First synthesis of non-symmetrical "phthalimidoyl active ester" bi-dentate cross-linking reagents having an acid chloride, 2-benzothiazole, or 1-benzotriazol group†

Md. Chanmiya Sheikh,[a] Shunsuke Takagi,[a] Mebumi Sakai,[a] Hitoshi Abe[b] and Hiroyuki Morita*[a]

Received 3rd September 2008, Accepted 15th October 2008
First published as an Advance Article on the web 24th October 2008
DOI: 10.1039/b815369h

For the purpose of modification of a variety of derivatives, including biologically important compounds, such as sugar derivatives and proteins *etc.*, we have first synthesized several non-symmetrical bi-dentate cross-linking reagents, namely 3-(phthalimidoyloxycarbonyl)butyric acid chloride (1), 4-(2-benzothiazolyloxycarbonyl)butyric-*N*-hydroxyphthalimide ester (4) and 4-(1-benzotriazoleoxa)butyric-*N*-hydroxyphthalimide ester (5).

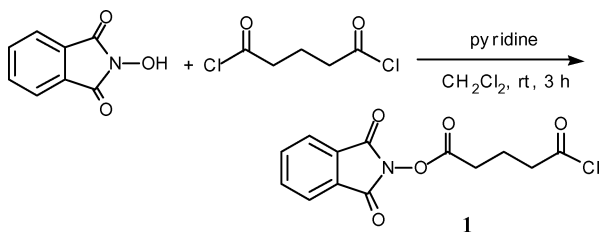
Recently, we have synthesized several antigens which were constructed from oxidized cholesterols bound to protein through MBS (*m*-maleimidobenzoyl *N*-hydroxylsuccinimide ester) cross-linking reagents, in order to develop a new diagnostic method for atherosclerosis and related diseases using the immuno-assay protocol.[1]

As common symmetrical cross-linking reagents having "active ester" groups,[2] the synthesized bifunctional linkers, such as DSG (disuccimidyl glutarate) and MBS, have become very useful in the area of chemical conjugation of particular biologically active molecules.[3] The MBS linker is particularly useful as a non-symmetrical cross-linking reagent, however, apparently its limitation is that the maleimidoyl group is fundamentally only useful for the Michael addition of an SH group, which is sometimes hard or laborious to introduce into the target molecule. In order to attain the modification of versatile compounds, new types of non-symmetrical cross-linking reagents having two different reactivities towards various common nucleophilic groups, such as hydroxyl, amine, thiol, carboxyl and so on, are quite interesting and challenging targets. We first targeted and succeeded in synthesizing the acid chloride linker with "active ester" moiety, 3-(phthalimidoyloxycarbonyl)butyric acid chloride (**1**) in a pure crystalline form.

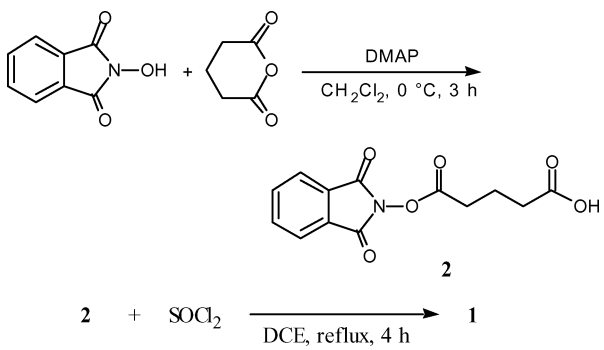
However, similarly to common acid chlorides, this linker is unstable and not safe for handling and longer storage. Therefore, we further targeted the synthesis of the non-symmetrical cross-linking reagents having two groups with different reactivities, 4-(2-benzothiazolyloxycarbonyl)butyric-*N*-hydroxyphthalimide ester (**4**) and 4-(1-benzotriazoleoxa)butyric-*N*-hydroxyphthalimide ester (**5**).

In this communication, we describe the first synthesis of a new class of non-symmetrical cross-linking reagents **1**, **4**, and **5**. We also preliminarily examined the introduction of the linkers **1**, **4**, and **5** into cholesterol through selective reaction with the 3β-OH, and successively with benzylamine as a protein model of the cholesterol antigen.

The acid chloride linker having an "active ester" **1**[4] was prepared by the following two procedures: (A) the one step reaction of *N*-hydroxyphthalimide (we selected *N*-hydroxyphthalimide instead of *N*-hydroxysuccimide for the reason of ease of monitoring the reaction by TLC) with glutaryl dichloride in the presence of pyridine in 44% yield (Scheme 1), or (B) the reaction of 1.2 equiv. of thionyl chloride with 4-(*N*-oxyphthalimidylcarbonyl)butyric acid (**2**) in 92% yield (Scheme 2), where compound **2** was obtained by the reaction of *N*-hydroxyphthalimide with glutaric anhydride in the presence of 4-dimethylaminopyridine (4-DMAP) in 87% yield. The target non-symmetrical chlorocarbonyl active ester linker (**1**) was obtained as a pure solid compound after repeated recrystallization from hexane-AcOEt.



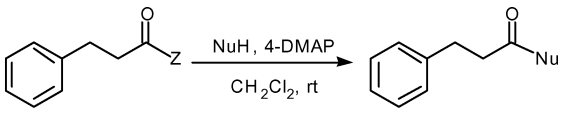
Scheme 1



Scheme 2

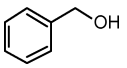
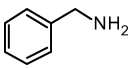
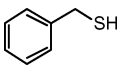
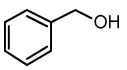
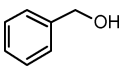
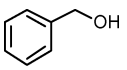
[a]*Department of Material Systems Engineering and Life Science, Faculty of Engineering, University of Toyama, 3190 Gofuku, Toyama, 930-8555, Japan. E-mail: morita@eng.u-toyama.ac.jp; Tel: +81-76-445-6851; Fax: +81-76-445-6703*
[b]*Department of Environmental Applied Chemistry, Faculty of Engineering, University of Toyama, 3190 Gofuku, Toyama, 930-8555, Japan. E-mail: abeh@eng.u-toyama.ac.jp; Tel: +81-76-445-6851; Fax: +81-76-445-6851*
† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b815369h

Table 1 The reactivity of several model "active ester" compounds towards nucleophiles



Z^a = OPhth (**6**), OBt (**7**), OBtz (**8**), Bt (**9**)

Nu : OBn (**10**), NHBn (**11**), SBn (**12**)

Entry	Substrate	NuH	Time	Product	Yield (%)[b]
1	Z = OPhth		24 h	**10**	82
2	Z = OPhth		16 h	**11**	75
3	Z = OPhth		12 h	**12**	77
4	Z = OBt		5 min	**10**	77
5	Z = OBtz		1 h	**10**	89
6	Z = Bt		6 h	**10**	83

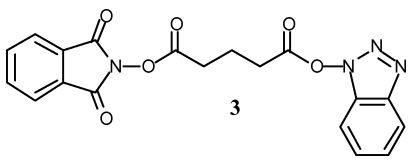
[a] OPhth=*N*-oxyphthalimidoyl, OBt=1-oxybenzotriazolyl, OBtz=2-oxybenzothiazolyl, and Bt=1-benzotriazolyl. [b] Isolated yields (not optimized).

The linker **1** is sensitive to moisture and decomposed gradually, however, it can be stored under N_2 for a long time.

Due to the activation of the acyl group (including sulfinyl and sulfonyl esters), it is well known that *N*-hydroxybenzotriazole[5] and *N*-benzotriazole[6–7] are quite effective and useful. Meanwhile, there has been no report about the reactivity difference between so-called "active ester" groups. In order to obtain more stable non-symmetrical cross-linkers having two different "active ester" groups than the chlorocarbonyl linker **1**, it is necessary and important to determine the reactivities of the "active ester" groups towards nucleophiles. Therefore, at first we determined the reactivity differences of several so-called "activated carbonyl" groups using model compounds (Table 1).

The results clearly indicate that the reactivity order towards benzyl alcohol is **7** >> **8** > **9** > **6** (cf. entries 1, 4, 5, and 6). In the case of **6**, the reactivities towards benzyl amine and benzyl thiol were also examined (entries 2 and 3). Expectedly, the reactivity order is BnSH > $BnNH_2$ > BnOH, as described in textbooks. From these results, we selected the combinations of benzotriazolyloxycarbonyl and *N*-phthalimidoxycarbonyl and 2-benzothiazolyloxycarbonyl groups as the candidates for the "active ester" groups of the bidentate cross-linkers. At first, we attempted to synthesize cross-linkers having benzotriazolyloxy carbonyl as one of the "active ester" groups, such as 4-(1-benzotriazolyloxycarbonyl)butyric-*N*-hydroxyphthalimide ester (**3**) (Fig. 1) by the reaction of **1** with *N*-hydroxybenzotriazole in the presence of DMAP.

The reaction proceeded to form **3**, however, we failed to isolate it in a pure form at this stage, probably due to the very reactive nature of the benzotriazolyloxycarbonyl group. Therefore, we targeted for

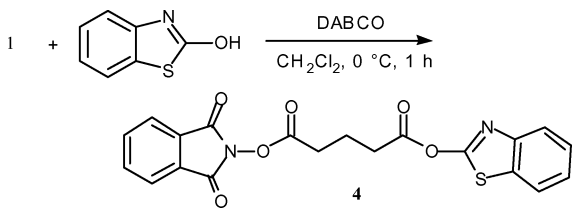


3

Fig. 1

synthesis the candidates having other combinations of PhthOCO-, BtzOCO-, and BtCO-groups as "activated" carbonyl groups.

Finally after extensive effort changing reaction conditions and isolation procedures, we successfully synthesized **4**[8] by the reaction of **1** with 2-hydroxybenzothiazole in the presence of DABCO at 0 °C in 46% yield (Scheme 3), and **5**[9] by the reaction of **2** with benzotriazole in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) at 0 °C in 84% yield (Scheme 4).

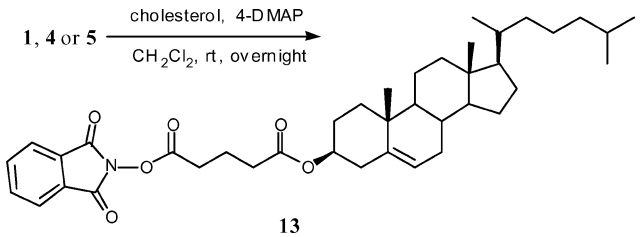


4

Scheme 3

In order to apply and test the ability of the new linkers thus prepared in an actual biological molecule system, we studied the reaction of the linkers **1**, **4**, and **5** with cholesterol in the

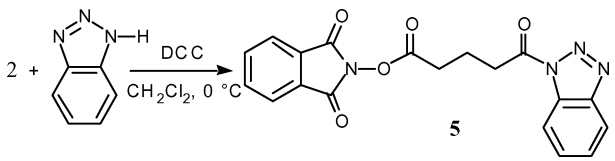
Table 2 Reaction of non-symmetrical cross linker with cholesterol



13

Entry	Substrate	Yield (%)[a]
1	**1**	54
2	**4**	68
3	**5**	27[b]

[a] Isolated yields (not optimized). [b] **5** was recovered in 40% yield.



5

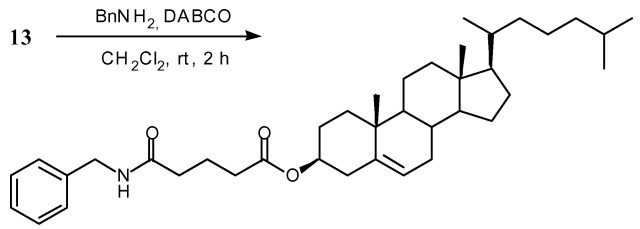
Scheme 4

presence of 4-DMAP as a base. The yields of the 3-cholesteroyl-4-(phthalimidoyloxycarbonyl)butyrate (**13**)[10] are summarized in Table 2.

As the result, a relatively higher yield was obtained in the case of **4** (entry 2) in a shorter time.

The low yield of **13** in the case of **5** (entry 3) will be explained by the relatively low leaving ability of the benzotriazole group (cf. Table 1).

Further, we examined the reaction of the compound **13** with benzylamine (a model compound for protein) as a model reaction for the synthesis of an antigen. The product 3-cholesteroyl-4-(benzylaminocarbonyl)butyrate (**14**)[11] was isolated by flash column chromatography on silica gel in a high yield of 91% (Scheme 5).



14

Scheme 5

In our previous report[1] we have demonstrated that the target monoclonal antibody was successfully obtained by in situ immunization with the antigen obtained by the reaction of the pre-antigen bearing MBS moiety (such as **13** in this report) with protein. Consequently, it is expected that many kinds of monoclonal antibodies will be easily attained by using the non-symmetrical cross-linkers described here.

It would be interesting to modify sugar derivatives, such as cellulose (include modified celluloses), starch (modified starches), cyclodextrin *etc.*, using the new non-symmetrical cross linkers **1**,**4**, and **5**. We are now aiming to introduce these linkers into filter papers and modified starches, in order to develop useful functionalized papers and starches, and have succeeded in modifying these materials in preliminary experiments.

Notes and References

1 (*a*) H. Morita and J. K. Byung, *Chem. Lett.*, 2000, **1**, 42–43; (*b*) H. Morita, J. K. Byung, S. Yamada, T. Funada and Y. Kadoma, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 357–359.

2 (*a*) For reviews and recent examples, see: G. M. Dubowchick and M. A. Walker, *Pharmacol. Ther.*, 1999, **83**, 67–123; (*b*) Lee H. Pai MD and Ira Pastan, *J. Am. Med. Assoc.*, 1993, **269**, 78; (*c*) V. K. Rusiecki and S. A. Warne, *Bioorg. Med. Chem. Lett.*, 1993, **3**, 707; (*d*) K. D. Janda, J. A. Ashley, T. M. Jones, D. A. McLeod, D. M. Schloeder and M. I. Weinhouse, *J. Am. Chem. Soc.*, 1990, **112**, 8886; (*e*) G. A. Pietersz, *Bioconjugate Chem.*, 1990, **1**, 89.

3 K. H. Dalton, G. M. Dubowchik and A. W. Michael, *Tetrahedron Lett.*, 2002, **43**, 1987–1990.

4 **3-(Phthalimidoyloxycarbonyl)butyric acid chloride (1)**: Method (A): Glutaryl dichloride (1457.4 mg, 8.62 mmol) was added to a solution of *N*- hydroxyphthalimide (469.81 mg, 2.87 mmol) and pyridine (749.07 µl, 2.87 mmol) in CH_2Cl_2 (7 ml) at rt under N_2 and stirred for 3 h. Hexane was added to the reaction mixture, than the precipitate was removed by glass funnel. The solution was concentrated by evaporation and purification by kugelrohr distillation and finally repeated re-crystallization from Hexane/AcOEt to yield acid chloride **1** as a colorless solid (373 mg, 44%); Method (B): *N*-hydroxyphthalimide (1000.0 mg, 6.13 mmol) and 4-DMAP (1123.9 mg, 9.19 mmol), were dissolved in CH_2Cl_2 (8 ml) and this solution was added to a solution of glutaric anhydride (1049.1 mg, 9.19 mmol) in CH_2Cl_2 (4 ml). The reaction mixture was stirred for 3 h under N_2 at 0 °C. Then, the reaction mixture was neutralized by 1 N HCl solution and extracted with CH_2Cl_2. The organic layer was washed with 1 N HCl (4×) and then H_2O, and dried over anhydrous $MgSO_4$, and concentrated under vacuum, to give **2** (87%) as a colorless solid. Thionyl chloride (316.1 µl, 4.33 mmol) was added to a solution of compound **2** (1000 mg, 3.60 mmol) in $ClCH_2CH_2Cl$ (13 ml) under N_2. The mixture was refluxed with stirring for 4 h. Then, the solvent was removed under vacuum. The residue was purified by repeated re-crystallization from Hexane-AcOEt to yield acid chloride **1** (979 mg, 92%); mp 88.5–90 °C (colorless solid from Hexane/AcOEt); ^{1}H NMR ($CDCl_3$, 400 Hz) δ 2.13–2.21 (m 2H), 2.80 (t, J = 7.9 Hz, 2H), 3.13 (t, J = 7.2 Hz, 2H), 7.79–7.83 (m, 2H), 7.88–7.92 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 19.9, 29.2, 45.2, 123.9, 124.0, 128.7, 134.7, 134.8, 161.7, 168.5, 173.1. ν_{max}/cm^{-1}1805, 1791, 1741 (CO). Elemental analysis (%) calc. for $C_{13}H_{10}ClNO_5$: C, 52.81; H, 3.41; N, 4.74. Found: C, 53.02; H, 3.56; N, 4.78.

5 A. R. Kartritzky, N. Shobana, J. Prnak, A. S. Afridi and F. Wei-Qiang, *Tetrahedron*, 1992, **48**, 7817–7822.

6 A. R. Katritzky, X. Lan, J. Z. Yang and V. Olga, *Chem. Rev.*, 1998, **98**, 409–548.

7 A. R. Katritzky, S. Ledoux, R. M. Witek and S. K. Nair, *J. Org. Chem.*, 2004, **69**, 2979.

8 **4-(2-Benzothiazolyloxycarbonyl)butyric-*N*-hydroxyphthalimide (4):** 2-hydroxybenzothiazole (25 mg, 0.17 mmol) and DABCO (37.9 mg, 0.34 mmol) were dissolved in CH_2Cl_2 (1 ml). This solution was added dropwise to a solution of **1** (50 mg, 0.17 mmol) in CH_2Cl_2 (2 ml) at 0 °C under N_2 and stirred for 1 h. Then, the reaction mixture was neutralized by dil. AcOH solution and extracted with CH_2Cl_2. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and concentrated under vacuum. The residue was purified with silica gel column chromatography (Hexane/AcOEt = 1:1) to yield **4** (31.9 mg, 46%); mp 150.3–150.6 °C (colorless solid from CH_2Cl_2/Hexane); ^{1}H NMR ($CDCl_3$, 400 Hz) δ 2.23–2.30 (m, 2H), 2.86 (t, J = 7.4 Hz, 2H), 3.32 (t, J = 7.0 Hz, 2H), 7.25–7.28 (m, 1H), 7.32–7.39 (m, 2H), 7.78–7.81 (m, 2H), 7.80–7.90 (m, 2H), 8.33 (d, J = 8.4 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 9.3, 29.9, 37.5, 117.8, 121.8, 121.9, 123.9, 125.5, 127.0, 128.8, 134.7, 161.8, 169.0, 172.9. ν_{max}/cm^{-1}1787, 1739, 1714 (CO); Elemental analysis (%) calc. for $C_{20}H_{14}N_2O_6S$: C, 58.53; H, 3.44; N, 6.83. Found: C, 57.94; H, 3.52; N, 6.81.

9 **4-(1-Benzotriazoleoxa)butyric-*N*-hydroxyphthalimide ester (5):** Compound **2** (1000mg, 3.82 mmol) was dissolved in CH_2Cl_2 (8 ml) and this solution was added to a solution of benzotriazole (456.4 mg, 3.82 mmol)

and DCC (948.1 mg, 4.59 mmol) in CH_2Cl_2 (17 ml). The solution was stirred for 1 h under N_2 at 0 °C. After condensation and filtration of the precipitate, purification was made by silica gel column chromatography (Hexane/AcOEt = 1:1) to yield **5** (1215 mg, 84%); mp 155.8–156.7 °C (colorless solid from CH_2Cl_2/Hexane); ^{1}H NMR ($CDCl_3$, 400 Hz) δ 2.37–2.44 (m, 2H), 2.95 (t, J = 7.2 Hz, 2H), 3.65 (t, J = 7.2 Hz, 2H), 7.50–7.54 (m, 1H), 7.65–7.69 (m, 1H), 7.78–7.82 (m, 2H), 7.87–7.90 (m, 2H), 8.12–8.15 (m, 1H), 8.29–8.31(m, 1H); ^{13}C NMR ($CDCl_3$) δ 19.2, 30.0, 34.1, 114.4, 120.2, 124.0, 126.2, 128.9, 130.5, 134.8, 146.2, 161.8, 168.9, 171.3. ν_{max}/cm^{-1}1812, 1785, 1752 (CO); Elemental analysis (%) calc. for $C_{19}H_{14}N_4O_5$: C, 60.32; H, 3.73; N, 14.81. Found: C, 60.46; H, 3.73; N, 14.85.

10 **3-Cholesteroyl-4-(phthalimidoyloxycarbonyl)butyrate (13):** *Typical procedure*: A stirred solution of DMAP (4.4 mg, 0.04 mmol) and cholesterol (14.1 mg, 0.04 mmol) was added dropwise to a solution of **5** (15 mg, 0.04 mmol) in CH_2Cl_2 (2 ml) at rt under N_2 and stirred overnight. Then the reaction mixture was neutralized by $NaHCO_3$ and extracted with CH_2Cl_2, and dried over anhydrous $MgSO_4$, and concentrated under vacuum, to give crude product which was purified by flash chromatography yielded **13** (16 mg, 68%); colorless solid; mp 83–85 °C (from CH_2Cl_2/Hexane); ^{1}H NMR ($CDCl_3$) δ 0.67 (s, 3H), 0.86 (dd, J = 1.6 Hz, 6.4 Hz, 6H), 0.91 (d, J = 6.4 Hz, 3H), 0.94–1.04 (m, 5H), 1.05–1.17 (m, 6H), 1.23–1.37 (m, 6H), 1.42–1.58 (m, 7H), 1.78–1.88 (m, 3H), 1.93–2.03 (m, 2H) 2.06–2.13 (m, 2H), 2.33 (d, J = 7.6 Hz, 2H), 2.47 (t, J = 7.2 Hz, 2H), 2.76 (t, J = 7.2 Hz, 2H), 4.60–4.68 (m, 1H), 5.38 (d, J = 4 Hz, 1H), 7.77–7.81 (m, 2H), 7.86–7.91 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 11.8, 18.7, 19.3, 19.9, 21.0, 22.5, 22.8, 23.8, 24.3, 27.8, 27.9, 28.2, 30.07, 31.8, 31.9, 33.1, 35.8, 36.2, 36.6, 36.9, 38.1, 39.5, 39.7, 42.3, 49.9, 56.1, 56.7, 74.2, 122.7, 123.9, 128.8, 134.8, 139.6, 161.8, 169.1, 171.9. ν_{max}/cm^{-1}1814, 1789, 1741 (CO). Elemental analysis (%) calc. for $C_{40}H_{55}NO_6$: C, 74.38; H, 8.58; N, 2.17. Found: C, 74.79; H, 8.71; N, 2.18.

11 **3-Cholesteroyl-4-(benzylaminocarbonyl)butyrate (14):** Colorless solid; mp 115–117 °C (from CH_2Cl_2/Hexane); ^{1}H NMR ($CDCl_3$) δ 0.67 (s, 3H), 0.86 (dd, J = 1.6 Hz, 7.2 Hz, 6H), 0.91 (d, J = 6.4 Hz, 3H), 0.94–1.04 (m, 5H), 1.08–1.21 (m, 6H), 1.24–1.39 (m, 6H), 1.42–1.61 (m, 7H), 1.78–1.87 (m, 3H), 1.94–2.02 (m, 4H), 2.25–2.29 (m, 4H), 2.35 (t, J = 7.0 Hz, 2H), 4.43 (d, J = 5.6 Hz, 1H), 4.55–4.63 (m, 1H), 5.36 (d, J = 4.4 Hz, 2H), 5.86 (s, 1H), 7.26–7.37 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 11.82, 18.7, 19.3, 20.9, 22.5, 22.8, 23.8, 24.2, 27.8, 27.9, 28.2, 31.8, 31.9, 33.6, 35.5, 35.8, 36.1, 36.6, 36.9, 38.1, 39.5, 39.7, 42.3, 43.6, 49.9, 56.1, 56.6, 74.0, 122.7, 127.5, 127.8, 128.7, 138.2, 139.5, 171.9, 172.6; ν_{max}/cm^{-1}1729, 1639 (CO); Elemental analysis (%) calc. for $C_{39}H_{59}NO_3$: C, 79.41; H, 10.08; N, 2.37. Found: C, 78.93; H, 9.87; N, 2.35.