Encapsulation of Guest Molecules into a Dendritic Box

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Dendrimers are well-defined, highly branched macromolecules that emanate from a central core and are synthesized through a stepwise, repetitive reaction sequence. The synthesis and characterization of dendritic boxes, based on the construction of a chiral shell of protected amino acids onto poly(propyleneimine) dendrimers with 64 amine end groups, is reported here. Nuclear magnetic resonance–relaxation and optical data show that a dense shell with solid-phase character is formed. Guest molecules were captured within the internal cavities of the boxes when these boxes were constructed in the presence of guest molecules. The diffusion of guest molecules out of the boxes into solution was unmeasurably slow because of the close packing of the shell. These monomolecular dendritic containers of 5-nanometer dimensions with physically locked-in guest molecules were characterized spectroscopically.

The interest in dendrimeric macromolecules arises from the distinctive properties of these highly branched, three-dimensional macromolecules with a branch point at each monomeric unit leading to structures that have a defined number of generations and functional end groups (*1*, *2*). The high degree of control over molecular weight and shape has led to the synthesis of unimolecular micelles (*3*) and spherical and cone-shape mesostructures (*4*), as well as stratified dendrimers that have generations of different structure (*5*). The diameters of the spherical dendrimers range from 3 to 10 nm, and so these structures could serve as "building blocks" of a new chemistry set (*6*).

After the initial reports on cascade molecules (*7*), proposals have been made for the construction and applications of guest-host systems made out of dendrimers (*1*, *2*, *8*). The concept of topological trapping by core-shell molecules is based on the fact that, at some stage in the synthesis of dendrimers, the space available for construction of the next generation is not sufficient to accommodate all of the atoms required for complete conversion (the so-called sterically induced stoichiometry) (*1*). Extending this principle in a more general fashion, dendrimers that have internal cavities with a dense outer shell may be synthesized by controlling the chemistry used in the last step. However, experimental verification of these guest-host core-shell systems has been lacking; only guests dissolved in dendrimers have been reported (*2*, *9*). Research into the procedures involved in trapping molecules into molecules is mainly done as part of the effort to construct self-assembled supramolecular architectures, including molecular containers and vesicles (*10*–*19*). We report here the synthesis of a dendritic box based on the concept of the construction of a chiral shell of protected amino acids onto poly(propyleneimine) dendrimers with 64 amine end groups.

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The flexible core of our core-shell unimolecular structures is based on poly(propyleneimine) dendrimers, which were synthesized by the divergent approach (*20*). A repetitive reaction sequence was used consisting of the double Michael addition of a primary amine to acrylonitrile followed by the heterogeneously catalyzed hydrogenation of the nitriles to primary amines. This approach yielded diaminobutane-based poly(propyleneimine) dendrimers with 4, 8, 16, 32, 64, and 128 primary amine end groups. The unmodified dendrimers are very flexible and have glass transition temperatures (T_g) of approximately −40°C and −65°C for the CN- and NH_2-terminated dendrimers, respectively (*20*).

The subsequent synthesis of the rigid shell is performed through a critical end group modification of the cascade polyamines with an appropriate bulky group, in this case an amino acid derivative. The *N*-hydroxy-succinimide ester of a *tert*-butyloxycarbonyl (tBOC)–protected amino acid is brought into reaction with the cascade polyamines in a CH_2Cl_2-triethylamine mixture (Fig. 1 and Table 1). Extended washing procedures were used to obtain pure modified dendrimers with molecular weights up to 24,000 starting from the fifth generation with 64 end groups. We purified

Table 1. Data for various amino acid–modified dendrimers. The yield is given as the chemical yield of the completely converted dendrimer, unless otherwise stated. The glass transition temperature (T_g) was measured with differential scanning calorimetry; the second heating run is reported.

Amino acid	Number of end groups										
	4		8		16		32		64		128
	Yield	T_g (°C)	Yield	T_g (°C)	Yield	T_g (°C)	Yield	T_g (°C)	Yield	T_g (°C)	Yield
L-Ala	95	35	81	41	80	34	61	36	90	37	X*
L-Leu	87	30	66	33	88	44	86	45	68	45	
L-Phe	94	55	82	52	81	41	89	38	82	39	Y†
D-Phe	83	55	96	52	82	41	89	38	82	39	
L-Tyr	66	56	89	57	88	53	80	51	83	53	
L-Trp	83	51	84	46	71	47	92	51	Z‡		

*A dendrimer in a chemical yield of 70% is isolated with a conversion of ≈70%. †A dendrimer in a chemical yield of 30% is isolated with a conversion of ≈40%. ‡A dendrimer in a chemical yield of 40% is isolated with a conversion of ≈60%.

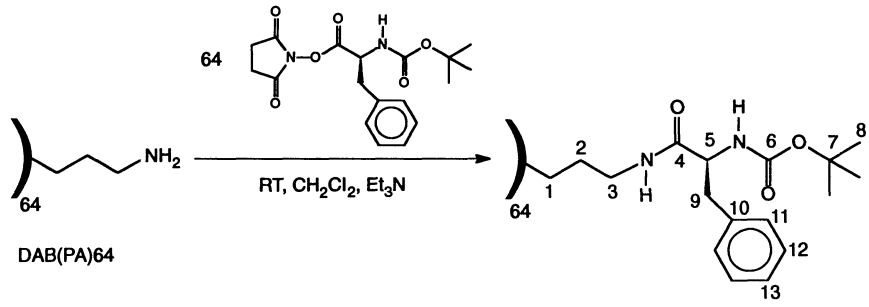


Fig. 1. Schematic presentation of the synthesis of amino acid–terminated dendrimers, including an atomic numbering of the shell of the dendritic box; Et, ethyl; DAB(PA)64, fifth generation of the poly(propyleneimine) dendrimer; RT, room temperature.

the modified dendrimers by making use of the large difference in solubility between the dendrimers that are fully modified (>95% of the end groups, which is the limit of detection at higher generations) and dendrimers with some remaining primary amines (*21*). In most cases at higher generations, it is not possible to completely remove all of the reagents or solvents (clathrate formation). The absence of racemization of the protected amino acids with this coupling procedure has been demonstrated by analysis of the amino acids isolated after acid-catalyzed hydrolysis of the modified dendrimers; an enantiomeric excess of >96% was determined by high-performance liquid chromatography with chiral stationary phases.

From Table 1 we can conclude that complete conversion is obtained for reactions between all poly(propyleneimine) dendrimers up to 64 end groups (fifth generation) and all modified amino acids, except for the bulky tryptophan residue. Attempts to derivatize cascade polyamines with 128 end groups have not been successful; with none of the natural amino acids was a complete conversion of this sixth-generation dendrimer observed, probably because of severe steric hindrance.

Structure elucidation of the dendrimers terminated with *N*-tBOC amino acids was performed with ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and by infrared, ultraviolet, and circular dichroism spectroscopy. The resonances in the ^{13}C NMR spectra show a significant line broadening for the higher generations. Furthermore, we have noticed, most surprisingly, that the optical rotation of several modified dendrimers of the fifth generation is vanishingly small. Both phenomena suggest a diminished molecular motion of the dendritic shell, whereas earlier work showed the large flexibility of the parent polyamine dendrimers (*20*).

In order to investigate the NMR line broadening in more detail (incomplete reaction can also give rise to such broadening), we performed spin-lattice (T_1) and spin-spin (T_2) relaxation measurements. The results for some selected atoms of the *N*-tBOC-L-phenyl-modified dendrimers (generations 1 to 5) are given in Fig. 2. The T_2 relaxation of a large number of atoms from the dendritic shell decreases steadily with increasing generations. The T_1 relaxation data, however, show a decrease up to the third generation, whereas for the higher generations an increase in relaxation times is recorded at 75 MHz. This minimum in T_1 is in sharp contrast to earlier reports in which a continuous decrease was found in T_1 relaxation for dendrimers (*22*) and for guests dissolved in dendrimers (*9*). Such an increase in T_1 relaxation is indicative of a decrease in molecular motion for the highest generations; an almost solid-phase be-

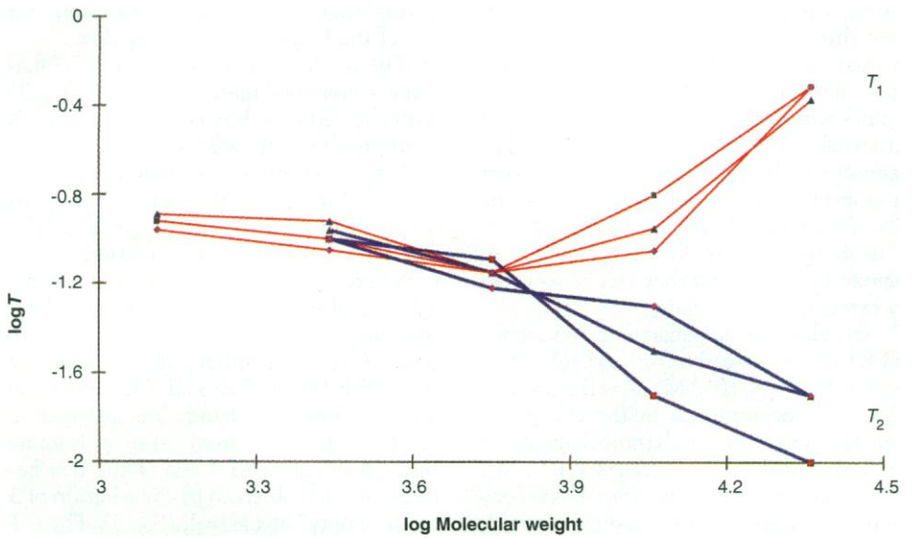
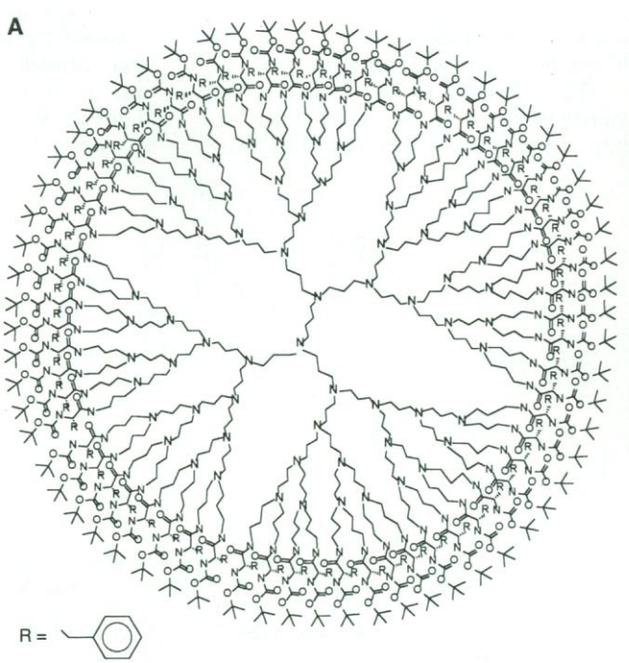


Fig. 2. Double logarithmic graph of relaxation data (carbon T_1 and T_2) versus molecular weight (generation). (Red) T_1 of atoms 1, 2, and 3; (blue) T_2 of atoms 1, 2, and 3. The carbon relaxation data were recorded on a Varian Gemini-300 at 75 MHz with standard software for these experiments (DOT1 and CPMGT2).



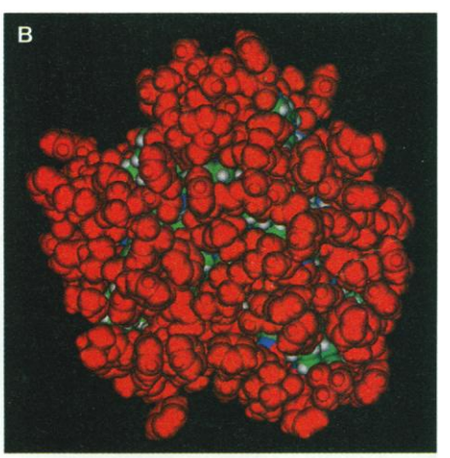


Fig. 3. (**A**) A two-dimensional and (**B**) a three-dimensional picture of the 64-Phe-box. We obtained the picture in (B) by modeling 64-Phe-box with QUANTA 3.3 and subsequently performing the minimalizations with CHARMm 22 with the steepest descent followed by the conjugate gradient methods.

havior of the shell in solution is proposed. Presumably, intramolecular hydrogen bonding between the Phe residues in the shell is contributing to this solid-phase character. An energy-minimized structure determined from CHARMm molecular mechanics calculations of the *N*-tBOC-L-Phe–terminated dendrimer of the fifth generation is presented in Fig. 3. All of the other experiments described below were performed with this dendrimer (64-Phe-box).

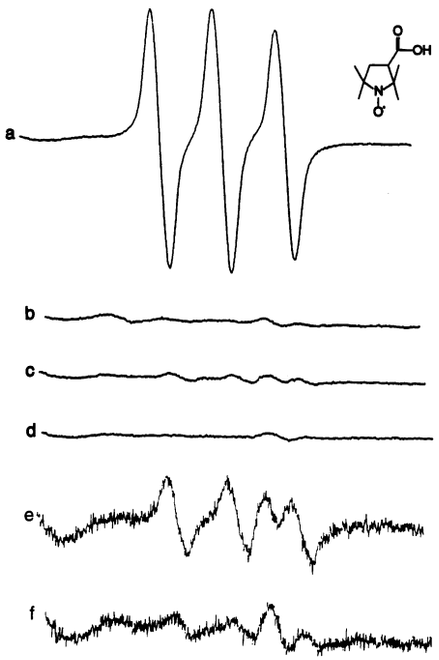


Fig. 4. The EPR spectra of 3-carboxy-proxyl (**1**) encapsulated in the 64-Phe-box. Curve a, **1**-in-box in CH_2Cl_2 after 16 washings with saturated Na_2CO_3. Curve b, test for clathrate formation; a mixture of 64-Phe-box and **1** in CH_2Cl_2 after 16 washings with saturated Na_2CO_3. Curve c, test for chemical bonding: attempts to encapsulate **1** during the synthesis of the 8-Phe dendrimer; dendrimer was dissolved in CH_2Cl_2 after standard workup. Curve d, same as curve c after four additional washings with saturated Na_2CO_3. Curve e, liberation of **1** from the 64-Phe-box after treatment of the box with 12 M HCl followed by the extraction of **1** out of the water layer with CH_2Cl_2. Curve f, result of washing the CH_2Cl_2 layer of the sample in curve e twice with saturated Na_2CO_3.

The experimental and modeling results presented above prompted us to propose that we have prepared molecules with a solid shell and a flexible core that will have internal cavities available for guest molecules; we call this a dendritic box. Because the Phe residues, which are the final branchings that together form the outer layer, are connected to the poly(propyleneimine) dendrimer in the final step, it should be possible to perform this coupling reaction in the presence of guest molecules. In fact, we encapsulated molecules with some affinity for the tertiary amines within the dendritic box. Traces of guests adhering to the surface are removed by extensive washing. Small amounts of solvent or reagent or both are also included in the box. When a dendrimer of lower generation is used, the shell is not dense enough to capture the guests and they can be removed by extraction.

An electron paramagnetic resonance (EPR) probe, 3-carboxy-proxyl (**1**), was used in low (4×10^{-3} M) as well as in high (0.5 M) concentrations in the encapsulation reaction with a poly(propyleneimine) dendrimer with 64 end groups (10^{-3} M). This procedure yielded dendritic boxes containing, on average, one and eight free radicals per box, respectively (*23*). Probe **1** is encapsulated into the 64-Phe-box (**1**-in-box) only and not into modified dendrimers with eight end groups (Fig. 4). After a number of washings, the EPR probe was completely removed from the dendrimer of lower generation, which shows that the radical is not chemically bound to the dendritic box but is physically locked into it.

We were able to encapsulate a large variety of dye molecules into the dendritic box by using the method described above. Some of the features of the dye-in-box system are illustrated with eriochrome black T (**2**). The absorption band with a maximum at 280 nm (λ_{max} = 280 nm) for free **2** in CH_2Cl_2 shifts to λ_{max} = 360 and 570 nm for encapsulated **2**-in-box in CH_2Cl_2. Furthermore, **2**-in-box exhibits the solubility profile of the dendritic box; whereas free **2** is easily dissolved in both water and acetonitrile, **2**-in-box does not dissolve at all in these solvents. Even after prolonged heating, standing for 3 months, or dialysis at room temperature of the suspension of H_2O and **2**-in-box, no coloration of the H_2O solution due to free **2** occurs. From these results, we conclude that the diffusion of **2** out of the box is unmeasurably slow.

The results of the interaction of 7,7,8,8-tetracyanoquinodimethane (TCNQ, **3**) with the dendritic box are shown in Fig. 5. A strong charge-transfer (CT) complex of **3** with the polyamine core is formed during the construction of the box and exhibits CT absorption bands with λ_{max} = 615, 670, 750, and 850 nm. The formation of CT complexes is accompanied by a very intense EPR signal for the radical anion of **3**. During the exhaustive washing procedure to remove free and adhered **3**, a decrease of the CT bands at 750 and 850 nm is observed. These CT bands are assigned to electron transfer from the polyamine through the shell to **3** out of the box because they are observed by the addition of **3** to an "empty" box as well (Fig. 5). The CT bands at 615 and 670 nm are assigned to the supramolecular isomer with both radical ions in the box. Moreover, this CT-complex **3**-in-box is remarkably stable.

Finally, rose bengal (**4**) was encapsulated into the dendritic box with an average load of one dye molecule per box, as determined by ultraviolet spectroscopy. A large difference in absorption and emission between **4**-in-box and free **4** was observed (Fig. 6). The emission of **4**-in-box was relatively insensitive to solvent

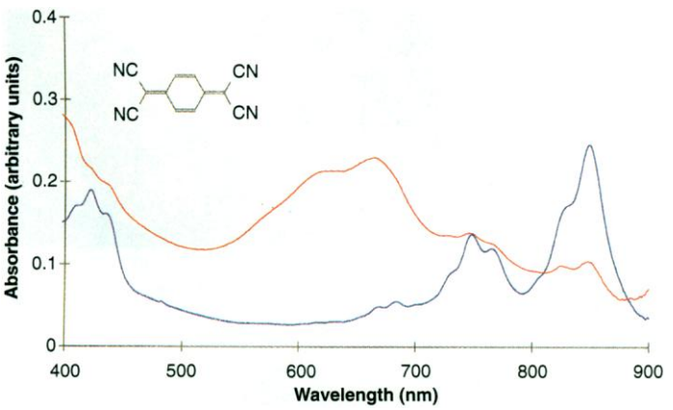


Fig. 5 (left). The ultraviolet spectra of TCNQ (**3**) with 64-Phe-box: red line, **3**-in-box in CH_2Cl_2 after 12 washings; blue line, clathrate between **3** and 64-Phe-box in CH_2Cl_2.

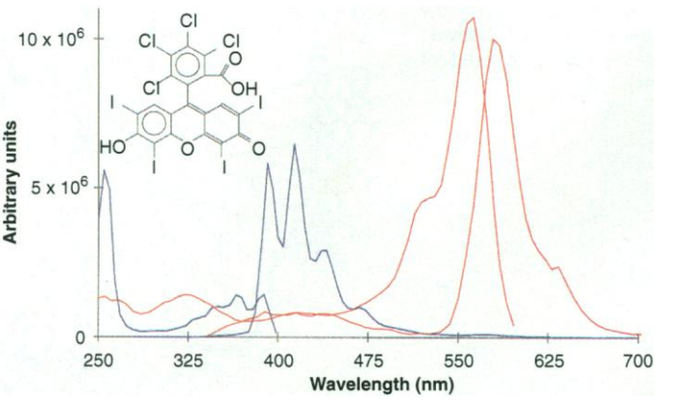


Fig. 6 (right). Fluorescence spectra of rose bengal (**4**) with 64-Phe-box: red line, **4**-in-box in CH_2Cl_2; blue line, free **4** in CH_2Cl_2.

effects. Hence, we believe that we have prepared a fluorescent sphere with an environment-independent emission profile.

These results strongly suggest that the procedures described here can be used to produce a unimolecular compartmented structure in which guest molecules are physically locked and for which the diffusion out of the box is unmeasurably slow. Obviously, it is important to know the detailed structure of the guest-in-box systems, but a discussion on this topic is purely speculative at this point. It can be argued that parts of the guest are within the shell domain or even sticking out of the box without, however, the possibility of being extracted. Both shell and core, as a consequence of the unimolecular structure, have a restricted mobility. (Chir)optical studies, including solvatochromic measurements made with probe molecules, will be required to obtain a more detailed insight into the exact nature of these new containers. A number of applications, such as fluorescent markers for pores in the nanometer range and controlled delivery, are foreseen. Moreover, with these systems it may be possible to study the photochemistry and photophysics of isolated molecules in a well-defined cage.

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21. A typical procedure for the construction of the dense shell is as follows. To a stirred solution of 72 mg (0.010 mmol) of amine dendrimer of the fifth generation (64 end groups) in 10 ml of CH_2Cl_2 with 0.1 ml of triethylamine was added 235 mg (0.65 mmol, 1.01 equivalent per NH_2 end group) of *N*-tBOC-L-Phe hydroxy succinimide ester. After it was stirred overnight, the reaction mixture was diluted with CH_2Cl_2 to 50 ml and subsequently washed with water (three times with 30 ml) and saturated Na_2CO_3 (three times with 30 ml), respectively. Drying (with Na_2SO_4) followed by evaporation of the solvent yielded 190 mg (0.0082 mmol, 82%) of the tBoc-L-Phe–modified dendrimer. All spectral data were in accordance with the proposed structure.
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23. A remarkable sharp triplet was observed for the box containing eight probe molecules. A comprehensive study of the mobility of the EPR probe and the nature of the radical assemblies is in progress (J. F. G. A. Jansen *et al.*, in preparation).

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DNA Sequence from Cretaceous Period Bone Fragments

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DNA was extracted from 80-million-year-old bone fragments found in strata of the Upper Cretaceous Blackhawk Formation in the roof of an underground coal mine in eastern Utah. This DNA was used as the template in a polymerase chain reaction that amplified and sequenced a portion of the gene encoding mitochondrial cytochrome b. These sequences differ from all other cytochrome b sequences investigated, including those in the GenBank and European Molecular Biology Laboratory databases. DNA isolated from these bone fragments and the resulting gene sequences demonstrate that small fragments of DNA may survive in bone for millions of years.

Biological molecules have varying stabilities over extended periods. Immediately after cell death, these molecules begin rapid degeneration. Nucleic acids have limited life expectancies under physiological conditions, and DNA is particularly susceptible to oxidative and hydrolytic damage. Alterations resulting in abasic sites and other base or sugar modifications quickly destabilize the molecule, producing strand breaks and other degradative changes (*1*). Under physiological conditions, it would be extremely rare to find preserved DNA that was tens of thousands of years old. If biological molecules are to be preserved over geologic time periods, they must be removed from physiological conditions soon after biological death and maintained in that condition. At the same time, the molecules must be protected from other extremes that may be responsible for the nonphysiological conditions, such as heat and pressure. These requirements would preclude the recovery of biological molecules from ancient sources in most instances. However, there have been reports of the persistence of amino acids associated with fossils and dinosaur bones in the sedimentary matrix (*2*, *3*), and recent reports of ancient DNA recovered from insects and plants trapped and preserved in amber have demonstrated the possibility of finding extremely old DNA from ancient organisms (*4*). In this report, we present evidence of the isolation and amplification of DNA from bone material recovered from a Cretaceous period coal bed.

Coal beds are the result of large accumulations of peat in ancient bogs that have been covered by silt, sand, and other muds, sealing the peat from further organic decomposition and eventually resulting in coalification. We recovered two bone fragments associated with coal beds from the Upper Cretaceous Blackhawk Formation of the Mesaverde Group that range in thickness from 1.5 to 8.2 m. These strata represent coastal plain and lower delta plain deposits formed along the western shoreline of a large inland sea known as the Mancos Sea (*5*, *6*). This sea covered much of the western interior of North America during the Cretaceous period. The rock formation is approximately 80 to 85 million years old (*7*). The coal is a high-volatile bituminous type that has an estimated depth of burial of

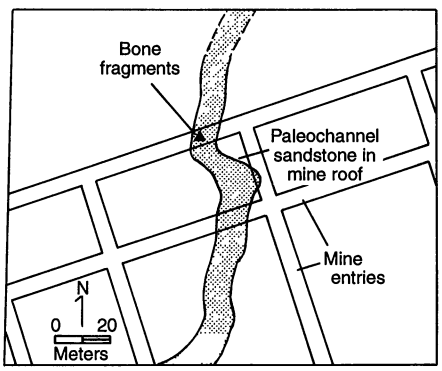

Fig. 1. Map of the mine area and entries, showing the probable path of the sandstone paleochannel in which the bone fragments were found.

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