excess of phosgene for 2 h. The excess phosgene was removed to provide the chloroformate AMNBOC-Cl (**5**) in pure form and in almost quantitative yield. This acid chloride was found to be highly reactive and sensitive towards hydrolysis, but it can be stored at 4 °C for weeks without significant decomposition.

While the synthesis of tubulysin derivatives with a central N-methylamide bond, such as in the case of pretubulysin, is straightforward,[25] the synthesis of other N-substituted derivatives is far from trivial for steric reasons.[26] An interesting approach towards the synthesis of N-substituted tubulysins was reported recently by Wessjohann et al.,[27] which took advantage of Ugi reactions.[28] The products obtained, called tubugis, showed excellent biological activities comparable to those of the natural products. The structure of the tubulysin side chain was defined by the structure of the isocyanide used. We used this concept for the synthesis of photoactivatable tubulysin derivatives, introducing the photolabile group via the isocyanide. The natural tubulysins vary primarily in the structure of the aliphatic acylal side chain. To introduce a photolabile protecting group at this position, a heterofunctionality is required in the side chain. Since it was not known what type of functionalities would be accepted, we decided to introduce not only a protected hydroxy group but also an amine and a carboxyl functionality. The latter ones should generate ionic side chains on cleavage, what should have a rather strong effect on the biological activity. The required isocyanides were obtained according to Scheme 2. The



Scheme 2. Syntheses of photolabile isocyanides: a) **4**, TBTU [*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate] (1.3 equiv), DMAP (0.3 equiv), DIPEA (2.0 equiv), CH_2Cl_2, RT, 16 h; b) 4 M HCl/dioxane, CH_2Cl_2, RT, 2 h; c) HCOOEt, NEt_3, TsOH (cat.), 52 °C, 20 h; d) $POCl_3$ (1.0 equiv), NEt_3 (3.0 equiv), CH_2Cl_2, 0 °C, 1 h; e) **5**, pyridine (1.0 equiv), THF, RT, 20 h.

photolabile, protected carboxylic acid derivative **7** could be obtained directly from Boc-Gly-OH and **4** using standard transformations. Generation of the isocyanide turned out to be the only critical step. Even when the reaction mixture had been stirred at 0 °C for 1 h, conversion was still not complete but the overall yield of 65% (for three steps) was acceptable. Prolonging the reaction time or running the reaction at higher temperature resulted in lower yield, caused by side reactions. The isocyanides containing a photolabile carbamate and carbonate group (**9a** and **9b**) were prepared in an analogous fashion, while completely anhydrous conditions were essential for good results. In addition, for the synthesis of reference samples, the analogous non-photolabile isocyanides were synthesized (see the Supporting Information).

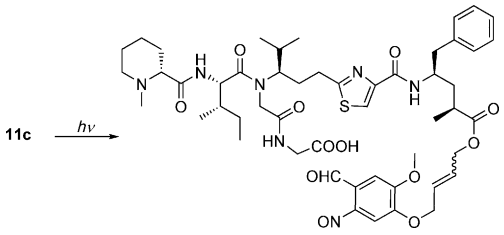


Scheme 3. Syntheses of photocleavable tubulysin derivatives: a) CH_2Cl_2, RT, 5 d; b) benzylidene[1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene]dichloro(tricyclohexylphosphine)ruthenium (Grubbs II catalyst, 15 mol%), HCl (5.0 equiv), CH_2Cl_2, 1. 40°C, 1 h; 2. RT, 1 h.

The Ugi reactions were carried out in CH_2Cl_2 (instead of the commonly used MeOH or trifluoroethanol) to avoid side reactions caused by nucleophilic attack of the solvent at the reaction intermediates (Scheme 3).[28] Paraformaldehyde was found to be more suitable than formaldehyde solution, because of its lower reactivity (fewer side reactions). Usage of the neutral form of the dipeptide serving as the acid component was crucial, since the yields dropped significantly when the corresponding amine hydrochloride was used. Under these conditions, the reaction time was comparatively long (5 days), but the reactions proceeded very cleanly and the desired products could be obtained in good yields.

The subsequent ring-closing metathesis also required some optimization studies.[29] In initial experiments using **10c** as the substrate, only traces of the cyclization product could be obtained, even when 0.6 equiv of Grubbs catalyst was used.[30] We assumed that the reaction failed because of coordination of the N-terminal tertiary amine on the Ru catalyst.[31] Possible changes to suppress this catalyst deactivation included the addition of $Ti(OiPr)_4$[32] and H^+.[33] After investigating several options we found that the addition of 5.0 equiv of HCl and the use of 15 mol% Grubbs II catalyst gave the best results. Under these conditions the desired product **11c** could be obtained in 72% yield (purity > 95%) after reversed-phase flash chromatography.[35] Similar results could be obtained for the other Ugi products under these conditions as well. This protocol provided cyclic tubulysin derivatives in only two steps from rather simple building blocks (see the Supporting Information).

To verify the photolabile characteristics of our products, cyclopeptide **11c** was irradiated for two hours with a UV-LED lamp (365 nm). The reaction was monitored and analyzed by HPLC/MS. According to the known mechanism,



Scheme 4. Expected product from the photocleavage of **11c**.

a *o*-nitrosobenzaldehyde derivative (Scheme 4) should be formed upon photocleavage.[16] This linear product should show the same molecular mass as **11c**. Indeed, besides traces of unreacted starting material **11c**, a new product with the same mass (*m*/*z* 503, $[M+2H]^{2+}$) could be identified. In addition, a peak at *m*/*z* 496 was observed, correlating with the product resulting from the reduction of the nitroso group to the corresponding aniline ($[M+2-14]^{2+}$). This is in agreement with the typical ionization pattern of aromatic nitroso compounds.[36]

To determine whether the biological activity of the cyclic compounds **11** is indeed lower than that of the potential cleavage products **12**, we measured their cytotoxicity (IC_{50}) towards a panel of tumor cell lines and compared the results with those obtained for the linear peptides **12** (Figure 4). The results obtained with the HCT-116 cell line are summarized in Table 1. Interestingly, the cyclic carbonate **11a** (IC_{50}: 120 ng mL^{-1}) and the ester **11c** (IC_{50}: 63 ng mL^{-1}) both

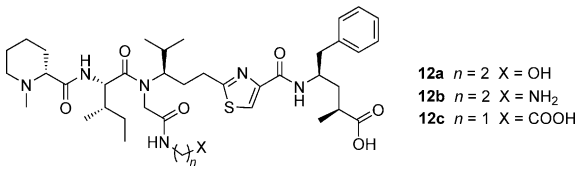


Figure 4. Potential cleavage products after irradiation and ester cleavage.

Table 1: Biological activity of derivatives **11a–c** and **12a–c** determined by MTT assay (HCT-116/CHO-K1 cell line, IC_{50} [μg mL^{-1}]).

Cyclic, photoactivatable tubulysin derivatives		Open-chain, deprotected reference samples	
11a	0.12/0.35	**12a**	0.02/0.10
11b	0.57/4.54	**12b**	2.12/6.34
11c	0.063/n.d[a].	**12c**	0.58/0.64

[a] n.d.: not determined.

showed comparatively high activity, while the carbamate **11b** was significantly less active (IC_{50}: 570 ng mL^{-1}).

When we examined the linear derivatives **12** closely it became apparent that charged side chains are probably not tolerated, since the activity of the amine **12b** as well as the carboxylic acid **12c** was significantly lower than that of the cyclic compounds (by a factor of 4–10). On the other hand, an

OH functionality seems to be accepted. The activity of the potential cleavage product **12a** (IC_{50}: 20 ng mL^{-1}) is in a pharmacologically relevant range and six times higher than that of **11a**.

The comparatively high cytotoxicity of the cyclic derivatives **11a** and **11c** is surprising, especially considering that the bulky side chains differ significantly from the tubulysin side chains. The reason for this is still unclear and the subject of further investigations. Most likely, the cyclization forces the derivatives into a conformation suitable for receptor binding, such that the carboxyl terminus and the side chain are in close proximity in the bound state. This would be in good agreement with the results of NMR studies described by Carlomagno et al.[37]

In conclusion we demonstrated that the photoactivation of cyclic drug candidates could be realized with tubulysin derivatives. The synthesis of photoactivatable peptides proceeded in only two steps from easily available building blocks. The biological data obtained provided important information about the influence of functionalized side chains on the biological activity. Ongoing research will focus on photoactivations in tumor cells and tumor tissues.

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