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A New Synthetic Route to 2,2':5',2"-Terthiophene-5-derivatives to Conjugate with Proteins and Monoclonal Antibodies

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Abstract: A number of α-terthienyl-5-derivatives which can be linked to proteins or other carrier molecules by using different functional groups have been synthesised and characterized. The conjugation of some of these compounds to carrier proteins and antibodies is described.

In the last few years, naturally occurring thiophene derivatives such as 2,2':5',2"-terthiophene (α-T) **1** have stimulated much interest because of their phototoxic activity against a great number of organisms.[1-5]

Chemical, biochemical and photophysical studies have shown that α-T is a powerful photodynamic sensitiser which efficiently generates singlet oxygen.[6]

Compound **1** causes dose-dependent phototoxicity to human skin when administered topically or intradermally.[7] In spite of its lack of selectivity but taking into account the great therapeutic potential of **1** against viral infections and cancer, we have aimed at developing convenient procedures to synthesise several derivatives of **1** which can be either covalently or non-covalently bound to carrier (bio)molecules in order to increase both accumulation rates and specific localization, and to reduce side effects to healthy tissues. The α-T derivatives which are described in this report can be coupled to carriers molecules such as: antibodies, peptides, or other carriers able to specifically recognize biological targets. In particular we describe here a simple strategy for binding α-T moiety to BSA, Concanavalin A, Avidin and to monoclonal and recombinant antibodies.

RESULTS AND DISCUSSION

Carrier biomolecules often bear reactive groups, which can be used for chemical conjugation. Amino groups (such as the ε-amino group of lysine side-chain, or the amino group of cytosine) are typical targets, since they allow the chemical functionalization of peptides, proteins and nucleic acids. We therefore choose the 5-formyl-2,2':5',2"-terthiophene **(2)**[8] (Scheme 1) as starting material for reaction with amino groups through a

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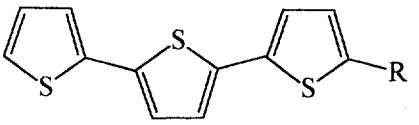
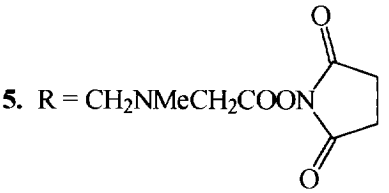
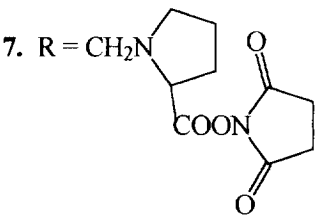
reductive amination of carbonyl group by using $NaBH_3CN$ as reducing agent.[9] Compound **3** was obtained by reaction of aldehyde **2** with methylamine. The amine **3** was chosen as the initial synthetic target because (i) it is a good example to illustrate the reductive amination of carbonyl group; (ii) the secondary amino group is a suitable site on which to build others interesting derivatives (see below).



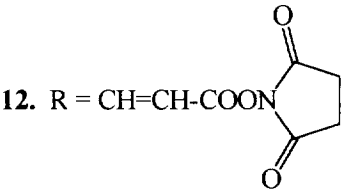
1. R = H
2. R = CHO
3. R = CH_2NHMe
4. R = CH_2NMeCH_2COOH



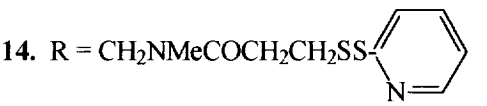
6. R = CH_2N COOH



8. R = $CH_2NMeCH_2CH_2OH$
9. R = $CH_2NHCH_2CH_2NHOCOCH_2Ph$
10. R = CH=CH-COOMe
11. R = CH=CH-COOH



13. R = $CH_2NMeCOCH_2Br$



15. R = $CH_2NMeCH_2CONHNH_2$

Scheme 1

This reaction, however, is not the best choice for our purposes, because the reaction conditions may lead to partial protein denaturation and to loss of functional properties.

A method to overcome this problem is to convert the carbonyl function into a more reactive and selective group. Particularly useful are the *N*-succinimidyl esters that require mild reaction conditions and can react also

with a secondary amino group (e. g. hystidine residues). Derivative **4** was therefore synthesised, by reductive amination of aldehyde **2** with *N*-methylaminoacetic acid and then was converted into the *N*-succinimidyl ester **5** by reaction with *N*-hydroxysuccinimide.

Compound **6** and then the derivative **7** were obtained in a similar manner.

Due to the large applicability of the reductive amination reaction, it is also possible to use bifunctional compounds in which the second function is not the carboxylic one. Indeed, the aminoalcohol **8** and the derivative **9** were obtained by reaction of aldehyde **2** with 2-(methylamino)ethanol and carbobenzoxyethylenediamine, respectively. The latter two compounds can be used as intermediates or to bind the phototoxic α-T moiety to substrates other than proteins.

The *N*-succinimidyl ester **12** is an example of highly reactive α-T derivatives with extended conjugation; it was obtained from the propenoic acid **11** using the normal reaction conditions. The acid **11** was prepared by hydrolysis of methylester **10** which was made available, in quantitative yield, by Horner reaction between aldehyde **2** and trimethylphosphono acetate.

With the aim of obtaining α-T derivatives which are able to react specifically with groups other than amino groups, the compound **13** was synthesised starting from amine **3** and *N*-succinimidyl bromoacetate. Compound **13** reacts with thiol groups in the proteins (e. g. cysteines' side chains) to yield stable thioether linkages. A unique reactive cysteine residue can often be engineered in recombinant proteins, allowing a selective functionalization at a specific site. For example, cysteine-containing peptidic tags can be genetically appended at the C-terminal extremity of recombinant antibody fragments.[10] The corresponding thiol group is the only reactive one in the protein, since the cysteine residues of the immunoglobulin domains are engaged in stable disulfide bridges. This approach has allowed the mild antibody functionalization with iodoacetamido- and maleimido-derivatives of organic compounds, as well as with radionuclides, with complete retention of immunoreactivity as measured by gel-retardation assays.[11]

For selective thiol functionalization, the disulfur derivative **14** was also synthesised by reaction of amine **3** with *N*-succinimidyl-3-[(2-pyridyl)dithio]propionate (SPDP), another crosslinking reagent.

Finally the hydrazide **15**, potentially reactive toward carbohydrates or for labeling DNA and RNA through cytosine residues, can be easily obtained from *N*-succinimidyl ester **5** by reaction with hydrazine hydrate.

A major limitation of phototherapy of tumors and other affections with photosensitisers is the lack of selectivity of presently available photosensitisers.[12] To overcome this problem, conjugation of photosensitisers to monoclonal antibodies has been proposed; immunophotokilling of tumors and bacteria has been tested *in vitro*[13] and *in vivo*.[14]

According to this approach, the α-T derivatives chemically bound to carriers should allow for a minor amount of administrated photosensitiser and for less negative side effects related to this therapeutic approach.

We have conjugated the α-T derivative **7** to proteins and antibodies. The lectins Concanavalin A and

Succinyl Concanavalin A were used as model compounds, because of their ability to bind to fungine cells.

The protein labelling ratio was determined following a modification of published procedure,[15] using the value of ε^{360}= 27000 LM^{-1} cm^{-1} for the α-T bound to proteins and taking into account the contribution at 280 nm which was determined to be 10% of ε^{360} value for the purpose of protein determination.

A labelling ratio of 10 mol of α-T derivative per mole of Concanavalin A and 1.5 mol of α-T derivative per mole of Succinyl Concanavalin A owing to a lower availability of the amino groups for the latter, were calculated. UV-A irradiation (2 Jcm^{-2}; 0.5 hrs) of *Candida albicans* suspension ($2.5x10^4$ $cellsml^{-1}$ in phosphate buffered saline) in the presence of Concanavalin A- α-T (labelling ratio 1:10) resulted in 100 % killing at $3x10^{-7}$ M (concentration of α-T) whereas Concanavalin A was not toxic up to $3.4x10^{-5}$ M while 10 % killing was achieved at $4x10^{-6}$ M concentration of α-T alone.

The Avidin-Biotin and the Streptavidin-Biotin complex systems[16] have found many applications over the years because of their high affinities. We sinthesised a biotinylated derivatives of α-T (compound **16**, see Fig.1) starting from the amine **3**. The Biotin-α-T conjugate **16** is capable of high-affinity Streptavidin binding (data not shown) and may be valuable for *in vitro* applications[17] as well as *in vivo* use.[18]

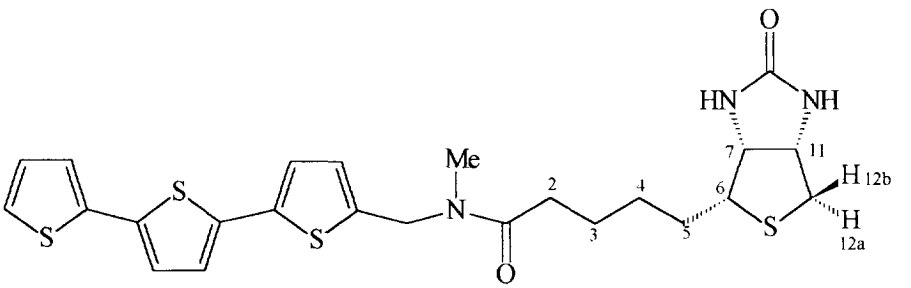


Figure 1. Structure of compound **16**, the numbers are referred to ^1H NMR assignments.

Good product yields were obtained in the reactions involving the labelling of Bovine Serum Albumin and monoclonal antibody 225-28S[19] with the ester **7**. Finally, the compound **13** was used to label the recombinant antibody scFv(HyHEL-10)[20] thus showing the usefulness of those compounds for labelling of various carriers.

CONCLUSIONS

We have shown that it is possible to synthesise in few steps α-T derivatives which are able to react with amino groups and thiol groups of proteins and are likely to be useful for the labelling of other (bio)molecules. These compounds may be valuable for both *in vitro* biochemical applications and *in vivo* use, for example for immuno-photodynamic therapy. Preliminary investigations of the photophysical properties of the conjugates indicate that the fluorescence of the α-T moiety is unaffected by attachment to carrier molecules. It is likely that

these conjugates will be able to efficiently generate $^1O_2$ on light irradiation as judged from the great efficacy demonstrated in *in vitro* experiments on several fungine and melanoma coltures.

EXPERIMENTAL

All melting points are obtained on Kofler apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AC-200 MHz instrument; chemical shifts are reported in ppm high frequency from tetramethylsilane as secondary reference standard and coupling constant in Hz. HPLCs were performed on a Perkin Elmer Series 3B machine equipped with a spectrophotometric detector LC 75. Reverse phase HPLC analyses were performed on a Vydac C_{18} column, at a flow rate of 4 ml min^{-1}. Silica gel 60 F_{254} plates (Riedel-de Haën) and silica gel 60 (Merck 230-400 mesh) were used for analytical and preparative TLC and for column chromatographies, respectively. Solvent were removed under reduced pressure. Gel filtration Sephadex columns were supplied by Pharmacia. *N*-Succinimidyl-3-[(2-pyridyl)dithio]propionate (SPDP) (Pierce), Concanavalin A (Sigma), Succinyl Concanavalin A (Sigma), Avidin (Boheringer) and compound **1** (Aldrich) are commercially available; compound **2** was obtained according to published procedures[8].

_N_-Methyl-_N_-(2,2':5',2"-terthien-5-yl)methylamine (3)

Methylamine hydrochloride (583 mg; 8.70 mmol) was added to a suspension of aldehyde **2** (400 mg; 1.45 mmol) in degassed methanol (50 ml). Then $NaBH_3CN$ was added (46 mg; 0.72 mmol) and the mixture was stirred, under nitrogen in the dark, for 2 h at room temperature. The solvent was evaporated and the residue (169 mg; 40%) was purified by column chromatography (chloroform-ethanol 93:7 v/v as eluant) to give amine **3** (Found: C, 57.71; H, 4.49; N, 4.79. $C_{14}H_{13}NS_3$ requires C, 57.69; H, 4.50; N, 4.81 %). ^{1}H NMR (DMSO-d_6) δ: 7.35 - 6.62 (m, 7H, α-T), 3.80 (s, 2H, CH_2), 2.29 (s, 3H, NMe), 1.89 (s, exch., 1H, NH).

_N_-Methyl-_N_-(2,2':5',2"-terthien-5-yl)methylaminoacetic acid (4)

N-Methylaminoacetic acid (89 mg; 2.73 mmol) was added to a suspension of aldehyde **2** (250 mg; 0.91 mmol) in degassed methanol/acetic acid (99:1) solution (50 ml). Then $NaBH_3CN$ was added (29 mg; 0.46 mmol) and the mixture was stirred, under nitrogen and in the dark, for 24 h at room temperature. After this time the abundant yellow precipitate was separated by filtration (254 mg; 80%). An analytical sample was prepared by crystallization from 2-propanol, m.p. 180 °C (dec.) (Found: C, 55.02; H, 4.27; N, 4.31. $C_{16}H_{15}NO_2S_3$ requires C, 54.98; H, 4.32; N, 4.00 %).

***N*-Succinimidyl *N*-methyl-*N*-(2,2':5',2"-terthien-5-yl)methylaminoacetate (5)**

To a vigorously stirred suspension of acid **4** (45 mg; 0.13 mmol) in DMF (3 ml) and dichloromethane (5 ml) were added *N*-hydroxysuccinimide (15 mg; 0.13 mmol) and a solution of dicyclohexylcarbodiimide (27 mg; 0.13 mmol) in dichloromethane (5 ml) at room temperature; after 20 h the white solid obtained, principally constituted by dicyclohexylurea, was filtered off and the clear filtrate was evaporate to dryness to give a yellow oil which solidified by adding petroleum ether (52 mg; 90%) (Found: C, 53.76; H, 4.08; N, 6.26. $C_{20}H_{18}N_2O_4S_3$ requires C, 53.79; H, 4.06; N, 6.27 %).

_N_-(2,2':5',2"-Terthien-5-yl)methyl-L-proline (6)

To a stirred suspension of aldehyde **2** (160 mg; 0.58 mmol) in methanol (30 ml) were added, L-proline (117 mg; 1.16 mmol) and molecular sieves (4Å, 300 mg), at room temperature. Then, under nitrogen in the

dark, was added $NaBH_3CN$ (100 mg; 1.59 mmol) and the mixture stirred for 12 h. After this time the solvent was evaporated and the greyish residue treated with water and filtered in order to eliminate molecular sieves. Compound **6** was obtained as a yellow solid, which darkens on light exposure (96 mg; 60%) m.p.157-163 °C (dec.) (methanol-DMF) (Found: C, 56.88; H, 4.74; N, 5.10. $C_{18}H_{17}NO_2S_3 \cdot 1/2$ DMF requires C, 56.84; H, 5.01; N, 5.10 %). ^{1}H NMR (DMSO-d_6) δ: 7.52 - 6.96 (m, 7H, α-T), 4.04 (AB sys, 2H, α-T-CH_2-N), 3.51 - 3.37 (m, 2H, Pro), 3.15 - 3.00 (m, 1H, Pro), 2.70-2.52 (m, 1H, Pro), 2.21 - 2.03 (m, 1H, Pro), 2.01 - 1.55 (m, 2H, Pro).

***N'*-Succinimidyl *N*-(2,2':5',2"-terthien-5-yl)methyl-L-pyrrolidin-2-ylcarboxamide (7)**

Operating as for compound **5**, starting from compound **6**, the required product was obtained (61 mg; 90%) as a yellow solid (Found: C, 55.92; H, 4.28; N, 5.91. $C_{22}H_{20}N_2O_4S_3$ requires C, 55.91; H, 4.27; N, 5.93 %). ^{1}H NMR ($CDCl_3$) δ: 7.26 - 6.87 (m, 7H, α-T), 4.10 (AB sys, 2H, α-T-CH_2-N), 3.78 (m, 1H, H-2 pirrolidine), 3.10 (m, 1H, pirrolidine), 2.84 (s, 4H, 2xCH_2 succinimide), 2.82 (m, 1H, pirrolidine), 2.39 - 1.80 (m, 4H, pirrolidine).

2-[*N*-Methyl-*N*-(2,2':5',2"-terthien-5-yl)methylamino]ethanol (8)

To a stirred suspension of aldehyde **2** (188 mg; 0.68 mmol) in methanol (50 ml), 2-(methylamino)ethanol (151 mg; 2.07 mmol) was added, at room temperature. Then, under nitrogen in the dark, $NaBH_3CN$ was added (21 mg; 0.34 mmol) and the mixture stirred for 72 h. After this time the solvent was evaporated and the oil residue purified by column chromatography (chloroform-methanol 9:1 v/v as eluant) to give **8** as a pale yellow solid (137 mg; 60%). An analytical sample was obtained by crystallization from AcOEt-cyclohexane m.p. 84-85 °C (Found: C, 56.67; H, 5.01; N, 4.16. $C_{16}H_{17}NOS_3$ requires C, 57.28; H, 5.11; N, 4.17 %). ^{1}H NMR ($CDCl_3$) δ: 7.22 - 6.80 (m, 7H, α-T), 3.77 (s, 2H, α-T-CH_2), 3.65 (t, 2H, J = 5.5 Hz, CH_2OH), 2.65 (t, 2H, J = 5.5 Hz, NCH_2), 2.47 (bs, exch., 1H, OH), 2.33 (s, 3H, NMe).

***N*-(2,2':5',2"-terthien-5-yl)methyl-*N'*-carbobenzoxyethylenediamine (9)**

To a stirred suspension of aldehyde **2** (180 mg; 0.65 mmol) in methanol (50 ml) containing HCl (0.1 ml) was added carbobenzoxyethylenediamine[21] (630 mg; 3.25 mmol) at room temperature. Then, under nitrogen in the dark, $NaBH_3CN$ (21 mg; 0.34 mmol) was added, and the mixture stirred for 72 h. After this time the solvent was evaporated and the residue was purified by preparative thin layer chromatography (chloroform-methanol 99:1 v/v as eluant) to give **9** as a pale yellow solid (177 mg; 60%) (Found: C, 60.74; H, 4.86; N, 6.14. $C_{23}H_{22}N_2O_2S_3$ requires C, 60.76; H, 4.88; N, 6.16 %). ^{1}H NMR ($CDCl_3$) δ: 7.27 (m, 5H, Ph) 7.25 - 6.72 (m, 7H, α-T), 5.10 (bs, exch, 1H, NH), 5.04 (s, 2H, CH_2O) 3.87 (s, 2H, α-T-CH_2), 3.24 (t, 2H, J = 5.8 Hz, CH_2NHCO), 2.75 (t, 2H, J = 5.8 Hz, α-T-CH_2NHCH_3).

Methyl (*E*)-3-(2,2':5',2"-terthien-5-yl)propenoate (10)

Sodium hydride (59 mg; 2.46 mmol) was added at 0 °C to a stirred solution of trimethylphosphono acetate (358 mg; 1.96 mmol) in anhydrous THF. After 30 min. the solution was allowed to warm to room temperature and stirred for further 15 min.; after this time the aldehyde **2** (500 mg; 1.81 mmol) was added and the reaction mixture was stirred for 2 h. The solvent was evaporated and the residue was taken up with water; the yellow solid obtained was filtered, dried (590 mg; 98%) and crystallized: m.p. 198-199 °C (ethyl acetate). Lit.[22]: m.p. 199-200 °C. (Found: C, 57.67; H, 3.47. $C_{16}H_{12}O_2S_3$ requires C, 57.80; H, 3.64 %). ^{1}H NMR ($CDCl_3$) δ: 7.73 (d, 1H, J = 15.8 Hz, α-T-CH=), 7.24 - 7.01 (m, 7H, α-T), 6.18 (d, 1H, J = 15.8 Hz, C*H*-COOMe), 3.80 (s, 3H, Me).

(*E*)-3-(2,2':5',2"-terthien-5-yl)propenoic acid (11)

10 ml of 20% KOH solution was added to a stirred suspension of ester **10** (100 mg; 0.30 mmol) in methanol (3 ml) and the reaction mixture refluxed for 56 h. The solvent was then evaporated and the residue extracted

with chloroform (3x10 ml) after treatement with HCl 6N (1 ml). The organic extract was washed with water (2x5 ml), dried (Na_2SO_4) and evaporated to give a yellow solid (19 mg; 20%) m.p. >300 °C (dec.) (Found: C, 56.60; H, 3.15. $C_{15}H_{10}O_2S_3$ requires C, 56.58; H, 3.17 %). ^{1}H NMR (DMSO-d_6) δ: 12.4 (bs, exch., 1H, COOH), 7.71 (d, 1H, *J* = 15.8 Hz, α-T-CH=), 7.57 - 7.05 (m, 7H, α-T), 6.15 (d, 1H, *J* = 15.8 Hz, =C*H*-COO).

N-Succinimidyl (*E*)-3-(2,2':5',2"-terthien-5-yl)propenoate (12)

To a stirred suspension of acid **11** (60 mg; 0.19 mmol) in DMF (6 ml) was added, *N*-hydroxysuccinimide (32 mg; 0.28 mmol) and a solution of dicyclohexylcarbodiimide (39 mg; 0.19 mmol) in dichloromethane (15 ml); at room temperature. After 48 h, the separated dicyclohexylurea was filtered off and the filtrate evaporated to dryness. The solid residue was then further treated with dichloromethane in order to eliminate the dicyclohexylurea, then the solution was washed with water and dried on Na_2SO_4. By evaporation of the solvent a yellow solid (71 mg; 95%) was obtained (Found: C, 54.95; H, 3.14; N, 3.34. $C_{19}H_{13}NO_4S_3$ requires C, 54.92; H, 3.15; N, 3.37 %). ^{1}H NMR ($CDCl_3$) δ: 7.73 (d, 1H, *J* = 15.8 Hz, α-T-CH=), 7.24 - 7.01 (m, 7H, α-T), 6.18 (d, 1H, *J* = 15.8 Hz, =CH-COO), 2.84 (s, 4H, 2xCH_2).

N-Methyl-N-(2,2':5',2"-terthien-5-yl)methybromo acetamide (13)

To a stirred and cooled solution (0 °C) of amine **3** (70 mg; 0.24 mmol) in degassed dichloromethane (50 ml), *N*-succinimidyl bromoacetate[23] (57 mg; 0.24 mmol) was added, under nitrogen in the dark. After 30 min. the solution was allowed to warm to room temperature and then kept under stirring for 2 h. The solvent was evaporated and the crystalline residue (94 mg; 95%) was purified by preparative layer chromatography (chloroform as eluant) to give the desidered compound **13** as a light yellow solid m.p. 107-109 °C (Found: C, 46.80; H, 3.37; N, 3.42. $C_{16}H_{14}BrNOS_3$ requires C, 46.60; H, 3.44; N, 3.39 %). ^{1}H NMR ($CDCl_3$) δ: 7.23 - 6.88 (m, 7H, α-T), 4.72 and 4.68 (s, 2H, α-T-CH_2), 3.95 and 3.89 (s, 2H, $COCH_2$), 3.10 and 3.02 (s, 3H, NMe).

N-Methyl-N-(2,2':5',2"-terthien-5-yl)methyl-3-[(2-pyridyl)dithio]propionamide (14)

To a stirred and cooled solution (0 °C) of amine **3** (117 mg; 0.40 mmol) in degassed dichloromethane (100 ml), *N*-succinimidyl-3-[(2-pyridyl)dithio]propionate (SPDP) (137 mg; 0.44 mmol) was added, under nitrogen and in the dark. After 1 h the solution was allowed to warm to room temperature and kept under stirrring for 48 h. The solvent was evaporated and the residue was purified by column chromatography (chloroform-methanol 49:1 v/v as eluant). The fastest running band was identified as disulphide **14** (137 mg; 70%) (Found: C, 53.90; H, 4.11; N, 5.72. $C_{22}H_{20}N_2OS_5$ requires C, 54.07; H, 4.12; N, 5.73 %). ^{1}H NMR ($CDCl_3$) δ: 8.44 (ddd, 1H, *J* = 4.0, 1.7 and 0.8 Hz, H-6 pyridine), 7.75 - 7.58 (m, 3H, H-3, H-4 and H-5 pyridine) 7.22 - 6.85 (m, 7H, α-T), 4.65 and 4.57 (s, 2H, α-T-CH_2), 3.13 (pt, 2H, *J* = Hz, SCH_2), 3.00 and 2.93 (s, 3H, NMe), 2.78 (pt, 2H, *J* = Hz, $COCH_2$).

N'-Methyl-N-(2,2':5',2"-terthien-5-yl)methylaminoacetic acid hydrazide (15)

To a vigorously stirred, cooled (0 °C) solution of hydrazine monohydrate (25 mg; 0.50 mmol) in THF was added dropwise a solution of compound **5** (223 mg; 0.50 mmol) in anhydrous THF. After 4 h the reaction mixture was evaporate (109 mg; 60%) and the oily residue purified by column chromatography (chloroform-methanol 19:1 v/v as eluant) to give a light yellow solid m.p. 158 °C (Found: C, 52.89; H, 4.71; N, 11.54. $C_{16}H_{17}N_3OS_3$ requires C, 52.87; H, 4.71; N, 11.56 %). ^{1}H NMR ($CDCl_3$) δ: 8.01 (bs, exch., 1H, NH), 7.18 - 6.71 (m, 7H, α-T), 3.75 (bs, exch., 2H, NH_2), 3.65 (s, 2H, α-T-CH_2), 3.08 (s, 2H, NCH_2CO), 2.27 (s, 3H, NMe).

N-Methyl-N-(2,2':5',2"-terthien-5-yl)methylbiotinamide (16)

To a suspension of Biotin (107 mg; 0.44 mmol) and *N*-hydroxysuccinimide (55 mg; 0.48 mmol) in DMF

(1.5 ml) dicyclohexylcarbodiimide (91 mg; 0.44 mmol) was added. The mixture was stirred at room temperature for 20 h and after this time a solution of the amine **3** (140 mg; 0.48 mmol) in dichloromethane (10 ml) was added. After 3 h at room temperature the resulting dicyclohexylurea was filtered off and the organic phase was washed with water and dried over Na_2SO_4. The solvent was evaporated (103 mg; 45%) and the obtained solid was purified by HPLC (H_2O/CF_3COOH 0.1% - MeOH). ^1H NMR ($CDCl_3$) δ: 7.37 - 6.78 (m, 7H, α-T), 5.25 (bs, exch., 2H, 2xNH), 4.65 (d, 2H, J = 10.0 Hz, CH_2), 4.50 (dd, 1H, J = 8.1 and 5.0 Hz, H-11 biotin), 4.32 (pt, 1H, J = 8.3 Hz, H-7 biotin), 3.18 (dt, 1H, J = 8.1 and 7.0 Hz, H-6 biotin), 2.92 (dd, 1H, J = 12.1 and 5.0 Hz, H-12a biotin), 2.71 (d, 1H, J = 12.1 Hz, H-12b biotin), 2.39 (t, 2H, J = 7.7 Hz, 2-CH_2), 1.98 - 1.42 (m, 6H, 3-CH_2, 4-CH_2 and 5-CH_2 biotin)

Conjugation of aldehyde 2 and succinimidoester 7 to Bovine Serum Albumin

A solution of Bovine Serum Albumin (BSA) in phosphate buffer (50 mg ml^{-1}), a solution of aldehyde **2** in DMSO (2 mg ml^{-1}) and a solution of ester **7** in DMSO (2 mg ml^{-1}) were prepared:

In two parallel experiments: 1.8 ml of BSA solution were allowed to react for 12 h at 4 °C in the dark, with 0.2 ml of the two solutions of the two α-T derivatives **2** and **7**. A small aliquot of the two reaction products were purified on Sephadex PD-10 columns. During the gel filtration, as expected for a conjugation, the yellowish coloured BSA-α-T derivative co-migrated completely with the protein fraction, showing that the reaction has been quantitative. The products purified by gel filtration were finally analysed by SDS PAGE electrophoresis,[24] using fluoresceine labelled BSA as reference. The bands in the gel were visualized by irradiation with UV light. This analysis revealed that the terthienylation of the BSA with the two reactants give a fluorescence well detectable by the characteristic two bands of the BSA, which migrated in an analogous way compared to the two bands of the fluoresceine labelled BSA.

Conjugation of succinimidoester 7 to Concanavalin A

To a solution of Concanavalin A (2 mg) in phosphate buffer (100 mM; pH 8; 250 μl) was slowly added a solution of succinimidoester **7** in DMF (100 μl; 16.5 mg/ml). The yellow suspension was gently shaken for 12 h at 0 °C in the dark. The mixture was then centrifuged, the supernatant was recovered and purified on a Sephadex G25 column collecting the fraction showing the characteristic fluorescence. The numbers of α-T residues was determined spectrophotometrically as reported previously and this value was found to be 10.

Conjugation of succinimidoester 7 to Succinyl Concanavalin A

Operating as above on Succinyl Concanavalin A, the ratio of moles of α-T per mole of protein was found to be 1.5.

Conjugation of succinimidoester 7 to Avidine

To a solution of Avidine (2 mg) in phosphate buffer (100 mM; pH 8; 500 μl) was slowly added a solution of succinimidoester **7** in DMSO (100 μl; 1.54 mg/ml). The obtained suspension was gently stirred for 12 h to 0 °C in the dark. The mixture was then centrifuged, the supernatant was collected and purified on a Sephadex G10 column collecting the fraction showing the characteristic fluorescence. The moles of α-T per mole of Avidin after terthienylation was spectrophotometrically determined to be 7.

Conjugation of succinimidoester 7 to monoclonal antibody 225-28S

The monoclonal antibody 225-28S[19] has been functionalized with ester **7** on the amino groups according to the following protocol:

50 μl of a solution of compound **7** (17 mg ml^{-1}) in DMSO (final conc $1.3x10^{-3}$ mM) was slowly added, by a variable volume pipette, to 1 mg of monoclonal 225-28S at a concentration of 5 mg ml^{-1} in 100 mM phosphate

buffer (pH 8). The milky suspension so obtained was gently shaken for 2 h at room temperature in the dark. After this time, the mixture was centrifuged, the supernatant recovered and purified on a Sephadex G25 column, collecting the fraction showing two absorption maxima at 280 and 360 nm due to the presence of antibody and α-T moiety, respectively. The labelling ratio estimated was 3 moles of α-T per mole of protein.

Conjugation of bromoacetamide 14 to recombinant antibody scFv(HyHEL-10)

A recombinant anti-lysozyme antibody HyHEL-10,[20] in scFv configuration[25] in which a cysteine residue has been cloned at the C-terminal end of the molecule as single site of thiol specific functionalization (scFv-cys), was derivatized with the bromo acetamide **14** in the following way.

The antibody scFv-cys at a concentration of 1 mg ml^{-1} in phosphate buffer was reduced for 15 min by addition of dithiotreitol (DTT) at a final concentration of 0.1 mM. To the solution of reduced antibody (0.9 ml) were added 0.1 ml of a solution of bromo acetamide **14** in DMSO (2 mg ml^{-1}; final concentration 0.48 mM), in order to both saturate the DTT present in solution and functionalizing the cysteines of the molecule of scFv-cys. The reaction was carried out for 2 h in ice, then stopped by addition of 0.1 ml of DTT 100 mM.

The terthienylated antibody was purified by gel filtration on Sephadex PD-10 column and the occurred terthienylation was checked by running a SDS PAGE using a molecular weight marker as the standard reference. The gel irradiated with UV light showed the presence of a fluorescent band of molecular weight of about 30000 dalton.

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