Drug Delivery

International Edition: DOI: 10.1002/anie.201601506
German Edition: DOI: 10.1002/ange.201601506

The Methylene Alkoxy Carbamate Self-Immolative Unit: Utilization for the Targeted Delivery of Alcohol-Containing Payloads with Antibody–Drug Conjugates

Robert V. Kolakowski, Karl T. Haelsig, Kim K. Emmerton, Chris I. Leiske, Jamie B. Miyamoto, Julia H. Cochran, Robert P. Lyon, Peter D. Senter, and Scott C. Jeffrey**

Abstract: *A strategy for the conjugation of alcohol-containing payloads to antibodies has been developed and involves the methylene alkoxy carbamate (MAC) self-immolative unit. A series of MAC β-glucuronide model constructs were prepared to evaluate stability and enzymatic release, and the results demonstrated high stability at physiological pH in a substitution-dependent manner. All the MAC model compounds efficiently released alcohol drug surrogates under the action of β-glucuronidase. To assess the MAC technology for ADCs, the potent microtubule-disrupting agent auristatin E (AE) was incorporated through the norephedrine alcohol. Conjugation of the MAC β-glucuronide AE drug linker to the anti-CD30 antibody cAC10, and an IgG control antibody, gave potent and immunologically specific activities in vitro and in vivo. These studies validate the MAC self-immolative unit for alcohol-containing payloads within ADCs, a class that has not been widely exploited.*

Interest in antibody–drug conjugate (ADC) technology has greatly increased in recent years because of the approval of ADCETRIS® (brentuximab vedotin)[1] and KADCYLA® (ado-trastuzimab emtansine)[2] for the treatment of various malignancies, and numerous other ADCs having promising preclinical and early-stage clinical profiles.[3] Consequently, considerable attention has been directed towards evaluating new payloads to expand the scope and utility of ADCs.[4] The most commonly used drugs for ADCs are the auristatins,[5] maytansines,[6] γ-calicheamicin,[7] camptothecins,[8] duocarmycin analogues,[9] pyrrolobenzodiazepine dimers,[10] and anthracyclines,[8b] which have demonstrated pronounced activities. Typically, these agents are linked through a reactive residue, and emphasis has been placed on maximizing linker stability in circulation.[6] To deliver amine-containing payloads, we and others have employed peptide-[11] or β-glucuronide-based linker systems,[12] both of which are stable in circulation and give facile drug release once the ADC is internalized into cancer cells (Figure 1). When an amine for conjugating is not present in a molecule of interest, we have, when possible, installed such residues synthetically.[8e] However, introducing an amine functional group may not always be synthetically feasible, and it may have a detrimental impact on the pharmacology of the resulting drug analogue.[4b]

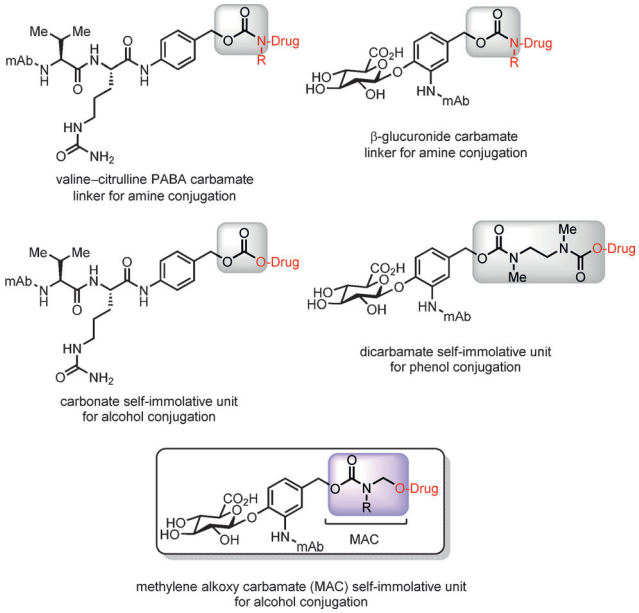


Figure 1. Methods for conjugation of amine- and alcohol-containing payloads for ADCs.

Several strategies have been explored for the utilization of alcohol-containing agents as ADC payloads. A carbonate of a camptothecin analogue, SN-38,[8b] has been linked to monoclonal antibodies (mAbs), but was found to be labile under physiological conditions. Self-immolative dicarbamates[8b,9,13] (Figure 1) and ether linkages[14] have also been employed, but these approaches are limited in scope, thus efficiently releasing only phenolic payloads.

We surmised that a general solution for utilizing alcohol-containing drugs within ADCs could involve a methylene alkoxy carbamate (MAC) construct, a method which has been used in several drug delivery strategies. One of the first applications involved a MAC-type unit to release 5-fluorouracil from a glucuronide prodrug.[15] Shabat and co-workers further developed the unit for the release of mercaptoacetic acid[16] and glucose.[17] Subsequently, Santi and co-workers utilized a MAC construct for β-elimination-promoted release of the potent cytotoxic agent SN-38.[18]

[*] Dr. R. V. Kolakowski, K. T. Haelsig, K. K. Emmerton, C. I. Leiske, J. B. Miyamoto, J. H. Cochran, Dr. R. P. Lyon, Dr. P. D. Senter, Dr. S. C. Jeffrey
Seattle Genetics, 21823 30th Dr SE, Bothell, WA 98021 (USA)
E-mail: rkolakowski@seagen.com
sjeffrey@seagen.com

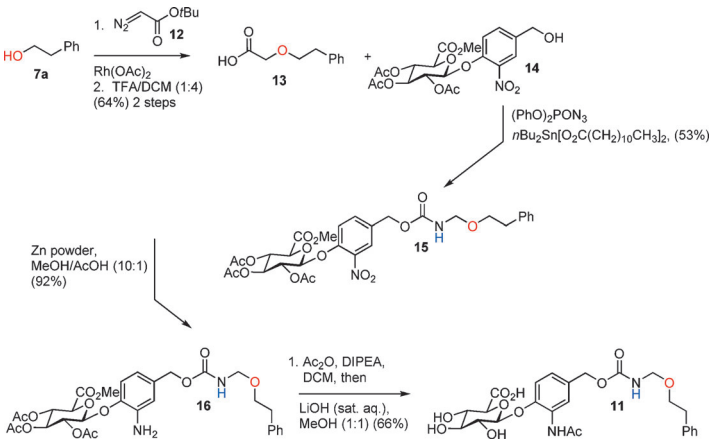
Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201601506.

The goal of our work was to utilize a MAC construct for antitumor ADCs. To achieve this goal, the MAC unit was configured for stability during prolonged serum exposure because of the long circulation time of ADCs. In addition, we utilized a β-glucuronidase-promoted release mechanism[12] to trigger MAC self-immolation, to enable rapid and efficient payload release upon target cell delivery. Herein, we describe a novel MAC construct of the potent microtubule-disrupting agent auristatin E[5] and demonstrate that the derived conjugates are stable under physiological conditions, highly potent, and immunologically specific in vitro and in vivo. The results support the use of this linker technology for alcohol-containing drugs within ADCs.

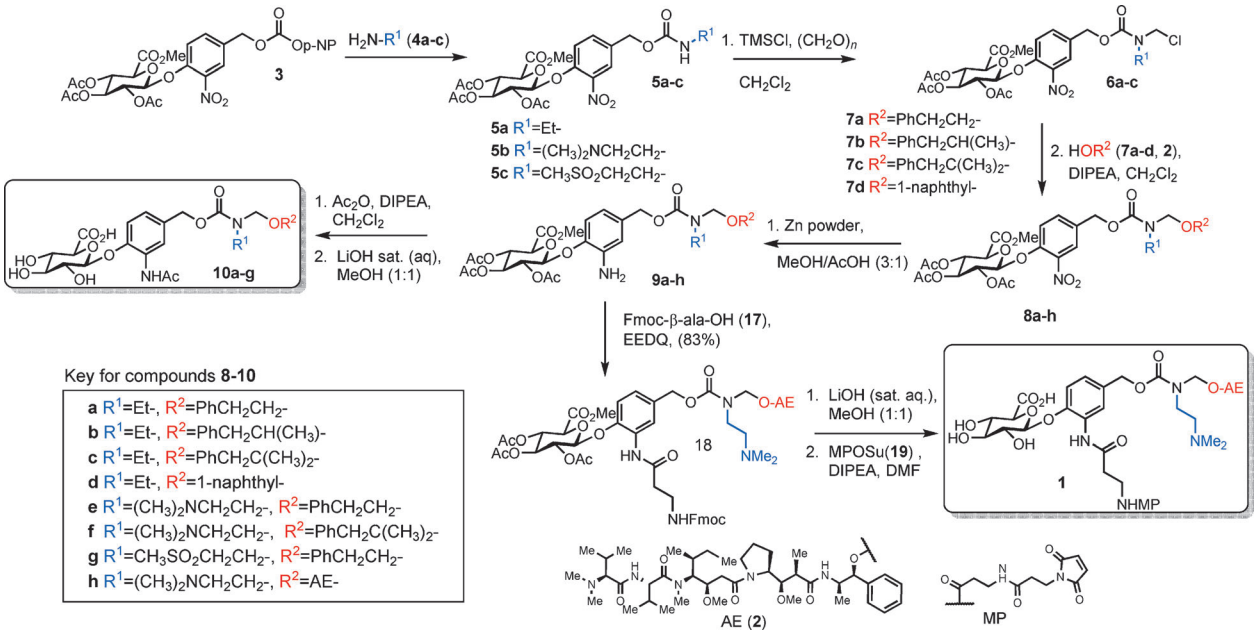
Our design of a MAC unit for ADCs centered on incorporating the payload alcohol oxygen atom in a stable manner to allow efficient release upon enzymatic processing. To explore the viability of the MAC approach for conjugating alcohols to ADCs, a series of model compounds was prepared starting with the known *para*-nitrophenylcarbonate **3** (Scheme 1).[12] Addition of ethyl amine (**4a**), *N*,*N*-dimethylethylenediamine (**4b**), and 2-(methylsulfonyl)ethylamine (**4c**) gave the compounds **5a–c**, respectively, which were converted into the corresponding chloromethyl adducts **6a–c** through reaction with chlorotrimethylsilane and paraformaldehyde.[19] The N-ethyl chloromethyl adduct **6a** was reacted with alcohols **7a–d**, to provide the corresponding intermediates **8a–d**. The carbamate **6b** was reacted with **7a** and **7c** to give compounds **8e** and **8f**, respectively. Reaction of the methanesulfonyl adduct **6c** with **7a** afforded **8g**. Nitro group reduction of **8a–g** gave the corresponding anilines **9a–g**, which were capped as acetamides by treatment with acetic anhydride, and saponification of the β-glucuronide protecting groups gave the target MAC β-glucuronides, the model compounds **10a–g**.

A final model compound was prepared by Curtius rearrangement[17a] to provide the secondary carbamate compound **11** (Scheme 2). Rhodium-acetate-promoted alkylation of **7a** with *tert*-butyl azidoacetate (**12**), followed by *tert*-butyl ester removal, gave the acid **13**, which was subjected to Curtius rearrangement conditions using diphenylphosphoryl azide. The intermediate isocyanate was trapped with the alcohol **14** in the presence of dibutyltin dilaurate to afford the carbamate **15**. Nitro group reduction gave the aniline **16**, which was acetylated and saponified, thus providing the model MAC construct **11**.

To assess stability, each of the MAC β-glucuronide model constructs **10a–g** and **11** was dissolved in phosphate-buffered

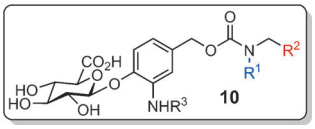


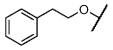
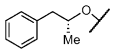
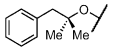
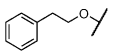
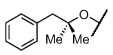
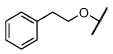
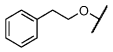
Scheme 2. Synthesis of a MAC-based substrate by a Curtius rearrangement. DCM = dichloromethane, TFA = trifluoroacetic acid.



Scheme 1. Synthesis of model MAC-linked test substrates and an auristatin E based drug linker. DIPEA = diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, EEDQ = ethyl-2-ethoxy-1,2-dihydro-1-quinolinecarboxylate, Fmoc = fluorenylmethoxycarbonyl, TMS = trimethylsilyl.

Table 1: MAC analogue structures and hydrolysis under physiological conditions.



Compound	R^1	R^2	R^3	Hydrolysis (%)[a] 7/14 days
10a	Et		Ac	44/n.d.
10b	Et		Ac	0/0
10c	Et		Ac	61/n.d.
10d	Et		Ac	0/0
10e			Ac	0/0
10f			Ac	0/0
10g			Ac	0/0
11	H		Ac	100/n.d.
1[b]		AE	MP	0/0

[a] In Gibco pH 7.4 (10X) PBS at 37 °C, as determined by LC-MS. [b] The compound **1** undergoes maleimide hydrolysis to the ring-open form during the course of the stability study. n.d. = not determined because of instability at the 7 day time point.

saline and incubated at 37 °C for up to 14 days. Changes were monitored by liquid chromatography/mass spectroscopy (LC/MS) and integration of the parent peak of each model compound was used to determine the degree of hydrolysis. The results for the substituted carbamate model compounds are shown in Table 1. The compounds **10a–d**, with N-ethyl carbamate residues derived from primary, secondary, tertiary, and 1-napthyl alcohols (**7a–d**), were differentially stable. The compounds **10b** and **10d** were completely stable up to 14 days, while the primary and tertiary alcohol constructs **10a** and **10c** showed significant hydrolysis at 7 days.

To stabilize the MAC linkage, we introduced both basic and electron-withdrawing groups proximal to the aminal linkage. Replacement of the R^1 ethyl group of **10a** and **10c**, with a dimethylaminoethyl group, resulted in the compounds **10e** and **10f**, respectively, which were stable for the 14 day incubation period. Likewise, incorporation of the methanesulfonylethyl group in **10g** also improved stability relative to the N-ethyl compound **10a** (and **10c**), presumably by reducing electron density at the carbamate nitrogen atom. High electron density at the carbamate nitrogen atom may explain why the secondary carbamate, **11**, was not stable and showed complete hydrolysis at 7 days.

Each model construct released the corresponding alcohol compound when treated with bovine liver β-glucuronidase. As anticipated, the intermediate aminal released from the β-glucuronide MAC construct could not be detected by LC/MS, presumably because of rapid decomposition and release of the free alcohol.

We next turned our attention toward the utility of the MAC unit for ADCs using the potent microtubule-disrupting agent AE (**2**).[5] The dimethylaminoethyl-containing construct was selected, based on the stability characteristics from the model compounds **10e** and **10f**. Reaction of the chloromethyl adduct **6b** with **2** gave the AE-alkoxy carbamate **8h** (Scheme 1). Nitro group reduction afforded **9h**, which was coupled to Fmoc β-alanine (**17**) using EEDQ, to give **18**. Saponification with concomitant removal of the Fmoc protecting group using aqueous lithium hydroxide, followed by maleimide installation using the *N*-hydroxysuccinimide ester **19**, gave the fully elaborated MAC β-glucuronide AE drug linker **1**. The compound **1** was incubated in phosphate-buffered saline at 37 °C, and like the model MAC constructs possessing the dimethylaminoethyl residue (**10e** and **10f**), no hydrolysis of the MAC-AE unit was observed over the 14 day incubation period (Table 1; see Figure S1 in the Supporting Information). As expected, β-glucuronidase treatment resulted in clean and facile formation of AE (**2**; see Figure S2).

The method for conjugating **1** was as previously described.[12] Briefly, the anti-CD30 mAb cAC10[1] was partially reduced with tris(2-carboxyethyl)phosphine and then purified by gel filtration, thus affording approximately four free thiol residues/mAb. Addition of a slight excess of **1** to the reduced mAb resulted in conjugation with an average of 4 drugs/mAb.

The cAC10-**1** conjugate was evaluated against CD30-positive and -negative cell lines (Table 2). As a positive control for the assay, the anti-CD30 ADC brentuximab vedotin, employing monomethyl auristatin E (MMAE) attached by a valine-citrulline *para*-aminobenzyl carbamate linker, was included[1] (Figure 1). The ADC cAC10-**1** was comparable to brentuximab vedotin in activity on both the antigen positive cells, and on the antigen negative cell line, in which both constructs were relatively inactive. Further evidence for antigen-dependent activity was obtained with the nonbinding hIgG-**1** conjugate, which was inactive ($IC_{50} >$ 1000 ng mL^{-1}) on all three cell lines tested. These results are consistent with the high degree of stability observed with **1** in aqueous buffer (Table 1). A stability assessment under physiological-type conditions was also obtained by incubating cAC10-**1** in mouse plasma at 37 °C. An LC/MS assay detected less than 1% AE (**2**) liberated after a 7 day incubation (see Figure S3).

Table 2: In vitro activity and immunologic specificity.[a]

ADC	Karpas 299 CD30 290K ALCL	L540cy CD30 433K HL	Ramos CD30 (−) Burkitt's
cAC10-**1**	0.5	2	>1000
hIgG-**1**	>1000	>1000	>1000
brentuximab vedotin	0.5	4	>1000

[a] Activity reported as IC_{50} value in ng mL^{-1} of ADC. Cells were treated with ADC for 96 h and viability was assessed using Cell TiterGlo. Anaplastic large cell lymphoma (ALCL), Hodgkin lymphoma (HL).

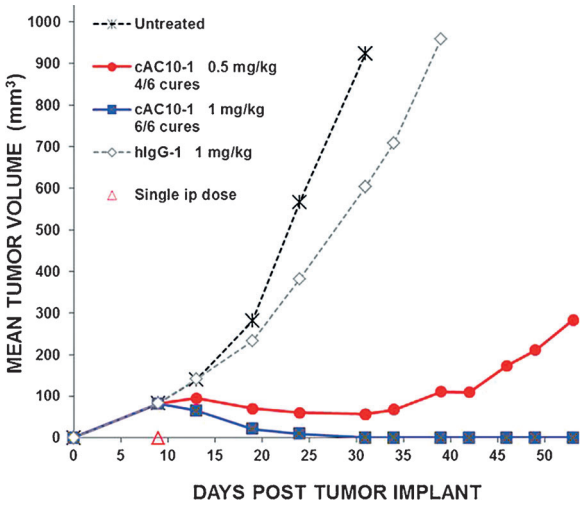


Figure 2. In vivo activity and immunologic specificity of cAC10-**1** in an L540cy xenograft model.

The MAC-AE ADCs were evaluated in a mouse xenograft model of Hodgkin lymphoma using the CD30-positive L540cy cell line (Figure 2). In this study, cAC10-**1** was fully curative at 1 mg kg^{-1} (6/6 animals), and partially curative at 0.5 mg kg^{-1} (3/6 animals). The effects were immunologically specific, since the nonbinding control ADC hIgG-**1** showed minimal activity at 1 mg kg^{-1}, with tumor growth similar to that of vehicle-treated animals.

There is a strong need to expand ADC linker technology to encompass a broad array of anticancer drugs. The known MAC self-immolative unit,[15–18] when combined with an enzyme-cleavable linker, offers a solution for stable conjugation and efficient release of alcohol-based payloads for ADCs. We demonstrated the utility of this linker technology both in vitro and in vivo with the potent microtubule-disrupting drug AE, and it provided a new ADC with pronounced levels of activity and immunological specificity. The stability of the linker ensures that a maximal amount of drug can be intratumorally delivered.[20] The MAC self-immolative unit should have broad utility as a means for attaching alcohol-containing payloads to mAbs for targeted delivery.

Keywords: alcohols · antibodies · cancer · drug delivery · glycoconjugate

[1] P. D. Senter, E. L. Sievers, *Nat. Biotechnol.* **2012**, *30*, 631 – 637.
[2] J. M. Lambert, R. V. Chari, *J. Med. Chem.* **2014**, *57*, 6949 – 6964.
[3] a) P. Polakis, *Pharmacol. Rev.* **2016**, *68*, 3 – 19; b) R. V. Chari, M. L. Miller, W. C. Widdison, *Angew. Chem. Int. Ed.* **2014**, *53*, 3796 – 3827; *Angew. Chem.* **2014**, *126*, 3872 – 3904.
[4] a) R. E. Wang, T. Liu, Y. Wang, Y. Cao, J. Du, X. Luo, V. Deshmukh, C. H. Kim, B. R. Lawson, M. S. Tremblay, T. S. Young, S. A. Kazane, F. Wang, P. G. Schultz, *J. Am. Chem. Soc.* **2015**, *137*, 3229 – 3232; b) P. J. Burke, J. Z. Hamilton, T. A. Pires, J. R. Setter, J. H. Hunter, J. H. Cochran, A. B. Waight, K. A. Gordon, B. E. Toki, K. K. Emmerton, W. Zeng, I. J. Stone, P. D. Senter, R. P. Lyon, S. C. Jeffrey, *Mol. Cancer Ther.* **2016**, in press.
[5] S. O. Doronina, B. E. Toki, M. Y. Torgov, B. A. Mendelsohn, C. G. Cerveny, D. F. Chace, R. L. DeBlanc, R. P. Gearing, T. D. Bovee, C. B. Siegall, J. A. Francisco, A. F. Wahl, D. L. Meyer, P. D. Senter, *Nat. Biotechnol.* **2003**, *21*, 778 – 784.
[6] W. C. Widdison, S. D. Wilhelm, E. E. Cavanagh, K. R. Whiteman, B. A. Leece, Y. Kovtun, V. S. Goldmacher, H. Xie, R. M. Steeves, R. J. Lutz, R. Zhao, L. Wang, W. A. Blattler, R. V. Chari, *J. Med. Chem.* **2006**, *49*, 4392 – 4408.
[7] A. D. Ricart, *Clin. Cancer Res.* **2011**, *17*, 6417 – 6427.
[8] a) M. A. Walker, G. M. Dubowchik, S. J. Hofstead, P. A. Trail, R. A. Firestone, *Bioorg. Med. Chem. Lett.* **2002**, *12*, 217 – 219; b) S. V. Govindan, T. M. Cardillo, R. M. Sharkey, F. Tat, D. V. Gold, D. M. Goldenberg, *Mol. Cancer Ther.* **2013**, *12*, 968 – 978; c) S. J. Moon, S. V. Govindan, T. M. Cardillo, C. A. D'Souza, H. J. Hansen, D. M. Goldenberg, *J. Med. Chem.* **2008**, *51*, 6916 – 6926; d) T. M. Cardillo, S. V. Govindan, R. M. Sharkey, P. Trisal, D. M. Goldenberg, *Clin. Cancer Res.* **2011**, *17*, 3157 – 3169; e) P. J. Burke, P. D. Senter, D. W. Meyer, J. B. Miyamoto, M. Anderson, B. E. Toki, G. Manikumar, M. C. Wani, D. J. Kroll, S. C. Jeffrey, *Bioconjugate Chem.* **2009**, *20*, 1242 – 1250.
[9] R. C. Elgersma, R. G. Coumans, T. Huijbregts, W. M. Menge, J. A. Joosten, H. J. Spijker, F. M. de Groot, M. M. van der Lee, R. Ubink, D. J. van den Dobbelsteen, D. F. Egging, W. H. Dokter, G. F. Verheijden, J. M. Lemmens, C. M. Timmers, P. H. Beusker, *Mol. Pharmacol.* **2015**, *12*, 1813 – 1835.
[10] a) S. C. Jeffrey, P. J. Burke, R. P. Lyon, D. W. Meyer, D. Sussman, M. Anderson, J. H. Hunter, C. I. Leiske, J. B. Miyamoto, N. D. Nicholas, N. M. Okeley, R. J. Sanderson, I. J. Stone, W. Zeng, S. J. Gregson, L. Masterson, A. C. Tiberghien, P. W. Howard, D. E. Thurston, C. L. Law, P. D. Senter, *Bioconjugate Chem.* **2013**, *24*, 1256 – 1263; b) L. R. Saunders, A. J. Bankovich, W. C. Anderson, M. A. Aujay, S. Bheddah, K. Black, R. Desai, P. A. Escarpe, J. Hampl, A. Laysang, D. Liu, J. Lopez-Molina, M. Milton, A. Park, M. A. Pysz, H. Shao, B. Slingerland, M. Torgov, S. A. Williams, O. Foord, P. Howard, J. Jassem, A. Badzio, P. Czapiewski, D. H. Harpole, A. Dowlati, P. P. Massion, W. D. Travis, M. C. Pietanza, J. T. Poirier, C. M. Rudin, R. A. Stull, S. J. Dylla, *Sci. Transl. Med.* **2015**, *7*, 302ra136.
[11] G. M. Dubowchik, R. A. Firestone, L. Padilla, D. Willner, S. J. Hofstead, K. Mosure, J. O. Knipe, S. J. Lasch, P. A. Trail, *Bioconjugate Chem.* **2002**, *13*, 855 – 869.
[12] S. C. Jeffrey, J. B. Andreyka, S. X. Bernhardt, K. M. Kissler, T. Kline, J. S. Lenox, R. F. Moser, M. T. Nguyen, N. M. Okeley, I. J. Stone, X. Zhang, P. D. Senter, *Bioconjugate Chem.* **2006**, *17*, 831 – 840.
[13] D. Shabat, H. N. Lode, U. Pertl, R. A. Reisfeld, C. Rader, R. A. Lerner, C. F. Barbas III, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 7528 – 7533.
[14] B. E. Toki, C. G. Cerveny, A. F. Wahl, P. D. Senter, *J. Org. Chem.* **2002**, *67*, 1866 – 1872.
[15] R. F. Madec-Lougerstay, C.-J. Monneret, *J. Chem. Soc. Perkin Trans. 1* **1999**, 1369 – 1375.
[16] E. Sella, R. Weinstain, R. Erez, N. Z. Burns, P. S. Baran, D. Shabat, *Chem. Commun.* **2010**, *46*, 6575 – 6577.
[17] a) N. Karton-Lifshin, D. Shabat, *New J. Chem.* **2012**, *36*, 386 – 393; b) N. Karton-Lifshin, U. Vogel, E. Sella, P. H. Seeberger, D. Shabat, B. Lepenies, *Org. Biomol. Chem.* **2013**, *11*, 2903 – 2910.
[18] D. V. Santi, E. L. Schneider, G. W. Ashley, *J. Med. Chem.* **2014**, *57*, 2303 – 2314.
[19] S. Majumdar, K. B. Sloan, *Synth. Commun.* **2006**, *36*, 3537 – 3548.
[20] S. C. Alley, D. R. Benjamin, S. C. Jeffrey, N. M. Okeley, D. L. Meyer, R. J. Sanderson, P. D. Senter, *Bioconjugate Chem.* **2008**, *19*, 759 – 765.

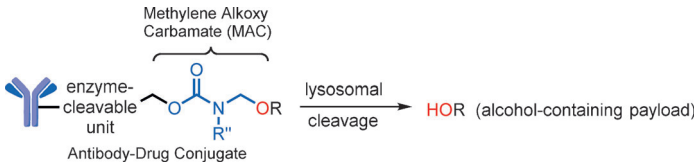
Received: February 11, 2016
Revised: April 4, 2016
Published online: ■■ ■■, ■■■■

Communications

Drug Delivery

R. V. Kolakowski,* K. T. Haelsig,
K. K. Emmerton, C. I. Leiske,
J. B. Miyamoto, J. H. Cochran, R. P. Lyon,
P. D. Senter, S. C. Jeffrey* ____ ■■■■–■■■■

The Methylene Alkoxy Carbamate Self-Immolative Unit: Utilization for the Targeted Delivery of Alcohol-Containing Payloads with Antibody–Drug Conjugates



MAC out: A strategy for the conjugation of alcohol-containing payloads to antibodies has been developed using the methylene alkoxy carbamate (MAC) self-immolative unit. A series of MAC β-glucuronide model constructs were prepared and demonstrated high stability at physiological pH, and efficiently released alcohol drug surrogates under the action of β-glucuronidase.