A novel strategy for bioconjugation: synthesis and preliminary evaluation with amphotericin B†

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A novel linker strategy is presented based on a double reductive amination of a dialdehyde to the amine of the amphotericin B mycosamine sugar and the biological activity of a series of conjugates is compared to the native amphotericin B.

Despite the fact that is was discovered well over 40 years ago, the mode of action of the polyene macrolide antimycotic agent amphotericin B remains enigmatic, puzzling biochemists and pharmacologists alike.[1,2]

Although there is clear evidence that its incorporation into biological and abiological membranes results in electrolyte efflux, there persists an ongoing debate as to the causal relationship of this observed effect on cell death. Indeed, numerous investigations reveal contradicting behaviour between model studies and those *in vivo*.[3]

We have been interested in developing biochemical probes based on amphotericin B (**1**, Fig. 1) as a means of studying several hypotheses concerning the mode of action of this important antifungal agent and to facilitate the study of processes believed to occur at the membrane. We have initiated a program in the design and synthesis of tailor-made amphotericin B conjugates bearing reporter groups that permit insight into the fate of amphotericin in cells and liposomes.[4,5] In our preliminary disclosure, we documented the preparation of an amphotericin B–fluorescein conjugate which exhibited interesting properties when employed as a probe in the fungal membrane. In this communication we report the synthesis of additional conjugates using a piperazine as a point of attachment, and we introduce a related conjugation strategy involving a 4-carboxypiperidinyl linker, which is

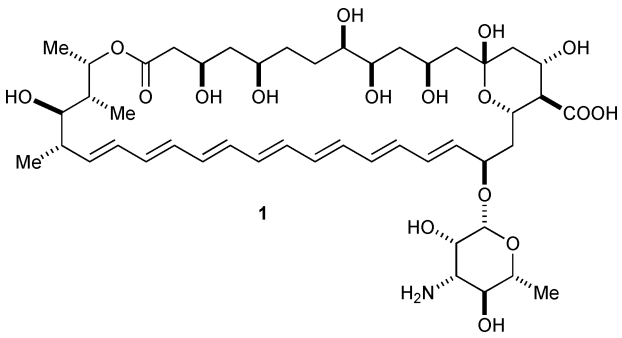
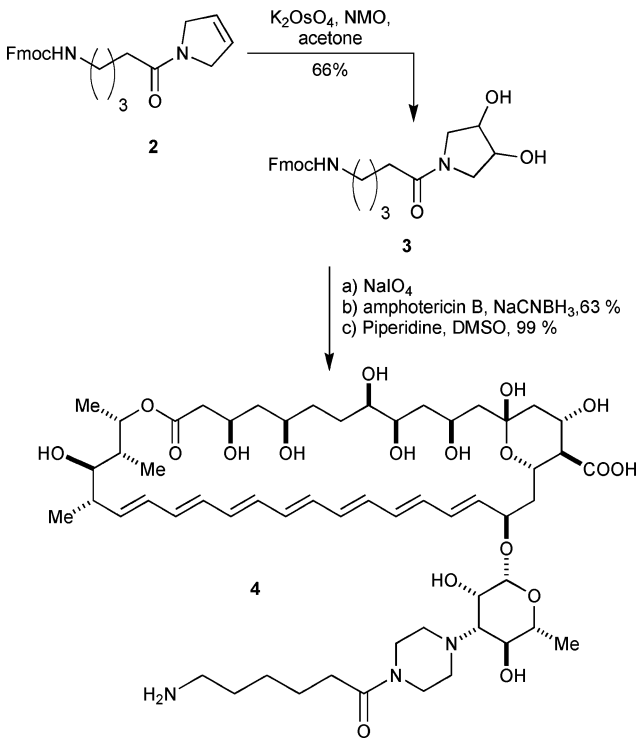


Fig. 1 Structure of the antifungal agent amphotericin B.



Scheme 1 Synthesis of aminohexanoyl piperazinyl amphotericin B.

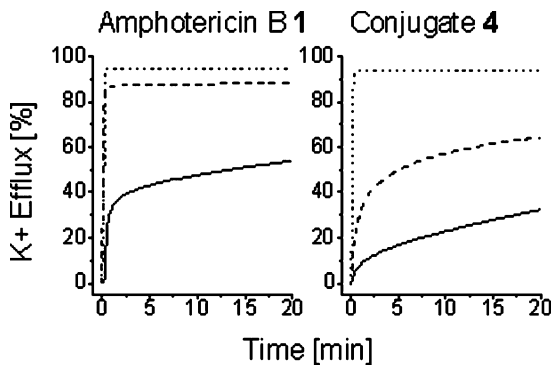


Fig. 2 K^+ release from POPC based liposomes measured *via* potentiometry. Substrate was added externally to lipid vesicles at a molar ratio of 1 : 200 (reagent : total lipid), solid line: POPC alone, dashed line: POPC admixed with 30 mol% cholesterol; dotted line: POPC admixed with 13 mol% ergosterol.

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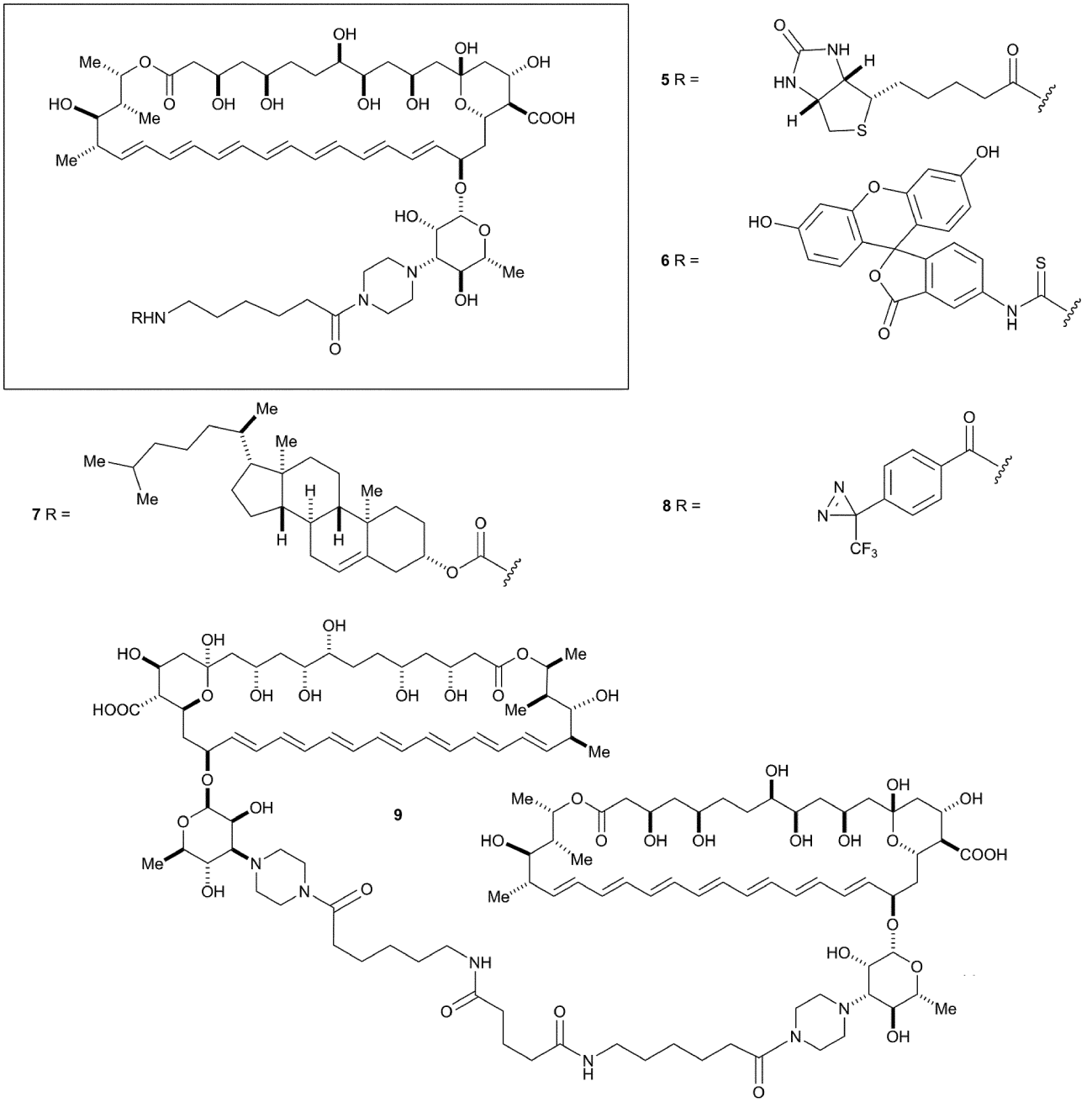


Fig. 3 Piperazinoyl-linked amphotericin B conjugates with reporter groups biotin (**5**), fluorescein (**6**), cholesterol (**7**), and photo-crosslinking diazirane (**8**), along with dimer **9**.

prepared through a short convenient sequence of reactions from commercially available 3-cyclopentene-1-carboxylic acid and 1-Fmoc-1,6-diaminohexane. Furthermore a preliminary validation on the potential utility of one of these conjugates is carried in yeast; we also document ion-flux studies in POPC vesicles (liposomes).

Earlier structure–activity relationship studies on amphotericin B underlined the importance of the basic amine for the mechanism of action. Simple acylations of this functional group have led to compounds with significantly lowered biological activity.[6] As such, this delimits the types of functionality that can be relied upon to prepare conjugates. Two different solutions to this

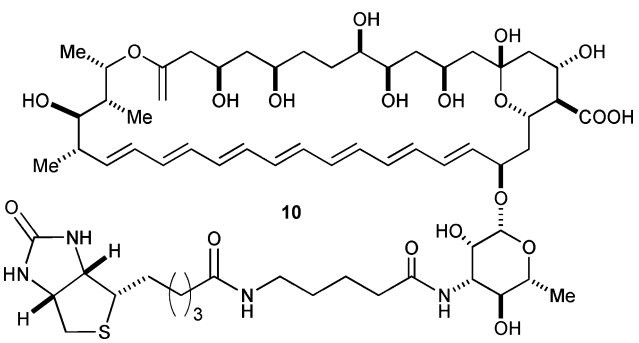


Fig. 4 Amide-linked biotin-amphotericin B.

problem have appeared recently. Murata and co-workers have reported the monoalkylation of amphotericin mycosamine sugar through reductive amination of an aldehyde.[7] We have documented the use of dialdehydes in a double reductive alkylation reaction of amphotericin mycosamine to afford novel piperazinyl linked structures (Scheme 1). This latter approach is convenient in that the formation of dialkylated products produced when monoaldehydes are employed are avoided, thus giving products that are easier to purify.

In order to evaluate the new linker we determined the minimal inhibitory concentration (MIC) required to inhibit growth of *Saccharomyces cerevisiae*. Amphotericin B (**1**) completely stopped cell growth when added at a concentration of 1 μM. Conjugate **4** retained activity and was toxic above a threshold of 1.6 μM.

We also prepared large (100 nm) unilamellar vesicles (LUVs) and directly measured the induced K^+ permeability of the LUVs with a K^+-selective electrode. This assay contrasts with that recently employed, which monitors the pH-sensitive shift corresponding to the ^{31}P NMR signal of a membrane probe.[6] For such a commonly employed assay, amphotericin B-induced leakage of K^+ ions from liposomes triggers a counter H^+ ion influx, levelling out a transmembrane pH gradient. We reasoned that the direct analysis of K^+ efflux *via* a K^+-selective electrode would have the benefit of fast on-line measurements with increased accuracy as well as have the advantage of maintaining the same physiologically-relevant pH throughout the efflux experiment. This is especially important since amphotericin B–liposome interactions are known to be very sensitive to pH change.[8]

In the vesicle assay that we have adapted for our purposes,[9] LUVs from POPC or from POPC with admixed sterols (mimicking conditions in natural biomembranes[10]) were prepared in a KCl solution and the vesicles were dialyzed against NaCl in order to create an ion gradient (K^+ inside, Na^+ outside). Freshly prepared valinomycin-based K^+-selective electrodes were characterized and employed as described earlier.[4c]

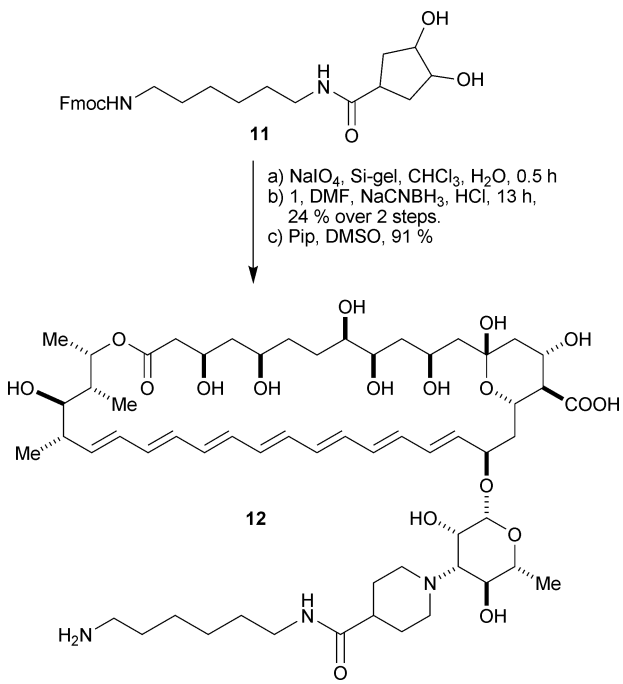
The parent amphotericin B (**1**) triggered an immediate release of K^+ ions from liposomes containing cholesterol or ergosterol (Fig. 2). Conjugate **4** showed an efflux pattern that was very similar to native amphotericin B in the case of ergosterol containing vesicles. However, a difference was found when comparing the efflux from cholesterol and non-sterol containing liposomes. The new conjugate **4** induced a clear distinction between the three vesicle systems, a fact that may show beneficial influence on the selectivity of the drug for fungal *versus* mammalian cells.

We set out to establish the utility of **4** by preparation of several amphotericin B conjugates incorporating typical affinity- (**5**), fluorescent- (**6**), photolabelling- (**8**) groups (Fig. 3) as well as amphotericin–cholesterol conjugate **7** and dimer **9**. Piperazine-linked amphotericin analog **4** was allowed to react with biotin *N*-hydroxysuccinimide ester to give **5**, with fluorescein isothiocyanate to furnish **6**, with cholesterol chloroformate to provide **7**, and with the *N*-hydroxysuccinimide ester of 4-(3-trifluoromethyl-3*H*-diazirin-3-yl)-benzoate ester to afford diazirane **8**. When **4** was condensed with an activated form of glutaric acid dimer **9** was isolated in 24% yield from amphotericin B, which is a considerable improvement over the yield that had been previously described for related dimeric compounds.[7]

Biotin conjugate **5** was selected for thorough examination in both liposomal (K^+ efflux) and cellular assays. For comparison purposes, amide-linked conjugate **10** was prepared (Fig. 4).

Both biotin-amphotericin B conjugates showed a K^+ release pattern similar to native amphotericin B (**1**) (Fig. 5). The piperazinyl-linked conjugate **5** however, retained a high toxicity against fungal cells (MIC = 20 μM) whereas the amide linked conjugate **10** showed a fivefold drop in activity (MIC = 100 μM) compared to **5**. This feature of probe **5** permits its use for subsequent investigations of channel-forming properties in the membrane.[11]

As an alternative approach we have examined the use of a 3-cyclopentene-1-carboxylic acid derivative, providing a diverse set of building blocks for the construction of amphotericin conjugates. The new linker strategy also allows for simple synthetic variations of the binding motive. The commercially available cyclopentene carboxylic acid is converted into the corresponding acid chloride (oxalyl chloride, DMF) and treated with monoprotected 1,6-diaminohexane in pyridine to afford a cyclopentenyl amide which following dihydroxylation furnishes **11**. Oxidation of this diol to the dialdehyde and double reductive amination with amphotericin B (**1**) installed the new linker. After deprotection the diaminohexanoyl pyridyl amphotericin B **12** was isolated in 91% yield (Scheme 2).



Scheme 2 Synthesis of diaminohexyl pyridyl amphotericin B.

Both conjugates **4** and **12** bearing the novel linkers retained the activity and were toxic above a threshold of 1.6 μM. The piperidyl conjugate **12** not only showed remarkably reduced release kinetics but a positive change was also found in the increased selectivity for cholesterol-over ergosterol-containing vesicles (Fig. 6).

In conclusion, we have established a new linker for bioconjugation through amines that allows for the synthesis of amphotericin B conjugates in multi-gram scale. In a broader sense, the linking strategy we document is suitable for bioconjugation in a number

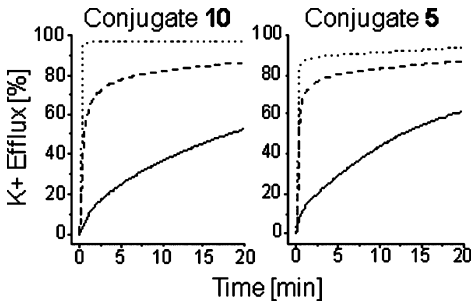


Fig. 5 K^+ release from POPC based liposomes measured *via* potentiometry. Substrate was added externally to lipid vesicles at a molar ratio of 1 : 200 (reagent : total lipid), solid line: POPC alone, dashed line: POPC admixed with 30 mol% cholesterol; dotted line: POPC admixed with 13 mol% ergosterol.

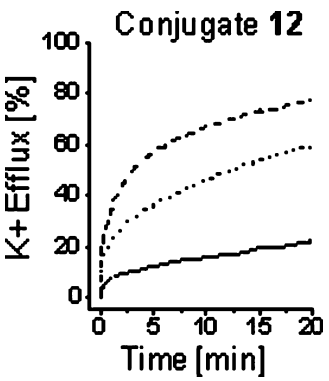


Fig. 6 K^+ release from POPC based liposomes measured *via* potentiometry. Substrate was added externally to lipid vesicles at a molar ratio of 1 : 200 (reagent : total lipid), solid line: POPC alone, dashed line: POPC admixed with 30 mol% cholesterol; dotted line: POPC admixed with 13 mol% ergosterol.

of other biologically important systems wherein functionalization through a primary amine is required yielding molecules with interesting new properties. A biotin conjugate was prepared as a benchmark case which displayed comparable biological activity to the parent compound and preserved K^+ efflux-inducing properties in a LUV assay. The new linker strategy and various prepared amphotericin B conjugates will be useful in shedding some more light on the mechanism of action of this important antifungal drug.

Acknowledgements

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Notes and references

1 (*a*) G Medoff, J Brajtburg and G. S Kobayashi, *Annu. Rev. Pharmacol. Toxicol.*, 1983, **23**, 303–330; (*b*) J Bolard, *Biochim. Biophys. Acta*, 1986, **864**, 257–304; (*c*) J Brajtburg, W. G Powderly, G. S. Kobayashi and G. Medoff, *Antimicrob. Agents Chemother.*, 1990, **34**(2), 183–188; (*d*) F. C. Szoka, Jr. and M. Tang, *J. Liposome Res.*, 1993, **3**(3), 363–375; (*e*) C. Hatch, W. Ayenew and S. C. Hartsel, *J. Liposome Res.*, 1993, **3**(3), 377–408; (*f*) J. Bolard, V. Joly and P. Yeni, *J. Liposome Res.*, 1993, **3**(3), 409–427; (*g*) S. C. Hartsel and J. Bolard, *Trends Pharmacol. Sci.*, 1996, **17**, 445–449; (*h*) I. Al-Mohsen and W. T. Hughes, *Ann. Saudi Med.*, 1998, **18**(1), 28–38; (*i*) B. E. Cohen, *Int. J. Pharm.*, 1998, **162**, 95–106; (*j*) I. M. Hann and H. G. Prentice, *Int. J. Antimicrob. Agents*, 2001, **17**, 161–169; (*k*) S. B. Zotchev, *Curr. Med. Chem.*, 2003, **10**, 211–223.
2 For a recent review of the biology and chemistry of amphotericin, see: D. M. Cereghetti and E. M. Carreira, *Synthesis*, 2006, 914–942.
3 (*a*) C. C. Hsu-Chen and D. S. Feingold, *Nature*, 1974, **251**, 656–659; (*b*) J. Brajtburg, J. Medoff and M. L. Sokol-Andersen, *J. Infect. Dis.*, 1986, **154**, 76–83; (*c*) W. H. Beggs, *Antimicrob. Agents Chemother.*, 1994, **38**, 363–364.
4 (*a*) J. Krüger and E. M. Carreira, *Tetrahedron Lett.*, 1998, **39**, 7013–7016; (*b*) J. Tholander and E. M. Carreira, *Helv. Chim. Acta*, 2001, **84**(3), 613–622; (*c*) A. Zumbuehl, P. Stano, M. Sohrmann, T. Vigassy, E. Pretsch, M. Peter, P. Walde, D. Jeannerat and E. M. Carreira, *Angew. Chem., Int. Ed.*, 2004, **43**, 5181–5185.
5 For additional recent studies with amphotericin, see: (*a*) A. Zumbuehl, P. Stano, D. Heer, P. Walde and E. M. Carreira, *Org. Lett.*, 2004, **6**, 3683–3686; (*b*) V. Paquet and E. M. Carreira, *Org. Lett.*, 2006, **8**, 1807–1809; (*c*) J. M. Manthorpe, A. M. Szpilman and E. M. Carreira, *Synthesis*, 2005, 3380–3388; (*d*) N. Matsumori, Y. Sawada and M. Murata, *J. Am. Chem. Soc.*, 2006, **128**, 11977–11984.
6 M. Chéron, B. Cybulska, J. Mazerski, J. Grzybowska, A. Czerwinski and E. Borowski, *Biochem. Pharmacol.*, 1998, **37**(5), 827–836.
7 N. Matsumori, N. Yamaji, S. Matsuoka, T. Oishi and M. Murata, *J. Am. Chem. Soc.*, 2002, **124**, 4180–4181.
8 A. W. Norman, R. A. Demel, B. de Krujiff, B., W. S. M. Geurts vanKessel and L. L. M. van Deenen, *Biochim. Biophys. Acta*, 1972, **290**, 1–14.
9 (*a*) P. van Hoogevest and B. de Kruijff, *Biochim. Biophys. Acta*, 1978, **511**, 397–407; (*b*) D. S. Orlov, T. Nguyen and R. I. Lehrer, *J. Microbiol. Methods*, 2002, **49**, 325–328.
10 J. Barwicz and P. Tancrède, *Chem. Phys. Lipids*, 1997, **85**, 145–155.
11 However, we believe that although LUVs can serve as excellent model systems in a narrowly defined area we suggest caution in drawing a direct correlation between liposomal and cell studies. In fact, in a previous paper we documented a case with a conjugate where potassium efflux from LUV is induced by a non-toxic compound up to 500 μM[4c].

