The synthesis and high-resolution NMR spectroscopy of ethyl *N*-(2-triphenylmethylthio)ethanoyl-*O*-{4′-[4″-(1″-bis(2‴-chloroethyl)amino)phenyl]butanoyl}-L-seryl-*S*-benzyl-L-cysteine: a chelate–chlorambucil complex for use as a ligand for ^{99m}Tc radio-imaging

Russell A. Bell, Donald W. Hughes, Colin J.L. Lock, and John F. Valliant

Abstract: A potential agent for the imaging of tumours has been synthesized from the antineoplastic agent chlorambucil. Standard peptide coupling techniques were used to synthesize a tripeptide covalently coupled to chlorambucil in 10 steps. The final product was characterized by high-resolution NMR spectroscopy.

Key words: ethyl *N*-triphenylmethylthioethanoyl-*O*-{4′-[4″-(1″-bis(2‴-chloroethyl)amino)phenyl]butanoyl}-L-seryl-*S*-benzyl-L-cysteine, NMR spectroscopy, synthesis, radio-imaging agent.

Résumé : Utilisant l'agent antinéoplasique, on a synthétisé un agent pouvant éventuellement servir dans l'imagerie des tumeurs. On a utilisé les techniques standard de couplage des peptides pour synthétiser, en 10 étapes, un tripeptide lié d'une façon covalente à du chlorambucil. On a caractérisé le produit final à l'aide de la spectroscopie RMN à haute résolution.

Mots clés : *N*-triphénylméthylthioéthanoyl-*O*-{4′-[4″-(1″-bis(2‴-chloroéthyl)amino)phényl}butanoyl]}-L-séryl-*S*-benzyl-L-cystéine d'éthyle, spectroscopie RMN, synthèse, agent pour radio-imagerie.

[Traduit par la rédaction]

Introduction

There are a number of approaches to the synthesis of technetium complexes that may act as radio-imaging agents. Of these, one of the most important is the synthesis of relatively small molecules that can chelate the technetium atom and that, typically, have three, four, or five coordinating atoms (1, 2). Examples are the ligands based on the *N*,*N*′-bis(mercaptoethyl) ethylenediamine framework, which has two nitrogen and two sulphur coordinating atoms (3). A second, important method is to bind one of these small chelating groups to a biologically significant molecule, often through a spacer chain. The biological molecule should have a well-defined receptor in the human body and, preferably, the nature of the binding site in the biological molecule should be known. The chelant group is bound to the biological molecule at a position distant from the binding site, and one hopes that the chelated metal is sufficiently far away that it does not interfere markedly with binding of the combined molecule to the receptor. Radio-imaging agents based on monoclonal antibodies, naturally occurring biological molecules, and drugs have all been prepared (4). An increasingly popular, third alternative is to synthesize relatively small polypeptides, with one end providing the site that binds to the receptor and the other end providing the chelant groups. In this paper we describe a combination of the two last procedures where we have used a tripeptide to provide the chelant group and the anti-cancer drug chlorambucil (5, 6) as the biological molecule.

Results and discussion

The first method used to try to synthesize **1** (see Fig. 1) was to make the tripeptide fragment and attach chlorambucil subsequently. This led, however, to an inseparable mixture, despite the coupling method used.[3] In the successful approach the pro-

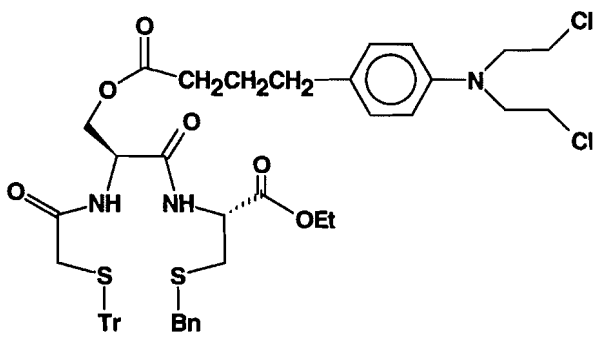
Received February 19, 1996.

R.A. Bell,[1] **C.J.L. Lock,**[2] **and J.F. Valliant.** Laboratories for Inorganic Medicine, Departments of Chemistry, Biochemistry, and Pathology, McMaster University, ABB-266A, Hamilton, ON L8S 4M1, Canada.
D.W. Hughes. McMaster University N.M.R. Facility, McMaster University, Hamilton, ON L8S 4M1, Canada.

[1] Author to whom correspondence may be addressed. Telephone: (905) 525-9140, ext. 23479. Fax: (905) 522-2509. E-mail: Bell@mcmaster.ca
[2] Deceased May 1, 1996.

[3] The methods used the following combinations of coupling reagents: oxalyl chloride – dimethylformamide , DCC–DMAP, EDAC-HCl–DMAP.

Fig. 1. Ethyl *N*-(2-triphenylmethylthio)ethanoyl-*O*-{4′-[4″-(1″-bis(2‴-chloroethyl)amino)phenyl]butanoyl}-L-seryl-*S*-benzyl-L-cysteine, **1**.



tected dipeptide L-Ser-L-Cys[4] was coupled to chlorambucil before the addition of the mercaptoethanoic acid fragment.

The synthesis of the dipeptide–chlorambucil molecule is outlined in Fig. 2. The ethyl ester of *S*-benzyl-L-cysteine, **3**, was produced as the tosylate salt. The free amine of the cysteine derivative was subsequently coupled to *N-tert*-butoxycarbonyl-L-serine, in 80% yield, with ethyl-3-(3-dimethyl-amino)propylcarbodiimide hydrochloride (EDAC-HCl). The use of the water-soluble carbodiimide resulted in a product that could be isolated by recrystallization. This was in contrast to reactions in which the coupling reagent was dicyclohexylcarbodiimide (DCC), where repeated chromatography was required in order to remove residual dicyclohexylurea. The protected dipeptide **5** contained a free hydroxyl group and was now ready for formation of the chlorambucil ester **6**. The initial approach, which had limited success, was the preparation of the ester through the acid chloride (8). The improved procedure used EDAC-HCl and 4-dimethylaminopyridine (DMAP) as coupling reagents and gave **6** in 71% yield.

N-Hydroxysuccinimido-2-(triphenylmethylthio)ethanoate, **8**, has been reported to be a useful material with which to introduce protected mercaptoethanoic acid into a molecule (9). The literature procedures for the protection and activation of mercaptoethanoic acid are low-yield reactions that leave the final product contaminated with starting material and (or) dicyclohexylurea. In our approach to the protection of mercaptoethanoic acid (Fig. 3), the acid and triphenylcarbinol were dissolved in a mixture of acetic acid and dichloromethane, followed by the addition of boron trifluoride etherate. The use of dichloromethane as a cosolvent allowed reaction to occur at ambient temperatures rather than at 80°C, as the literature suggested, and resulted in a very pure product that was obtained in high yield. Compound **8** was produced by coupling **7** and *N*-hydroxysuccinimide in acetronitrile with EDAC-HCl: the product precipitated in high yield and purity in a short reaction time.

The addition of the final fragment, **8**, required the removal of the carbamate on the dipeptide–chlorambucil adduct (Fig. 4) and this was accomplished with trifluoroacetic acid and triethylsilane (9). The product, **9**, after extraction with acqueous sodium carbonate solution, was coupled to **8** and gave the tripeptide–chlorambucil adduct, **1**. The overall yield from *S*-benzyl-L-cysteine was 30%. Compound **1** was subjected to high-resolution NMR analysis.

NMR spectroscopy

The Bruker DRX-500 spectrometer, with its gradient capability, allowed the acquisition of two-dimensional spectra in remarkably short time periods. The gradient-COSY (10–13) and HSQC (14–16) spectra for compound **1** were each acquired in 5.5 min on approximately 15 mg of sample. The majority of the aliphatic ^{1}H and ^{13}C signals of **1** were assigned (Tables 1 and 2) by the aforementioned two-dimensional techniques and these were consistent with assignments made for the synthetic precursors. There remained several assignments in both the proton and carbon atom spectra of **1** that could not be made unambiguously. Consequently, a Heteronuclear Multiple Bond Correlation (HMBC) (17) experiment and a Heteronuclear Multiple Quantum Coherence – Total Correlation Spectroscopy (HMQC–TOCSY) (18) experiment were performed (Figs. 5 and 6).

The use of the HMBC pulse sequence with a low-pass *J* filter allowed proton resonances to be correlated with neighbouring carbon atom resonances through spin coupling interactions of $^{2}J_{H-C}$ and $^{3}J_{H-C}$ (Fig. 5). The initial use of the HMBC experiment was to assign the four carbonyl signals that corresponded to the two amide and two ester groups. The ethyl ester carbonyl peak (C-24) was assigned by correlation of the quartet of the methylene group of the ester (4.146 ppm) and the carbon atom signal at 169.9 ppm. The amide carbonyl signals were assigned by the observation of a two-bond correlation with the adjacent α proton signal. The α proton chemical shifts had been assigned previously to the appropriate amino acid by use of the COSY experiment. The remaining carbonyl signal belonged to the ester between the chlorambucil unit and the serine hydroxyl group. The HMBC experiment also facilitated the assignment of all the carbon atom signals in the aromatic systems as well as confirming the assignments of H-6, H-12, and H-19.

The HMQC–TOCSY experiment (Fig. 6) was used to corroborate the ^{1}H and ^{13}C assignments. For example, the proton resonance at 4.382 ppm was assigned as the α proton of serine (H-9). In the HMQC–TOCSY experiment, H-9 exhibited a HMQC correlation to C-9, the carbon to which it is directly bound. As a result of the TOCSY portion of the pulse sequence, H-9 also showed correlation with H-8 and H-10, the amide and β protons of serine, respectively. The proton signal assigned as H-10 also correlated to its directly bound carbon

[4] In this paper we have used a number of abbreviations. These are: AcOH, acetic acid; ACQ, acquire; BIRD, bi-linear rotation decoupling; Bn, benzyl; Boc, *tert*-butoxycarbonyl; COSY, correlation spectroscopy; DCC, dicyclohexylcarbodiimide; DCI, direct injection chemical ionization; DMAP, dimethylaminopyridine; EDAC, ethyl-3-(3-dimethylamino)propylcarbodiimide; Et, ethyl; FID, free induction decay; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; HRDEI, high-resolution direct injection electron impact; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; TFA, trifluoroacetic acid; TLC, thin–layer chromatography; TOCSY, total correlation spectroscopy; Tr, triphenylmethyl; TsOH, *p*-toluenesulphonic acid. In addition, the standard three-letter abbreviations for amino acids are used (7).

Fig. 2. The synthetic pathway to ethyl *N-tert*-butoxycarbonyl-*O*-{4′-[4″-(1″-bis(2‴-chloroethyl)amino)phenyl]butanoyl}-L-seryl-*S*-benzyl-L-cysteine, **6**.

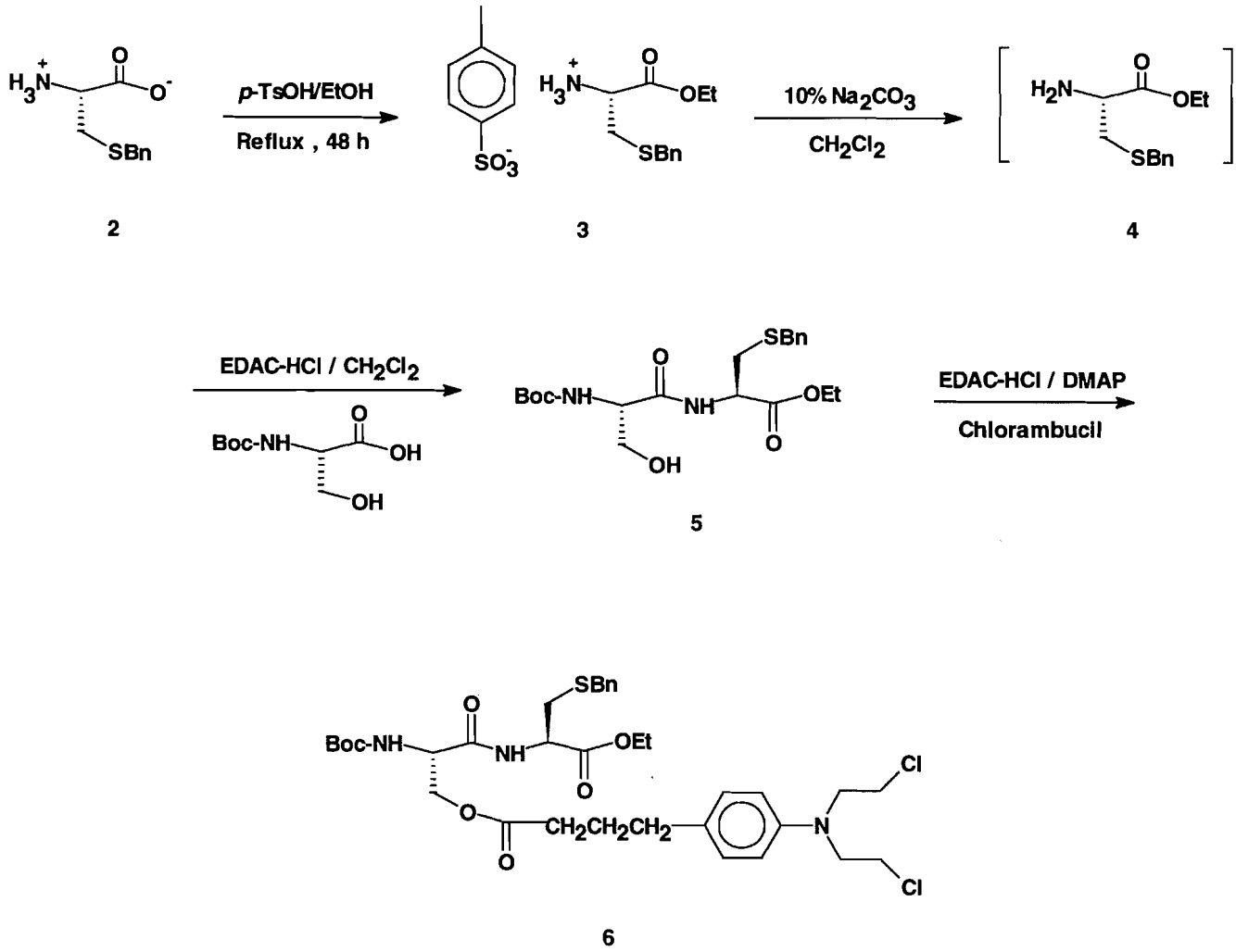
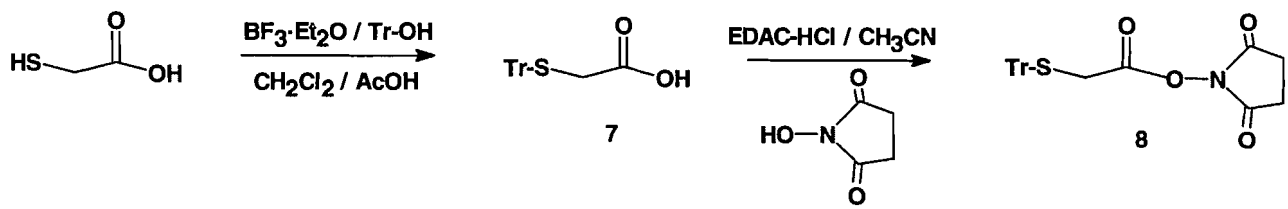


Fig. 3. Synthesis of *O*-(*N*-hydroxysuccinimido)-2-(triphenylmethylthio)ethanoate, **8**.



atom. As a result, all the proton and carbon atom signals within the serine portion of the molecule could be assigned. The results of the HMQC–TOCSY experiment were consistent with the assignment made in Tables 1 and 2.

The one-dimensional NOE difference spectra and the two-dimensional NOESY (19) spectra of **1** in $CDCl_3$ were entirely consistent with the assignments presented above. The presence of strong NOEs between H-9 and H-22, and between H-8 and H-6 and the complete absence of NOEs between H-9 and H-6, and H-9 and H-23, showed the anticipated preponderance of the *Z* geometric isomers of the two amide groups. Otherwise the molecule appeared to be relatively flexible with no particularly demanding conformational preferences. Thus the *ortho* protons on each of the aromatic rings showed NOEs to side-chain protons that were on carbon atoms one, two, and three bonds removed from the ring but there was no evidence for NOEs over longer distances. Likewise there were no observable NOEs between one set of aromatic protons and another that might have arisen from any possible stacking of the aromatic rings.

Fig. 4. The deprotection of **6** to yield **9** and subsequent coupling to **8** to yield ethyl *N*-(2-triphenylmethylthio)ethanoyl-*O*-{4′-[4″-(1″-bis(2‴-chloroethyl)amino)phenyl]butanoyl}-L-seryl-*S*-benzyl-L-cysteine, **1**.

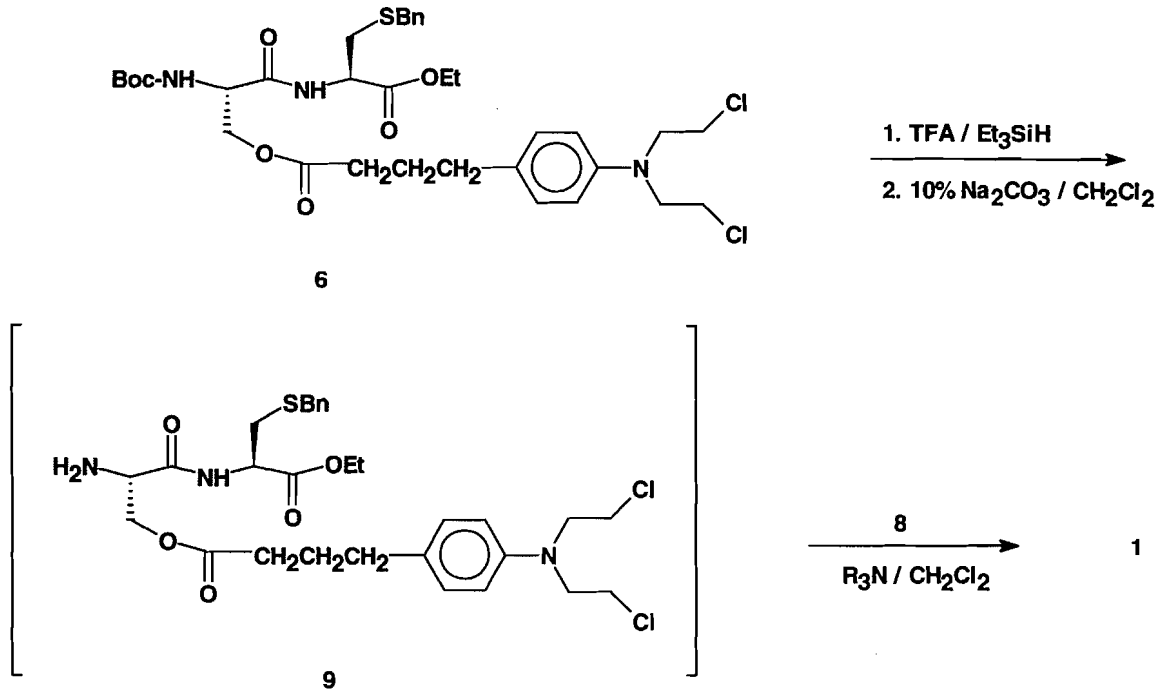


Fig. 5. The gradient HMBC spectrum of **1**.

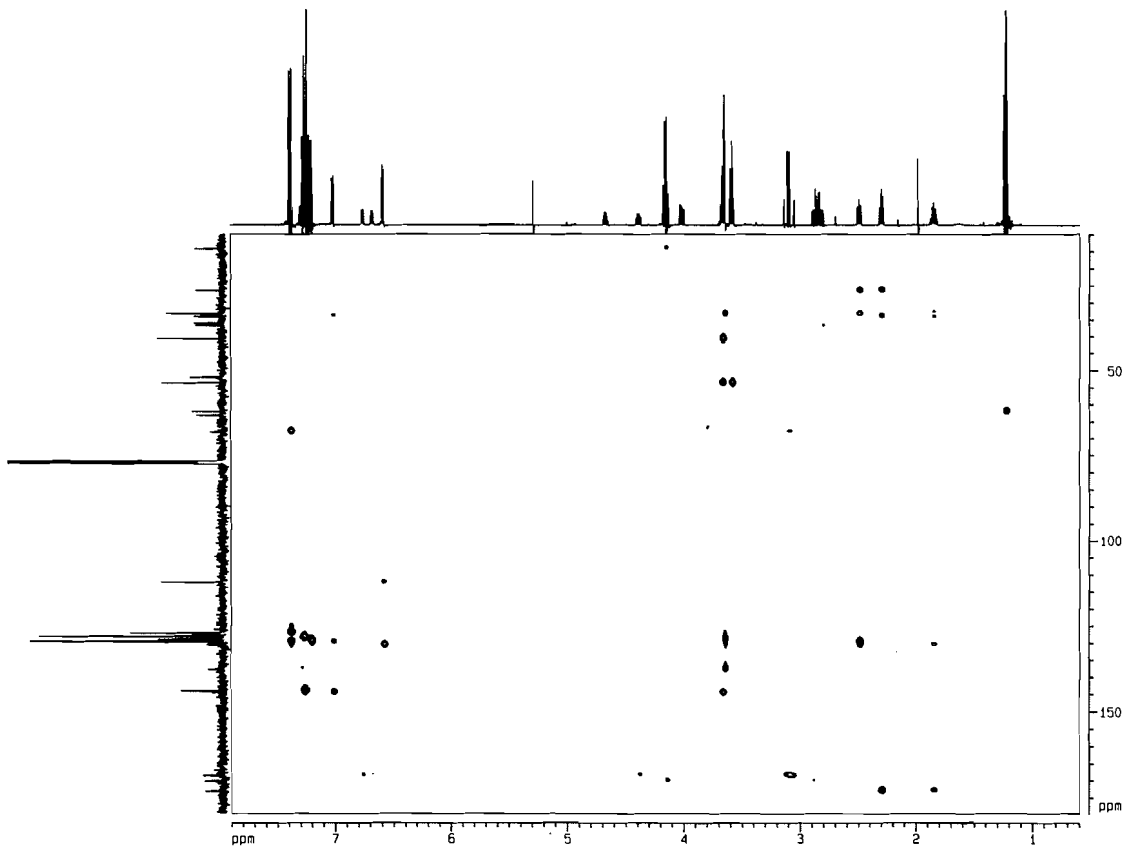
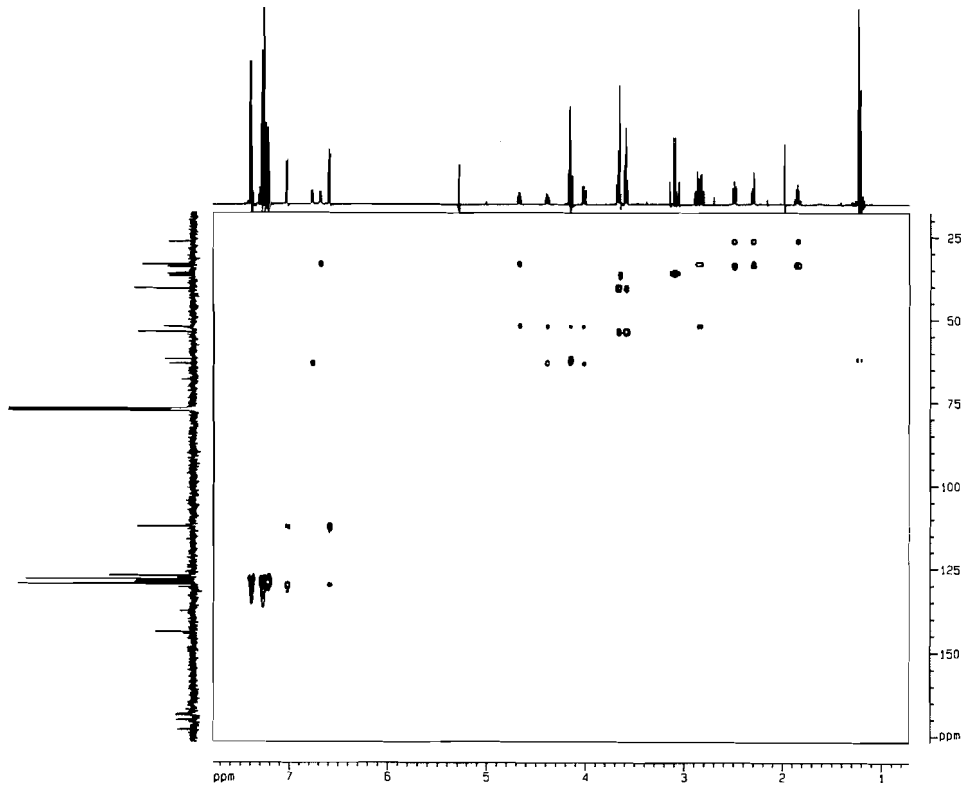


Fig. 6. The HMQC–TOCSY spectrum of compound **1**.



Thus, the synthesis of **1** was completed in 10 steps with 30% overall yield. A combination of two-dimensional NMR techniques was used to assign completely the proton and carbon atom spectra. As a result of the spectrometer's gradient capability the entire collection of spectra, ^{1}H, ^{13}C, COSY, HMQC, HMBC, HMQC–TOCSY, were collected within 6 h on a moderately dilute sample (15 mg/mL). The use of this compound as a reagent for the early detection of breast cancer is currently being investigated.

Experimental section

Analytical TLC was performed on silica gel 60-F_{254} (Merck) with detection by long-wavelength ultraviolet light. Chromatography was performed with a chromatotron (Harrison Research model 7924T) that used a 4 mm plate (EM Science silica gel 60 PF_{254} that contained gypsum). All commercial reagents were used as supplied. Solvents were distilled, under nitrogen, from calcium hydride. Nitrogen was dried by passing it through calcium sulphate. All reactions were protected from light and carried out under a slow flow of nitrogen. Solvents were evaporated with a rotary evaporator (20 Torr; 1 Torr = 133.3 Pa) at elevated temperatures (30–50°C).

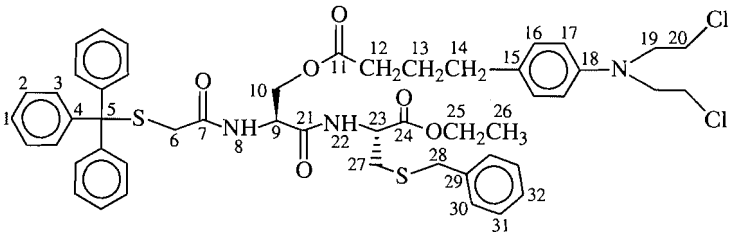
The NMR spectra of **1** were recorded on a Bruker Avance DRX-500 spectrometer. Proton spectra were acquired at 500.130 MHz with a 5 mm broadband inverse probe with triple axis gradient capability. Spectra were obtained in eight scans in 32K data points over a 4.006 kHz spectral width (4.096 s acquisition time). Sample temperature was maintained at 30°C by a Bruker Eurotherm variable temperature unit. Gaussian multiplication (line broadening: −1.5 Hz, Gaussian broadening: 0.2) was used to process the free induction decay (FID), which was zero-filled to 64K before Fourier transformation.

Proton COSY two-dimensional NMR spectra were recorded in the absolute value mode with the pulse sequence 90°–t_1–45°–ACQ and included pulsed field gradients for coherence selection. Spectra were acquired in one scan for each of the 256 FIDs that contained 2K data points in $F2$ over the previously mentioned spectral width. The ^{1}H 90° pulse width was 6.6 μs. A 1.0 s relaxation delay was employed between acquisitions. Zero-filling in $F1$ produced a 1K × 1K data matrix with a digital resolution of 3.91 Hz/point in both dimensions. During two-dimensional Fourier transformation a sine-bell squared window function was applied to both dimensions. The transformed data were then symmetrized.

Carbon-13 NMR spectra were recorded at 125.758 MHz with a 5 mm broadband inverse probe with triple axis gradient capability. The spectra were acquired over a 28.986 kHz spectral width in 32K data points (0.577 s acquisition time). The ^{13}C pulse width was 4.0 μs (30° flip angle). A relaxation delay of 0.5 s was used. Exponential multiplication (line broadening: 4.0 Hz) was used to process the FID, which was zero-filled to 64K before Fourier transformation.

Inverse detected ^{1}H–^{13}C two-dimensional chemical shift correlation spectra were acquired in the phase-sensitive mode and used the pulsed field gradient version of the HSQC pulse sequence. The FIDs in the $F2$ (^{1}H) dimension were recorded over a 3.655 kHz spectral width in 1K data points. The 128 FIDs in the $F1$ (^{13}C) dimension were obtained over a 21.368 kHz spectral width. Each FID was acquired in two scans. The fixed delays during the pulse sequence were a 1.0 s relaxation

Table 1. The proton NMR assignments for ethyl *N*-(2-triphenylmethyl-thio)ethanoyl-*O*-{4′-[4″-(1″-bis(2‴-chloroethyl)amino)phenyl]butanoyl}-L-seryl-*S*-benzyl-L-cysteine, **1**.



Chemical shift, δ	Proton	*J*(Hz)
7.390–7.180	H-aryl	
7.016	H-16	$^3J_{16,17} = 8.8$
6.761	H-8	$^3J_{8,9} = 7.0$
6.679	H-22	$^3J_{22,23} = 7.6$
6.581	H-17	
4.663	H-23	
4.382	H-9	
4.146	H-25	
4.151	$H\text{-}10_A$[a]	
4.009	$H\text{-}10_B$	$^3J_{AX} = 4.9$ $^3J_{BX} = 6.4$ $^2J_{AB} = -11.2$
3.646	H-28	
3.678–3.563	H-19, H-20	
3.117	$H\text{-}6_A$	$^2J_{6A,6B} = -15.9$
3.065	$H\text{-}6_B$	
2.872	$H\text{-}27_A$	
2.814	$H\text{-}27_B$	$^3J_{AX} = 4.9$ $^3J_{BX} = 5.7$ $^2J_{AB} = -13.9$
2.491	H-14	$^3J_{13,14} = 7.8$
2.299	H-12	$^3J_{12,13} = 7.5$
1.848	H-13	
1.225	H-26	$^3J_{25,26} = 7.1$

[a]In the case of diastereotopic pairs of protons, the symbols A and B refer to the downfield and upfield signals, respectively, where these could be resolved.

delay and a polarization transfer delay of 1.786 ms. The 90° ^{1}H pulse was 6.6 μs while the ^{13}C 90° pulse was 11.6 μs. The data were processed with a sine-bell squared window function shifted by π/2 in both dimensions and linear prediction to 256 data points in *F*1 followed by zero-filling to 1K.

The pulsed field gradient version of the HMBC pulse sequence was used to acquire the inverse detected ^{1}H–^{13}C two-dimensional chemical shift correlation spectra through two- and three-bond coupling interactions in the absolute value mode. The FIDs in the *F*2 (^{1}H) dimension were recorded over a 3.655 kHz spectral width in 1K data points. The 128 FIDs in the *F*1 (^{13}C) dimension were obtained over a 21.368 kHz spectral width. Each FID was acquired in two scans. The fixed delays during the pulse sequence were a 1.0 s relaxation delay, a 3.3 ms delay for the low-pass *J*-filter, and 0.08 s delay to allow evolution of the long-range coupling. The 90° ^{1}H pulse was 6.6 μs while the ^{13}C 90° pulse was 11.6 μs. The data were processed with a sine-bell window function in both dimensions and linear prediction to 256 data points in *F*1 followed by zero-filling to 1K.

The HMQC–TOCSY spectra were acquired in the phase-sensitive mode. The FIDs in the *F*2 (^{1}H) dimension were recorded over a 4.006 kHz spectral width in 1K data points. The 128 FIDs in the *F*1 (^{13}C) dimension were obtained over a 21.368 kHz spectral width. Each FID was acquired in 32 scans. The fixed delays during the pulse sequence were a 1.0 s relaxation delay, a 0.3 s delay between the BIRD pulse and HMQC pulse sequence, and 3.571 ms for polarization transfer. The TOCSY spin lock was 100 ms. The 90° ^{1}H pulse was 6.6 μs while the ^{13}C 90° pulse was 11.6 μs. The ^{1}H spin lock 90° pulse width was 27.0 μs. The data were processed with a sine-bell squared window function shifted by π/2 in both dimensions and linear prediction to 256 data points in *F*1 followed by zero-filling to 1K.

Proton–proton NOE difference spectra were obtained by subtraction of a control FID from an on-resonance FID. The decoupler in the control FID irradiated a position in the spectrum where there was no proton signal. The on-resonance FID was obtained while the proton of interest was selectively saturated. In both cases the same decoupler power and duration of saturation (5.0 s) were used. This saturation period also served as the relaxation delay for both the control and on-resonance

Table 2. The carbon-13 NMR assignments for ethyl *N*-triphenylmethylthioethanoyl-*O*-{4′-[4″-(1″-bis(2‴-chloroethyl)amino)phenyl]butanoyl}-L-seryl-*S*-benzyl-L-cysteine, **1**.

Chemical shift (ppm)	Carbon atom	Chemical shift (ppm)	Carbon atom
173.0	C-11	170.0	C-24
168.5	C-7	168.2	C-21
144.4	C-18	143.9	C-4
137.5	C-29	130.5	C-15
129.6	C-16	129.5	C-3
128.9	C-30	128.6	C-31
128.2	C-2	127.3	C-32
127.1	C-1	112.2	C-17
67.9	C-5	63.1	C-10
61.9	C-25	53.6	C-20
52.0	C-9	51.9	C-23
40.5	C-19	36.6	C-28
36.0	C-6	33.9	C-14
33.2	C-27, C-12	26.4	C-13
14.1	C-26		

FIDs. The decoupler was gated off during acquisition. Eight scans were acquired for both the control and on-resonance FIDs and were repeated four times for a total of 32 scans for the final difference spectrum. A 90° ^{1}H pulse width of 6.6 μs was used. The FIDs were processed with exponential multiplication (line broadening: 4.0 Hz) and were zero-filled to 64K during Fourier transformation. The sample was not degassed.

Two-dimensional NOESY spectra were acquired in the phase-sensitive mode with use of the pulse sequence: 90°–t_1–90°–τ–90°–ACQ. Phase-sensitive data were obtained with time proportional phase incrementation (TPPI) (20, 21). The mixing time τ was 0.8 s. In the *F*2: dimension 2K data points were used during the acquisition of the 256 FIDs. Each FID was acquired in 32 scans over 4.006 kHz spectral width using a 1.0 s relaxation delay. Zero-filling in the *F*1 dimension produced a 1K × 1K data matrix after 2-D Fourier transformation of the phase-sensitive data. This resulted in an *F*2 digital resolution of 3.91 Hz/point. During the 2-D Fourier transform a sine-bell window function shifted by π/2 was applied to both dimensions. The transformed data were not symmetrized.

The compounds used in this study were dissolved in $CDCl_3$ (Isotec, Inc.) to a concentration of approximately 15.0 mg/mL. Chemical shifts are reported in ppm relative to TMS. The residual solvent signals at 7.24 and 77.0 ppm were used as internal references for the ^{1}H and ^{13}C spectra, respectively.

All other NMR spectra were recorded on a Bruker AC-200 spectrometer. Proton spectra were acquired at 200.133 MHz with a 5 mm dual frequency probe. Spectra were obtained in eight scans in 16K data points over a 2.403 kHz spectral width (3.408 s acquisition time). Spectra were acquired at ambient probe temperature. The free induction decay (FID) was processed with exponential multiplication (line broadening: 0.1 Hz) and was zero-filled to 32K before Fourier transformation.

Carbon-13 NMR spectra were recorded at 50.323 MHz with the 5 mm dual frequency probe. The spectra were acquired over a 12.195 kHz spectral width in 16K data points (0.672 s acquisition time). The ^{13}C pulse width was 1.5 μs (42° flip angle). A 0.5 s relaxation delay was used. The FIDs were processed with exponential multiplication (line broadening: 3.0 Hz) and zero-filled to 32K before Fourier transformation.

Synthetic procedures

2-(Triphenylmethylthio)ethanoic acid, 7

Triphenylcarbinol (24 g, 92.3 mmol) and mercaptoethanoic acid (8.5 g, 92.4 mmol) were dissolved in dichloromethane (50 mL) and glacial acetic acid (50 mL). A deep red solution was formed when boron trifluoride etherate (16 mL, 130 mmol) was added. The solution was allowed to stir at room temperature for 1 h, during which a precipitate formed. The dichloromethane was removed in vacuo and water (100 mL) was added to the residue. The product was collected by filtration and washed with water (3 × 100 mL), acetonitrile (50 mL), and cold diethyl ether (20 mL). The product was recrystallized from benzene. Yield: 26.68 g, 87%; mp 155–157°C (lit. (9) mp 158.5–160.0°C); TLC: R_f = 0.33 (10:90 v:v CH_3OH/CH_2Cl_2); ^{1}H NMR (200 MHz) ($CDCl_3$, δ: 7.25 (m, 15H, aryl), 2.90(s, 2H, S-CH_2); ^{13}C NMR (50 MHz) ($CDCl_3$), δ: 175.77 (COOH), 144.09–128.05 (C-aryl), 67.44 (CPh_3), 34.67 (S-CH_2).

O-*(*N*-Hydroxysuccinimido)-2-(triphenylmethylthio)-ethanoate, 8*

N-Hydroxysuccinimide (1.72 g, 15 mmol) was added to 2-(triphenylmethylthio)ethanoic acid (5.01 g, 15 mmol) in acetonitrile (20 mL). Solid ethyl-3-(3-dimethylamino)propylcarbodiimide hydrochloride (EDAC-HCl) (3.16 g, 16.5 mmol) was added to the mixture and the solution became transparent after 5 min. Shortly thereafter a precipitate was seen. After the suspension was allowed to stir for 2 h the precipitate was collected by filtration. The colourless solid (3.88 g, 60%) was washed with cold acetonitrile (15 mL). After cooling the filtrate to 4°C overnight an additional crop of product was collected by filtration (1.62 g, 25%); mp 183–185°C; TLC: R_f = 0.46 (2:98 v:v CH_3OH/CH_2Cl_2); ^{1}H NMR (200 MHz) ($CDCl_3$, δ: 7.17 (m, 15H, H-aryl), 3.08 (s, 2H, S-CH_2) 2.47 (s, 4H, CH_2-C=O)$_2$); ^{13}C NMR (50 MHz) ($CDCl_3$), δ: 165.0 ((CH_2-*C*=O)$_2$), 163.00 (SCH_2*C*OON), 143.49 (C-ipso), 129.42 (C-*ortho*), 128.17) C-*meta*), 127.08 (C-*para*), 67.98 (*C*Ph_3), 31.35 (S-CH_2), 25.45 ((CH_2-C=O)$_2$).

Ethyl S*-benzyl-*L*-cysteine* p*-toluenesulphonate, 3*

p-Toluenesulphonic acid (10.8 g, 56.8 mmol) was added to *S*-benzyl-L-cysteine (3.0 g, 4.2 mmol) in absolute ethanol (100 mL). The mixture was heated to reflux for 48 h. The solution was then evaporated to dryness and diethyl ether (100 mL) was added. The resulting white precipitate was collected by filtration and washed with ether (200 mL). Yield: 4.67 g, 80%; mp 131–134°C: ^{1}H NMR (200 MHz) (CD_3OD), δ: 7.299 (m, 9H, H-aryl), 4.155 (q, 2H, O*C*$H_2$$CH_3$), 4.102 (m, 1H, H_2N-*C*H), 3.752 (s, 2H, S*C*H_2Ph), 2.886 (m, 2H, CH-*C*H_2*S*), 2.319 (s, 3H, CH_3-Ph), 1.232 (t, 3H, OCH_2*C*H_3); ^{13}C NMR (50.3 MHz) (CD_3OD), δ: 169.12 (*C*O_2Et), 143.15 (C-SO_3H (*p*TsOH-*para* C) 141.90 (Bn-ipso C), 138.61 (*p*TsOH-ipso C), 130.16 (pTsOH-*meta* C), 129.85 (Bn-*ortho* C), 129.66 (*p*TsOH-*ortho*), 128.42 (Bn-*para* C), 126.95 (Bn-*meta* C),

63.86 (OCH_2CH_3), 53.29 (H_2N-*C*H), 36.79 (SCH_2Ph), 32.06 ($CHCH_2S$), 21.31 (*p*TsOH-CH_3), 14.30 (OCH_2CH_3).

Ethyl N-tert-*butoxycarbonyl-L-seryl*-S-*benzyl-L-cysteine, 5*

Aqueous sodium carbonate (40 mL of 10%) was added to a suspension of **3** (5.0 g, 12.2 mmol) in dichloromethane (80 mL). The mixture was shaken until everything dissolved. The aqueous layer was back-extracted with dichloromethane (2 × 40 mL) and the organic layers combined and dried over anhydrous sodium sulphate. The organic layers were combined and evaporated to dryness. The resulting yellow oil was then diluted with dichloromethane (50 mL). *N-tert*-Butoxycarbonyl-L-serine (2.27 g, 11.1 mmol) and EDAC-HCl (2.33 g, 12.2. mmol) were added to this solution. The solution was stirred under nitrogen and protected from light for 16 h. The solution was extracted with 1 N HCl (2 × 20 mL), 1 N $NaHCO_3$ (2 × 20 mL), and distilled water (2 × 20 mL). The organic layer was evaporated to dryness and the resulting solid recrystallized from acetonitrile. Yield: 3.7 g, 80%; mp 78–79°C; TLC: R_f = 0.58 (10:90 v:v CH_3OH/CH_2Cl_2); ^1H NMR (200 MHz) ($CDCl_3$), δ: 7.308 (s, 5H, H-aryl), 5.485 (d, ^3J = 7.15, 1H, amide NH), 4.740 (m, 1H, $CHCH_2S$), 4.155 (q, 2H, OCH_2CH_3), 4.066 (m, 1H, $CHCH_2OH$), 3.688 (s, 2H, SCH_2Ph), 3.650 (m, 2H, $CHCH_2OH$), 2.851 (m, 2H, $CHCH_2S$), 1.751 (bs, OH), 1.436 (s, 9H, $C(CH_3)_3$), 1.243 (t, $^3J_{18,19}$ = 7.12, 3H, OCH_2CH_3); ^{13}C NMR (50 MHz) ($CDCl_3$), δ: 171.18 (*C*OOEt), 170.54 (amide C=O), 155.78 (carbamate C=O), 137.42 (C-ipso), 128.86 (C-*ortho*), 128.03 (C-*meta*), 127.17 (C-*para*), 80.36 (*Ct*Bu), 62.89 (OCH_2CH_3), 61.91 (CH_2OH), 55.34 ($CHCH_2S$), 51.74 ($CHCH_2OH$), 36.27 (SCH_2Ph), 32.97 ($CHCH_2S$), 28.19 ($C(CH_3)_3$), 13.95 (OCH_2CH_3); MS (NH_3-DCI), *m/z* (RI%): 444 (15, M+1+ NH_3), 427 (100, M+1), 327 (30, M+1−Boc).

Ethyl N-tert-*butoxycarbonyl*-O-*{4'-[4''-(1''-bis(2'''-chloroethyl)amino)phenyl]butanoyl}-L-seryl*-S-*benzyl-L-cysteine, 6*

A solution of EDAC (100 mg, 0.52 mmol) and DMAP (6 mg, 10 mol%) in dichloromethane (5 mL) was added to **5** (200 mg, 0.47 mmol) and chlorambucil (136 mg, 0.45 mmol) in dry dichloromethane (15 mL). The reaction was stirred under a nitrogen atmosphere and protected from the light for 6 h. The solution was then extracted with 1 N HCl (2 × 10 mL), 1 N $NaHCO_3$ (2 × 10 mL), and distilled water (2 × 10 mL). The organic layer was concentrated and the product purified by chromatography (1% CH_3OH in CH_2Cl_2) to yield a colourless oil. Yield: 237 mg, 71%; TLC: R_f = 0.80 (5:200 v:v CH_3OH/CH_2Cl_2); ^1H NMR (200 MHz) ($CDCl_3$), δ: 7.269 (s, 5H, H-aryl), 6.997 (d, 2H, aniline-*meta*), 6.997 (d, 1H, amide NH), 6.584 (d, ^3J = 8.77, 2H, aniline-*ortho*), 5.210 (m, ^3J = 7.18, 1H, Boc-NH), 4.710 (m, 1H, $CHCH_2O$), 4.431 (m, 3H, CH-CH_2O and $CHCH_2S$), 4.154 (q, 2H, OCH_2CH_3), 3.659 (s, 2H, SCH_2Ph), 3.609 (m, 8H, CH_2CH_2Cl), 2.893 (m, 2H, $^3J_{AX}$ = 4.90, $^3J_{BX}$ = 5.71, $^2J_{AB}$ = −19.6, CH_2S), 2.497 (t, 2H, ^3J = 7.77, CH_2Ph), 2.307 (t, ^3J = 7.68, 2H, $C(O)CH_2$), 1.852 (m, 2H, $CH_2CH_2CH_2$), 1.434 (s, 9H, $C(CH_3)_3$), 1.233 (t, ^3J = 7.16, 3H, OCH_2CH_3); ^{13}C NMR (50 MHz), δ: 173.18 ($C(O)CH_2CH_2CH_2$), 170.12 ($C(O)OCH_2CH_3$), 168.96 (amide-C(O)), 154.37 (Boc-C(O)), 144.26 (aniline-ipso), 137.52 (aniline-*para*), 130.33 (benzyl-ipso), 129.60 (benzyl-*ortho*), 128.85 (benzyl-*meta*), 128.52 (aniline-*meta*), 127.21 (benzyl-*para*), 112.08 (aniline-*ortho*), 80.60 ($C(CH_3)_3$), 63.79 ($CH_2OC(O)$), 61.85 (OCH_2CH_3), 53.49 (CH_2Cl), 51.79 ($CHCH_2S$ and $CHCH_2O$), 40.43 (NCH_2), 36.47 (SCH_2Ph), 33.80 (CH_2Ph), 33.21 ($C(O)CH_2$ and $CHCH_2S$), 28.18 ($C(CH_3)_3$), 26.41 ($CH_2CH_2CH_2$), 14.00 (OCH_2CH_3); MS (HRDEI), calcd.: 711.2540; found: 711.2526.

Ethyl N-*(2-triphenylmethylthio)ethanoyl*-O-*{4'-[4''-(1''-bis(2'''-chloroethyl)amino)phenyl]butanoyl}-L-seryl*-S-*benzyl-L-cysteine, 1*

Triethylsilane was added dropwise to a solution of **6** (50 mg, 0.070 mmol) in trifluoroacetic acid, TFA (5 mL), until the yellow solution became colourless. The reaction mixture, which was protected from light, was allowed to stir for 2 h before the TFA was removed in vacuo. The resulting oil was diluted with dry dichloromethane (20 mL) and the reaction mixture extracted with aqueous Na_2CO_3 (10 mL, 10%). The aqueous layer was back-extracted with dichloromethane (2 × 10 mL) and the organic layers combined and evaporated to dryness. The resulting oil was diluted to 10 mL with dichloromethane and compound **8** (28 mg, 0.064 mmol) was added together with freshly distilled diisopropylethylamine (9.1 mg, 0.070 mmol). The reaction was stirred under nitrogen and protected from light for 24 h. The product was isolated by chromatography (1% CH_3OH in CH_2Cl_2). Yield: 33 mg, 50%; TLC: R_f = 0.71 (2:98 v:v CH_3OH/CH_2Cl_2). Anal. calcd.: C 64.7, H 5.9, N 4.5%; found: C 65.2, H 6.1, N 4.6%. For NMR data see Tables 1 and 2.

Acknowledgements

We acknowledge, with thanks, financial support of this work by the Natural Sciences and Engineering Research Council of Canada and the grant of an NSERC postgraduate fellowship to J.F.V.

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