Tetrahedron Vol. 47, No 28, pp. 5231-5236, 1991
Printed in Great Britain

0040-4020/91 $3.00+.00

THE SYNTHESIS OF NOVEL BIFUNCTIONAL LINKER MOLECULES

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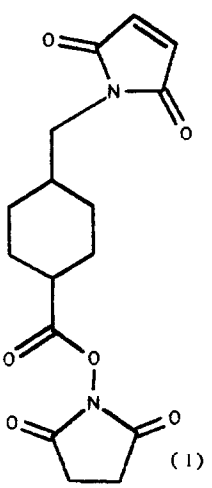
(*Received in UK* 27 *February* 1991)

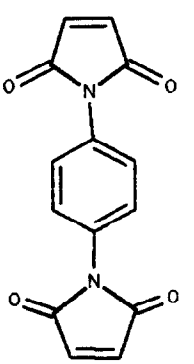
We describe the synthesis of the novel bifunctional linker molecule 2-methyl, 2-(3-carboxypropyl)-cyclopent-4-en-1,3-dione and various related compounds. These compounds are highly thiol-specific.

As part of a drug targeting programme, we are interested in the design and synthesis of bifunctional linker molecules. These will be attached at one end to a monoclonal antibody or other biomolecule, and at the other end to a drug or complexed radioisotope. A number of such compounds have already been described, and the maleimides (1)[1] and (2)[2] are typical. Both of these are thiol-specific, but in common with many other linkers, they are only stable over a narrow range of pH. We reasoned that heterobifunctional compounds like (3) should be both thiol-specific and relatively stable over a wide pH range. Our synthesis of the parent compound (3a) is shown in Scheme One.

Ozonolysis of 2-methyl-2-(prop-2-enyl)-cyclopentane-1,3-dione in dichloromethane at -78°, followed by reaction with dimethylsulphide yielded the expected aldehyde (4) in good yield (94%). Reaction of this with tert-butoxycarbonylmethylidene, triphenylphosphorane provided the trans-ester (5) (60%), and catalytic hydrogenation yielded the saturated ester (6). Introduction of the 4,5 double bond was achieved using phenylselenyl chloride (in ethyl acetate without added base), followed by oxidation with 30% aqueous hydrogen peroxide (64% yield). Finally, the ester (7) was hydrolysed using trifluoroethanoic acid to yield the target compound (3a) (78% after recrystallisation). The entire reaction sequence was repeated numerous times on the multi-gramme scale.

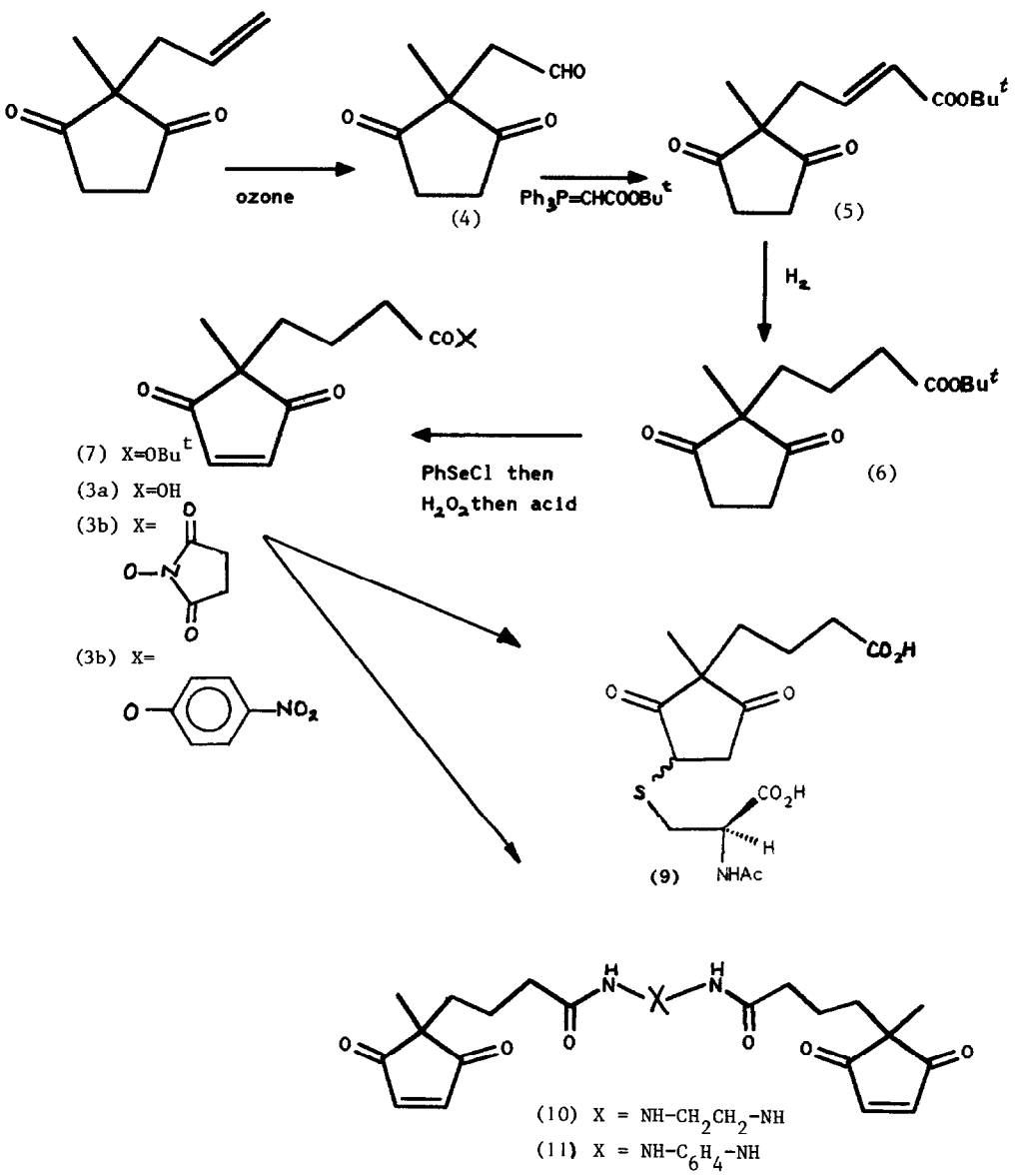


(1)



(2)

SCHEME ONE

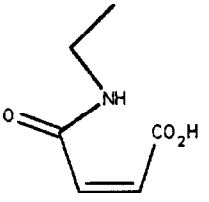

ozone
(4)
$Ph_3P=CHCOOBu^t$
(5)
H_2
(6)
PhSeCl then
H_2O_2 then acid
(7) X=OBu^t
(3a) X=OH
(3b) X=
(3b) X=
(9)
NHAc
(10) X = NH-CH_2CH_2-NH
(11) X = NH-C_6H_4-NH

To facilitate the attachment of the linker to a drug or complexed radioisotope, it was necessary to prepare activated esters, and both the N-hydroxysuccinimidyl-(3b) and p-nitrophenyl esters (3c) were prepared. This involved reaction of the acid (3a) in dichloromethane with DCC, followed by addition of the requisite alcohol, and subsequent purification by flash chromatography.

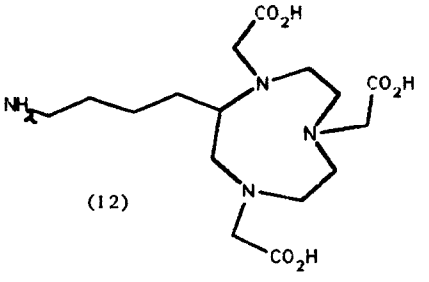
With these heterobifunctional linker molecules in hand, assessment of their reactivity and utility could be undertaken. The acid (3a) was reacted with N-acetylcysteine or with (S)-alanyl-(S)-proline in phosphate buffer at pH 5.5, 6.5, and 7.5. After 10 minutes, at all pH's, the reaction with N-acetylcysteine was greater than 90% complete as judged by hplc, but there was no reaction with the dipeptide even after 48 hours. In contrast, although N-ethylmaleimide exhibited similar selectivity and rapidity of reaction at pH 5.5, 6.5, and 7.5. It was converted into the acyclic form (8) at higher pH, and this reacted with both N-acetylcysteine and with the dipeptide. In separate experiments, the acid (3a) and N-acetylcysteine were reacted in buffers (pH 4 and 9), and the ^{1}H-n.m.r. spectrum of adduct (9) (mixture of all four possible stereoisomers) was checked over a one month period. No changes were observed thus establishing that the adduct is stable.

Reaction of either (3b) or (3c) with ethylene diamine or 1,4-benzenediamine produced the bis-amides (10) and (11). These same bis-amides were also produced from acid (3a) in conjunction with isobutylchloroformate or DCC and the appropriate diamine. These bis-cyclopentene diones were also thiol specific.

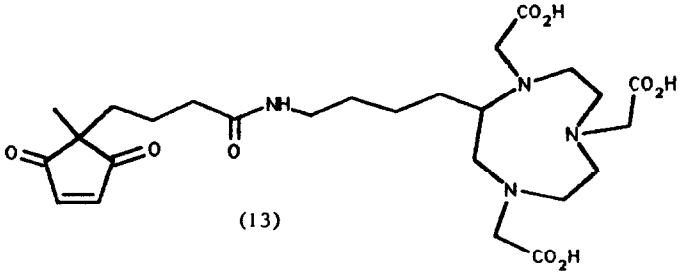
Finally, reaction of (3b) with the aminobutyl macrocycle (12) yielded the expected amide (13) and we hope to exploit this complexing agent for targeting of monoclonal antibody/radioisotope complexes to tumours (cf. ref. 3).



(8)



(12)



(13)

Experimental

IR spectra were recorded with a Perkin-Elmer 881 double beam grating spectrophotometer. NMR spectra were recorded with a Perkin-Elmer R34 (220MHz) instrument or Varian T60 (60MHz) instrument (at Reading), or with a Bruker WH 400 (400MHz) instrument (at the University of Warwick). Mass spectra were obtained at the University of Swansea using a VG ZAB-E high resolution mass spectrometer. Flash chromatography was performed using Crosfield Sorbsil C60 (40-60 microns). Solvents were purified according to Perrin[4], and petrol refers to the petroleum fraction with b.pt. 40-60°C.

2-Formylmethylene-2-methylcyclopentane-1,3-dione (4).
2-Propenyl-2-methylcyclopentane-1,3-dione (5g, 0.032 mole) was dissolved in dichloromethane (70ml) and ozonised at -78°C until a blue colour persisted. The reaction mixture was kept at this temperature until TLC analysis showed that reaction was complete. After purging the reaction mixture with nitrogen, dimethyl sulphide (2ml) was added, and the mixture allowed to come to RT. After concentration it was used for the next stage without purification. Yield 4.8g (ca. 90%).

Rf 0.43 (4:1, dichloromethane:ether); IR (neat) 2975, 2940, 1720 and 1690 cm^{-1}; NMR ($CDCl_3$, 60MHz) 1.10 (s, Me), 2.90 (s, ring methylenes), 3.17 (s, other methylene), 8.90 (s, CHO) ppm.

2-(3-Carboxyprop-1-yl)-2-methylcyclopent-4-ene-1,3-diene (3a).
The aldehyde (4) (4.8g, ca. 31mmole) was dissolved in dichloromethane (50ml) and treated with tert-butoxycarbonyl-methylidene triphenylphosphorane (13.2g, 35mmole), and the reaction mixture held at RT for 20 hours. After concentration and flash chromatography (eluent ether:petrol 1:1) the ester (5) was obtained as a pale yellow solid (5.1g, 62%) and this was dissolved in ethyl acetate (200 ml), and hydrogenated in the presence of 10% palladium on charcoal (ca. 500mg) in a Parr hydrogenator. The catalyst was filtered off using Celite, and the crude hydrogenation product was dissolved in ethyl acetate (150ml), and phenyl selenenyl chloride (3.2g, 16mmol) was added. The solution was stirred at room temperature for 4 hours prior to addition of THF (15ml) and 30% hydrogen peroxide (2ml). The mixture was then stirred for 3 hours prior to work-up. The organic layer was washed several times with water, dried and concentrated to yield a red oil which was purified by flash chromatography using petrol:ether (4:1) as elutant to provide the cyclopentenedione (7) as a light yellow oil (3.4g, *ca.* 65% for the 2 steps). This was dissolved in trifluoroethanoic acid (2ml), and the solution stirred at RT for 20 hours. Concentration yielded an oily solid which was taken up in dichloromethane, and the product precipitated following addition of ether. Recrystallisation from dichloromethane/ether yielded the desired acid (3a) (2.4g, 92%) as a yellow solid. M.pt. 110-11°; IR (dichloromethane) 3450, 3070, 2985, 2945, 1750 and1720 cm^{-1}; ^1H NMR ($CDCl_3$, 220 MHz) 1.20 (s, Me), 1.48 (m, 2-H), 1.78 (m, 1-H), 2.32 (t, J 7.7 Hz, 3-H), 7.30 (s, olefinic H), and 12.5 (br, acid H); ^{13}C NMR ($CDCl_3$) 19.02 (Me), 19.79 (2-C), 33.67 and 33.88 (1-C and 3-C), 50.22 (quaternary ring C), 148.34 (olefinic C), 178.63 (acid C=O), 207.51 (ring C=O); required for $C_{10}H_{12}O_4$: C 61.21% and H 6.16%, and found: C 61.1% and H 6.3%.

The activated esters (3b) and (3c) were prepared by the following procedure. The acid (3a) (5 mmole) in dry THF (25ml) containing triethylamine (0.7ml, 5mmole), was treated with iso-butylchloroformate (0.7ml, 5mmole) at -10°. After one hour at RT, the precipitate of triethylamine hyhdrochloride was removed by filtration, and the filtrate was reacted with a solution of the requisite alcohol (N-hydroxysuccinimide or p-nitrophenol) (5mmole) and triethylamine (0.25ml, 1.8mmole) dissolved in dry THF (25ml). After 4 hours at RT, the product was isolated and purified by flash chromatography eluting in both instances with ether:dichloromethane (1:3).

4-(2-Methylcyclopent-4-ene-1,3-dione-yl)butanoic acid, N-succinimidyl ester (3b).
Yield 51%; R_f 0.47 (ether:dichloromethane 1:3); m.pt. 104-6°; IR (dichloromethane) 3070, 2980, 2940, 1820, 1755 and 1720 cm^{-1}; ^1H NMR ($CDCl_3$, 220 MHz) 1.18 (s, Me), 1.60 (m, 3-H), 1.80 (m, 4-H), 2.57 (t, J 7.7 Hz, 2-H), 2.84 (s, succinimide CH_2), and 7.28 (s, olefinic H); required for $C_{14}H_{15}NO_6$: C 57.33%, H 5.15%, and N 4.77%, found C 57.2%, H 5.3%, and N 4.7%.

4-(2-Methyl-cyclopent-4-ene-1,3-dione-yl)butanoic acid, p-nitrophenyl ester (3c).
Yield 78%; R_f 0.68 (ether:dichloromethane 1:3); m.pt. 97-8°; IR (dichloromethane) 3070, 2980, 2940, 1770, 1710, 1600, 1530, 1495, and 1350 cm^{-1}; ^1H NMR ($CDCl_3$, 220 MHz) 1.20 (s, Me), 1.60 (m, 3-H), 1.82 (m, 4-H), 2.56 (t, J 7.7 Hz, 2-H), 7.28 (d, J 8.8 Hz, aryl H), 7.30 (s, olefinic H), and 8.28 (d, J 8.8 Hz, aryl H); required for $C_{16}H_{15}NO_6$: C 60.56%, H 4.76%, and N 4.41%, found C 60.7%, H 4.9%, and N 4.5%.

Di-N-[4-(2-methylcyclopent-4-ene-1,3-dionyl)] butanoyl-1,2-diaminoethane (10)
The acid (3a) (0.4g, 2 mmole) dissolved in dichloromethane (15 ml) containing DCC (0.4g, 2 mmole) was reacted with 1,2-diaminoethane (0.065ml, 1mmole). The reaction mixture was stirred at RT for 19 hours prior to filtration, and purification by flash chromatography (dichloromethane:methanol 9:1 as eluent). A yellow solid was obtained (0.11g, 26%); m.pt. 99-101°C; IR (dichloromethane) 3470, 3340, 3060, 2980, 2940, 1710 and 1670 cm^{-1}; H NMR (220 MHz, $CDCl_3$) 1.16(s, Me) , 1.42 (m, 3-H), 1.70 (m, 4-H), 2.12 (t, J 7.7 Hz, 2-H), 3.34 (m, CH_2N), 6.30 (s, NH), and 7.28 (s, olefinic H); required for $C_{22}H_{28}N_2O_6$: C 63.44%, H 6.77%, N 6.72%, found C 63.3%, H 7.0%, N 6.5%.

Di-N-[4-(2-methylcyclopent-4-ene-1,3-dionyl)]butanoyl-1,4-diaminobenzene (11)
The acid (3a) (1 mmole) in dry THF (10 ml) containing triethylamine (1.08 mmole) was treated with isobutylchloroformate (1.07 mmole) at -10°C. After one hour at RT, the mixture was filtered, and the filtrate treated with 1,4-diaminobenzene (0.5 mmole) and triethylamine (0.25ml) in THF (5ml). After 5 hours at 4°C, the reaction mixture was concentrated and then applied to a silica column and the product eluted with dichloromethane:methanol (20:1). Yield 345; m.pt. 121-3°C; IR (nujol) 3468, 3342, 1715, 1675, and 1600 cm^{-1}; ^1H NMR ($CDCl_3$, CD_3OD, 400 MHz) 1.15 (s, Me), 1.49 (m, 3-H), 1.74 (m, 4-H), 2.24 (t, J 7.7 Hz, 2), 7.35 (s, olefinic H), 7.45 9s, aryl H); required for $C_{26}H_{28}N_2O_6$: C 67.24%, H 6.03%, N 3.21%, found C 67.4%, H 5.9%, N 3.3%.

Reaction of (3a) with N-acetylcysteine

The acid (3a, 1mmole) was treated with N-acetylcysteine (5mmole) in water at pH 5.5, 6.5 or 7.5. HPLC analysis showed complete disappearance of (3a) after 10 minutes. Concentration and nmr analysis (D_2O, CD_3OD, 300 MHz) revealed four separate methyl signals (δ 1.0-1.2) and no olefinic hydrogens. The major signals due to stereoisomers of (9) were: δ 1.0-1.2 (4S, 3H, Me), 1.35 (m, 2H, 2-H), 1.60 (m, 2H, 1-H), 2.15 (m, 2H, 3-H), 2.25-3.15 (m, 3H, ring CH_2).

Reaction of (3b) with the macrocycle (12)

The N-hydroxysuccinimidyl ester (3b) (343 µl of a solution containing 30 mg/ml in dioxan, 1.2 eq.) was treated with the macrocycle (220 µl of a solution containing 75 mg/ml of 6M.KOH, 1 eq.) in PIPES buffer (150 µl) and the reaction was followed by HPLC. Purification by semi-preparative HPLC and concentration by freeze drying yielded 5 mg of (13). ^1H NMR (D_2O 220MHz) 0.90(s,Me), 1.4(m,3-H+2xCH_2), 1.72(m,4-H), 1.90(t,J 7.8Hz,2-H), 3.0-3.4(11H,CH_2N + CHN), 3.55-4.0(8H,CH_2NHCO + CH_2CO_2H), 7.23(s,olefinic H). FAB/MS $M+H^+$ 553.

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Acknowledgements

We thank the SERC and Celltech Ltd. for a CASE studentship held by Susan Billington.