Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1343–1346

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

A New Method for Chemoselective Conjugation of Unprotected Peptides to Dauno- and Doxorubicin

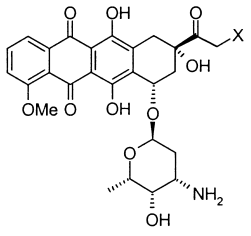
Paolo Ingallinella, Annalise Di Marco, Marina Taliani, Daniela Fattori* and Antonello Pessi

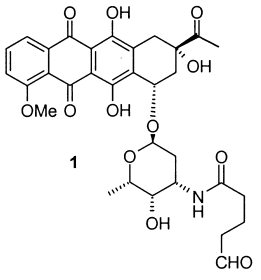
Istituto di Ricerche di Biologia Molecolare P. Angeletti (IRBM), Via Pontina Km 30.600, Pomezia, Rome, Italy

Received 7 June 2000; revised 13 March 2001; accepted 29 March 2001

Abstract—A new approach for chemoselective ligation of peptides to dauno- and doxorubicin through an oxime bond is presented. The method does not require protecting groups on the peptide moiety.

Daunorubicin and doxorubicin are widely used as antineoplastic agents in tumor treatment. However, toxic dose-related side effects, such as nephrotoxicity and cardiotoxicity, limit their clinical application. Different approaches have been adopted in order to increase their therapeutic index. One way of reducing the therapeutic dose is tumor targeting obtained by attaching the cytotoxic compound to carrier peptides that show affinity to the tumor tissue[1a] or are selectively cleaved in the presence of tumor cells.[1b]





Several ways of conjugating these drugs to peptides have been published to date: formation of an amide bond on the sugar amino group,[2] formation of an ester bond on the primary hydroxyl of doxorubicine,[3] alkylation of the sugar amino group through reductive amination,[4] and introduction of a maleimide moiety for further ligation with a cysteine-containing peptide.[5] Such methods, however, are not compatible with the entire variety of amino acid functionalities on the reacting peptide, so that some residues have to be avoided, or selectively protected, with the ensuing solubility problems. Furthermore, the instability of the glycosidic bond to the acidic conditions normally used for N^{α} deprotection (Boc synthesis) or resin cleavage (Fmoc synthesis) precludes a solid-phase approach to the problem.

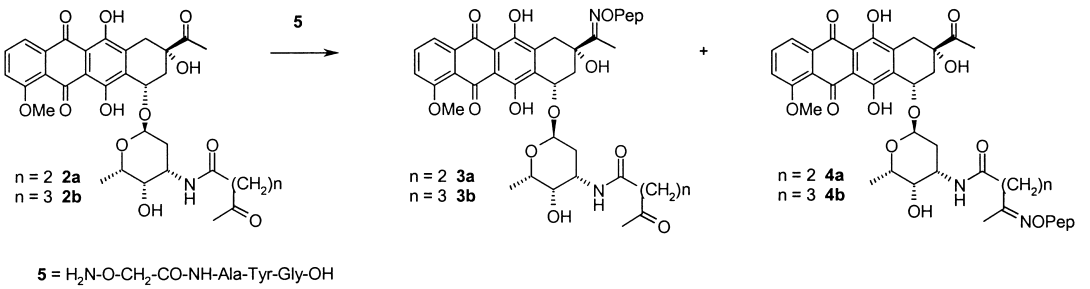
Our goal was to develop a general method to produce conjugates between daunorubicin or doxorubicin and a peptide of any sequence. We decided to take advantage of oxime ligation, used to link totally unprotected peptidic fragments.[6] Since the precursor *O*-alkylhydroxylamine is easily obtained through coupling (Boc-protected) aminoxyacetic acid to a free amino group of the peptide,[6] our efforts were addressed to introducing the partner carbonyl function in the anthracyclinone moiety. Both dauno- and doxorubicin already contain a ketone, but modifications of this carbonyl to form a methyl oxime have been shown to dramatically reduce the drug cytotoxicity.[7]

We first synthesized derivative **1** of daunorubicin, containing a very reactive aldehydic group. The compound however proved unstable and prone to intramolecular cyclization in aqueous solution. The less reactive keto derivative **2a** (from levulinic acid) and **2b** (from 5-oxohexanoic acid) were then isolated and characterized.[8]

A first test was run[9] according to the standard conditions for oxime ligation[6] that is aqueous solution buffered at pH 4, with the model peptide **5** (H_2N–O–CH_2–CO–NH–Ala-Tyr-Gly–OH). We obtained two products resulting from condensation either with the exocyclic

*Corresponding author. Tel.: +39-6-9118-522; fax; +39-6-9109-3225; e-mail: dfattori@menarini-ricerche.it

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00223-2



Scheme 1. Ligation of the test peptide **5** with daunorubicin derivatives **2a** and **2b**.

methyl ketone (**3a** and **3b**) or with the desired methyl ketone of the sugar side chain (**4a** and **4b**) (Scheme 1). The ratios were 1:1 for **2** and 1:2 in favor of the desired product **4b** for **2b**.[10] Changing the solvent from water to dimethylformamide (DMF) or the buffer counterion from sodium to potassium did not alter these ratios. Since oxime formation is acid catalyzed, we then examined regioselectivity as a function of the pH.

The amount of the desired isomer (**4a** and **4b**) increased with increasing pH for both **2a** and **2b** (Figs. 1 and 2). Moreover, while **2a** reached a plateau (**3a**/**4a** ratio of 55:45 at pH 6), derivative **2b** yielded $>80\%$ of **4b** at the same pH.

This result prompted us to apply the same scheme to doxorubicin. The 5-oxohexanoic derivative **6** was prepared[11] and the regioselectivity of the condensation with **5** was studied as a function of the pH (Scheme 2).

As shown in Figure 3, running the reaction at pH 6 allowed quantitative recovery of **8** in this case.

To test the method with larger peptides including all the variety of side chains, we prepared the conjugates of **2b** and **6** with the 33-mer peptide **9**, derivatized with aminoxyacetic acid on the ε-amino group of the C-terminal lysine. Both reactions were run in aqueous buffer at pH 6, using a 5-fold excess of the anthracyclinone: the target conjugates were smoothly produced in 24 h and isolated by preparative HPLC. The yields were 43 and 34%, respectively, for **10** and **11**,[12] with aminoxyacetic acid on the ε-amino group of the C-terminal lysine.

AEGEFALSETAKRWRLLFYRAGVGNAEDPAKG
GK(COCH$_2$ONH$_2$)CONH$_2$ (**9**)

↓ **2b** or **6** (DMF, pH 6)

AEGEFALSETAKRWRLLFYRAGVGNAEDPAKG
GK(COCH$_2$ON=X)CONH$_2$ (**10** or **11**)

X = anthracyclinone

Finally, we prepared a conjugate of **2b** with a peptide containing a disulfide bridge (**12**), since constrained peptides of this kind are frequently selected from phage libraries.[13]

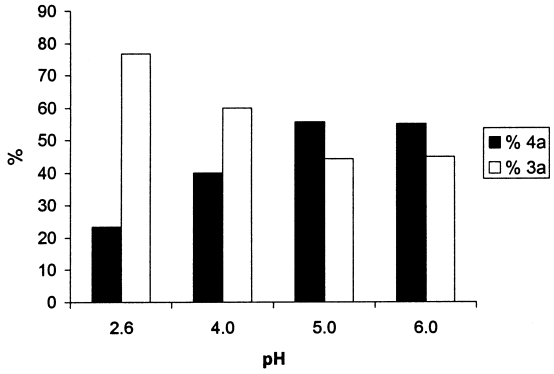


Figure 1. Observed regioselectivities as a function of pH for the ligation of **5** with **2a**.

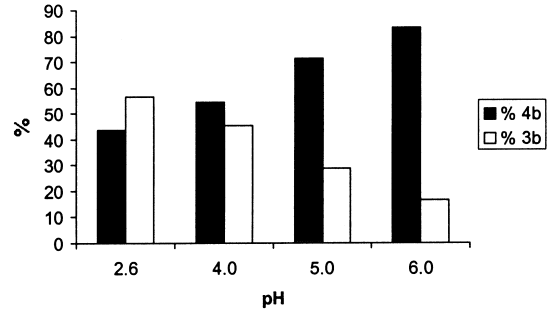


Figure 2. Observed regioselectivities as a function of pH for the ligation of **5** with **2b**.

AEGEFMRASRCNNPCPWMAGADPAKGGK(COC
H$_2$ONH$_2$)CONH$_2$(**12**)

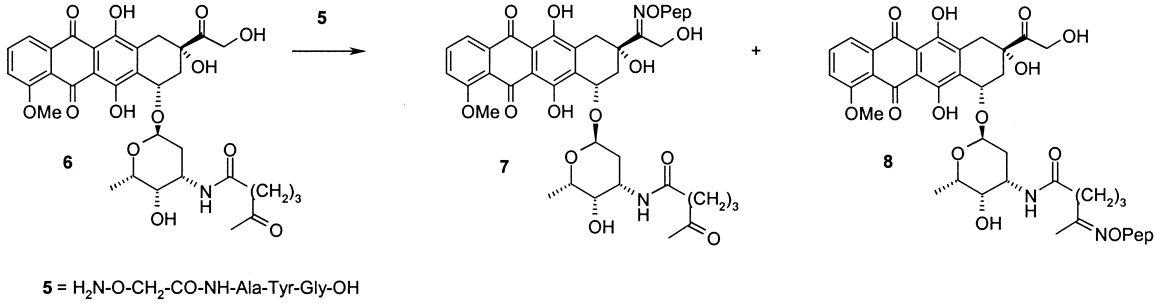
↓ **2b** (DMF, pH 6)

AEGEFMRASRCNNPCPWMAGADPAKGGK(COC
H$_2$ON=X)CONH$_2$(**13**)

X = anthracyclinone

Derivative **13** was obtained in 23% yield after HPLC purification.[12]

In conclusion, we have developed an efficient and general method for the preparation of peptide-dauno-/doxorubicin conjugates by chemoselective



Scheme 2. Ligation of the test peptide **5** with doxorubicin derivative **6**.

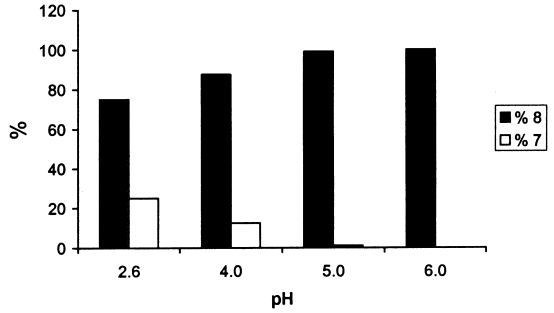


Figure 3. Observed regioselectivities as a function of pH for the ligation of **5** with **6**.

ligation. Our method makes use of an oxime bond, which is known to be reasonably stable both in vitro and in vivo,[14,15] and is applicable to totally unprotected precursors of any sequence.

Acknowledgements

The authors wish to thank S. Pesci for NMR, S. Orrù, F. Bonelli and A. Alfieri for LC–MS, O. Gonzalez, U. Koch, R. Laufer, V. G. Matassa, and Elisabetta Bianchi for useful discussions, Professor F. Arcamone for the generous gift of daunorubicin, and J. Clench for manuscript correction.

References and Notes

1. (a) Arap, W.; Pasqualini, R.; Rusoshlati, E. *Science* **1998**, *279*, 377. (b) DeFeo-Jones, D.; Garsky, V.; Wong, B. K.; Feng, D.-M.; Bolyar, T.; Haskell, K.; Kiefer, D. M.; Leander, K.; McAvoy, E.; Lumma, P.; Wau, J.; Senderak, E. T.; Motzel, S. L.; Keenan, K.; van Zwieten, M.; Lin, J. H.; Freidinger, R.; Huff, J.; Oliff, A.; Jones, R. E. *Nat. Med.* **2000**, *6*, 1248.
2. Trouet, A.; Masquelier, M.; Baurain, R.; Deprez De Campeneere, D. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 626.
3. Nagy, A.; Schally, A.; Halmos, G.; Armatis, P.; Cai, R. Z.; Csernus, V.; Kovacs, M.; Koppan, M.; Szepeshazi, K.; Kahan, Z. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1794.
4. Farquhar, D.; Cherif, A.; Bakina, E.; Nelson, J. A. *J. Med. Chem.* **1998**, *41*, 965.
5. Langer, M.; Kratz, F.; Beck-Sickinger, A. G. 3rd Lausanne Conference on Bioorganic Chemistry, 1999.
6. (a) Tuchscherer, G. *Tetrahedron Lett.* **1993**, *34*, 8419. (b) Rose, K. *J. Am. Chem. Soc.* **1994**, *116*, 30. (c) Canne, L. E.; Ferré-D'Amaré, A. R.; Burley, S. K.; Kent, S. B. H. *J. Am. Chem. Soc.* **1995**, *117*, 2998.
7. Yamamoto, K.; Acton, E. M.; Henry, D. W. *J. Med. Chem.* **1972**, *15*, 872.
8. **2a** and **2b** were prepared through coupling of daunorubicin with the corresponding acid in standard conditions (PyBOP, HOBt, DIPEA in DMF, rt) and purified by silica gel chromatography (CH_2Cl_2/MeOH 9:1). **2a**, ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.94 (m, 2H), 7.64 (m, 1H), 7.52 (d, 1H), 5.52 (s, 1H), 5.22 (dd, 1H), 4.96 (dd, 1H), 4.72 (d, 1H), 4.17 (m, 1H), 3.99 (s, 3H), 3.99 (m, 1H), 3.37 (m, 1H), 3.00 (m, 3H), 2.57 (m, 2H), 2.25 (s, 3H), 2.17 (m, 2H), 2.05 (s, 3H), 1.84 (m, 1H), 1.74 (m, 2H), 1.42 (m, 1H), 1.13 (dd, 3H). ES-MS analysis: $[M+H^+]$ m/z 626. **2b**, ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.89 (m, 2H), 7.66 (m, 1H), 7.47 (d, 1H), 5.53 (s, 1H), 5.21 (dd, 1H), 4.95 (dd, 1H), 4.71 (d, 1H), 4.16 (m, 1H), 4.00 (s, 3H), 3.39 (m, 1H), 3.37 (m, 1H), 3.00 (m, 2H), 2.37 (m, 2H), 2.26 (s, 3H), 2.03 (m, 2H), 2.03 (s, 3H), 1.84 (m, 1H), 1.74 (m, 2H), 1.63 (m, 2H), 1.42 (m, 2H), 1.15 (dd, 3H). ES-MS analysis: $[M+H^+]$ m/z 640.
9. In a typical experiment 2.6 μmol of **5** and 3.9 μmol of keto-derivative were dissolved in 1 mL citrate buffer (pH 2.6) or KOAc buffer (pH 4.0, 5.0, 6.0) and the reaction was monitored by HPLC (RP C-18 column, flow rate of 1 mL/min, linear 5–70% gradient of buffer B (0.1% TFA/CH_3CN) in buffer A (0.1% TFA/H_2O) over 20 min, UV detection at 214 and 490 nm).
10. Structure assignment was done on the basis of the MS fragmentation pattern and of the modification of the methyl ketone ^{1}H NMR chemical shift upon oxime formation. The ratio between regioisomers was calculated by integration of the area of the respective HPLC peak (detection: UV, 214 nm).
11. **6** was prepared through coupling of doxorubicin with the 5-oxohexanoic acid in standard conditions (PyBOP, HOBt, DIPEA in DMF, rt) and purified by silica gel chromatography (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.95 (m, 2H), 7.67 (m, 1H), 7.49 (d, 1H), 5.47 (s, 1H), 5.24 (dd, 1H), 4.97 (m, 1H), 4.85 (dd, 1H), 4.72 (d, 1H), 4.57 (m, 2H), 4.17 (m, 1H), 4.00 (s, 3H), 4.00 (m, 1H), 3.40 (m, 1H), 3.00 (m, 2H), 2.37 (m, 2H), 2.35–2.17 (m, 4H), 2.05 (s, 3H), 1.82 (m, 1H), 1.75 (m, 2H), 1.62 (m, 2H), 1.45 (m, 1H), 1.12 (dd, 3H). ES-MS analysis: $[M+H^+]$ m/z 656.
12. Peptides were assembled by machine-assisted Fmoc/*t*-Bu chemistry using standard deprotection/coupling cycles (Atherton, E.; Sheppard, R. C. *Solid Phase Peptide Synthesis, a Practical Approach*; IRL Press: Oxford, 1989). The disulfide bridge was formed as described in Tam, J. P.; Wu, C.-R.; Liu, W.; Zhang, J.-W. *J. Am. Chem. Soc.* **1991**, *113*, 6657: the peptide was stirred overnight in an aqueous solution of DMSO (15%, pH 7.2) at a concentration of 0.10–0.15 mg/mL; after completion of the reaction, the oxidized peptide was isolated by preparative HPLC. **11**: ES-MS analysis: $[M+H^+]$ m/z 4390, calcd 4389; **12**: ES-MS analysis: $[M+H^+]$ m/z 4406, calcd 4405; **14**: ES-MS analysis: $[M+H^+]$ m/z 3643, calcd 3642.
13. Cortese, R.; Monaci, P.; Nicosia, A.; Luzzago, A.; Felici, F.; Galfre', G.; Pessi, A.; Tramontano, A.; Sollazzo, M. *Curr. Opin. Biotechnol.* **1995**, *6*, 73.

14. (a) Braslawsky, G. R.; Kadow, K.; Knipe, J.; McGoff, K.; Edson, M.; Kaneko, T.; Greenfiels, R. S. *Cancer Immunol. Immunother*. **1991**, *33*, 367. (b) van Dijk, J.; Zwagemakers, M. A. *J. Med. Chem*. **1977**, *20*, 1199.

15. The test peptide derivative **4b** was used to further analyze the specific oxime linkage present in our derivatives with respect to ex vivo stability and toxicity: (a) **4b** was incubated with human plasma, and no traces of the starting ketone **2b** were detected after 2.5 h (analysis by LC–MS); (b) **4b** was incubated with Jurkat cells up to a concentration of 100 μmol, without observing any significant toxicity.