Benzocaine-modified maleic anhydride–cyclohexyl-1,3-dioxepin copolymer: preparation and potential medical applications

Constantin V. Uglea* and Alina Vatajanu
Institute of Biological Research, Blvd. Copou 20A, Iassy 6600, Romania

and Offenberg Hary
University 'Alexandru I. Cuza', Iassy, Department of Organic Chemistry, Blvd. Copou 11, Iassy 6600, Romania

and Alexandru Grecianu
Veterinary Medical School, Iassy 6600, Romania

and Raphael M. Ottenbrite
Department of Chemistry, Virginia Commonwealth University, Richmond, VA 23284, USA

and Ioan I. Negulescu
Louisiana State University, Department of Chemistry, Baton Rouge, LA 70803-1804, USA
(Received 2 July 1992; revised 23 November 1992)

This paper presents a new synthetic polycarboxylic compound, i.e. a benzocaine-modified maleic anhydride–cyclohexyl-1,3-dioxepin copolymer. *p*-Aminobenzoic acid is linked via an anaesthesine (*p*-aminoethylbenzoate, benzocaine) to the carboxylic groups of the copolymer, both as a spacer and to increase the hydrophobicity of the polymer through the phenyl group. The structure of the modified copolymer is characterized by thin-layer chromatography and by i.r. and n.m.r. spectroscopy.

(Keywords: copolymer; benzocaine; anhydride)

INTRODUCTION

Polyanions can enter the host and behave in a similar way to certain proteins, glycoproteins and polynucleotides, which modulate a variety of biological responses related to host defence reactions. These effects include enhanced host resistance to bacteria and fungi, enhanced immune response, inhibition of adjuvant arthritis and, depending on the polymer size, either depression or stimulation of the functional phagocytic activity of the reticuloendothelial system.

The action of polyanions as mitotic inhibitors, their functional role in neoplastic processes and the role of polynucleotides in immunology and resistance to viruses have been reported. Cytotoxic lymphocytes, cytotoxic antibodies and 'activated' macrophages have all been shown to inhibit or destroy tumour cells[1].

Macrophages may be 'activated' *in vivo* to become cytotoxic to tumour cells *in vitro*. Normal peritoneal macrophages are not cytotoxic but they may be activated by natural agents such as the bacteria *C. parvum* and bacille Calmette–Guérin (BCG) and, most importantly, by synthetic polyanionic copolymers such as the cyclocopolymer of maleic anhydride and divinyl ether (2:1 ratio) known as Pyran polymer. A common finding among all these activated macrophage populations is that they are cytotoxic or cytostatic to tumour cells, such as Lewis lung and Ehrlich ascites, while they have no apparent effect on normal cell populations such as new-born-mouse fibroplasts. Therefore, polyanion-activated macrophages possess the unique capability of discriminating between normal cells and tumour cells[2–4].

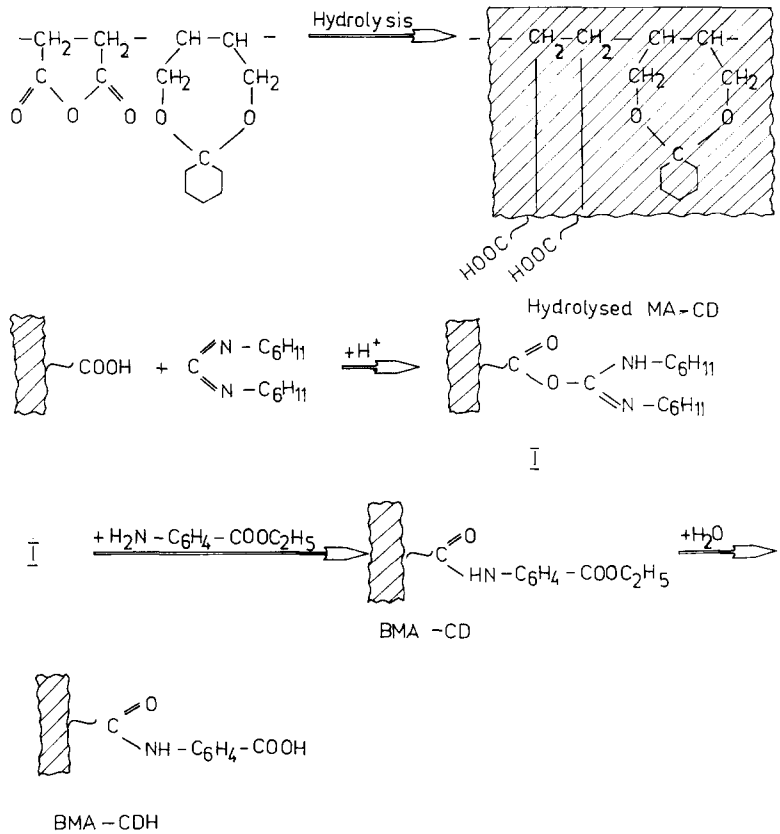
EXPERIMENTAL

Hydrolysis of maleic anhydride–cyclohexyl-1,3-dioxepin copolymer

The maleic anhydride–cyclohexyl-1,3-dioxepin copolymer (MA-CD)[5] (1 g) was added to a solution of 2 N NaOH (25 ml) and the mixture was refluxed until homogeneous (1 h). The polymer solution was poured into 2 N HCl (25 ml) to precipitate the polymer. The polymer was isolated by filtration and washed repeatedly with water to remove the NaCl, then dried in vacuum at 50°C. The copolymer in the carboxylate form had a

* To whom correspondence should be addressed

0032-3861/93/153298-04



Scheme 1 Synthesis of benzocaine-modified maleic anhydride–cyclohexyl-1,3-dioxepin copolymer

molecular weight (g.p.c.) of 11 700, with a molecular mass distribution ranging from 7000 to 20 000 daltons.

Modification of MA-CD copolymer

The copolymer in the acid form (0.9 g) and *p*-aminoethylbenzoate (benzocaine) (2.2 g) were dissolved in pyridine (40 ml) and the mixture was cooled in an ice bath. *N,N'*-dicyclohexylcarbodiimide (Sigma, 3.0 g) was added and the reaction mixture was stirred at 0°C for 5 h and subsequently at room temperature for 48 h. Dicyclohexylurea precipitated as a by-product of the reaction and was filtered off. The pyridine from the filtrate was evaporated under vacuum (50°C, 10 mmHg). Dilute HCl was added to form a water-soluble hydrochloride salt of the unreacted benzocaine. The modified copolymer solution was extracted with ethyl acetate, then neutralized with 5% $NaHCO_3$, washed with water and dried over anhydrous Na_2SO_4. The benzocaine-modified MA-CD (BMA-CD), prepared according to *Scheme 1*, was recovered by removing the solvent. The modified copolymer was obtained in the acid form (BMA-CDH) by mild hydrolysis of BMA-CD. Thin-layer chromatography on silica gel plates (Merck GP254) and petroleum ether:ethyl acetate (1:1) eluent were used to assess the purity of the modified copolymer. N.m.r. spectroscopy (Jeol JNM-C-60-HL; solvent-deuterated pyridine, internal standard tetramethylsilane) and i.r. spectroscopy (Specord IRH, Karl Zeiss Jena; NaBr pellet) were used to elucidate the structure of the BMA-CD product. The encapsulation of the anionic copolymers in liposomes was carried out by the solvent-vaporization technique[6].

Biological tests

The anaesthetic effect of both the unlinked benzocaine and BMA-CD was studied on isolated single fibres of frog muscle. The preparation of single muscle fibres and the anaesthetic tests were carried out according to Edman and Kiessling[7] and Isac[8]. The anti-tumour activity of synthesized products was tested *in vitro* using tumour cell cultures (HELA line) and a normal cell culture of monkey renal cells (BSC). Vials containing a cellular monolayer grown after 72 h were inoculated with 2 ml of copolymer samples of various concentrations (0.25–5 mg ml^{-1}). The vials were kept at 37°C for 24–72 h. The cytotoxic effect was assessed by direct microscopy and the May–Guwald–Glemsa method[9].

RESULTS AND DISCUSSION

The synthetic polyanionic polymer that has received the most interest is the Pyran copolymer, first reported by Butler[10]. Pyran showed significant anti-tumour activity as well as a wide range of other biological activities which have been well documented[11]. Therefore this polycarboxylic copolymer was chosen as a standard for this study of *in vitro* cytotoxic activity of anionic products.

Recently, liposomes have gained acceptance as potential drug carriers. Since they have many advantages over drug delivery systems and are rapidly taken up by macrophages, it was thought that an augmented effect could be obtained by encapsulation of the polyanionic polymers in liposomes[12]. It was also observed that Pyran and other MA copolymers, particularly MA-CD, did not perturb the liposomal assembly[13]. Consequently, the

cytotoxic effect of anionic polymers was also assessed for liposome-encapsulated materials.

The formation of a benzocaine-modified copolymer according to *Scheme 1* was determined from the i.r. spectra of the product by the disappearance of absorption characteristic of –COOH groups (1735 cm^{-1}) and the appearance of new bands for benzocaine as well as bands characteristic of amide groups (1640 cm^{-1}) formed by the coupling reaction. The ^1H n.m.r. spectrum of the same product contained new signals of aromatic protons from the phenyl ring (two multiplets at 7.15 and 8.2 ppm), amide proton signals (5.7 ppm) and signals for the ethyl ester group protons centred at 1.7 ppm, as compared to the n.m.r. spectrum of the parent product (*Figures 1* and *2*).

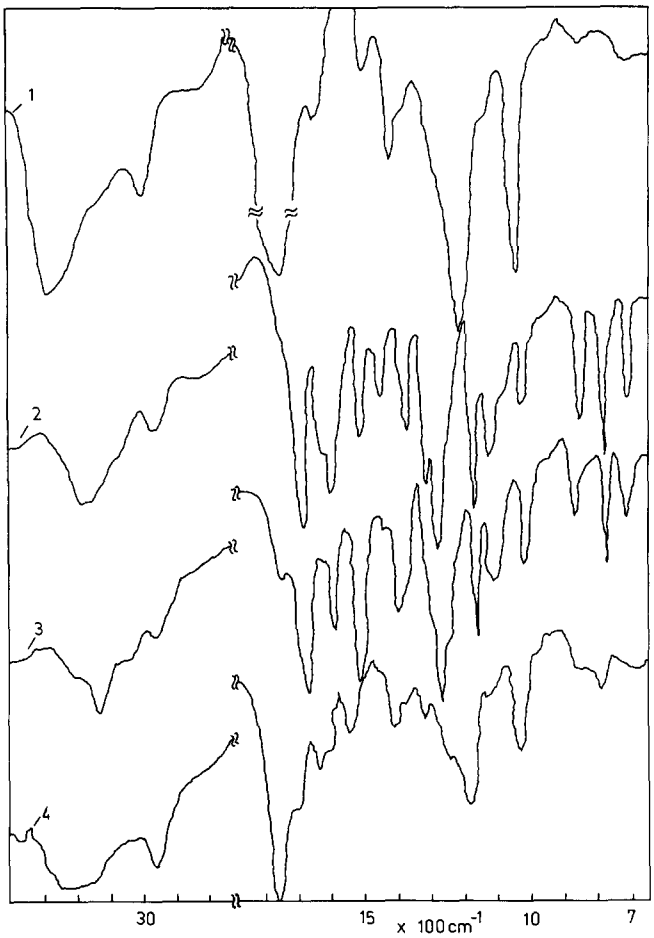


Figure 1 I.r. spectra of modified MA-CD copolymer: 1, hydrolysed MA-CD copolymer; 2, *p*-aminoethylbenzoate; 3, BMA-CD copolymer; 4, BMA-CDH copolymer

Benzocaine was chosen as a spacer, since its detachment from the macromolecular support through hydrolysis of the amide link can be evaluated *in vitro* through its anaesthetic effect. The effect of benzocaine was studied on an isolated single fibre of frog muscle. Benzocaine (10^{-4} M) caused isometric contractions of the muscle preparation and the total duration of cellular biopotential was markedly prolonged. At higher concentrations (10^{-3} M) the resting potential decreased and the 'spike' of the action potential disappeared. A dose-effect study showed that the maximum anaesthetic effect was obtained at a concentration of around 10^{-3} M. At the same concentration the benzocaine had a marked anaesthetic effect comparable to that of the benzocaine-modified copolymer, showing that benzocaine itself was not released by hydrolysis from the macromolecular support.

The cytotoxic effects were also assessed (*Table 1*). Besides Pyran, succinic acid was also tested as a micromolecular polycarboxylic derivative. As expected,

Table 1 *In vitro* cytotoxic effect (represented by +) of polycarboxylic derivatives and related products

		Concentration (mg ml^{-1})								
		2.0			1.0			0.5		
		Contact time (h)								
Agent	Cell type	24	48	72	24	48	72	24	48	72
Succinic acid	HELA	+	+	+	–	–	–	–	–	–
	BSC	+	+	+	–	–	–	–	–	–
MA-CD	HELA	+	+	+	+	+	+	+	+	+
	BSC	–	–	–	–	–	–	–	–	–
BMA-CD	HELA	–	–	–	–	–	–	–	–	–
	BSC	–	–	–	–	–	–	–	–	–
BMA-CDH	HELA	+	+	+	+	+	+	+	+	+
	BSC	–	–	–	–	–	–	–	–	–

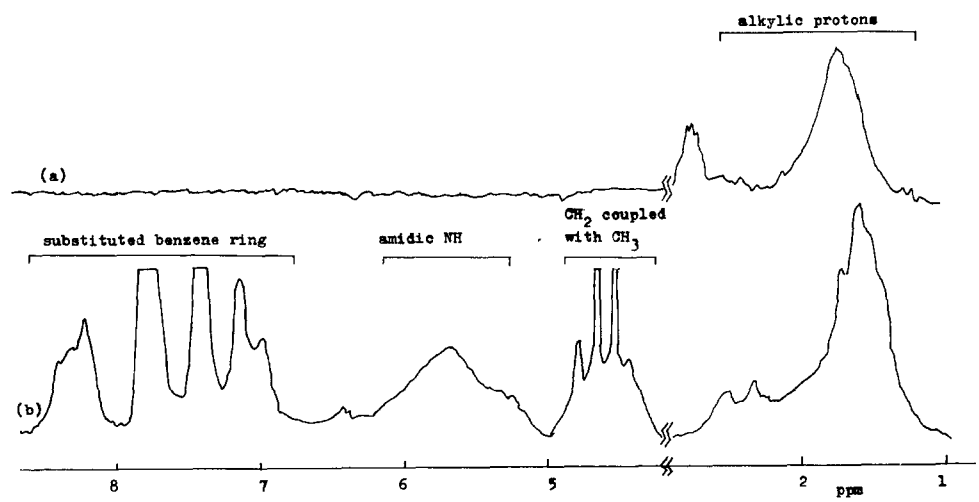


Figure 2 ^1H n.m.r. spectra of (a) hydrolysed MA-CD copolymer and (b) BMA-CD copolymer

Table 2 Relative *in vitro* cytotoxic effect (represented by +) of liposome-encapsulated polycarboxylic products

Dose (ml/ml)	Cell type	Pyran			BMA-CDH			Liposome BMA-CDH		
		Contact time (h)								
		24	48	72	24	48	72	24	48	72
0.5/1.5	HELA	+	+	+	+	+	+	+	+	+
	BSC	–	–	–	–	–	–	–	–	–
0.2/1.8	HELA	–	+	+	–	–	–	+	+	+
	BSC	–	–	–	–	–	–	–	–	–

only polycarboxylic polymers had positive and differentiating cytotoxic effects. Moreover, the introduction of a spacer, i.e. the decrease of negative charge by placing the carboxylic groups away from the polymer backbone, did not markedly affect the cytotoxic effect of the polyanionic modified copolymer (i.e. hydrolysed BMA-CD). The anaesthetic test proved that the spacer is not readily released in *in vitro* conditions. Therefore, the cytotoxic activity of BMA-CD with the benzoic carboxyl groups contributes activity to the parent MA-CD.

Encapsulation of the polycarboxylic product in liposomes had a beneficial effect (*Table 2*). The encapsulated BMA-CDH showed a three-fold increase in cytotoxic activity.

CONCLUSIONS

The preparation and biological characterization of BMA-CD and BMA-CDH copolymers have been presented. Structural analysis confirmed that the BMA-CD and BMA-CDH copolymers were indeed prepared.

Concerning the relation between structure and cytostatic activity, the results obtained show that the macromolecular character of the compounds represents the key factor of the positive and differentiating cytotoxic effects. Moreover, the introduction of a spacer, i.e. the decrease of groups away from the polymer backbone, did not markedly affect the cytotoxic effect of the polyanionic modified copolymer.

Encapsulation of the polycarboxylic product in liposomes had a beneficial effect; the encapsulated BMA-CDH showed a three-fold increase in cytotoxic activity.

REFERENCES

1 Donaruma, G., Ottenbrite, R. M. and Vogl, O. (Eds) 'Anionic Polymeric Drugs', J. Wiley and Sons, New York, 1980
2 Ottenbrite, R. *J. Macromol. Sci. Chem.* 1985, **A22**, 819
3 Kus, K., Ottenbrite, R. M. and Caplan, A. D. *J. Biol. Resp. Med.* 1985, **4**, 46
4 Ottenbrite, R. M. and Butler, G. B. *Drugs Pharm. Sci.* 1984, **24**, 247
5 Ottenbrite, R. M. and Kaplan, A. M. *Ann. NY Acad. Sci.* 1985, **446**, 160
6 Deamer, D. and Bangham, A. B. *Biochem. Biophys. Acta* 1976, **443**, 629
7 Edman, A. B. and Kiessling, I. *Biochem. Biophys. Acta* 1975, **442**, 549
8 Isac, M. *Rev. Roumaine Chim.* 1978, **18**, 232
9 Schertz, L. G. and Marsh, C. J. in 'Cancer' (Ed. F. F. Becker), Plenum Press, New York, 1977, Vol. 5, Ch. 2
10 Butler, G. B. *J. Polym. Sci.* 1960, **48**, 279
11 Ottenbrite, R. M. *Proc. ACS Div. Polym. Mater. Sci. Eng.* 1988, **58**, 228
12 Ottenbrite, R. M., Sunamoto, J., Sato, J. and Oka, M. *Polym. Prepr.* 1985, **26** (1), 212
13 Ottenbrite, R. M. and Sunamoto, J. *Polym. Prepr.* 1986, **27** (1), 44